新規試験法提案書

皮膚感作性試験代替法
Direct Peptide Reactivity Assay (DPRA):
ペプチド結合性試験

平成27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 27 年 3 月 20 日

No.　2014-02

皮膚感作性試験代替法 Direct Peptide Reactivity Assay (DPRA)：
ペプチド結合性試験に関する提案

平成 26 年 12 月 17 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：　ペプチド結合性試験で陽性の結果が得られた場合、その化学物質を強い感作性物質に分類することは行政上可能である。しかしながら、まれに偽陽性の結果が生じることに留意しなければならない。一方、ペプチド結合性試験の陰性結果が得られた場合、その化学物質の皮膚感作性は偽陰性の可能性があり、DPRA 単独でその皮膚感作性を判定することは難しい。その他の情報源と組み合わせて評価することにより、ペプチド結合性試験は行政的に利用可能であると考える。

この提案書は、OECD TG 442C; *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)および JRC Scientific and Policy report, EURL ECVAM Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testingをもとに、皮膚感作性試験資料編纂委員会によりまとめられた文書を用いて、JaCVAM評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として皮膚感作性試験代替法　ペプチド結合性試験の使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

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平賀秀明	（独立行政法人　医薬品医療機器総合機構）
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横関博雄	（日本皮膚アレルギー・接触皮膚炎学会）
吉田武美	（日本毒性学会）
吉田　緑	（国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部）
吉村　功	（座長推薦）
渡部一人	（日本製薬工業協会）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

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小島　肇　（国立医薬品食品衛生研究所　安全性生物試験研究センター　薬理部
新規試験表評価室）：事務局

JaCVAM statement on
Direct Peptide Reactivity Assay for Skin Sensitisation Testing

At a meeting held on 17 December 2014 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: When using the Direct Peptide Reactivity Assay (DPRA) in a regulatory context, it is reasonable for substances that give positive results to be classified as a strong sensitiser, i.e., a Category 1 substance under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). It is, however, necessary to bear in mind that this assay occasionally yields false positive results for certain substances. Conversely, it is quite possible that the DPRA will yield false negative results for some chemicals, which means that it would be unreasonable to use this assay as a standalone test for assessing skin sensitisation potency. We therefore conclude that the use of the DRPA in a regulatory context requires that the assessment also take into account information from other sources.

This statement was prepared following a review of OECD TG No. 442C "*In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)" as well as a JRC Scientific and Policy Report "EURL ECVAM Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing". The JaCVAM Regulatory Acceptance Board acknowledges that the results of this review as well as of a study of materials prepared by the JaCVAM Editorial Committee indicate that this assay is useful in a regulatory context.

Based on the above, we propose that regulatory agencies performing safety assessment of skin sensitisation potency consider using the DPRA Assay as an alternative to testing with laboratory animals.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

20 March 2015

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

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

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

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

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

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

ペプチド結合性試験：Direct Peptide Reactivity Assay (DPRA)

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ペプチド結合性試験（Direct Peptide Reactivity Assay：DPRA）の評価会議報告書

JaCVAM 評価会議

平成 27 年（2015 年）2 月 6 日

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JaCVAM 評価会議

大野泰雄　（運営委員会推薦）：座長

五十嵐良明（国立医薬品食品衛生研究所　生活衛生化学部）

一鬼　勉　（日本化学工業協会）

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渡部一人　（日本製薬工業協会）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

ペプチド結合性試験（Direct Peptide Reactivity Assay ：DPRA）は、化学物質の皮膚感作性を予測する試験法であり、従来のモルモットの皮膚感作性試験（OECD TG406）、マウスの局所リンパ節試験（Local Lymph Node Assay: LLNA、OECD TG429）および LLNA の変法である LLNA:DA (OECD TG442A)ならびに LLNA:BrdU-ELISA (OECD TG442B)と比べ､動物を用いないという特徴を有する。DPRA は、皮膚感作性成立の初期段階の反応であるハプテンとタンパク質の結合性を調べることにより、皮膚感作性の有無を予測する試験法である。European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)によるバリデーション研究を基にした ESAC（ECVAM Scientific Advisory Committee） による第三者評価が終了し OECD による専門家会議を経て、OECD TG442C として 2015 年 2 月 5 日に採択された [1)]。JaCVAM 評価会議は、皮膚感作性試験資料編纂委員会は、上記資料をもとに作成した皮膚感作性試験評価報告書を用いて、DPRA の妥当性について検討した。

１．試験法の定義

名称： ペプチド結合性試験（Direct Peptide Reactivity Assay ：DPRA)

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

試験法の概略： DPRA は、生体における皮膚感作性発現までの過程の化学物質とタンパク質との安定な結合物の形成性を評価する“動物を用いない *in chemico* 試験”である。DPRA では、皮膚内のタンパク質の代わりに 7 個のアミノ酸から成る合成ペプチドのシステイン含有ペプチドとリジン含有ペプチドの 2 種類（いずれも市販品）を使用する。化学物質とそれぞれのペプチドを混合することにより反応させ、混合 24 時間後における未反応のペプチド量を HPLC で分離定量する。試験成立条件を満たした化学物質の反応性は、測定ごとのペプチド減少率から平均値を算出し、予測モデルに従って High、Moderate、Low および Minimal の 4 段階に分類される。その結果、反応性が Low、Moderate および High に分類される化学物質は皮膚感作性あり、Minimal に分類される化学物質は皮膚感作性なしと予測される。

２．評価に用いた資料および評価内容の科学的妥当性

DPRA は、EURL ECVAM 主導のバリデーション試験とそれに続く ESAC による独立した第三者評価において評価され、代替試験法として科学的に妥当であると報告されている [2)]。本邦においては、JaCVAM 皮膚感作性試験資料編纂委員会が、DPRA 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、OECD TG442C [1)]、EURL ECVAM のバリデーション報告書 [3)]のみならず現在まで

公開されている情報[4)]を基に検証した。検証の結果[5)]、DPRA には感作性発現機序における初期の重要なイベントであるタンパク質と化学物質の結合反応を検出するという科学性があり、*in chemico* の皮膚感作性試験代替法として妥当であると考えられた。

３．本試験法の有用性と適用限界

DPRA は、動物を用いない *in chemico* の試験法で、3Rs の精神に合致している。また、１アッセイ当たりの経費も、LLNA と比較して 1/10 程度で実施可能であり、試験期間も LLNA より短期間であることから、本試験法は簡便性および経済性の面から有用といえる。更に、DPRA は、感作性発現機序における初期の重要なイベントであるタンパク質と化学物質の結合反応を検出するものであり、化学物質の感作性を判断する上で重要な情報を与える。

しかしながら、DPRA の施設内再現性において、United Nations (UN)　Globally Harmonized System of Classification and Labelling of Chemicals (GHS) 区分 1B の物質（弱い感作性物質）では再現性が得られなかったため達成基準（85%）を満たせず、DPRA では弱い感作性物質に対しては判定を誤る懸念がある。また、施設間再現性においては、ペプチドと共有結合を生じうる官能基を持たない金属塩を除くことにより再現性は 82%となり、達成基準 (80%) を上回る。従って、DPRA において金属塩は適用範囲外とすべきである。DPRA は液相中で反応を行うため、化学物質は指定された数種類の溶媒に溶解する必要があり、疎水性の高い化学物質は正しく判定されない可能性がある。また、DPRA の検証試験における感度は、試験適用外と考えられる金属塩を除いた場合でも 75%であり、DPRA で陰性の結果が得られた場合は、偽陰性の可能性を考慮し、補完し得る他の試験法との組み合わせで確認する必要がある。これまでの結果から、皮膚感作性が Moderate または Weak の化学物質、感作性の獲得に代謝的あるいは非代謝的活性化を必要とするプロハプテンあるいはプレハプテン、および疎水性の高い化学物質は偽陰性を示す可能性が高いため、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、行政上の利用および社会的受け入れの可能性

社会的受け入れ性：

DPRA は、HPLC 分析が可能な施設であれば容易に実施できる試験法であり、また化学物質との反応に用いる 2 種類の合成ペプチドも市販されており、入手に制限はない。DPRA の実施に当たっては、化学物質の性質を見極め、得られた結果の解釈に注意を払う必要がある。しかし、DPRA は皮膚感作性成立の初期段階の化学物質とタンパク質の結合反応を検出しており、化学物質の感作性を考える上で重要な情報を与えていること、また、簡便かつ安価な試験法であることから、DPRA の社会的受け入れ性は高いものと考える。

行政上の利用性：

DPRA で陽性の結果が得られた場合、その化学物質を UN GHS 区分 1 に分類（強い感作性物質）することは行政上可能である。しかしながら、まれに偽陽性の結果が生じることに留意しなければならない。一方、DPRA の陰性結果が得られた場合、その化学物質の皮膚感作性は偽陰性の可能性があり、DPRA 単独でその皮膚感作性を判定することは難しい。Integrated Approaches to Testing and Assessment: IATA)* を構成するその他の情報源と組み合わせて評価することにより、DPRA は行政的に利用可能であると考える。

*:OECD にてガイダンス案が検討中

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皮膚感作性試験評価報告書

Direct Peptide Reactivity Assay (DPRA)：

ペプチド結合性試験

平成 27 年 2 月 6 日

JaCVAM 皮膚感作性試験資料編纂委員会

JaCVAM 皮膚感作性試験資料編纂委員会

委員長　筒井尚久（田辺三菱製薬株式会社）
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　　武吉正博（一般財団法人　化学物質評価研究機構）
　　森本隆史（住友化学株式会社）

要旨

皮膚感作性は化学物質の安全性評価において重要な評価項目であり、従来、モルモットやマウスを用いる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* 試験の代替法が推奨されており、動物実験により安全性評価された成分を含む化粧品の輸入販売が禁止されたことから（2013 年 3 月全面施行）、動物を用いない *in vitro* 試験法の開発が強く望まれている。Direct Peptide Reactivity Assay（DPRA：ペプチド結合性試験）は感作性成立初期段階の反応であるタンパク質と化学物質の結合反応を機器分析によって評価する手法である。本報告書は、この DPRA について、その手順や有用性と限界を The 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及び EURL ECVAM Scientific Advisory Committee（ESAC）第三者評価（ピアレビュー）報告書などを基に作成したものである。

DPRA はシステイン含有ペプチド（Ac-RFAACAA-COOH）と被験物質及びリジン含有ペプチド（Ac-RFAAKAA-COOH）と被験物質をそれぞれ混合・反応させ、24 時間後における未反応のペプチド量を基に被験物質の反応性を分類する手法であり、高速液体クロマトグラフィー（HPLC）分析が可能な施設であれば容易に実施することができる。本試験法の先行バリデーション試験における施設内再現性は 73%-100%であり、3 施設中 1 施設において、GHS 区分 1B（弱い感作性物質）の物質で再現性が低かったため、達成基準（85%）に達しておらず、弱い感作性物質では判定がぶれる懸念があった。一方、施設間再現性は 75%であり、達成基準（80%）を満たさなかったが、適用範囲外の金属塩を除いた場合に達成基準を上回った。

接触皮膚炎のリスクを動物で予測する試験法として知られているマウスを用いる局所リンパ節試験（Local Lymph Node Assay: LLNA）の試験結果を参照し、先行バリデーション試験における正確度（Accuracy）は 78%、感度（Sensitivity）は 71%、特異度（Specificity）は 92%と報告されている。本試験適用外の金属塩を除いた場合にでも感度は約 75%であり、陰性の結果が得られた場合は、偽陰性の可能性を考慮し、補完し得る他の試験法により確認しなければならない。

DPRA は感作性発現機序における初期の重要なイベントであるタンパク質と化学物質の結合反応を検出しており、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。LLNA の 1/10 程度の経費で実施可能であり、動物を用いない *in chemico* 試験法であることから、有用性は高い。しかしながら、本法は代謝系を欠く化学的試験法であり、活性化に代謝系や非生物的活性化を必要とする感作性物質、弱い感作性物質や金属塩、疎水性の高い物質などは正しくその感作性が検出されない可能性がある。以上の事実を踏まえ、資料編纂委員会は、証拠の重み付けや他の試験法（LLNA、モルモットを用いる皮膚感作性試験）と組み合わせでの評価を推奨する。

１．緒言

皮膚感作性を評価することは化学物質の安全性評価において重要である。化学物質の皮膚での接触皮膚炎のリスクを動物で予測する試験法としてモルモットを用いる皮膚感作性試験（OECD TG406）やマウスを用いる局所リンパ節試験（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）を用いず ATP 量を測定する LLNA:DA（OECD TG442A）や Bromodeoxyuridine 量を測定する LLNA:BrdU-ELISA（OECD TG442B）がある。このように、現在 OECD からガイドラインとして公表されている試験法は、動物を用いた *in vivo* の試験法のみである。

EU における欧州化学品規則（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* 試験等による代替法が推奨されており、動物実験により安全性が評価された成分を含んだ化粧品の輸入及び販売が禁止された（2013 年 3 月全面施行）。そのため、化学物質の皮膚感作性を評価する代替法の開発が強く求められている。

