新規試験法提案書

皮膚感作性試験代替法
ADRA：アミノ酸誘導体結合性試験

令和 6 年 2 月

国立医薬品食品衛生研究所

新規試験法提案書

令和 6 年 2 月 26 日

No.　2023-01

皮膚感作性試験代替法ADRA法に関する提案

令和 5 年 12 月 12 日に国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の提案がなされた。

提案内容：本試験法は、感作性発現機序における初期の重要な事象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。ただし、本試験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、本試験法は他の代替法と同様、単独で皮膚感作性を評価できず、OECD ガイドライン 497 に記載されているような細胞を用いる他の KE を対象にした代替法や構造活性相関（Quantitative Structure-Activity Relationship: QSAR）等の in silico 手法と組み合わせての利用を推奨するものである。

この提案書は、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 ADRA 法の使用を提案するものである。

西川秋佳

JaCVAM 評価会議　議長

平林容子

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

西 川 秋 佳 （国立医薬品食品衛生研究所 安全性生物試験研究センター 病理部／
名古屋徳洲会総合病院）：座長
小 島 幸 一 （一般財団法人 食品薬品安全センター）
中村るりこ （独立行政法人 製品評価技術基盤機構）
西 村 次 平 （独立行政法人 医薬品医療機器総合機構）
平 林 容 子 （国立医薬品食品衛生研究所 安全性生物試験研究センター）
松 本 一 彦 （名古屋市立大学大学院）

任期：令和 4 年 4 月 1 日～令和 6 年 3 月 31 日

JaCVAM 運営委員会

平 林 容 子　（国立医薬品食品衛生研究所 安全性生物試験研究センター）：委員長
石 井 孝 司　（国立感染症研究所）
稲 角 嘉 彦　（厚生労働省 医薬・生活衛生局 医薬品審査管理課 化学物質安全対策室）
小川久美子　（国立医薬品食品衛生研究所 安全性生物試験研究センター 病理部）
諫 田 泰 成　（国立医薬品食品衛生研究所 安全性生物試験研究センター 薬理部）
北 嶋　聡　（国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部）
杉 山 圭 一　（国立医薬品食品衛生研究所 安全性生物試験研究センター 変異遺伝部）
髙 橋 祐 次　（国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部 動物管理室）
束 野 正 明　（厚生労働省 医薬・生活衛生局 医薬品審査管理課 化学物質安全対策室）
林　亜紀子　（厚生労働省 医薬・生活衛生局 医薬品審査管理課）
本 間 正 充　（国立医薬品食品衛生研究所）
真 木 一 茂　（独立行政法人 医薬品医療機器総合機構）
増 村 健 一　（国立医薬品食品衛生研究所 安全性生物試験研究センター 安全性予測評価部）
横 田 雅 彦　（独立行政法人 医薬品医療機器総合機構）
足利太可雄　（国立医薬品食品衛生研究所 安全性生物試験研究センター 安全性予測評価部 第二室）：事務局

JaCVAM statement on the ADRA, an alternative method for evaluating skin sensitization

At a meeting held on 12 December, 2023 at 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: This test method detects the covalent bond reaction between a protein and a chemical substance, an early key event in the sensitization mechanism and provides important information for determining skin sensitization potential of chemical substance. However, as this test method is an in chemico test that lacks a metabolic system, sensitization potential of chemical substances show below may not be correctly detected. Weak sensitizers, metal salts, highly hydrophobic substances, sensitizers that require a metabolic system or activation by an abiotically. Based on the above facts, this test method alone, like other alternative methods, cannot evaluate skin sensitization potential; thus, we recommend its use in combination with cell based alternative methods for other key events as described in OECD Guideline 497 or in silico methods such as quantitative structure–activity relationship (QSAR).

This statement was released following a review prepared by the skin sensitization test JaCVAM Editorial Committee to acknowledge that the results of the review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.

Based on the above, we proposed the ADRA method as a useful means for assessing skin sensitization potential during safety assessments by regulatory agencies.

Nishikawa Akiyoshi
Chairperson,
JaCVAM Regulatory Acceptance Board.

Hirabayashi Yoko
Chairperson,
JaCVAM Steering Committee.

February 26, 2024

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:

Nishikawa Akiyoshi (Division of Pathology, Center for Biological Safety and Research:CBSR, NIHS / Nagoya Tokushukai General Hospital) : Chairperson
Hirabayashi Yoko (CBSR, NIHS)
Kojima Koichi (Food and Drug Safety Center)
Matsumoto Kazuhiko (Nagoya City University)
Nakamura Ruriko (National Institute of Technology and Evaluation)
Nishimura Jihei (Pharmaceuticals and Medical Devices Agency)

Term: From 1st April 2022 to 31st March 2024

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

Hirabayashi Yoko (CBSR, NIHS): Chairperson
Hayashi Akiko (Ministry of Health, Labour and Welfare)
Honma Masamitsu (NIHS)
Inazumi Yoshihiko (Ministry of Health, Labour and Welfare)
Ishii Koji (National Institute of Infectious Diseases)
Kanda Yasunari (Division of Pharmacology, CBSR, NIHS)
Kitajima Satoshi (Division of Cellular and Molecular Toxicology, CBSR, NIHS)
Maki Kazushige (Pharmaceuticals and Medical Devices Agency)
Masumura Kenichi (Division of Risk Assessment, CBSR, NIHS)
Ogawa Kumiko (Division of Pathology, CBSR, NIHS)
Sugiyama Keiichi (Division of Genetics and Mutagenesis, CBSR, NIHS)
Taquahashi Yuhji (Animal Management Section of the Division of Toxicology, CBSR, NIHS)
Tsukano Masaaki (Ministry of Health, Labour and Welfare)
Yokota Masahiko (Pharmaceuticals and Medical Devices Agency)
Ashikaga Takao (Division of Risk Assessment, CBSR, NIHS): Secretary