現在、ペプチドとの結合反応を利用した Direct Peptide Reactivity Assay（DPRA：ペプチド結合性試験）、単球系細胞の活性化を利用した human Cell Line Activation Test（h-CLAT）及び Myeloid U937 Skin Sensitization Test（MUSST）、ケラチノサイト細胞系の標的遺伝子を用いた ARE-Nrf2 Luciferase Test Method（KeratinoSens™法）などの皮膚感作性試験の動物を用いない動物実験代替法が提案されており、The European Union Reference Laboratory for Alternatives to Animal Testing（EURL ECVAM）等において先行バリデーション研究が行われている。

DPRA は、化学物質がペプチドと共有結合し免疫応答を引き起こす抗原となる性質を利用し、未反応のシステイン或いはリジンを含むペプチドを高速液体クロマトグラフィー（HPLC）で測定する試験法である。本法の先行バリデーション研究の結果については、EURL ECVAM Scientific Advisory Committee（ESAC）による第三者評価（ピアレビュー）が完了している。

JaCVAM 皮膚感作性試験資料編纂委員会（以下、委員会）が DPRA 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性は次の 4 つの Key event から成るとされている [1)]。

1）化学物質とタンパク質のシステイン残基あるいはリジン残基との共有結合

2）ケラチノサイトにおける炎症性応答及び Antioxidant/electrophile response element (ARE)-dependent pathway による遺伝子発現

3）樹状細胞の活性化（特異的細胞表面マーカーの発現、ケモカインやサイトカインの産生）
4）リンパ節における T 細胞の増殖

DPRA は、上述した皮膚感作性の AOP における『化学物質とタンパク質の共有結合』に対応した動物を用いない *in chemico* 試験である。他にも *in chemico* の皮膚感作性試験がいくつか報告されている [2)-4)]。

DPRA では、皮膚内のタンパク質の代わりに合成ペプチドであるシステイン含有ペプチド（Ac-RFAACAA-COOH）とリジン含有ペプチド（Ac-RFAAKAA-COOH）の 2 種類を使用する。化学物質とそれぞれのペプチドを混合し、反応させ、混合 24 時間後における未反応のペプチド量を高速液体クロマトグラフィー（HPLC）で分離定量する。その結果を基に、化学物質の反応性を 4 段階（High, Moderate, Low, Minimal）に分類する。

３．試験手順／判定 [5)]

3-1.　使用するペプチドおよび陽性対照の調製

・システイン含有ペプチド/リン酸緩衝溶液（システイン含有ペプチド溶液）
　システイン含有ペプチド（Ac-RFAACAA-COOH、純度：90-95%）は、リン酸緩衝液（pH7.5）に 0.667 mM の濃度になるように溶解させる。
・リジン含有ペプチド/酢酸アンモニウム緩衝溶液（リジン含有ペプチド溶液）
　リジン含有ペプチド（Ac-RFAAKAA-COOH、純度：90-95%）は、酢酸アンモニウム緩衝液（pH10.2）に 0.667 mM の濃度になるように溶解させる。
・陽性対照：Cinnamic Aldehyde（CAS No:104-55-2、純度≧95%）
　Cinnamic Aldehyde を、Acetonitrile に 100 mM の濃度で溶解させ、その後被験物質と同様に両ペプチド溶液と混合する。

3-2.　方法

以下のフローチャートに従い、実施する。

1. 被験物質を100mMの濃度で以下のいずれかの媒体に溶解させる（被験物質溶液）。
【媒体】Acetonitrile、Water、 Acetonitrile : Water(1:1)、Isopropanol、Acetone、
Acetone : Acetonitrile(1:1)、DMSO: Acetonitrile(1:9)、
DMSO: Acetonitrile(1:1)

2. 被験物質溶液とﾍﾟﾌﾟﾁﾄﾞ溶液を混合する

2-A. 被験物質溶液とｼｽﾃｲﾝ含有ﾍﾟﾌﾟﾁﾄﾞ溶液を10:1で混合(n=3)
- Sample:被験物質溶液＋ｼｽﾃｲﾝ含有ﾍﾟﾌﾟﾁﾄﾞ溶液
- Co-elusion Control:被験物質溶液＋ﾘﾝ酸緩衝液
- Reference Control: 媒体＋ｼｽﾃｲﾝ含有ﾍﾟﾌﾟﾁﾄﾞ溶液

2-B.被験物質溶液とﾘｼﾞﾝ含有ﾍﾟﾌﾟﾁﾄﾞ溶液を50:1で混合(n=3)
- Sample:被験物質溶液＋ﾘｼﾞﾝ含有ﾍﾟﾌﾟﾁﾄﾞ溶液
- Co-elusion Control:被験物質溶液＋酢酸ｱﾝﾓﾆｳﾑ緩衝液
- Reference Control: 媒体＋ﾘｼﾞﾝ含有ﾍﾟﾌﾟﾁﾄﾞ溶液

3. 各混合液を24±2時間ｲﾝｷｭﾍﾞｰﾄ（暗室、25±2.5℃）する

4. HPLCで分析する*

5. 未反応ﾍﾟﾌﾟﾁﾄﾞのPeak面積から、以下の式により
ﾍﾟﾌﾟﾁﾄﾞ減少率（Percent Peptide Depletion）を算出する

$$\text{Percent Peptide Depletion} = \left\{1 - \left(\frac{\text{Peptide Peak Area in Sample}}{\text{Mean Peptide Peak Area in Reference Controls}}\right)\right\} \times 100$$

*分析条件（推奨）

推奨カラム：Zorbax SB-C18 （3.5 μm, 2.1mm×100mm）等

温度：30℃

UV 検出波長：220 nm

流速：0.35 mL/min

移動相： （A）0.1%トリフルオロ酢酸水

（B）0.085%トリフルオロ酢酸アセトニトリル

時間（分）	A%	B%
0	90	10
10	75	25
11	10	90
13	10	90
13.5	90	10

3-3. 試験成立の条件

1) 試験成立には、以下の条件を満たさなければならない。

・システイン含有ペプチドおよびリジン含有ペプチドのそれぞれを用いて、0.0167~0.534mM の範囲の 6 濃度にて標準曲線の作成を行い、その相関係数が 0.99 より大きくなければならない。

・陽性対照である Cinnamic aldehyde の結果は、システイン含有ペプチドでは 3 回の繰り返しによる平均ペプチド減少率は 60.8%~100%で、標準偏差は 14.9%より小さく、リジン含

有ペプチドでは 3 回繰り返しによる平均ペプチド減少率は 40.2%~69.0%で、標準偏差は 11.6%より小さくなければならない。

・それぞれのペプチドごとに 3 種類の Reference Control（A, B, C）を設ける。Reference Control A（n=3）は、分析前の HPLC システム適合性の確認のためのもの、 Reference Control B（n=6）は分析時間中の Reference Control の安定性を確認するためのもの、 Reference Control C（n=3）は使用された溶媒がペプチドの減少に影響しないことを確認するためのものである。Reference Control A の平均ペプチド濃度は 0.50±0.05mM となり、9 つの Reference Control B および C ではペプチドピーク面積の変動係数が 15.0%より小さくなければならない。

2) 被験物質を含む Sample の結果では、以下の条件が満たされなければならない

・繰り返しで行う分析値の最大標準偏差は、システイン含有ペプチド減少率では 14.9%より小さく、リジン含有ペプチド減少率では 11.6%より小さくなければならない。

・3 つの Reference Control C の平均濃度は 0.50±0.05mM でなければならない。

3-4. 評価

被験物質の反応性は、測定ごとのペプチド減少率（Percent Peptide Depletion）から、平均値を算出し、以下の DPRA 分類予測モデル（表 1）に従って反応性を分類する [5]。反応性の分類で、Low、Moderate および High に分類される化学物質は陽性、No or Minimal に分類される化学物質は陰性と予測する [6]。ちなみに、ペプチドと被験物質の溶出時間が重なった場合（Co-elution）、ペプチド減少率（Percent Peptide Depletion）の算出が不可能となるが、その溶出時間の重なりが、リジン含有ペプチドでのみ認めた場合には、システイン含有ペプチドの結果から「システイン 1:10 のみの予測モデル」に従い、反応性を分類し、予測する。

表 1　分類予測モデル

システイン 1:10 およびリジン 1:50 の予測モデル

システインの減少率とリジンの減少率の平均値	反応性の分類	DPRA 予測
0%≦減少率の平均値≦6.38%	No or Minimal	陰性
6.38%<減少率の平均値≦22.62%	Low	陽性
22.62%<減少率の平均値≦42.47%	Moderate	
42.47%<減少率の平均値≦100%	High	

システイン 1:10 のみの予測モデル

システインの減少率	反応性の分類	DPRA 予測
0%≦減少率≦13.89%	No or Minimal	陰性
13.89%<減少率≦23.09%	Low	陽性
23.09%<減少率≦98.24%	Moderate	
98.24%<平均減少率≦100%	High	

４．精度

本法は、EURL ECVAM において、表 2 に示す 24 物質を用いて先行バリデーション試験が実施され、技術移転性、施設内再現性及び施設間再現性が評価されている。

4-1. 技術移転性

16 物質を用いて主導施設の P&G 社から Ricerca 社と IVMU（EURL ECVAM の研究ユニット）への技術移転性について評価が行われている。その結果、設備が整っていて訓練されたスタッフが居れば 3 日間のトレーニングで P&G 社から Ricerca 社と IVMU への DPRA の技術移転が可能であった。また、DPRA は難しい操作手順を必要とせず、装置や試薬は購入可能であり、標準操作手順書（Standard Operating Procedure: SOP）が詳細に作成されていることから、委員会は、本邦では汎用 HPLC 及びその技術を保有する施設で容易に実施可能と考える。

4-2. 施設内再現性（表 3-1）

15 物質を用いた 3 施設の施設内での皮膚感作性（S）と非感作性（NS）の一致度は、P&G 社：73.3%, Ricerca 社：100%, IVMU：86.7%であった。達成基準は 85%に設定されており、P&G 社はこの値を下回った。P&G 社は、GHS 区分 1A の 6 物質はすべて再現性のある結果であったが、1B の 3 物質中 2 物質（Benzyl Salicylate 及び R(+)Limonene）で再現性が得られなかったため、達成基準を下回ったと考えられた。また、Minimal, Low, Moderate, High の 4 クラスの一致度は P&G 社：66.7%、Ricerca 社：100%、IVMU：73.3%であった。

4-3. 施設間再現性（表 3-2）

24 物質の 3 施設の施設間再現性は皮膚感作性（S）と非感作性（NS）の一致度では 75%となり達成基準の 80%を下回った。24 物質の中には金属塩である Beryllium sulfate 及び Nickel chloride が含まれており、金属塩は対象ペプチドとの共有結合を生じうる官能基を持たないことから、本法の適用範囲外と判断されるため、これら 2 物質を除いた場合の施設間再現性は 82%であった。また、24 物質での Minimal, Low, Moderate, High の 4 クラスでは、施設間再現性は 62.5%であった。

５．正確度（感度及び特異度）

精度と同様に、表 2 に示す 24 物質を用いた EURL ECVAM の先行バリデーション試験の成績を基に既知の LLNA の成績との一致度、感度及び特異度が評価されている。

24 物質全ての成績を基に評価を行った場合、実施 3 施設の成績の積算による感度（Sensitivity）は 70.8%、特異度（Specificity）は 91.7%、正確度（Accuracy）は 77.8%であった。試験施設毎の成績は、試験施設 1（P&G）では感度は 68.8%、特異度は 100%、正確度は 79.2%、試験施設 2（Ricerca）では感度は 68.8%、特異度は 100%、正確度は 79.2%、試験施設 3（IVMU）では感度は 75%、特異度は 75%、正確度は 75%であった。また、本法の正確度に関して開発施設である P&G から提出されたヒストリカルデータでは 86%、既報

では 80%～89%と報告されている。

24 物質の試験物質の中には金属塩である Beryllium sulfate 及び Nickel chloride が含まれており、金属塩は対象ペプチドとの共有結合を生じうる官能基を持たないことから、本法の適用範囲外と判断されるため、これらを除く 22 物質による評価を行った場合、実施 3 施設の成績の積算による感度は 76.2%、特異度は 91.7%、正確度は 81.8%となる。

適用範囲外と考えられる金属塩を除外した場合でも、全てあるいは一部の施設で 5 物質（Dihydroeugenol、Chlorpromazine HCl、Benzylsalicylate、Benzylcinnamate、R（+）-Limonene）が偽陰性と評価されるが、これらの物質の中で Dihydroeugenol 及び Limonene はプロハプテンあるいはプレハプテンとして知られており、本試験系が代謝系を有さない単純な化学反応を検出する試験系であることが偽陰性となる一因と思われる。

なお、全て或いは一部の施設で偽陰性と判定される Chlorpromazine HCl、Benzylsalicylate 及び Benzylcinnamate の 3 物質、一部の施設で偽陽性と判定される Benzyl alcohol 及び Methylsalicylate の 2 物質について、バリデーション報告書では、その理由について明確な説明はなされていない。しかし、これらの中で偽陰性と判定される 3 物質の cLogP の値はいずれも 3.2 以上であり、その疎水性の高さが水系での反応を必要とする本法で偽陰性となる一要因と推察される。一方で 2-Mercaptobenzothiazole（cLogP : 3.42）は高疎水性にもかかわらず正しく陽性と判定されているが、これは本物質の持つ SH 基がリジン残基と高い反応性を有することに起因すると思われる。

表 2　供試物質リスト [6)]

No.	Chemical Name	CAS	State	cLogP*	LLNA	LLNA potency category**	GHS potency category
1	Benzoquinone	106-51-4	Solid	0.96	+	extreme	1A
2	4-Phenylenediamine	106-50-3	Solid	0.43	+	strong	1A
3	Kathon CG (1.2% CMI)	26172-55-4	Liquid	-	+	extreme	1A
4	Beryllium sulfate	7787-56-6	Solid	-	+	extreme	1A
5	Formaldehyde	50-00-0	Liquid	-0.69	+	strong	1A
6	Chloramine T	127-65-1	Solid	-	+	strong	1A
7	Chlorpromazine HCl	69-09-0	Solid	4.89	+	strong	1A
8	2-Mercaptobenzothiazole	149-30-4	Solid	3.42	+	moderate	1A
9	Dihydroeugenol	2785-87-7	Liquid	2.84	+	moderate	1B
10	1-Thioglycerol	96-27-5	Liquid	-0.5	+	moderate	1B
11	Imidazolidinylurea	39236-46-9	Solid	-1.28	+	weak	1B
12	Methylmethacrylate	80-62-6	Liquid	1.14	+	weak	1B
13	Benzylsalicylate	118-58-1	Liquid	3.2	+	moderate	1B
14	Nickel chloride	7718-54-9	Solid	-	-	no category	1B
15	Benzylcinnamate	103-41-3	Solid	3.89	+	weak	1B
16	R(+)-Limonene	5989-27-5	Liquid	3.01	+	weak	1B
17	Glycerol	56-81-5	Liquid	-1.33	-	no category	NC
18	2,4-Dichloronitrobenzene	611-06-3	Solid	3.06	-	no category	NC
19	Benzyl alcohol	100-51-6	Liquid	1.02	-	no category	NC
20	Methylsalicylate	119-36-8	Liquid	1.46	-	no category	NC
21	Isopropanol	67-63-0	Liquid	0.38	-	no category	NC
22	Dimethylisophthalate	1459-93-4	Solid	2.12	-	no category	NC
23	4-Aminobenzoic acid	150-13-0	Solid	0.78	-	no category	NC
24	Xylene	1330-20-7	Liquid	3.01	+	weak	NC

+：LLNA 陽性、-：LLNA 陰性、NC：GHS 区分外。

*：ChemBio Draw Ultra 11.0 (Cambridge Soft) を用いて算出。

**：ECETOC Technical Report #87 の分類による。

Direct Peptide Reactivity Assay (DPRA) , ECVAM Validation Study Report（EUROPEAN COMMISSION, 2012）記載の情報を基に編集。

表 3-1　施設内再現性実験成績 [6)]

Chemical	Reference result (GHS category)	P&G			Ricerca			IVMU		
		Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
Kathon CG (1.2% CMI)	+(1A)	S_{LYS}	S_{LYS}	S_{LYS}	S	S	S	S	S	S
Beryllium sulfate	+(1A)	S	S	S	NS	NS	NS	NS	NS	NS
Formaldehyde	+(1A)	S	S	S	S	S	S	S	S	S
Chloramine T	+(1A)	S_{LYS}	S_{LYS}	S_{LYS}	S_{LYS}	S_{LYS}	S_{LYS}	S	S	S
Chlorpromazine HCl	+(1A)	NS	NS	NS	NS	NS	NS	NS	NS	NS
2-Mercaptobenzothiazole	+(1A)	S_{LYS}	S_{LYS}	S_{LYS}	S	S	S	S	S	S
Benzylsalicylate	+(1B)	NS	NS	NS	S	S	S	NS	NS	NS
Nickel chloride	+(1B)	NS	NS	S	NS	NS	NS	S	NS	S
Benzylcinnamate	+(1B)	NS	NS	NS	NS	NS	NS	S	NS	NS
R(+)-Limonene	+(1B)	S	NS	S	S	S	S	S	S	S
Methylsalicylate	-(NC)	NS	NS	S	NS	NS	NS	S	S	S
Isopropanol	-(NC)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Dimethylisophthalate	-(NC)	NS_{LYS}	NS_{LYS}	NS_{LYS}	NS	NS	NS	NS_{LYS}	NS_{LYS}	NS_{LYS}
4-Aminobenzoic acid	-(NC)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Xylene	-(NC)	NS	NS	S	NS	NS	NS	NS	NS	NS

+：LLNA 陽性、-：LLNA 陰性、NC：GHS 区分外。

S_{LYS} 或いは NS_{LYS}：リジン含有ペプチドにて Co-elution が生じたため、システイン含有ペプチドの結果のみから、判定

S:皮膚感作性ありと判定、NS:皮膚感作性なしと判定

Direct Peptide Reactivity Assay (DPRA) , ECVAM Validation Study Report（EUROPEAN COMMISSION, 2012）記載の情報を基に編集。

表 3-2　施設間再現性実験成績 [6)]

Chemical	Reference result (GHS category)	P&G	Ricerca	IVMU
Benzoquinone	+(1A)	S	S_{LYS}	S
4-Phenylenediamine	+(1A)	S	S_{CL}	S
Kathon CG (1.2% CMI)	+(1A)	**S**	**S**	**S**
Beryllium sulfate	+(1A)	**S**	**NS**	**NS**
Formaldehyde	+(1A)	**S**	**S**	**S**
Chloramine T	+(1A)	**S**	**S**	**S**
Chlorpromazine HCl	+(1A)	**NS***	**NS**	**NS**
2-Mercaptobenzothiazole	+(1A)	**S**	**S**	**S**
Dihydroeugenol	+(1B)	NS	NS_{LYS}	S
1-Thioglycerol	+(1B)	S_{LYS}	S_{CL}	S_{CL}
Imidazolidinylurea	+(1B)	S	S	S
Methylmethacrylate	+(1B)	S_{CL}	S_{CL}	S
Benzylsalicylate	+(1B)	**NS**	**NS**	**S**
Nickel chloride	+(1B)	**NS**	**S**	**NS**
Benzylcinnamate	+(1B)	**NS**	**NS**	**NS**
R(+)-Limonene	+(1B)	**S**	**S**	**S**
Glycerol	-(NC)	NS	NS	NS
2,4-Dichloronitrobenzene	-(NC)	NS	NS	NS
Benzyl alcohol	-(NC)	NS_{LYS}	NS	S
Methylsalicylate	-(NC)	**NS**	**NS**	**S**
Isopropanol	-(NC)	**NS**	**NS**	**NS**
Dimethylisophthalate	-(NC)	**NS**	**NS**	**NS**
4-Aminobenzoic acid	-(NC)	**NS**	**NS**	**NS**
Xylene	-(NC)	**NS**	**NS**	**NS**

+：LLNA 陽性、-：LLNA 陰性、NC：GHS 区分外。

LYS 或いは CL：Co-elution が生じたことを示す。

S:皮膚感作性ありと判定、NS:皮膚感作性なしと判定

*太字の成績は前出の施設内再現性データの多数決判定により決定されたもの。

Direct Peptide Reactivity Assay (DPRA) , ECVAM Validation Study Report（EUROPEAN COMMISSION, 2012）記載の情報を基に編集。

６．評価可能な物質の範囲

Gerberick らの 82 化学物質を用いた評価では、表 4 に示す通り、様々な化学物質の皮膚感作性の予測が可能であることが示されている。ただし、LLNA で Weak sensitizer に分類される 5 物質（α-Hexylcinnamaldehyde, α-Amylcinnamaldehyde, Oxalic acid, Benzyl benzoate, 2,2,6,6-Tetramethyl-3,5-heptanedione） と Modelate sensitizer に分類される 1 物質（Nonanoyl chloride）は、DPRA では Minimal となり、陰性に分類されている。したがって、感作性ポテンシャルの弱い物質は、偽陰性と判定される場合があることに留意する必要がある。

一部のプレハプテン（例、4-Phenylenediamine）は正しく判定されるが、プレハプテン及びプロハプテンのすべてを評価可能な物質とする根拠は十分にはない。また、本試験法は、主にシステイン基やリジン基と反応する化学物質を対象とする。一方、タンパク質と配位結合を形成する金属類は、評価可能な物質の範囲から外れる。

表 4　82 化学物質の評価結果 [7)]

Chemical Name	EC3 value	LLNA categoly	Reactivity based on Cys(1:10) and Lys(1:50) data	Chemical Name	EC3 value	LLNA categoly	Reactivity based on Cys(1:10) and Lys(1:50) data
Diphenylcyclopropenone	0.0003	Extreme	High	Oxalic acid	15	Weak	Minimal
Oxazolone	0.003	Extreme	High	Benzyl benzoate	17	Weak	Minimal
Benzoyl peroxide	0.004	Extreme	High	4-Allylanisole	18	Weak	Low
Kathon CG	0.008	Extreme	High	Lilial	19	Weak	Low
Bandrowski's base	0.008	Extreme	High	Cyclamen aldehyde	22	Weak	Low
5-Chloro-2-methyl-4-isothiazolin-3-one	0.009	Extreme	High	Imidazolidinyl urea	24	Weak	Moderate
p-Benzoquinone	0.0099	Extreme	High	5-Methyl-2,3-hexanedione	26	Weak	Low
Tetrachlorosalicylanlide	0.04	Extreme	Moderate	2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	Minimal
2,4-Dinitrochlorobenzene	0.05	Extreme	High	Ethyleneglycol dimethacrylate	28	Weak	High
Glutaraldehyde	0.1	Strong	High	Ethyl acrylate	28	Weak	High
Fluorescein isothiocynate	0.14	Strong	High	Hydroxycitronellal	33	Weak	Low
Phthalic anhydride	0.16	Strong	Moderate	Glycerol	Not calculated	Non sensitizer	Minimal
Lauryl gallate	0.3	Strong	High	Hexane	Not calculated	Non sensitizer	Minimal
Propyl gallate	0.32	Strong	High	Diethyl phthalate	Not calculated	Non sensitizer	Minimal
CD3	0.6	Strong	High	Octanoic acid	Not calculated	Non sensitizer	Minimal
Trimellitic anhydride	0.6	Strong	Low	2-Hydroxypropyl methacrylate	Not calculated	Non sensitizer	Low
Formaldehyde	0.61	Strong	Moderate	1-Butanol	Not calculated	Non sensitizer	Minimal
Metol	0.8	Strong	High	4-Hydroxybenzoic acid	Not calculated	Non sensitizer	Minimal
2-Hydroxyethyl acrylate	1.4	Moderate	High	6-Methyl coumarin	Not calculated	Non sensitizer	Minimal
Glyoxal	1.4	Moderate	High	Methyl salicylate	Not calculated	Non sensitizer	Minimal
Vinyl pyridine	1.6	Moderate	Moderate	Chlorobenzene	Not calculated	Non sensitizer	Minimal
2-Mercaptobenzothiazole	1.7	Moderate	High	Lactic acid	Not calculated	Non sensitizer	Minimal
Nonanoyl chloride	1.8	Moderate	Minimal	1-Bromobutane	Not calculated	Non sensitizer	Low
2-Methyl-2H-isothiazol-3-one	1.9	Moderate	High	2-Acetylcyclohexanone	Not calculated	Non sensitizer	Low
1,2-Benzisothiazolin-3-one	2.3	Moderate	High	4-Methoxyacetophenone	Not calculated	Non sensitizer	Minimal
Methyl-2-nonynoate	2.5	Moderate	High	Ethylbenzoylacetate	Not calculated	Non sensitizer	Minimal
Cinnamaldehyde	3	Moderate	High	Ethyl vanillin	Not calculated	Non sensitizer	Minimal
Phenylacetaldehyde	3	Moderate	Moderate	Isopropanol	Not calculated	Non sensitizer	Minimal
Benzylideneacetone	3.7	Moderate	High	Propylene glycol	Not calculated	Non sensitizer	Minimal
2,4-Heptadienal	4	Moderate	High	Sulfanilamide	Not calculated	Non sensitizer	Minimal
Squaric acid	4.3	Moderate	Moderate	Isopropyl myristate	Not calculated	Non sensitizer	Minimal
Trans-2-hexanal	5.5	Moderate	High	Benzaldehyde	Not calculated	Non sensitizer	Minimal
Diethyl maleate	5.8	Moderate	High	Methylparaben	Not calculated	Non sensitizer	Minimal
2-Phenylpropionaldehyde	6.3	Moderate	Moderate	Nonanoic acid	21 (False +)	Non sensitizer	Minimal
Perillaldehyde	8.1	Moderate	Moderate	Propyl paraben	Not calculated	Non sensitizer	Minimal
Palmitoyl chloride	8.8	Moderate	Moderate	Rsorcinol	Not calculated	Non sensitizer	Minimal
1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	Low	Salicylic acid	Not calculated	Non sensitizer	-
a-Hexylcinnamaldehyde	11	Weak	Minimal	Sulphanilic acid	Not calculated	Non sensitizer	Minimal
a-Amylcinnamaldehyde	11	Weak	Minimal	Vanillin	Not calculated	Non sensitizer	Minimal
2,3-Butanedione	11	Weak	High	Coumarin	Not calculated	Non sensitizer	Minimal
Farnesal	12	Weak	Low	Vinylidene dichloride	Not calculated	Non sensitizer	Minimal