皮膚感作性試験代替法 ADRA :アミノ酸誘導体結合性試験に関する提案

添付資料一覧

添付資料 1

評価会議報告書

皮膚感作性試験代替法　**ADRA:**アミノ酸誘導体結合性試験

JaCVAM 評価会議

令和 5 年(2023 年)12 月 12 日

JaCVAM 評価会議

西 川 秋 佳　（国立医薬品食品衛生研究所 安全性生物試験研究センター 病理部／
名古屋徳洲会総合病院）：座長
小 島 幸 一　（一般財団法人 食品薬品安全センター）
中村るりこ　（独立行政法人 製品評価技術基盤機構）
西 村 次 平　（独立行政法人 医薬品医療機器総合機構）
平 林 容 子　（国立医薬品食品衛生研究所 安全性生物試験研究センター）
松 本 一 彦　（名古屋市立大学大学院）

任期：令和 4 年 4 月 1 日～令和 6 年 3 月 31 日

JaCVAM 評価会議は、皮膚感作性試験資料編纂委員会により作成された「皮膚感作性試験代替法 Amino acid Derivative Reactivity Assay（ADRA：アミノ酸誘導体結合性試験）評価報告書」[1]をもとに本試験法の科学的妥当性、社会的および行政的な受け入れ性について検討した。

1. 試験法の定義および科学的妥当性

名称：アミノ酸誘導体結合性試験（Amino acid Derivative Reactivity Assay：ADRA）

代替する対象毒性試験：マウスを用いる局所リンパ節試験（Local Lymph Node Assay: LLNA）[2]

科学的妥当性：

当該試験法は、感作性発現機序における初期の重要な Key Event（KE）であるタンパク質と化学物質の結合反応をもとに皮膚感作性の有無を検出できる *in chemico* 皮膚感作性試験であり[3]、LLNA を代替できる試験として、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）試験法ガイドライン（Test Guideline: TG）442C に収載されている[4]。

求核試薬としてナフタレン環を導入したシステインあるいはリジンを用い、被験物質と求核試薬を混合・反応させた後、24 時間後における未反応の求核試薬量を紫外線（Ultra Violet: UV）検出器および蛍光（Fluorescence: FL）検出器を搭載した高速液体クロマトグラフィー（High Performance Liquid Chromatography：HPLC）で分析することから、科学的には妥当な手法である。

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

社会的受け入れ性：

本試験法は、汎用 HPLC およ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、本試験法は動物を用いない *in chemico* の手法であり、3Rs の精神と合致している。さらに 1 化合物あたりの試験消耗品費用は LLNA と比べて安価である。ADRA の試験期間は LLNA や他の代替法と比べて短く、試験法として簡便性・経済性の面から有用と考えられる。

行政上の利用性：

本試験法は、感作性発現機序における初期の重要な事象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。ただし、本試験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、本試験法は他の代替法と同様、単独で皮膚感作性を評価できず、OECD ガイドライン 497[5]に記載されているような細胞を用いる他の KE を対象にした代替法や構造活性相関（Quantitative Structure-Activity Relationship: QSAR）等の *in silico* 手法と組み合わせての利用を推奨するものである。

参考文献（最終確認日：2023 年 9 月 20 日）

1) JaCVAM　皮膚感作性試験資料編纂委員会：皮膚感作性試験代替法 Amino acid Derivative Reactivity Assay（ADRA：アミノ酸誘導体結合性試験）評価報告書（2023 年 6 月 7 日）
2) OECD (2010) OECD Guidelines for the Testing of Chemicals No. 429. The Local Lymph Node Assay (LLNA), Organisation for Economic Cooperation and Development, Paris. Available at: https://www.oecd-ilibrary.org/environment/test-no-429-skin-sensitisation_9789264071100-en
3) Fujita M, Yamamoto Y, Tahara H, Kasahara T, Jimbo Y, Hioki T, (2014) Development of a prediction method for skin sensitisation using novel cysteine and lysine derivatives, Journal of Pharmacological and Toxicological Methods, 70: 94-105.
4) OECD (2023 OECD Guidelines for the Testing of Chemicals No. 442C. *In chemico* skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins. Organisation for Economic Cooperation and Development, Paris. Available at: https://www.oecd-ilibrary.org/environment/test-no-442c-in-chemico-skin-sensitisation_9789264229709-en
5) OECD (2021) OECD Guideline for the Testing of Chemicals No. 497. Defined Approaches on Skin Sentitisation. Organization for Economic Cooperation and Development, Paris. Available at: https://www.oecd-ilibrary.org/environment/guideline-no-497-defined-approaches-on-skin-sensitisation_b92879a4-en

添付資料 2

評価報告書

皮膚感作性試験代替法　ADRA:アミノ酸誘導体結合性試験

皮膚感作性試験資料編纂委員会

令和 5 年(2023 年)12 月 12 日

皮膚感作性試験資料編纂委員会

福 山 朋 季　（委員長：麻布大学）
安 達 玲 子　（国立医薬品食品衛生研究所）
大 竹 利 幸　（株式会社資生堂）
小 島 幸 一　（一般財団法人　食品薬品安全センター）
小 島　　肇　（国立医薬品食品衛生研究所）
瀬 崎 拓 人　（三井化学株式会社）
武 吉 正 博　（一般財団法人　化学物質評価研究機構）

要旨

皮膚感作性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対象とした代替法であるペプチド結合性試験（Direct Peptide Reactivity Assay：DPRA）は、システインあるいはリジンを含むペプチドと化学物質の結合反応を機器分析によって評価する簡単な手法であり、2015 年に OECD 試験法ガイドライン（Test Guidelien : TG）に収載された。しかし、DPRA は、反応液中での被験物質の析出や共溶出が起こりやすく、且つ混合物の感作性評価ができない等の課題があった。日本で開発されたアミノ酸誘導体結合性試験（Amino acid Derivative Reactivity Assay : ADRA）は、DPRA と同じ原理であるが、求核試薬としてナフタレン環を導入したシステインあるいはリジンを用い、被験物質と混合・反応させた後、紫外線（Ultra Violet : UV）検出器および蛍光（Fluorescence : FL）検出器を搭載した高速液体クロマトグラフィー（High Performance Liquid Chromatography：HPLC）で分析することで、これらの課題を克服することができる。ADRA の使用方法は、被験物質の情報や状態によって異なる。分子量が既知の被験物質については、4 mM に調製した被験物質溶液を用いた ADRA（4 mM）、混合物のような分子量が未知の被験物質については、0.5 mg/mL に調製した被験物質溶液を用いた ADRA（0.5 mg/mL）を使用する。共溶出が認められる場合は、FL 検出器を用いた ADRA-FL 法が使用できる。

ADRA は、最初、1 mM に調製した被験物質溶液を用いた ADRA（1 mM）が、2019 年に OECD の TG に収載された。その後、混合物の感作性評価を可能にするための ADRA（0.5 mg/mL）、求核試薬を蛍光検出する ADRA-FL 法および偽陰性物質の数を減らすために開発された ADRA（4 mM）が盛り込まれた OECD TG 改定版が 2022 年に発出された。

ADRA（0.5 mg/mL）および ADRA（4 mM）のバリデーション研究は、5 施設において、10 化合物および 8 化合物の習熟度確認物質を対象に、施設内および施設間再現性試験が実施された。なお、HPLC による求核試薬の測定は、UV 検出器と FL 検出器の両方で実施し、ADRA-UV 法と ADRA-FL 法の同一性についても検証が行われた。ADRA（0.5 mg/mL）および ADRA（4 mM）の施設内再現性は、ADRA-UV 法あるいは ADRA-FL 法のどちらで測定しても 100%であり、施設間再現性も、ADRA-UV 法あるいは ADRA-FL 法のどちらで測定しても 100%であり、達成基準（85%）を満たした。また、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、ADRA（0.5 mg/mL）および ADRA（4 mM）における正確度（Accuracy）、感度（Sensitivity）および特異度（Specificity）はいずれも 100%と報告されている。

ADRA は感作性発現機序における初期の重要な事象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。また、ADRA は、LLNA の 1/20 程度、DPRA の 1/2 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、本資料編纂委員会は、他の Key Event（KE）を対象とした代替法や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）等の *in silico* 手法と組み合わせた評価を推奨する。

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経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）TG としてモルモットを用いる皮膚感作性試験（OECD TG 406）やマウスを用いる局所リンパ節試験（Local Lymph Node Assay: LLNA）がある。LLNA には放射性化合物（Radioisotope：RI）の取込量を測定する LLNA-RI 法（OECD TG 429[1]）のほか、RI を用いず Adenosine triphosphate（ATP）量を測定する LLNA: DA 法（OECD TG 442A[2]）や Bromodeoxyuridine（BrdU）量を測定する LLNA: BrdU-ELISA 法および LLNA: BrdU-FCM 法（OECD TG 442B[3]）がある。

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 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および販売が禁止された（2013 年 3 月全面施行）。そのため、化学物質の皮膚感作性を評価する代替法の開発が進み、動物を用いない多くの代替法が OECD TG として承認されてきている。

皮膚感作性に伴う化学的および生物学的機序に関する最新の知見は、有害性発現経路（Adverse Outcome Pathway：AOP）として提示されている。皮膚感作性は、その AOP に基づく 4 つの Key Event（KE）を経て成立し、下記のように、KE1～KE3 には動物を使わない代替法が開発され、OECD TG 化されている。

KE1: 化学物質とタンパク質の共有結合、DPRA、ADRA および kDPRA（OECD TG 442C）[4]

KE2: 角化細胞活性化に関連する ARE Nrf2 依存性ルシフェラーゼの発現、KeratinoSens™ および LuSens（OECD TG 442D）[5]

KE3: 特異的細胞表面マーカーの発現およびケモカインやサイトカインの産生、皮膚感作性関連遺伝子群の発現プロファイルを指標とした樹状細胞の活性化、h-CLAT、U-SENS™、IL-8 Luc assay および GARD™ skin（OECD TG 442E）[6]

KE4: リンパ節における T 細胞の増殖、LLNA（OECD TG 429[1]、TG 442A[2]および TG 442B[3]）

DPRA は、求核試薬として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）を用い、それぞれを被験物質と混合・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、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[4, 7, 8]。しかし、DPRA で用いる求核試薬のペプチドは、特異的な UV 吸収や FL がなく、ペプチドの検出波長として 220 nm を用いるため、DPRA の被験物質の調製濃度は、100 mM と高濃度となる。DPRA は反応液中での被験物質の析出[9]、HPLC の検出時における被験物質と求核試薬の共溶出[10]、システイン含有ペプチド二量体の多発など試験精度に影響を及ぼす事例[11]や重量濃度で調製された被験物質溶液や混合物の感作性評価ができないなどの限界があった。

そこで、求核試薬の測定における検出感度を上げるために、求核試薬として、システインおよびリジンに検出部位として高い UV 吸収率を持ち、かつ特定波長の光照射によって強い FL を発するナフタレン環を導入した N-(2-(1-naphthyl)acetyl)-L-cysteine（NAC）および α-N-(2-(1-naphthyl)acetyl)-L-lysine（NAL）を用いた ADRA が開発された（図 1）[12, 13]。NAC（N-

Acetyl Cystein)および NAL の検出部位であるナフタレン環は 281 nm と比較的長波長側に極大吸収波長をもつため、220 nm で検出している DPRA と比較して検出特異性が高く、被験物質由来の夾雑ピーク(吸収)の影響を受けにくいだけでなく、FL 検出器による特異的な検出・定量が可能である[14, 15]。

ADRA は、最初、1 mM に調製した被験物質溶液を用いた ADRA(1 mM)のバリデーション研究が始まり、Japanese Center for the Validation of Alternative Methods(JaCVAM)による第三者評価(ピアレビュー)を経て[16-18]、OECD 専門家会議で議論された後、2019 年に OECD の TG に収載された。その後、混合物の感作性評価を可能にするために、0.5 mg/mL の被験物質溶液を用いた ADRA(0.5 mg/mL)[19]、求核試薬を FL 検出する ADRA-FL 法[14, 15]、偽陰性物質の数を減らすために開発された ADRA(4 mM)[20]が、2022 年に OECD TG に盛り込まれ、改定された(Annex 1 参照)。

JaCVAM 皮膚感作性試験資料編纂委員会(以下、委員会)が ADRA 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. 試験法の原理と方法