７．有用性と限界

本法は汎用 HPLC 及びその技術を保有する施設で容易に実施可能である。また、本法は動物を用いない *in chemico* の手法であり、科学的な目的のために実施される動物実験に関し、「動物の愛護及び管理に関する法律」及び 3Rs の精神と合致している。さらに DPRA 及び LLNA について使用する消耗品費を試算したところ、1 アッセイ当たりの消耗品費は LLNA

で約 10 万円であるのに対し、DPRA では約 1 万円と 1/10 程度の経費で実施可能であり、実験期間も LLNA より短期間で実施可能であることから、試験法として簡便性・経済性の面から有用と思われる。

しかしながら、本法は液相中での反応を必要とするため、被験物質は少なくとも適切な溶媒（Acetonitrile、Water、Acetonitrile:Water (1:1)、Isopropanol、Acetone、Acetone:Acetonitrile (1:1)、DMSO:Acetonitrile (1:9) 、DMSO:Acetonitrile (1:1)）に 100mM の濃度で溶解する必要がある。また、本法の感度は試験適用外と考えられる金属塩を除いた場合でも約 75%であり、1/4 の化合物が偽陰性と評価されるため、この試験法単独で皮膚感作性の代替法として考えるのは難しい。特に下記の化合物が偽陰性となる可能性が高いため試験結果の解釈には注意が必要と考えられる。

1）LLNA で moderate または weak の化合物

LLNA で weak sensitizer に分類される 5 物質（α-Hexylcinnamaldehyde, α-Amylcinnamaldehyde, Oxalic acid, Benzyl benzoate, 2,2,6,6-Tetramethyl-3,5-heptanedione） と Modelate sensitizer に分類される 1 物質（Nonanoyl chloride）は、DPRA では Minimal となり、陰性に分類される。

2）プロハプテン、プレハプテン

本試験系は代謝系を有さない単純な化学反応を検出する試験系であることから、感作性の獲得に代謝的或いは非代謝的活性化を必要とするプロハプテン或いはプレハプテン（例：Dihydroeugenol 及び Limonene）は偽陰性と評価される可能性がある。

3）疎水性の高い物質等

正確度（感度及び特異度）で述べたとおり、水系での反応を必要とする本法では疎水性の高い物質（例：Chlorpromazine HCl）は偽陰性と判定される場合がある。さらにシステインやリジン以外のアミノ酸と優先的に反応するような求電子残基を有する物質に関しても正しく判定されない可能性がある。

以上のことから、DPRA により感作性陰性と判断された場合は、その物性等により偽陰性となる可能性を考慮し、補完し得る他の試験法により確認する必要がある。

先行バリデーション試験の成績から DPRA により陽性と判断された場合は、感作性陽性と判断することは可能と考えるが、本法の特異度は約 90%であり、希に偽陽性の結果が生じる可能性があることにも留意する必要がある。なお、本法は結合強度を Minimal, Low, Moderate, High の 4 クラスの分類する方法も提案されているが、施設間再現性は 62.5%と低く、結合強度の分類には適さないと考える。

一方、DPRA は感作性発現機序における初期の重要なイベントであるタンパク質と化学物質の結合反応を検出しており、化学物質の感作性を判断する上で重要な情報を与えることから、証拠の重み付けや他の試験法と組み合わせでの評価を推奨する。

８．結論

DPRA は、簡便性・経済性の面から有用な動物実験代替法である。

本試験法の先行バリデーション試験における施設内再現性は、3 施設中 1 施設において、GHS 区分 1B（弱い感作性物質）の物質で再現性が得られなかったため達成基準に達しておらず、弱い感作性物質では判定がぶれる懸念がある。一方、施設間再現性は、適用範囲外の金属塩を除いた場合に達成基準を上回った。

本試験法適用外の金属塩を除いた場合の先行バリデーション試験における感度は約 75%であるため、陰性の結果が得られた場合は、偽陰性の可能性を考慮し、補完し得る他の試験法により確認しなければならなく、DPRA のみで皮膚感作性を陰性と判定することはできない。

先行バリデーション試験における本法の特異度は約 90%であり、陽性の結果が得られた場合は、感作性陽性と判断することは可能だが、希に偽陽性の結果が生じる可能性があることに留意しなければならない。

なお、本法は結合強度を Minimal, Low, Moderate, High の 4 クラスに分類する方法も提案されているが、施設間再現性は 62.5%と低く、結合強度の分類には適さない。

DPRA は感作性発現機序における初期の重要なイベントであるタンパク質と化学物質の結合反応を検出しており、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。LLNA の 1/10 程度の経費で実施可能であり、動物を用いない *in chemico* 試験法であることから、有用性は高い。しかしながら、本法は代謝系を欠く化学的試験法であり、活性化に代謝系や非生物的活性化を必要とする感作性物質、弱い感作性物質や金属塩、疎水性の高い物質などは正しくその感作性が検出されない可能性がある。以上の事実を踏まえ、委員会は、証拠の重み付けや他の試験法（LLNA、モルモットを用いる皮膚感作性試験）と組み合わせでの評価を推奨する。

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)

INTRODUCTION

1. A skin sensitiser refers to a substance that will lead to an allergic response following skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). This Test Guideline (TG) provides an *in chemico* procedure (Direct Peptide Reactivity Assay – DPRA) to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1).

2. There is general agreement regarding the key biological events underlying skin sensitisation. The existing knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), from the molecular initiating event through the intermediate events to the adverse effect namely allergic contact dermatitis in humans or contact hypersensitivity in rodents. Within the skin sensitisation AOP, the molecular initiating event is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins.

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

4. More recently mechanistically based *in chemico* and *in vitro* test methods have been considered scientifically valid for the evaluation of the skin sensitisation hazard of chemicals. However, combinations of non-animal methods (*in silico*, *in chemico*, *in vitro*) within Integrated Approaches to Testing and Assessment (IATA) will be needed to be able to fully substitute for the animal tests currently in use given the restricted AOP mechanistic coverage of each of the currently available non-animal test methods (2) (7).

5. The DPRA is proposed to address the molecular initiating event of the skin sensitisation AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (8). Cysteine and lysine percent peptide depletion values are then used to categorise a substance in one of four classes of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers (9).

6. The DPRA has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-lead validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) and was considered scientifically valid (10) to be used as part of an IATA to support the discrimination between skin sensitisers and non-sensitisers for the purpose of hazard classification and labelling. Examples on the use of DPRA data in combination with other information are reported in the literature (11) (12) (13) (14).

7. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

8. The correlation of protein reactivity with skin sensitisation potential is well established (15) (16) (17). Nevertheless, since protein binding represents only one key event, albeit the molecular initiating event of the skin sensitisation AOP, protein reactivity information generated with testing and non-testing methods may not be sufficient on its own to conclude on the absence of skin sensitisation potential of chemicals. Therefore, data generated with this Test Guideline should be considered in the context of integrated approaches such as IATA, combining them with other complementary information e.g., derived from *in vitro* assays addressing other key events of the skin sensitisation AOP as well as non-testing methods including read-across from chemical analogues.

9. The test method described in this Test Guideline can be used, in combination with other complementary information, 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 DPRA may be used on its own to classify a chemical into UN GHS category 1.

10. The DPRA test method proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within laboratories and 80% between laboratories (10). Results generated in the validation study (18) and published studies (19) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers is 80% (N=157) with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA results. The DPRA is more likely to under predict chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (18) (19). However, the accuracy values given here for the DPRA 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 the species of interest, i.e. humans. On the basis of the overall data available, the DPRA 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 physico-chemical properties (8) (9) (10) (19). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

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 DPRA to the testing of substances and/or mixtures. This Test Guideline is

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

not applicable for the testing of metal compounds since they are known to react with proteins with mechanisms other than covalent binding. A test chemical should be soluble in an appropriate solvent at a final concentration of 100 mM (see paragraph 18). However, test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result. Limited information is currently available on the applicability of the DPRA to mixtures of known composition (18) (19). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraph 18). Before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the defined molar ratio of test chemical and peptide. For this purpose a new prediction model based on a gravimetric approach will need to be developed. In cases where evidence can be demonstrated on the non-applicability of the Test Guideline to other specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

12. The test method described in this Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) are reported to be in some cases correctly detected by the test method (18). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignement to a higher reactivity class (see paragraphs 29 and 30).

13. As described, the DPRA assay supports the discrimination between skin sensitisers and non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (11) when used in integrated approaches such as IATA. However further work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

14. The DPRA is an *in chemico* method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours incubation with the test chemical at 25±2.5ºC. The synthetic peptides contain phenylalanine to aid in the detection. Relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Cysteine- and lysine peptide percent depletion values are then calculated and used in a prediction model (see paragraph 29) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

15. Prior to routine use of the method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 2.

PROCEDURE

16. This Test Guideline is based on the DPRA DB-ALM protocol n^{o} 154 (20) 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 method in the laboratory. The following is a description of the main components and procedures for the DPRA. If an alternative HPLC set-up is used, its equivalence to the validated set-up described in the DB-ALM protocol should be demonstrated (e.g. by testing the proficiency substances in Annex 2).

Preparation of the cysteine or lysine-containing peptides

17. Stock solutions of cysteine (Ac-RFAA**C**AA-COOH) and lysine (Ac-RFAA**K**AA-COOH) containing synthetic peptides of purity higher than 85% and preferably in the range of 90-95%, should be freshly prepared just before their incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer whereas the final concentration of the lysine peptide should be 0.667 mM in pH 10.2 ammonium acetate buffer. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this Test Guideline, up to 26 analysis samples (which include the test chemical, the positive control and the appropriate number of solvent controls based on the number of individual solvents used in the test, each tested in triplicate), can be accommodated in a single HPLC run. All of the replicates analysed in the same run should use the identical cysteine and lysine peptide stock solutions. It is recommended to prove individual peptide batches for proper solubility prior to their use.

Preparation of the test chemical

18. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay following the solubilisation procedure described in the DPRA DB-ALM protocol (20). An appropriate solvent will dissolve the test chemical completely. Since in the DPRA the test chemical is incubated in large excess with either the cysteine or the lysine peptides, visual inspection of the forming of a clear solution is considered sufficient to ascertain that the test chemical (and all of its components in the case of testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are, acetonitrile, water, 1:1 mixture water:acetonitrile, isopropanol, acetone or 1:1 mixture acetone:acetonitrile. Other solvents can be used as long as they do not impact on the stability of the peptide as monitored with reference controls C (i.e. samples constituted by the peptide alone dissolved in the appropriate solvent; see Annex 3). As a last option if the test chemical is not soluble in any of these solvents attempts should be made to solubilise it in 300 µL of DMSO and dilute the resulting solution with 2700 µL of acetonitrile and if the test chemical is not soluble in this mixture attempts should be made to solubilise the same amount of test chemicals in 1500 µL of DMSO and dilute the resulting solution with 1500 µL of acetonitrile. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing in an appropriate solvent to prepare a 100 mM solution. For mixtures and multi-constituent substances of known composition, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight should be determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution. For polymers for which a predominant molecular weight cannot be determined, the molecular weight of the monomer (or the apparent molecular weight of the various monomers constituting the polymer) may be considered to prepare a 100 mM solution. However, when testing mixtures, multi-constituent substances or polymers of known composition, it should be considered to also test the neat chemical. For liquids, the neat chemical should be tested as such without any prior dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. For solids, the test chemical should be dissolved to its maximum soluble concentration in the same solvent used to prepare the apparent 100 mM solution. It should then be tested as such without any further dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. Concordant results (reactive or non-reactive) between the apparent 100 mM solution and the neat chemical should allow for a firm conclusion on the result.

Preparation of the positive control, reference controls and coelution controls

19. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. Other suitable positive controls preferentially providing mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition reference controls (i.e. samples containing only the peptide dissolved in the appropriate solvent) should also be included in the HPLC run sequence and these are used to verify the HPLC system suitability prior to the analysis (reference controls A), the stability of the reference controls over time (reference control B) and to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion (reference control C) (see Annex 3). The appropriate reference control for each substance is used to calculate the percent peptide depletion for that substance (see paragraph 26). In addition a co-elution control constituted by the test chemical alone for each of the test chemicals analysed should be included in the run sequence to detect possible co-elution of the test chemical with either the lysine or the cysteine peptide.

Incubation of the test chemical with the cysteine and lysine peptide solutions

20. Cysteine and lysine peptide solutions should be incubated in glass autosampler vials with the test chemical at 1:10 and 1:50 ratio respectively. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, in this case one cannot be sure how much test chemical remained in the solution to react with the peptide. Therefore, in such a case, a positive result could still be used, but a negative result is uncertain and should be interpreted with due care (see also provisions in paragraph 11 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 25±2.5ºC for 24±2 hours before running the HPLC analysis. Each test chemical should be analysed in triplicate for both peptides. Samples have to be visually inspected prior to HPLC analysis. If a precipitate or phase separation is observed, samples may be centrifuged at low speed (100-400xg) to force precipitate to the bottom of the vial as a precaution since large amounts of precipitate may clog the HPLC tubing or columns. If a precipitation or phase separation is observed after the incubation period, peptide depletion may be underestimated and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result.