OECD TG 442C に収載されている ADRA と DPRA は、KE1 の『化学物質とタンパク質の共有結合』に対応した動物および細胞を用いない *in chemico* 試験である。

多くの感作性物質は、タンパク質のシステインまたはリジンに結合することから、ADRA は、図 1 に示すように、システインおよびリジンの検出部位として高い UV 吸収や FL を持つナフタレン環を導入した NAC および NAL の 2 種類を使用する。被験物質とそれぞれの求核試薬を混合し、反応させ、24 時間後における未反応の求核試薬量を HPLC で分離定量する(詳細は Annex 2 参照)。ADRA では NAC 二量体を HPLC で定量することができるため、NAC のクオリティチェックに利用できるだけでなく、酸化促進作用のある被験物質による NAC 二量体の生成についても予測することができる。求核試薬を検出する HPLC システムには、UV 検出器と FL 検出器を直列につなぐことにより、同時検出することができる。これにより、もし UV 検出器で共溶出が認められた場合でも、FL 検出器で測定した値を採用することができる。ただし、NAC 二量体は、UV 吸収を持つが、FL は消失するため、原則的に UV 検出器で得られた値を採用する。

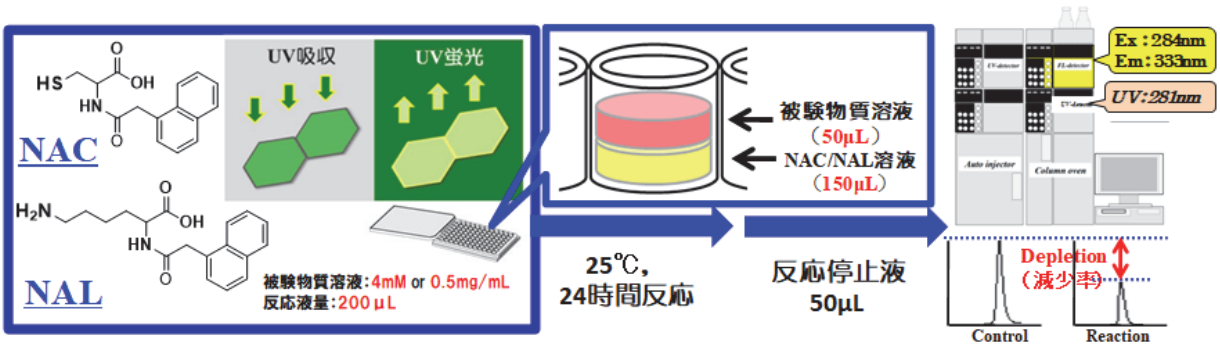


図 1　ADRA の概要

3．精度

ADRA（1 mM）は、4 施設に技術移転を実施し、その後に施設内再現性（Phase Ⅰ）および施設間再現性（Phase Ⅱ）の試験が実施されている。詳細な結果は Annex 3（表 S3-1～S3-5）に示す。

3-1. ADRA（1 mM）の技術移転性

基本的な実験設備と HPLC 技術を持つ参加施設に、リード施設（富士フイルム）から 1 日のトレーニングで技術移転は可能であった。プレトレーニング試験として、代表的な感作性物質 4 化合物および非感作性物質 1 化合物、計 5 化合物の評価が行われ（表 S3-1）、4 施設とも 5 化合物の評価はすべて、リード施設の結果と一致した（表 S3-2）。

プレトレーニング試験を終了した参加施設には、トレーニング試験が実施された。トレーニング試験の化合物は、NAC および NAL（以下、NAC/NAL）の減少率の平均値が、閾値に近い化合物を含む 6 化合物の感作性物質と 4 化合物の非感作性物質から成る 10 化合物のデータセットが選択され（表 S3-3）、4 施設の施設内再現性は、それぞれ 100%、90%、100%および 100%、施設間再現性は、80%となり、それぞれ達成基準（70%）を満たした（表 S3-4）。なお、プレトレーニング試験およびトレーニング試験では、減少率の閾値は、5.05 が使われた。これらの試験の後、NAC の二量体の増加を防ぐため、反応液に EDTA を添加することが決まったため、この条件で 82 化合物の ADRA を再実施し、得られた減少値から閾値を計算したところ、閾値が 4.9 に変更になった。従って、バリデーション研究以降の減少率の閾値は、4.9 が使用された。

3-2. ADRA（1 mM）の施設内再現性および施設間再現性試験 [16-18)]

ADRA（1 mM）のバリデーション研究は、4 施設において、10 化合物を対象に施設内再現性試験が実施され、これらの 10 化合物のデータを含む 40 化合物を対象に施設間再現性試験が実施された。4 施設の施設間再現性の結果は、100%（10/10）、100%（10/10）、100%（7/7）、90%（9/10）であった。40 化合物の施設間再現性（3 施設換算）は 91.9%であり、達成基準（80%）を満たした（表 1 および表 S3-5）。なお、ADRA の 4 施設におけるバリデーション研究で検証された 40 種類の化合物の LLNA の結果に対する本試験法の正確度は 86.9%（139/160）、感度は 81.5%（88/108）、特異度は 98.1%（51/52）であった。

Dihydroeugenol は、参加施設間で結果がバラついたが、これは、Dihydroeugenol がプレハプテンであり、一部の施設で試験中に酸化して反応性が増加したためと考えられた。Citral および Hydroxycitronellal も参加施設間で結果がバラついたが、これは NAC/NAL の減少率の平均値が閾値付近であったためと考えられた。Dextran も 1 施設のみが陽性の判定であったが、化学構造上は、NAC や NAL と反応する部位がなく、基本的に非反応性であると考えられる。しかしながら、グルコースなどの還元糖の 1 位のアルデヒド基は、それらの 5 位の水酸基と反応して環状構造を形成し、水溶液中では一部が開環し、部分的にアルデヒド基を生成することが知られており [21)]、このアルデヒド基と求核試薬の NAC が反応し、Sciff-base を形成する可能性がある。同様に、Dextran も水溶液中で末端に存在する環状構造の一部が開環して生じたアルデヒド基と NAC が結合し、減少率がわずかに上昇したことが一部の施設で偽陽性が生じた原因と推察される。しかし、改定された TG では、ポリマーは 0.5 mg/mL