Preparation of the HPLC standard calibration curve

21. A standard calibration curve should be generated for both the cysteine and the lysine peptides. Peptide standards should be prepared in a solution of 20% or 25% acetonitrile:buffer using phosphate buffer (pH 7.5) for the cysteine peptide and ammonium acetate buffer (pH 10.2) for the lysine peptide. Using serial dilution standards of the peptide stock solution (0.667 mM), 6 calibration solutions should be prepared to cover the range from 0.534 to 0.0167 mM. A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an r^2>0.99.

HPLC preparation and analysis

22. The suitability of the HPLC system should be verified before conducting the analysis. Peptide depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 220 nm signal). The appropriate column is installed in the HPLC system. The HPLC set-up described in the validated protocol uses a Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated at 30°C with 50% phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for at least 2 hours before running. The HPLC analysis should be performed using a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile

over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Equal volumes of each standard, sample and control should be injected. The column should be re-equilibrated under initial conditions for 7 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the cysteine and lysine peptides, including the injection volume, which may vary according to the system used (typically in the range from 3-10 µL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated (e.g., by testing the proficiency substances in Annex 2). Absorbance is monitored at 220 nm. If a photodiode array detector is used, absorbance at 258 nm should also be recorded. It should be noted that some supplies of acetonitrile could have a negative impact on peptide stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 220 peak area and the 258 peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90%<mean[2] area ratio of control samples<100% would give a good indication that co-elution has not occurred.

23. There may be test chemicals which could promote the oxidation of the cysteine peptide. The peak of the dimerised cysteine peptide may be visually monitored. If dimerisation appears to have occurred, this should be noted as percent peptide depletion may be over-estimated leading to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 29 and 30).

24. HPLC analysis for the cysteine and lysine peptides can be performed concurrently (if two HPLC systems are available) or on separate days. If analysis is conducted on separate days then all test chemical solutions should be freshly prepared for both assays on each day. The analysis should be timed to assure that the injection of the first sample starts 22 to 26 hours after the test chemical was mixed with the peptide solution. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this Test Guideline, up to 26 analysis samples can be accommodated in a single HPLC run (see also paragraph 17). An example of HPLC analysis sequence is provided in Annex 3.

DATA AND REPORTING

Data evaluation

25. The concentration of cysteine or lysine peptide is photometrically determined at 220 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

26. The percent peptide depletion is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C (see Annex 3) according to the formula described below.

$$\text{Percent peptide depletion} = \left[1 - \left(\frac{\text{Peptide peak area in replicate injection}}{\text{Mean peptide peak area in reference controls } C}\right)\right] \times 100$$

[2] For mean it is meant arithmetic mean throughout the document.

Acceptance criteria

27. The following criteria should be met for a run to be considered valid: a) the standard calibration curve should have an r^2>0.99, b) the mean percent peptide depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69.0% for the lysine peptide and the maximum standard deviation (SD) for the positive control replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion and c) the mean peptide concentration of reference controls A should be 0.50±0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B and C in acetonitrile should be <15.0%. If one or more of these criteria is not met the run should be repeated.

28. The following criteria should be met for a test chemical's results to be considered valid: a) the maximum standard deviation for the test chemical replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion, b) the mean peptide concentration of the three reference controls C in the appropriate solvent should be 0.50±0.05 mM. If these criteria are not met the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

29. The mean percent cysteine and percent lysine depletion value is calculated for each test chemical. Negative depletion is considered as "0" when calculating the mean. By using the cysteine 1:10/lysine 1:50 prediction model shown in Table1, the threshold of 6.38% average peptide depletion should be used to support the discrimination between skin sensitisers and non-sensitisers in the framework of an IATA. Application of the prediction model for assigning a test chemical to a reactivity class (i.e. low, moderate and high reactivity) may perhaps prove useful to inform potency assessment within the framework of an IATA.

Table1: Cysteine 1:10/lysine 1:50 prediction model[1]

Mean of cysteine and lysine % depletion	Reactivity Class	DPRA Prediction[2]
0% ≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 12.

30. There might be cases where the test chemical (the substance or one or several of the components of a multi-constituent substance or a mixture) absorbs significantly at 220 nm and has the same retention time of the peptide (co-elution). Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and the peptide. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated (e.g., by testing the proficiency substances in Annex 2). When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible. If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides then the analysis should be reported as

"inconclusive". In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.

Table2: Cysteine 1:10 prediction model[1]

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction[2]
0% ≤ Cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < Cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < Cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < Cys % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 12.

31. There might be other cases where the overlap in retention time between the test chemical and either of the peptides is incomplete. In such cases percent peptide depletion values can be estimated and used in the cysteine 1:10/lysine 1:50 prediction model, however assignment of the test chemical to a reactivity class cannot be made with accuracy.

32. A single HPLC analysis for both the cysteine and the lysine peptide should be sufficient for a test chemical when the result is unequivocal. However, in cases of results close to the threshold used to discriminate between positive and negative results (i.e. borderline results), additional testing may be necessary. If situations where the mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or the cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

Test report

33. The test report should include the following information

Test chemical

- Mono-constituent substance
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
    - Treatment prior to testing, if applicable (e.g., warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available.
- Multi-constituent substance, UVCB and mixture:
    - Characterisation as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;

- Physical appearance, water solubility and additional relevant physicochemical properties, to the extent available;
- Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
- Treatment prior to testing, if applicable (e.g., warming, grinding);
- Concentration(s) tested;
- Storage conditions and stability to the extent available.

Controls

- Positive control
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, water solubility, 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;
  - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Solvent/vehicle
  - Solvent/vehicle used and ratio of its constituents, if applicable;
  - Chemical identification(s), such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other solvents / vehicles than those mentioned in the Test Guideline are used and to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent for each test chemical;
  - For acetonitrile, results of test of impact on peptide stability.

Preparation of peptides, positive control and test chemical

- Characterisation of peptide solutions (supplier, lot, exact weight of peptide, volume added for the stock solution);
- Characterisation of positive control solution (exact weight of positive control substance, volume added for the test solution);

- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test solution).

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler;
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient.

System suitability

- Peptide peak area at 220 nm of each standard and reference control A replicate;
- Linear calibration curve graphically represented and the r^2 reported;
- Peptide concentration of each reference control A replicate;
- Mean peptide concentration (mM) of the three reference controls A, SD and CV;
- Peptide concentration of reference controls A and C.

Analysis sequence

- For reference controls:
    - Peptide peak area at 220 nm of each B and C replicate;
    - Mean peptide peak area at 220 nm of the nine reference controls B and C in acetonitrile, SD an CV (for stability of reference controls over analysis time);
    - For each solvent used, the mean peptide peak area at 220 nm of the three appropriate reference controls C (for the calculation of percent peptide depletion);
    - For each solvent used, the peptide concentration (mM) of the three appropriate reference controls C;
    - For each solvent used, the mean peptide concentration (mM) of the three appropriate reference controls C, SD and CV.
- For positive control:
    - Peptide peak area at 220 nm of each replicate;
    - Percent peptide depletion of each replicate;
    - Mean percent peptide depletion of the three replicates, SD and CV.
- For each test chemical:
    - Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
    - Presence of co-elution;
    - Description of any other relevant observations, if applicable;
    - Peptide peak area at 220 nm of each replicate;
    - Percent peptide depletion of each replicate;

- Mean of percent peptide depletion of the three replicate, SD and CV;
- Mean of percent cysteine and percent lysine depletion values;
- Prediction model used and DPRA prediction.

Proficiency testing

- If applicable, the procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

Discussion of the results

- Discussion of the results obtained with the DPRA test method;
- Discussion of the test method results in the context of an IATA if other relevant information is available.

Conclusion

LITERATURE

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ANNEX 1

DEFINITIONS

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

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).

Calibration curve: The relationship between the experimental response value and the analytical concentration (also called *standard curve*) of a known substance.

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

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.

Molecular Initiating Event: Chemical-induced perturbation of a biological system at the molecular level identified to be the starting event in the adverse outcome pathway.

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

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

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

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.

Reference control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

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

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

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (21).

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

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

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

System suitability: Determination of instrument performance (e.g., sensitivity) by analysis of a reference standard prior to running the analytical batch (22).

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 (21).

ANNEX 2

PROFICIENCY SUBSTANCES

***In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay**

Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected DPRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining cysteine and lysine depletion values that fall within the respective reference range for 8 out of the 10 proficiency substances for each peptide. These proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the DPRA are available, and that they were used in the EURL ECVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the Direct Peptide Reactivity Assay

Proficiency substances	CASRN	Physical state	*In vivo* prediction[1]	DPRA prediction[2]	Range[3] of % cysteine peptide depletion	Range[3] of % lysine peptide depletion
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	90-100	15-45
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	Positive	60-80	10-55
Formaldehyde	50-00-0	Liquid	Sensitiser (strong)	Positive	30-60	0-24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	0-7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	0-25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	0-7	0-5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	0-7	0-5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	0-7	0-5.5
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	0-7	0-5.5

[1]The *in vivo* hazard and (potency) predictions are based on LLNA data (19). The *in vivo* potency is derived using the criteria proposed by ECETOC (23).

[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 11.

[3] Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Cinnamic aldehyde, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Cinnamic aldehyde, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Cinnamic aldehyde, rep 3 Sample 1, rep 3 Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (i.e. samples constituted only by the peptide dissolved in the appropriate solvent) should be included in the analysis sequence:
Reference control A: used to verify the suitability of the HPLC system.
Reference control B: included at the beginning and at the end of the analysis sequence to verify stability of reference controls over the analysis time.
Reference control C: included in the analysis sequence to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion.

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EURL ECVAM Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing

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

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

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

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EURL ECVAM RECOMMENDATION

on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing

November 2013

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

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

Coordinator/Project leader of the validation study was Silvia Casati. Coordinator of the ESAC Peer Review and EURL ECVAM Recommendation was Claudius Griesinger.

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

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

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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 Recommendation, 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.

EXECUTIVE SUMMARY

EURL ECVAM fully endorses the ESAC opinion (Annex I) on the ECVAM-coordinated validation study of the Direct Peptide Reactivity Assay (DPRA) that assessed mainly protocol transferability and within- and between-laboratory reproducibility. The study was conducted in view of the DPRA's possible use as a component of an integrated approach for testing the potential of chemicals to cause skin sensitisation resulting in Allergic Contact Dermatitis (ACD). On the basis of the ESAC Opinion on the DPRA study, EURL ECVAM makes the following Recommendations:

(1) Haptenation, i.e. the covalent binding of low-molecular weight substances ("haptens") to proteins present in skin is considered a prominent mechanism through which chemicals or their metabolites become antigenic. Haptenation has been described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitisation which summarises the key events known to be involved in chemically-induced ACD (OECD, 2012). Therefore, information from peptide reactivity assays such as the DPRA is relevant for the assessment of the skin sensitisation potential of chemicals.

(2) The EURL ECVAM study showed that the DPRA is transferable to suitably equipped laboratories that are proficient in high performance liquid chromatography (HPLC) analysis and the results obtained demonstrated within- and between-laboratory reproducibility of 87% and 75%, respectively.

(3) Full evaluation of the predictive capacity and applicability domain of the DPRA were outside the scope of the EURL ECVAM study. However, based on the study results and excluding metal compounds for which the test is not applicable, the accuracy of the DPRA for distinguishing sensitisers from non-sensitisers was 82% (sensitivity of 76%, specificity of 92%) which is in agreement with published information from previous studies (Gerberick *et al*., 2007; Bauch et al., 2012; Natsch et al., 2013).

(4) In addition to supporting identification of sensitisers/non-sensitisers, the DPRA may also be able to contribute to the assessment of sensitising potency, e.g. by supporting, within an integrated approach, the subcategorisation of sensitisers according to the United Nations Globally Harmonised System of Classification and Labelling of Chemicals (UN GHS; UN, 2011). More work however is required to determine to which extent DPRA results relate to potency categories.

(5) As the DPRA is an *in chemico* test method lacking metabolic capacity, substances that require metabolic (pro-haptens) or abiotic activation (pre-haptens) may not be detected by the DPRA. In addition, the DPRA specifically detects peptide reactivity associated with lysine and cysteine amino acids. These factors should be taken into account when considering negative results.

(6) In view of the mechanistic complexity of skin sensitisation, DPRA data should always be considered in combination with other information in the context of integrated approaches such as Weight of Evidence (WoE) or Integrated Testing Strategies (ITS). Complementary information may be derived from test methods addressing other key events involved in skin sensitisation (OECD 2012) as well as non-testing methods including read-across information. Thus, EURL ECVAM recommends the development of integrated approaches for identifying and characterising skin sensitisation hazard (EURL ECVAM 2013) and potency. These approaches should be tailored to specific needs (e.g. depending on various sectorial and regulatory requirements).

(7) To support development of integrated approaches employing peptide reactivity assays (such as DPRA) and other sources of information, the applicability of the DPRA should be further characterised, e.g. through a retrospective analysis of existing data and, in case of identified data gaps, by generating additional information through targeted prospective testing. In particular, as pre-haptens are not consistently misclassified by the DPRA, the assay's applicability to these substances should be further investigated. Additionally, attention should be given to substances with electrophilic residues that react preferentially with amino acids other than cysteine or lysine.