に調製した被験物質溶液を用いることとなり、Dextran の場合、本実験条件の 1/8000 程度の濃度となるため、偽陽性になることはないと思われる。

なお、DPRA のバリデーション研究は、ADRA と試験デザインや化合物の種類が違うため直接の比較はできないが、3 施設の施設内再現性試験の正確度は、66.7%、3 施設の施設間再現性の正確度は 82%と報告されている [22]。従って、ADRA の施設内および施設間再現性はそれらを上回ることが示されている。

表 1 ADRA の施設内および施設間再現性値

施設内再現性（Phase I 10物質）

Lab.A : **100%** [=10/10]　Lab.B : **100%** [=10/10]　Lab.C : **100%** [=7/7]　Lab.D : **90%** [=9/10]

施設間再現性（Phase I 10物質 *1 ＋ Phase II 30物質）

91.9%（3施設換算値*2）

*1 Phase Iの10物質については3回（Lab. C の3物質においては2回）の試験結果におけるAve. scoreの平均値から最終的な判定を行った。
*2 施設間再現性は通常3施設の結果から計算するため以下の計算方法を用いて4施設の結果から3施設に換算した。
(1) 4施設のうち1施設 を除いた3施設の結果から施設間再現性を計算する（a) Lab.A除外、b) Lab.B除外、c) Lab.C除外、d) Lab.D除外の4パターン）。
(2) (1)の4つのパターンの施設間再現性をそれぞれ計算し、4つの施設間再現性の平均値を施設間再現性（3施設換算値）とする）。

3-3. ADRA（0.5 mg/mL）の施設内再現性および施設間再現性試験 [23]

ADRA（0.5 mg/mL）のバリデーション研究は、ADRA（1 mM）のバリデーション研究に参加した 4 施設にリード施設を加えた 5 施設で実施された。また、通常の UV 検出器を用いた測定の他に、同じサンプルで FL 検出器を用いた測定も実施し、ADRA-UV 法と ADRA-FL 法の同一性についても相関解析による検証が行われた。ADRA（0.5 mg/mL）の技術移転試験は、被験物質溶液の濃度を 1 mM から 0.5 mg/mL に変更しただけなので、実施されなかった。また、FL 検出器による検出法の技術移転は、UV 検出器の代わりに FL 検出器を使用しただけなので、各施設で NAC および NAL の検量線の測定が正常にできるかどうかの確認のみが実施された。5 施設のうち、1 施設は、2 種類の HPLC システムを用意できなかったために、この施設については、FL 検出器のみ使用された。0.5 mg/mL の被験物質溶液を用いた ADRA-FL 法は、5 施設により 10 種類の習熟度確認物質について施設内および施設間再現性試験が実施され、施設内再現性は 5 施設すべてで 100% 、施設間再現性もすべてで 100%であった（表 S3-6-1、S3-6-2 および S3-6-3）。また、0.5 mg/mL の被験物質溶液を用いた ADRA-UV 法は、4 施設により 10 種類の習熟度確認物質について施設内および施設間再現性試験が実施され、施設内再現性は 4 施設すべてで 100% 、施設間再現性もすべてで 100%であった。0.5 mg/mL 溶液を用いた ADRA-UV 法と ADRA-FL 法における NAC/NAL の減少率の平均値の相関係数は $r = 0.99$（$p < 0.001$）であり極めて強い相関が確認されたことから、両試験法の結果が同等であることが示された。

3-4. ADRA（4 mM）の施設内再現性および施設間再現性試験 [24]

ADRA（4 mM）のバリデーション研究も、ADRA（0.5 mg/mL）のバリデーション研究と同様に ADRA（1 mM）に参加した 4 施設にリード施設を加えた 5 施設で実施された。試験デザイ

ン等は、ADRA（0.5 mg/mL）のバリデーション研究と同じであるが、このバリデーション研究との違いは、FL 検出器と UV 検出器を HPLC システムに直列につないで同時検出している点であった。4 mM の被験物質溶液を用いた ADRA-FL 法および ADRA-UV 法は、5 施設により 8 種類の習熟度確認物質について施設内および施設間再現性試験が実施され、施設内再現性試験は両測定法とも 5 施設すべてで 100%、5 施設による施設間再現性もすべてで 100%であった（表 S3-7-1 および S3-7-2）。図 2 は、Farnesal を除く 8 種類の習熟度確認物質と、ADRA（4 mM）および ADRA（1 mM）で結果の違う 4 種類の化合物と 2 種類の陽性対照に対して、ADRA（4 mM）を実施したときの ADRA-UV 法と ADRA-FL 法の相関図である。NAC/NAL の減少率の相関係数は、いずれも $r = 1.00$（$p < 0.001$）であり極めて強い相関が確認されたことから、両試験法の結果が同等であることが示された。

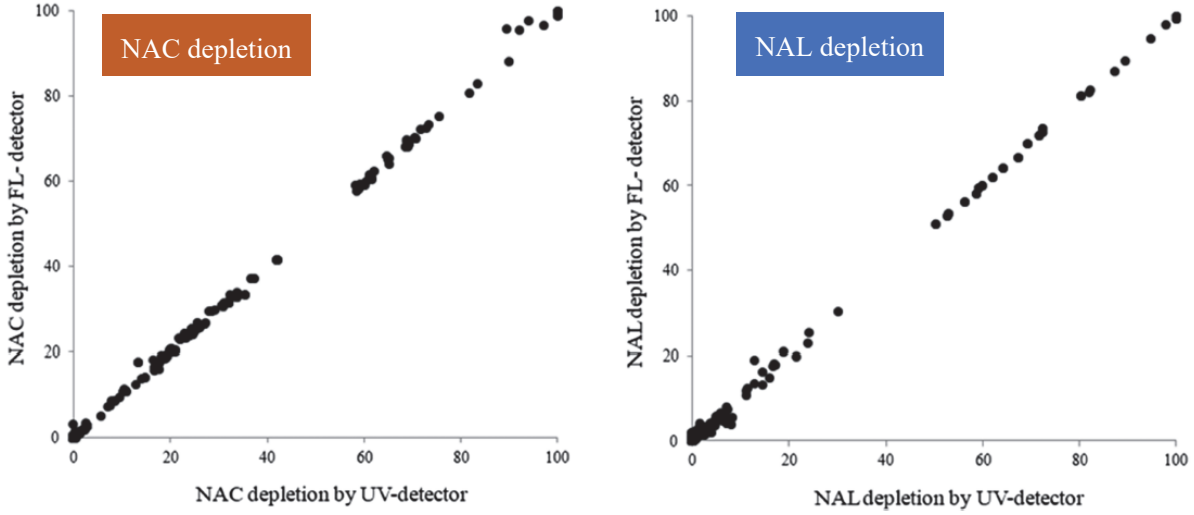


図 2　ADRA（4 mM）バリデーション研究における FL 検出と UV 検出の NAC/NAL 減少率の同等性の検証 [24)]

4. 正確度、感度および特異度

ADRA（1 mM）、ADRA（0.5 mg/mL）および ADRA（4 mM）のバリデーション研究の結果は、表 2 に示した。

ADRA（1 mM）の 4 施設におけるバリデーション研究で検証されたコード化された 40 種類の化合物の LLNA の結果に対する本試験法の正確度は 86.9%（139/160）、感度は 81.5%（88/108）、特異度は 98.1%（51/52）であった [18)]。なお、DPRA のバリデーション研究は ADRA と試験デザインや化合物の種類が異なるので直接の比較はできないが、3 施設の LLNA の結果に対する本試験法の正確度は 77.8%、感度は 70.8%、特異度は 91.7%であったことから [22)]、ADRA の正確度、感度および特異度は、感作性を予測する上で十分であることが示された。

ADRA（0.5 mg/mL）の 5 施設における 10 種類の習熟度確認物質のバリデーション研究では、UV 検出の正確度は 100%（40/40）、感度は 100%（24/24）、特異度は 100%（16/16）、また、FL 検出の正確度は 100%（50/50）、感度は 100%（30/30）、特異度は 100%（20/20）であった [23)]。なお、この試験の化合物のコード化は実施されていなかった。

ADRA（4 mM）の 5 施設におけるコード化された 8 種類の習熟度確認物質のバリデーション研究では、正確度、感度、特異度はいずれも 100%（40/40）であった[24]。

なお、ADRA（4 mM）は、被験物質を従来の 1 mM から 4 mM に濃度を高くすることで、ADRA（1 mM）で偽陰性と判定される化合物の数を減らす効果があることが報告されている[20]。リード施設だけでなく、他施設でもこの効果が再現するかを確認するために、ADRA（1 mM）において偽陰性化合物と判定される 4 化合物について、ADRA（4 mM）のバリデーション研究の中で検証された[24]。ADRA（1 mM）で偽陰性、ADRA（4 mM）で LLNA の結果と一致して陽性と判定される 4 化合物（*m*-Aminophenol、3-Propylidenephthalide、Ethyleneglycol dimethacrylate および n-Butyl glycidyl ether）について、5 施設において、3 回の試験が実施され、1 施設において、*m*-Aminophenol が 1 回の試験だけ陰性判定になった。そのため、正確度は、98.3%（59/60）であったが、その効果が複数の施設でも確認された（表 S3-8-1 および表 S3-8-2）。

これらのバリデーション以外にも、リード施設で 136 化合物のデータセット（バリデーション化合物を含む）についても、ADRA（1 mM）、ADRA（0.5 mg/mL）および ADRA（4 mM）が実施され、それぞれの予測結果についてまとめられた（Annex 5、表 S5-1 および S5-2）。

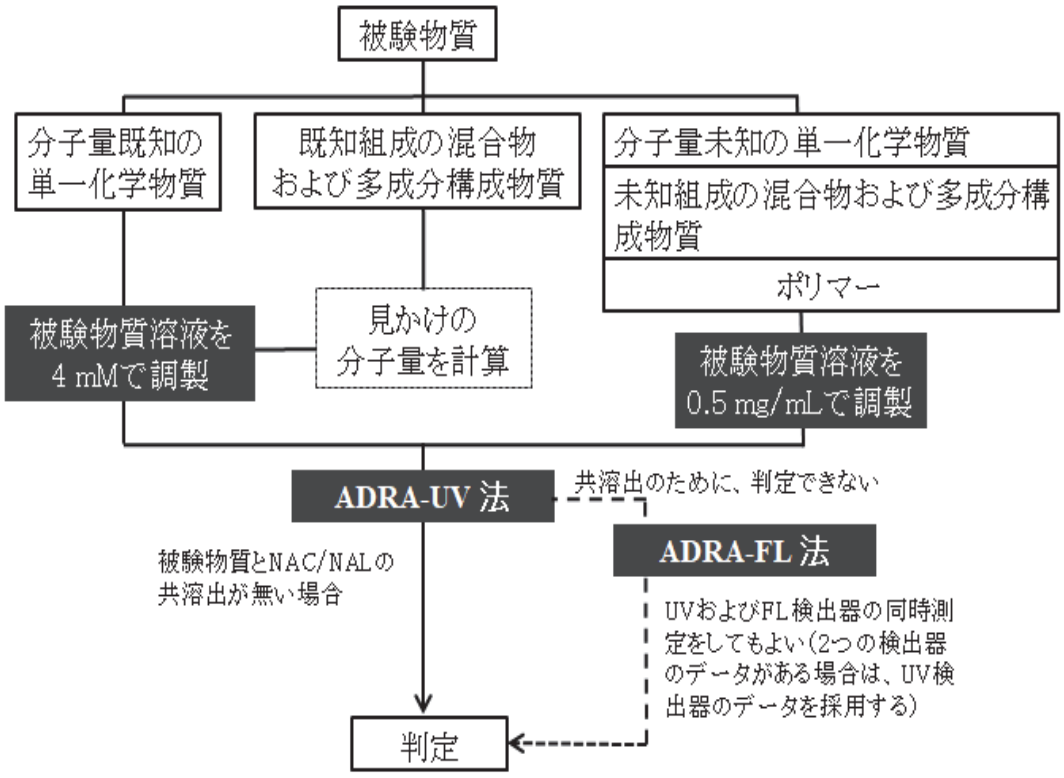
表 2 ADRA（1 mM）、ADRA（0.5 mg/mL）および ADRA（4 mM）のバリデーション研究のまとめ