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

1. Introduction

1) The assessment of skin sensitisation potential is an important component in the safety evaluation of substances and represents a standard requirement of legislation on chemicals in the EU. These include: the Classification Labelling and Packaging of substances and mixtures (CLP) Regulation (EU, 2008a), the REACH Regulation (EU, 2006), the Plant Protection Products (PPP) Regulation (EU, 2009a), the Biocides Regulation (EU, 2012) and the Cosmetics Directive (EU, 2009b). As outlined in the EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification (EURL ECVAM, 2013), determining the skin sensitisation hazard properties of substances is a key requirement satisfying already the majority of regulatory needs, e.g. under the CLP and REACH Regulations in the EU. Other regulatory contexts can require an understanding of the relative potency of skin sensitisers with regard to both induction as well as elicitation of contact dermatitis in order to support a full risk assessment and appropriate risk management measures (e.g. setting of appropriate thresholds).

2) Currently there are only *in vivo* regulatory accepted test methods to generate data satisfying regulatory requirements on skin sensitisation. For instance, in the frameworks of the Organisation for Economic Cooperation and Development (OECD) and the EU Test Methods Regulation (EU, 2008b), there are four accepted guidelines, describing: the Buehler Test and Guinea-pig Maximisation Test (GPMT), TG406 (OECD, 1992; EU test method B.6), the Local Lymph Node Assay (LLNA), TG429 (OECD, 2010a; EU test method B.42) and its non-radio-isotopic variants, the Local Lymph Node Assay: DA and the Local Lymph Node Assay: BrdU Elisa , TG 422A and TG 422B respectively (OECD, 2010b; OECD 2010c).

3) The key mechanistic events underpinning the skin sensitisation process that leads to Allergic Contact Dermatitis (ACD) in humans are well understood and have been recently summarised in the OECD report on "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins"(OECD 2012). These include 1) the covalent binding of the chemical to the skin protein (haptenation), 2) events in keratinocytes including the production of danger signals and release of pro-inflammatory mediators 3) the maturation and mobilisation of dendritic cells (DC), the immuno-competent cells in the skin, and 5) the antigen presentation to naïve T-cells and the 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 events. It is plausible that the initial event of haptenation is the major determinant of the skin sensitisation process and thus the protein-binding properties of a chemical should be intrinsically linked to its sensitisation potential and potency (Roberts & Aptula, 2008).

4) There is general agreement within the scientific community that, in the near future, it is unlikely that one single alternative method will be able to provide sufficient information to replace the use of animals for this endpoint (Adler et al., 2011). Instead it is held that information from different alternative testing and non-testing methods used in combination will need to be integrated to address this health endpoint (Jowsey *et al.*, 2006; Adler et al., 2011). These methods should address different key events leading to skin sensitisation thus covering the mechanistic complexity of this endpoint. Nevertheless, it should not be ruled out *a priori* that skin sensitisation testing may, in the future, be addressed by one single test method.

5) EURL ECVAM coordinated a validation study of DPRA following a modular approach (Hartung et al., 2004) which had the following objectives:

- To fully assess the reliability of the DPRA protocol, i.e. its transferability and within- and between-laboratory reproducibility.
- To conduct a preliminary evaluation of the ability of the DPRA to discriminate skin sensitising from non-sensitising chemicals as defined by the Globally Harmonised System (GHS) for the classification and labelling of substances for skin sensitisation and as implemented in the European Union CLP Regulation concerning both substances and mixtures. Characterisation of preliminary predictive capacity was performed in view of determining the potential contribution of the method to contribute to decisions on hazard within integrated approaches.
- To consider the ability of the DPRA to contribute to sub-categorisation of skin sensitising chemicals, e.g. into Sub-category 1A and Sub-category 1B as adopted in the 3rd revised version of the GHS.

6) After completion of the study and finalisation of the Validation Study Report (EC-ECVAM-2012), EURL ECVAM requested the ECVAM Scientific Advisory Committee (ESAC) to provide an ESAC Opinion on the study. An ESAC Working group (WG) was subsequently established which drafted an ESAC WG report, which then formed the basis of the ESAC Opinion (see annex) adopted by the ESAC on 17. 12. 2012.

2. Test Method definition

7) The correlation of protein reactivity with skin sensitisation potential is well recognised (Landsteiner and Jacobs, 1936; Dupuis & Benezra, 1982; Lepottevin et al., 1998). Chemical covalent binding to nucleophilic centres in skin proteins is regarded to be the molecular initiating event in the skin sensitisation AOP (OECD, 2012) without which skin sensitisation would not occur. Thus, chemicals capable of reacting with proteins either directly or after biotic or abiotic transformation may have the potential to act as a contact allergen. It should be noted that, in its current design, the DPRA does not provide a measure of reaction rate constant.

8) The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. Depletion of the peptide in the reaction mixture is measured by HPLC using UV detection. Average peptide depletion data for cysteine and lysine are then interpreted by using a classification model developed on the basis of a dataset of chemicals with known reactivity properties, in which chemicals are classified as having minimal, low, moderate or high reactivity. Substances with low to high reactivity are associated with substances that have skin sensitisation potential while those categorised as having minimal reactivity are considered to lack skin sensitisation potential. Due to the absence of a metabolic competent system in the assay, the DPRA is not suitable for the evaluation of pro-haptens, which require metabolic activation to act as sensitisers. The ability of the DPRA to detect pre-haptens, which require abiotic activation (e.g. through

oxidation by air), is not clear, although some pre-haptens are reported to be correctly identified by the DPRA.

9) As a result of the ECVAM-coordinated study (EC EURL ECVAM, 2012), the standardised protocol was found to be transferable and reproducible within and between laboratories experienced in HPLC analysis. Some minor aspects of the protocol have been refined as a result of the experienced gained in the validation study.

10) EURL ECVAM will publish in its DataBase service for ALternative Methods (DB-ALM, see http://ecvam-dbalm.jrc.ec.europa.eu), a comprehensive protocol including a detailed description of the test method and all necessary technical details needed by an end-user laboratory to implement it in a self-sufficient manner.

3. Overall performance of the Direct Peptide Reactivity Assay

Reference data

A key criterion employed for selecting the validation test chemicals was availability of high quality in vivo testing data from the murine LLNA and the GPMT with concordant classification from these two assays. The set of chemicals used in the study consisted of one third of non-sensitisers and two thirds of sensitisers with a balanced representation of potency classes (weak, moderate strong and extreme). Reference chemicals from the LLNA performance standards (OECD 2010a) were included in the chemical set. Additional details can be found in the Validation Study Report (EC EURL ECVAM, 2012).

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

Transferability and Reproducibility

11) On the basis of the results obtained during the study, it is evident that the DPRA can be readily transferred to new laboratories that are properly equipped and experienced with HPLC instruments and techniques.

12) The assessment of the reproducibility was performed on the basis of concordance in classification (sensitiser/non-sensitiser). The experimental data generated in the study indicate that the within-laboratory reproducibility (ranging from 73% in the lead laboratory to 100% in one of the two naïve laboratory) and the between-laboratory reproducibility (75%) are acceptable for the proposed future use of the DPRA (i.e. in combination with other complementary methods).

Preliminary evaluation of predictive capacity based on the ring trial data

13) Full evaluation of the predictive capacity of the DPRA was not within the scope of the EURL ECVAM study. However, the accuracy of the DPRA for dichotomous classification (sensitiser/non-sensitiser) on the basis of all 24 chemicals tested (including two metals, one pro-hapten, dihydroeugenol, and two pre-haptens, 4-phenylendiamine and R(+)-Limonene), was 79% (sensitivity=71%, specificity=92%). When excluding the two metal compounds (which are considered outside the applicability domain and can be readily excluded from testing during practical application of the assay), the accuracy was 82% (76% sensitivity and 92% specificity). Thus, the predictive capacity determined in the study is consistent with published information from a larger set of data (Gerberick *et al*., 2007). Importantly, substances reported as false negatives in the EURL ECVAM study were generally substances with a low sensitisation potency *in vivo.*

14) In relation to the ability of the DPRA to categorise substances in reactivity classes, data from the validation study does not support the use of the DPRA as a standalone method for potency categorisation. This is consistent with published information. However the study results indicate that the assignment of a chemical to a DPRA reactivity category may have the potential to contribute to the determination of its potency.

4. Limitations

4.1 Technical limitations

15) **Solubility of test substances:** Peptide depletion values for substances with limited solubility in the solvents prescribed by the DPRA SOP cannot be derived with sufficient accuracy. Despite the fact that all the chemicals selected for the EURL ECVAM study were found to be compatible with the test system, limitations with the testing of insoluble chemicals have been reported in the submission to ECVAM.

16) **Co-elution:** In those instances, mainly attributable to specific instrument settings, where the test substance or the reaction products elute at the same time as the peptide (co-elution), an accurate measurement of peptide depletion cannot be made. The DPRA SOP provides instructions on how to approach different instances of co-elution, allowing in certain cases an estimation of the peptide depletion and reactivity class assignment. However there might be circumstances were this type of approximation is not appropriate.

17) **Cysteine dimerisation:** Accurate determination of peptide depletion can also be hampered by substances promoting the oxidation of the thiol group in the cysteine peptide that leads to the formation of the cystine dimer. In such cases depletion of the peptide would be overestimated.

4.2 Limitations with regard to applicability

The following limitations of DPRA should be taken into consideration:

18) **Restriction to lysine and cysteine:** The DPRA is designed to measure reactivity of the electrophile towards two amino acids: the thiol group of *cysteine* and the primary amino groups of *lysine*. As other amino acids are not present in the assay, chemicals with preferential reactivity towards amino acids other than cysteine or lysine (e.g. nucleophilic sites in histidine), may lead to false negative results when tested in the DPRA. However, when considering this limitation, it should be also kept in mind that the relative percentages of substances reacting preferably with amino acids other than cysteine and lysine is at present unclear and that the cysteine and lysine peptides represent softer to harder model nucleophiles (OECD, 2012; Schwöbel et al., 2011) which would cover different reaction mechanisms

19) **Metal compounds:** DPRA is not designed to accommodate the spectrum of reaction mechanisms considered to be associated with sensitising metals. For example Nickel, the most important metal allergen, is postulated to form coordination bonds with nucleophilic residues in histidine. However, metal compounds can be readily excluded from testing based on chemical structure and, therefore, this limitation can be easily addressed by simply avoiding the DPRA for the testing of metal compounds.

20) **Pro-haptens and pre-haptens**: The DPRA is not designed to detect the sensitising properties of pro-haptens which require bioactivation, or pre-haptens which require abiotic transformation. Nevertheless, pre-haptens are in some cases reported to be correctly detected as sensitisers by the DPRA, as was the case in the EURL ECVAM study (n=2). However, the reasons why specific pre-haptens are detected while others are not remain unclear. To address the issue of pre- and pro-haptens, *in silico* expert systems such as TIMES-SS (Patlewicz et al., 2007; Roberts et al., 2007) and the OECD QSAR toolbox (www.qsartoolbox.org) could prove useful. Notably, a variation of the DPRA including an additional protocol step which mimics oxidative activation (co-incubation with horseradish peroxidase and hydrogen peroxide) to detect pre- and pro- haptens is under development. (Gerberick et al., 2009).

21) **Oxidation:** Some substances that have oxidative properties (e.g. oxidative colourants) without necessarily causing haptenation may lead to possible false positive results when tested in the DPRA.

5. Suggested regulatory use

22) 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. The DPRA is a reliable test method that provides information on peptide reactivity, which is considered to be the molecular initiating event of skin sensitisation (OECD 2012). Therefore, peptide depletion values generated with the DPRA could be used to support read-across from chemical analogues or combined with information from other non-animal methods in the context of a Weight of Evidence (WoE) approach or Integrated Testing Strategy (ITS). The extent of information needed to complement a DPRA result will depend on the intended application (e.g. hazard

identification, classification or potency assessment) and context (availability and quality of other information).

23) For the purpose of hazard identification (i.e. identifying substances with sensitising potential), it is plausible that an unequivocal depletion value in the DPRA combined with the presence of a structural alert or positive QSAR prediction for skin sensitisation may prove sufficient for decision making, thus justifying the waiving of an animal test. To conclude on the absence of sensitising potential, additional information would be needed to increase confidence, such as *in vitro* data on downstream events. In any case a negative DPRA result should be interpreted with care, taking into consideration the possibility of false negatives due to (1) possible reactivity with amino acid residues other than cysteine and lysine, (2) the lack of metabolic capacity of the assay leading to possible misclassification of pro-haptens as well as (3) the uncertain capacity of the DPRA to correctly pick up pre-haptens. For hazard assessment purposes, possible uses of DPRA data in the context of a WoE or ITS have been reported in several scientific publications (Ball et al., 2011; Bauch et al., 2012).

24) Use of results generated with the DPRA for potency prediction has also been proposed (Jaworska *et al*., 2011; Nukada *et al*., 2012). Results from the EURL ECVAM study showed that the limited number of substances with high DPRA reactivity fell into Category 1A of the UN GHS (UN, 2011), suggesting the potential application of DPRA results for potency sub-categorisation. However, further efforts are required to explore how DPRA data may support potency assessment, possibly in combination with data from other methods.

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

6. Follow-up activities recommended by EURL ECVAM

(1) In view of further testing with the DPRA, EURL ECVAM recommends that the revised protocol available at EURL ECVAM's DB-ALM service (http://ecvam-dbalm.jrc.ec.europa.eu) be used.