	期間	施設数	化合物数		再現性		備考
			施設内	施設間	施設内	施設間	
ADRA（1 mM） UV検出器のみ	'16 10月～ '17 3月	4	10 （3回実施）	40 （1回実施）	90.0～100.0 （平均 97.3%）	91.3	施設内のLab Cは、2回目の3化合物が欠測。施設間再現性103化合物は、施設内の結果を利用（多数決判定）
ADRA（0.5mg/mL） UVおよびFL検出器のみ	'19 10月～ '20 2月	5	10 （3回実施）	10 （3回実施）	100.0（UV） 100.0（FL）	100.0（UV） 100.0（FL）	化合物は、10個の習熟化合物を使用。反応したサンプルをUV検出器で、10倍希釈したサンプルをFL検出器で測定
ADRA（4 mM） UVおよびFL検出器のみ	'20 12月～ '21 3月	5	10 （3回実施）	10 （3回実施）	100.0（UV） 100.0（FL）	100.0（UV） 100.0（FL）	化合物は、10個の習熟化合物を使用。反応したサンプルをUV検出器およびFL検出器で同時測定

5. ADRA を用いた皮膚感作性評価手順

ADRA を用いた感作性評価フローを、図 3 に示した。被験物質が、分子量既知の単一化学物質の場合は、4 mM 被験物質溶液を調製し、ADRA（4 mM）を実施する。また、組成が既知の混合物および多成分構成物質は、それぞれの構成成分の分子量および濃度から見かけの分子量を算出し、その分子量を用いて 4 mM の被験物質溶液を調製し、ADRA（4 mM）を実施する。見かけの分子量の算出方法の例は Annex 4 に示した。分子量不明の単一化合物、組成が不明の混合物および多成分構成物質、ポリマーは、0.5 mg/mL の被験物質溶液を調製し、ADRA（0.5 mg/mL）を実施する。固体の混合物の場合は、適切な溶媒を選択し、0.5 mg/mL 被験物質溶液を調製する。液体の混合物の場合は、溶媒に溶解している混合物の重量濃度を基にして、TG に規定されている溶媒で 0.5 mg/mL に調製する。なお、液体混合物において、混合物の成分が溶解している溶媒が ADRA の TG に記載されていない場合は、その溶媒が、ADRA 試験系に影響がないことを示す必要がある。被験物質溶液と求核試薬を反応させ、反応停止液を添加したサンプルは、UV 検出器を用いた ADRA-UV 法で測定し、その値を ADRA の測定値とする。もし、ADRA-UV 法で共溶出が生じた場合は、ADRA-FL 法で測定

した値を採用してもよい。また、あらかじめ UV および FL 検出器を装備した HPLC システムで UV 値と FL 値を同時測定することも可能である。ただし、その場合には、上記のような理由がない限り、ADRA-UV 法で測定した値を採用する。混合物の感作性評価について、OECD は、KE1 の代替法については、なるべくモル濃度で被験物質を調製することを推奨している[4]。分子量が分からない混合物についても、大部分を占める成分の分子量が分かれば、その分子量を使用して被験物質溶液の調製が可能であることが記載されている（OECD TG442C　Appendix　Ⅱ、ADRA、段落 16）。



ADRA, amino acid derivative reactivity assay; UV, ultraviolet; FL, fluorescence;

図 3　ADRA を用いた皮膚感作性評価フロー

6. 評価可能な物質の範囲

136 物質を対象に ADRA を実施した評価では、Annex 5、表 S5-1 および S5-2 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[20]。

LLNA で陽性と判定される 98 物質は、4 mM に調製した被験物質溶液を用いた ADRA（4 mM）では 23 物質、ADRA（1 mM）では 34 物質、および ADRA（0.5 mg/mL）では 24 物質が偽陰性と判定された。偽陰性と判定された物質のうち、Strong sensitizer に分類されるのは Hexyl salicylate のみであり、多くは LLNA で moderate あるいは weak に分類される化合物であることから、DPRA と同様に感作性ポテンシャルの弱い物質の一部に偽陰性判定が生じる可能性があることに留意する必要がある。

一部のプレハプテンは正しく判定されるが、プロハプテンおよびプレハプテンは、DPRA と同様に原理的に適用範囲外と考えられる。また、本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しており、非共有結合により複合体を形成する Nickel（II）sulfate などの化合物は、DPRA と同様に評価可能な物質の範囲から外れる。

7. 有用性と限界

本法は汎用 HPLC およ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、「動物の愛護および管理に関する法律」および 3Rs の精神と合致している。さらに 1 化合物あたりの試験消耗品費用を試算したところ、DPRA では約 1 万円であるのに対し、 ADRA では約 5 千円と半分の経費であった。一方、LLNA は 1 化合物当たりの消耗品費は約 10 万円である。被験物質の必要量が DPRA に比して少量（1/25）で済み、その秤量から反応開始までの時間（DPRA の約半分）や、用いる反応容器が DPRA ではガラス製オートサンプラー用バイアルであるのに対して ADRA では 96 穴マイクロタイタープレートであることなど、操作性にも勝り簡便である。また、TG 442D および 442E に収載されている *in vitro* の試験法は、細胞の前培養なども含めると複数週の時間が必要である。これに対して、ADRA の試験期間は 2 日から 3 日で実施可能であり、試験法として簡便性、経済性や迅速性などの面から有用と考えられる。

本法は水系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（Water、Acetonitrile、Acetone および 5% DMSO 含有 Acetonitrile）に 4 mM あるいは 0.5 mg/mL の濃度で溶解する必要がある。また、本法の正確度は、約 75%であり、1/4 の化合物の判定が誤って評価される可能性があるため、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。特に下記の化合物の類似化合物が陰性判定となった場合、偽陰性の可能性があるため、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 にあたる弱い感作性物質 [19)]