(2) The predictive capacity of the DPRA for assessing the sensitisation potential of substances should be further evaluated in the context of its use as part of integrated approaches to testing and assessment.

(3) DPRA data should be analysed to understand the potential of the method to contribute to the potency assessment of substances, including sub-categorisation according to GHS (i.e. categories 1A and 1B). Use of existing human data and data from the LLNA are likely to be useful for this purpose. A study correlating DPRA data with potency categories derived from LLNA has recently been published (Natsch et al., 2013).

(4) To support ITS development and to increase confidence in the DPRA method, additional prospective testing with the DPRA should be tailored towards better understanding of its applicability domain to better define how the method performs with (a) weak sensitisers, (b) pre-haptens and (c) chemicals that have selective reactivity towards amino acids other than cysteine and lysine. The reason for false positive predictions also deserves further investigation.

(5) EURL ECVAM supports the development of an OECD Test Guideline for the DPRA. A project proposal has already been submitted to the OECD and included in the OECD 2012 work program. As this test is best employed in combination with complementary methods, it should be considered in the current initiative being undertaken at OECD to develop a guidance document on Integrated Approaches for Testing and Assessment (IATA) for skin sensitisation.

(6) EURL ECVAM recommends continued investment in the development of the next generation of peptide reactivity assays which can potentially address some of the limitations of the DPRA method.

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Annex 1 ESAC Opinion

Opinion of the EURL ECVAM Scientific Advisory Committee (EASC) on the ECVAM-led study of the Direct Peptide Reactivity Assay (DPRA)

Ispra, 17 December 2012

Summary of the ESAC Opinion

The ESAC was asked to provide an opinion on an EURL-ECVAM led prevalidation study assessing the transferability and reproducibility (within- and between-laboratories) of the DPRA 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.

The ESAC considered the scientific work presented of good quality. Overall, the conclusions made by the working group (WG) correspond well with the conclusions formulated in the report by the Validation Management Group (VMG).

- The within laboratory reproducibility (WLR) of the test method with respect to concordance of classification (S/NS) was considered acceptable.
- The data were considered strong enough to support transferability of the test to properly equipped, trained and staffed laboratories with the appropriate analytical capabilities.
- The between laboratory reproducibility (BLR) of the test method with respect to concordance of classification was considered sufficient when compounds outside the applicability domain were excluded.
- The preliminary predictive capacity as evaluated in this study is consistent with the published data (references 6, 7).
- The potential for use of DPRA reactivity information in potency sub-categorisation requires further examination with a larger dataset.

The ESAC had some questions about the statistical calculations underlying the determination of an adequate sample size to analyse reproducibility as a primary study goal. The ESAC was concerned that possible limitations of the assay were not described in sufficient detail in the validation study report.

The predictive capacity, applicability domain and limitations of the test are not defined yet, but the available data suggest that the test is a useful tool for early decision making during product development (screening) and a component in a weight-of-evidence (WoE) approach or integrated testing strategy (ITS).

The ESAC recommends that the possible limitations of the DPRA should be further investigated specifically in relation to pre-/pro-haptens, either by additional prospective testing or through analysis of existing information as there may be a risk of false negative results associated with these chemicals.

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 DPRA and the potential regulatory use of the test method.

(1) Study design – transferability, reliability and relevance

- The ESAC was requested to review whether the prevalidation study was conducted appropriately in view of the objective of the study:
  - Reproducibility of the DPRA method within one laboratory (WLR);
  - Transferability to other laboratories;
  - Reproducibility in other 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.

(3) Possible contribution of test method to integrated approach

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 sensitisers, (b) to make additional recommendations (as required) on the proper scientific use of the test method within such a strategy taking specific aspects of this method into account (e.g. applicability, limitations etc.) and (c) to identify possible further information required (i.e. are there gaps) to be able to conclude on the plausibility of the suggested use (including within an ITS).

2. Detailed opinion of the ESAC

The ESAC was asked to provide an opinion on an EURL-ECVAM led study assessing the transferability and reproducibility (within- and between-laboratories) of the DPRA (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.

(1) Study design – transferability, reliability and relevance.

The WLR was assessed at the level of concordance in prediction (S/NS). WLR for the three laboratories was in the range from 73% to 100% . The lowest value derived from the lead laboratory. The ESAC concluded that, in the context of the study, and in view of the fact that both naive laboratories exceeded the target of 85% as chosen by the VMG, the WLR was sufficient.

- The definition of the reproducibility target (85%) was based upon i) the background and specific objectives of the validation study; ii) the standards of performance that can realistically be expected from an *in vitro* test and standards of performance which have been considered acceptable in previous validation studies; iii) the proposed used of the *in vitro* tests (i.e. as a partial replacement method to become part of a toolbox of tests to be used in combination); and iv) the power of the design of the validation study.
- Transferability activities were divided into Training, Transferability and Qualification Runs The WLR was formulated for each partner to include 1) concordance in prediction, 2) depletion values for cysteine and lysine, as well as 3) control values. The data were considered strong enough to support transferability of the test to properly equipped, trained and staffed laboratories with the appropriate analytical capabilities.
- During the transfer and blind testing phase one laboratory had difficulties in meeting the acceptance criteria defined in the SOP, due to the Reference Control C being marginally outside the acceptance criteria. The cause of the problem could not be identified. The ESAC recommends that these acceptance criteria should be re-examined.
- The BLR was assessed in terms of 1) concordance in prediction and 2) depletion values for cysteine and lysine. Eighteen of the 24 chemicals were consistently classified (S/NS) by the three laboratories resulting in a BLR reproducibility of 75%, which is below the target (80%). The reproducibility assessment included 3 chemicals (beryllium sulphate, nickel chloride and dihydroeugenol) that were considered by the VMG as outside the applicability domain of the test. The exclusion of these three substances considered to fall outside the applicability domain would lead to a BLR of 87.5%. For 15 out of the 24 chemicals the laboratories assigned the same reactivity class resulting in a BLR of 62.5%. Data variability was observed for results from chemicals with low or no reactivity.
- The secondary goals included a <u>preliminary</u> evaluation of the ability of the test to discriminate skin sensitizers from non-sensitizers, and a preliminary consideration of the ability to contribute to sub-categorization of skin sensitising chemicals (GHS sub-category 1A and 1B). The validation study report (VSR) did not present a summary of the predictive capacity based on all 24 chemicals tested, since the VMG

judged three of them (beryllium sulphate, nickel chloride, dihydroeugenol) to fall outside the applicability domain. The two pre-haptens (4-phenylendeiamine and R(+)-Limonene) were included in the analysis as the VMG felt that there was insufficient evidence to exclude them from the evaluation of the predictive performance. The predictive capacity for all 24 substances was 77.8% (sensitivity: 70.8%; specificity: 91,7%) while 82.4% (sensitivity: 73.5%; specificity: 91,7%) for the 19 substances (since the WG felt that other two substances would fall outside the applicability domain, PPD and limonene).

- 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) and Predictive Capacity (Module 5).
- Overall, the chosen statistical approach was considered appropriate. In the calculations of suitable sample size it was not clear for the ESAC why a power of 75% was chosen for the BLR, especially as a more conventional 80% power was used for the WLR. However, since more chemicals than the minimum number were tested in both cases the actual power of the study was considered sufficient.

(2) Conclusions of the study

- Overall, the study design and the quality of the selected chemicals (N=24) were considered appropriate for the purpose of addressing the first objective of the study: Assessing the WLR and BLR of the DPRA.
- Overall, the conclusions made by the WG correspond well with the conclusions drawn by the VMG as described in the VSR, indicating that these conclusions are supported by the results shown in the report.
  - The WLR of the test method with respect to concordance of classification (S/NS) met the target of 85% and was considered sufficient for the purpose of this study.
  - The data were considered strong enough to support transferability of the test to properly equipped, trained and staffed laboratories with the appropriate analytical capabilities.
- In spite of a BLR (75%) below the target of 80%, the BLR of the test method with respect to concordance of classification was considered sufficient after the removal of the compounds outside the applicability domain. The potential for use of DPRA reactivity information in potency sub-categorisation requires further examination with a larger dataset.
  - The number of chemicals (N=24) did not provide support for a firm conclusion about the predictive capacity of the test method. The preliminary data were, however, considered promising.
  - The number of chemicals did not allow drawing a conclusion about the applicability domain of the test. Empirically the applicability domain seems to exclude pre-/pro-haptens and metal salts.

- Chemicals that preferably react with amino acids other then cysteine and lysine may fall outside the applicability domain. In addition, some pre-/pro-haptens were reported as correctly identified. Finally, the data seem to indicate that the test method has problems identifying weak sensitizers. The uncertainty about the applicability domain may result in an unacceptable level of false negative results.

(3) Possible use of the test method within an integrated approach

As outlined in the VSR and the ECVAM request for ESAC advice, the DPRA cannot be used as a stand-alone test method in a regulatory context but should be considered for use in an Integrated Testing Strategy (ITS). On the basis of the present report, especially negative outcomes have to be considered with care.

- As pre-haptens are not consistently correctly predicted by the DPRA, there remains uncertainty about whether to consider pre-haptens as part of the applicability domain of the method or not.
- Unless there are sufficiently accurate assays available identifying chemicals as pre-/pro-haptens in view of excluding them from routine testing using the DPRA, such compounds will be tested in the DPRA and may cause false negative results.
- The selection of cysteine and lysine-containing peptides selects for the majority, but not all, reactive chemicals.

Regarding reactivity class, the data obtained did not support the possibility to use DPRA as a stand-alone test method 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 (VSR page 8).

Information generated by the DPRA can be used to support regulatory decision making when used in the context of a weight-of-evidence approach or ITS. It is important to use the test in a context that allows confident conclusions about the protein-reactivity of the chemical, especially when the chemical in question is negative in the DPRA. As such the method may be helpful to address testing requirements of e.g. the REACH legislation and the 7th Amendment of the Cosmetic Directive.

Its inclusion into future ITSs can be considered for the purpose of an eventual full replacement of current *in vivo* hazard identification assays.

<u>Recommendations:</u>

The DPRA addresses a key mechanism (haptenation) in the development of skin sensitization/allergic contact dermatitis. Overall the provided data support transferability and reproducibility of the test to qualified laboratories. The predictive capacity of the test is not defined yet, but the preliminary data profiles the test as a useful tool for early decision making during product development (screening) and a component in a weight-of-evidence approach or ITS for safety/hazard assessment.

The ESAC recommends that the limitations of the DPRA (risk of false negative results) are further investigated specifically in relation to pre-/pro-haptens either by additional prospective testing or through analysis of existing information.

3. Informative background to the Mandate and Opinion

Skin sensitizsation 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.

The *Direct Peptide Reactivity Assay* DPRA is addressing one of the key upstream events in the cascade of mechanisms leading to the induction of skin sensitisation. It measures the ability of chemicals to react with proteins (haptenation). There is good evidence that haptenation is a determinant step in the induction of skin sensitisation. Chemical allergens are usually low molecular weight chemicals which are not immunogenic per se. However, chemical allergens (or their metabolites, oxidation products) have electrophilic properties that allow them to bind covalently with the nucleophilic side chains of amino acids of skin proteins to form an immunogenic conjugate. Already in 1936 this correlation between the reactivity of chemicals with proteins and their skin sensitisation potential was described (Landsteiner and Jacobs, Ref.5) and has in the meantime been extensively described in the literature. This knowledge is being exploited for the development of several *in chemico* reactivity assays with relevance for the testing of sensitisation potential, amongst these the DPRA assay.

The DPRA is designed to screen the ***sensitisation potential*** of chemicals by measuring peptide depletion with UV-HPLC, following incubation of the test chemicals with synthetic heptapeptides containing either cysteine (peptide/chemical ratio in the reaction mixture 1:10) or lysine residues (peptide/chemical ratio in the reaction mixture 1:50) (Gerberick 2004, Ref.6). The average of peptide depletion values for cysteine and lysine are used to classify chemicals into four reactivity categories: minimal, low, moderate and high reactivity (Gerberick 2007, Ref.7). Based on the known correlation between haptenation/chemical reactivity and sensitisation potential, it is assumed that these reactivity classes as predicted by the DPRA may contribute to the characterisation of sensitiser potency.

The possible predictive capacity of the DPRA is supported by the data of the original DPRA submission. On the basis of 133 chemicals, the DPRA classified chemicals as sensitisers or non-sensitisers (in relation to LLNA data) with an accuracy of 86% (87% sensitivity, 83% specificity).

4. References

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EURL ECVAM Scientific Advisory Committee

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- Prof. Ruud A. WOUTERSEN

ESAC Working Group Skin Sensitisation

- Dr. Erwin ROGGEN (ESAC member, Chair of ESAC WG and rapporteur)
- Prof. Walter PFALLER (ESAC member, ESAC Vice Chair)
- Prof. A. Wallace HAYES (ESAC member)
- Dr. Maja ALECSIC (external expert)
- Dr. Emanuela CORSINI (external expert)
- Dr. David LOVELL (external expert)
- Dr. Michael WOOLHISER (external expert)
- Prof. Yong HEO (external expert, ICATM nomination)

Coordination

- Dr. Claudius GRIESINGER *(EURL ECVAM Coordinator for ESAC peer reviews and EURL ECVAM recommendations)*
- Dr. Alexandre ANGERS *(specific support)*

Annex 2 EURL ECVAM request for ESAC advice

EURL ECVAM request for ESAC advice on an ECVAM-coordinated study concerning the transferability and reliability of the Direct Peptide Reactivity Assay (DPRA) for skin sensitisation testing

1. TYPE OF REQUEST

<table>
<tr><td colspan="2">Request Type</td><td>Identify request ("YES")</td></tr>
<tr><td colspan="2">1) ESAC Peer Review
of a Prevalidation Study or Validation Study</td><td>YES</td></tr>
<tr><td colspan="3">If R1)applies please specify further:</td></tr>
<tr><td>►Prevalidation Study</td><td colspan="2">YES

At present (January 2012) ECVAM is conducting a study of three test methods for skin sensitisation testing: 1) the Direct Peptide Reactivity Assay (DPRA), 2) the human Cell Line Activation Test (h-CLAT) and 3) the Myeloid U937 Skin Sensitisation Test (MUSST).

The study assesses transferability and reproducibility of these test methods in view of their possible future use (e.g. as partial replacement methods) within an integrated approach for skin sensitisation hazard identification aiming at the full replacement of the currently used regulatory in vivo assays for this purpose. In addition the data generated in this study will inform possible future evaluations on the predictive capacity of these assays.

While assessment of the h-CLAT and MUSST test methods are foreseen to be completed in 2012, the evaluation of the DPRA test method was finalised in 2011 and the adopted Validation Study Report is foreseen to be available by January 2012. ESAC review will commence in February 2012 employing the same ESAC WG which is currently (January 2012) peer reviewing the KeratioSens submission.</td></tr>
<tr><td>Prospective Validation Study</td><td colspan="2">No</td></tr>
<tr><td>Retrospective Validation Study</td><td colspan="2">No</td></tr>
<tr><td>Validation Study based on Performance Standards</td><td colspan="2">No</td></tr>
<tr><td colspan="2">2) Scientific Advice on a test method submitted to ECVAM for validation
(e.g. the test method's biological relevance etc.)</td><td>No</td></tr>
<tr><td colspan="2">3) Other Scientific Advice</td><td>No</td></tr>
</table>

(e.g. on test methods, their use; on technical issues such as cell culturing, stem cells etc.)	

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

Prevalidation of the Direct Peptide Reactivity Assay (DPRA) for skin sensitisation testing

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Background to skin sensitization and current predictive tests

Skin sensitisation is the toxicological endpoint associated with substances that have the intrinsic ability to cause Allergic Contact Dermatitis, ACD in humans. ACD represents the most common manifestation of immunotoxicity in humans, i.e. adverse effects of xenobiotics involving the immune system. The identification of the ***skin sensitization potential*** represents an important component of the safety assessment of any new substance and especially 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 integrated testing strategies, their capacity to produce reproducible results needs to be demonstrated as a first step.

2) Background to the DPRA, h-CLAT, MUSST

DPRA:

The *Direct Peptide Reactivity Assay* DPRA is addressing one of the key upstream events in the cascade of mechanisms leading to the induction of skin sensitisation. It measures the ability of chemicals to react with proteins (haptenation). There is good evidence that haptenation is a determinant step in the induction of skin sensitisation. Chemical allergens are usually low molecular weight chemicals which are not immunogenic per se. However, chemical allergens (or their metabolites, oxidation products) have electrophilic properties that allow them to bind covalently with the nucleophilic side chains of amino acids of skin proteins to form an immunogenic conjugate. Already in 1936 this correlation between the reactivity of chemicals with proteins and their skin sensitisation potential was described (Landsteiner and Jacobs,

Ref.5) and has in the meantime been extensively described in the literature. This knowledge is being exploited for the development of several *in chemico* reactivity assays with relevance for the testing of sensitisation potential, amongst these the DPRA assay.

The DPRA is designed to screen the ***sensitisation potential*** of chemicals by measuring peptide depletion with UV-HPLC, following incubation of the test chemicals with synthetic heptapeptides containing either cysteine (peptide/chemical ratio in the reaction mixture 1:10) or lysine residues (peptide/chemical ratio in the reaction mixture 1:50) (Gerberick 2004, Ref.6). The average of peptide depletion values for cysteine and lysine are used to classify chemicals into four reactivity categories: minimal, low, moderate and high reactivity (Gerberick 2007, Ref.7). Based on the known correlation between haptenation/chemical reactivity and sensitisation potential, it is assumed that these reactivity classes as predicted by the DPRA may contribute to the characterisation of sensitiser potency.

The possible predictive capacity of the DPRA is supported by the data of the original DPRA submission. On the basis of 133 chemicals, the DPRA classified chemicals as sensitisers or non-sensitisers (in relation to LLNA data) with an accuracy of 86% (87% sensitivity, 83% specificity).

h-CLAT & MUSST

The h-CLAT and MUSST are based on the use of Dendritic Cell (DC)-like cell lines. Using flow cytometry, these test methods monitor the induction of cell surface markers associated with DC activation, following exposure to the chemical. In the MUSST, changes in CD86 expression in the U937 cell line are detected; in the h-CLAT modulation of both CD86 and CD54 expression are recorded in THP-1 cells.

3) Study goals and design

In the first quarter of 2009 the DPRA was formally submitted to ECVAM together with other two test methods namely the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitisation Test (MUSST), developed by companies associated with the European Cosmetics Association (Colipa) and optimized within Colipa ring trials. Following detailed scientific assessment of the information submitted, ECVAM judged the three methods to be mature enough to enter the ECVAM validation process.

In September 2009 a formal study on the three above mentioned test methods was launched, with the main overall objective to evaluate their transferability and reliability (reproducibility within and between laboratories) when challenged with 24 coded chemicals.

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

a) A preliminary evaluation of the ability of each of the three tests to reliably discriminate skin sensitising (S) from non-sensitising (NS) chemicals as defined by the Globally Harmonised System (GHS, Ref. 6) for the classification and labelling of substances for skin sensitisation (category 1; no category) and as implemented in the European Commission Regulation (EC) No 1272/2008 (Ref.8) on classification, labelling and packaging (CLP) of substances and mixtures.

b) Where possible, a preliminary consideration of the ability of each of the three tests to contribute to sub-categorisation of skin sensitising chemicals, e.g. into Sub-category 1A (strong sensitisers) and Sub-category 1B (other sensitisers) as adopted in the 3rd revised version of the GHS.

The study experimental design foresees the testing of the 24 coded test items in each of the three participating laboratories for the assessment of the between-laboratory reproducibility. A subset of 15 of these chemicals are being tested two additional times in each laboratory for the

evaluation of within-laboratory reproducibility.

With respect to ECVAM's modular approach of validation (Hartung et al., 2004, Ref.10) the study will provide information on module 1) test definition, module 2) within laboratory reproducibility, module 3) transferability and module 4) between laboratory reproducibility. In addition, the data generated will provide preliminary information on module 5) predictive capacity; however, the number of chemicals tested is based on statistical considerations related to the evaluation of the reproducibility, and a larger sample size would be required for module 5 to be considered fulfilled.

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8. OECD, Organisation for Economic Cooperation and Development (1992) Skin Sensitisation Guidelines for Testing of Chemicals No. 406, 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 ECVAM with respect to the development of recommendations regarding the reliability (transferability, within and between laboratory reproducibility) of the DPRA and the potential regulatory use of the test method.

	In addition the ESAC should advice with regard to the possible necessary further work required (in relation to predictive capacity, applicability, limitations of the test method) to assess the potential contribution of the DPRA to a future (not yet designed) testing strategy or test battery that would aim to achieve full replacement of the currently used animal tests for skin sensitisation hazard assessment. Moreover, based on the evaluation of the data submitted, the ESAC should provide advice on the potential usefulness of the DPRA test method within a testing strategy for skin sensitisation testing and the proper scientific use of the test method within such a testing strategy (e.g. with respect to its specific applicability and limitations). It is explicitly noted that the ESAC is not requested to suggest the precise placing of the submitted method in a hypothetical ITS, but rather to provide advice on the characteristics of the method relevant for its subsequent integration into an ITS at a later point in time (i.e. when other buildings blocks of such an ITS are known).

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed in view of achieving the objective of the advice?*	1) **DESIGN & CONDUCT OF STUDY:** The ESAC is requested to review whether the prevalidation study was conducted appropriately in view of the objective of the study. The study objective was to assess (1) the reproducibility of the DPRA method within one laboratory (2) its transferability to other laboratories (3) its reproducibility in other laboratories (BLR). (4) Furthermore, the study aimed at assessing, in a preliminary manner, the predictive capacity of the test method When reviewing the design and conduct of the study, the following issues should be addressed in particular: Clarity of the test definition (module 1) Clarity of the definition of the study objective Appropriateness of the study design in view of study objective, *inter alia:* 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? Appropriateness of the **study execution** (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled? Were provisions specified for retesting? Was the number of repetitions sufficient? etc.) 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:

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

4.3 TIMELINES

Timelines concerning this request *When does ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	ESAC 37, 6/7 October 2012 or before through written procedure
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	YES, **before** ESAC 36 (20/21 March 2012)
	Request to be presented to ESAC at ESAC plenary meeting	NO

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)**
	1) ESAC Rapporteur	NO
	2) ESAC Working Group	**YES**
	3) Invited Experts	YES
	Ad 3): 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 (1-3):	

5.2 DELIVERABLES AS PROPOSED BY ECVAM

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

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

Count	Description of document	Available (YES/NO)	File name
1	OECD, Organisation for Economic Cooperation and Development (1992) Skin Sensitisation Guidelines for Testing of Chemicals No. 406, Paris	YES	ER2011-03_TG 406
2	OECD, Organisation for Economic Cooperation and Development (2002) The Local Lymph Node Assay. Guidelines for Testing of Chemicals No. 429, Paris	YES	ER2011-03_TG 429
3	OECD, Organisation for Economic Cooperation and Development (2010a) Skin Sensitization: Local Lymph Node Assay: DA, Guidelines for Testing of Chemicals No. 442A, Paris	YES	ER2011-03_TG 442A
4	OECD, Organisation for Economic Cooperation and Development (2010b) Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, Guidelines for Testing of Chemicals No. 442B, Paris	YES	ER2011-03_TG 442B
5	Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP, (2004) Development of a peptide reactivity assay for screening contact allergens. Toxicol Sci. 81; 332-43.	YES	ER2011-03_scientific paper on DPRA
6	Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP, (2007). Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol Sci. 97, 417-27.	YES	ER201-03_DPRA Classification Tree Model

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

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

7.2 TITLE OF THE ESAC WORKING GROUP

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

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific review of the relevant studies concerning four skin sensitisation test methods (DPRA, MUSST, h-CLAT, Keratinosens). The review needs to address the questions put forward to ESAC by ECVAM.
The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions of the Validation Management Team (VMT) / test method submitter are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLE OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Secretariat a detailed **ESAC Working Group Report** outlining its analyses and conclusions**.** A reporting template has been appended (Appendix 1) intended to facilitate the drafting of the report.
The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.
The WG is further asked to prepare a draft ESAC opinion as basis for the discussions by the entire ESAC, which shall adopt its opinion to the extent possible by consensus and on the basis of the ESAC WG report as well as all documents that were made available to the WG as well as to all ESAC members.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The following timelines have been proposed by ECVAM. These should be agreed upon during the face-to-face meeting (Item 1 in the table):

Item	Proposed date/time	Action	Deliverable
1	1-3 February	Face to face meeting	1)Input on draft mandate 2) Initial drafting of report
2	20. / 21. March	Progress report of WG Chair at ESAC36	Presentation / oral summary
3	14 May	Final report to be delivered to ESAC Coordinator/Secretariat.	Final draft report
4	8 June	Feedback from ESAC to WG on draft report (written procedure)	Feedback on final draft report
5	29 June	Final draft report available	Final report

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

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

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

APPENDIX 1 REPORTING STRUCTURE FOR THE *ESAC WG REPORT*

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

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable. However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies. The current template is version 5.

END OF EURL ECVAM RECOMMENDATION

European Commission
EUR 26383– Joint Research Centre – Institute for Health and Consumer Protection

Title: EUR 26383 - EURL ECVAM Recommendation on the **Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing**

Luxembourg: Publications Office of the European Union

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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 documented by the OECD's recent report summarising the key biological events leading to skin sensitisation ("Adverse Outcome Pathway" (AOP) for skin sensitisation). The molecular initiating event defined within this AOP is the covalent binding of chemicals with skin proteins. Thus peptide reactivity assays may provide valuable information in the context of integrated approaches such as Weight of Evidence (WoE) or Integrated Testing Strategies (ITS) for skin sensitisation hazard and safety assessment. Based on these considerations, EURL ECVAM coordinated a validation study on the Direct Peptide Reactivity Assay (DPRA) addressing mainly the test method's transferability and within- and between-laboratory reproducibility. Following independent scientific peer review by the EURL ECVAM's Scientific Advisory Committee (ESAC) and having considered the input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the DPRA may prove a valuable component of a WoE or ITS for skin sensitisation hazard assessment. In addition to this, the DPRA may also be able to contribute to the assessment of sensitising potency, e.g. by supporting sub-categorisation of sensitisers according to UN GHS. However it is recognised that further efforts are required to explore how DPRA data may contribute to potency assessment.

LB-NA-26383-EN-N

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