LLNA で weak sensitizer に分類される 12 物質（α-Hexylcinnamaldehyde、α-Amylcinnamaldehyde、Oxalic acid、Benzyl benzoate、Benzyl cinnamate、N,N-Dibutylaniline、Phenyl benzoate、Cinnamyl alcohol、Benzocaine、Linalool、d,l-Citronellol および Aniline）と Moderate sensitizer に分類される 10 物質（Ethylenediamine、Methyl pyruvate、Benzyl salicylate、Diethyl sulfate、Tropolone、4-Chloroaniline、10-Undecenal、12-Bromo-1-dodecanol、dl-α-Tocopherol および 5-Methyl-2-phenyl-4H-pyrazol-3-one）は、ADRA では陰性となるため、これらの化合物の類似物が陰性判定の場合、注意が必要である。

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（例：Ethylenediamine、Cinnamyl alcohol、Benzocaine など）は偽陰性と評価される可能性がある。

3）金属塩

本試験系は、KE1 すなわちタンパク質と共有結合する可能性のある化合物を検出する試験系であり、金属塩は、共有結合以外の機序によりタンパク質と反応することが知られているため、本試験法は適用外となる。ただし、一部の金属塩（塩化コバルトなど）は本試験系において陽性になることが確認されている。

4）疎水性の高い物質等

水系での反応を必要とする本法では、被験物質が反応液中で析出した場合、陽性の結果であれば、その判定を受け入れることは可能であるが、陰性の結果の場合、被験物質が十分に反応していない可能性があるので、陰性結果をそのまま受け入れることはできず、十分な考察が必要である。ADRA は、反応に必要な被験物質溶液の濃度が 4 mM（または 0.5 mg/mL）であり、DPRA の 100 mM と比較すると 1/10 以下と低い濃度のため、反応液中で被験物質が析出することは稀である。白濁した場合も析出した場合と同様に、陽性結果であれば、その判定を受け入れることは可能であるが、陰性結果はそのまま受け入れることはできず、十分な考察が必要である [9]。

以上のことから、DPRA を含む他の代替法と同様に、ADRA 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、必要に応じて補完し得る他の KE を対象にした代替法等により確認する必要がある。ADRA 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、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。ADRA は感作性発現機序における初期の重要な事象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代替法と組み合わせでの評価を推奨する [25-27]。

8. 結論

ADRA は、簡便性・経済性の面から有用な動物実験代替法である。ADRA は、当初、1 mM に調製した被験物質を用いた試験法のバリデーション研究が、40 化合物を対象にして実施された。DPRA のバリデーション研究と ADRA のバリデーション研究は、施設数、化合物の種類および化合物数が異なるため、直接比較することはできないが、ADRA の施設内および施設間再現性は、90～100%および 91.9%であり、DPRA（施設内：87～100%、施設間：75%）よりも高い。実際に、本試験に使用する濃度である 4 mM あるいは 0.5 mg/mL に調製した被験物質溶液を用いた ADRA のバリデーション研究は、1 mM の ADRA で施設内および施設間の再現性が十分に検証された後に、10 化合物あるいは 8 化合物の習熟度確認物質を対象に実施されており、両濃度とも施設内および施設間再現性は 100%であった。

本試験法（被験物質溶液濃度：4 mM および 0.5 mg/mL）のバリデーション研究における感度、特異度および正確度はすべて 100%であるが、化合物数が少ないので、リード施設が実施した 136 化合物の試験データに注目すると、LLNA の結果に対する ADRA（4 mM）の感度、特異度および正確度は、76%、79%および 76%であり、ADRA（0.5 mg/mL）の感度、特異度および正確度は、74%、79%および 76%であった。また、ヒトの結果に対する ADRA（4 mM）の感度、特異度および正確度は、83%、86%および 84%であり、ADRA（0.5 mg/mL）の感度、特異度および正確度は、81%、86%および 83%であった（Annex 5、表 S5-1）。このように、LLNA の結果に対する ADRA（4 mM）および ADRA（0.5 mg/mL）の正確度、感度および特異度は約 75%、ヒトの結果に対する ADRA（4 mM）および ADRA（0.5 mg/mL）の正確度、感度および特異度は、約 85%である。

ADRA は感作性発現機序における初期の重要な KE 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。ADRA の 1 化合物あたりの消耗品の費用は、LLNA の 1/20 程度、DPRA の 1/2 の経費で、短期間で

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、必要に応じて、細胞を用いる他の KE を対象にした代替法や QSAR 等の *in silico* 手法と組み合わせでの評価を推奨する。

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Annex 1.　ADRA を用いた皮膚感作性評価における試験法の変遷

ADRA では、さまざまな濃度の被験物質溶液を使用して、その感作性について評価されてきた。ADRA は、DPRA と同じ原理に基づく試験方法として開発されたため、当初、感作性評価は、DPRA と同じ 100 mM の被験物質溶液を使って評価された[12]。しかしながら、求核試薬の検出感度が高いため、100 mM の被験物質溶液を用いる ADRA では、反応後のサンプルを希釈してから HPLC にかける必要があった。100 mM の被験物質溶液の濃度では、難溶解性被験物質を評価する際に、反応液中で被験物質析出が危惧されるだけでなく、HPLC 測定毎に希釈作業を要する。そのため、適切な被験物質の濃度を決めるために、DPRA 法を確立するために使用された 82 化合物のデータセット[7]を用いて検討したところ、1 mM の被験物質溶液を用いる ADRA（1 mM）法が DPRA と同等の予測精度を示したため、ADRA（1 mM）を標準法とした[13]。これにより、被験物質溶液および求核試薬の濃度は、当初の 1/100 に減少し、反応溶液中の被験物質の析出頻度[9]や HPLC 分析における共溶出[10]の頻度を著しく低減することができた。2016 年に、この ADRA（1 mM）を OECD TG に収載するためのバリデーション研究を始めるために、バリデーション参加施設に技術移転試験を実施したところ、ほとんどの参加施設で求核試薬 NAC の二量体の増加が認められた。その原因を探索したところ、洗浄したガラス器具に残留した成分が NAC の二量体化を促進したことが疑われたため、実験に使用するすべての器具をディスポーザブルの製品（ポリプロピレン製）に変えた。その後も、一部の施設で NAC の二量体の増加が発生し、検討の結果、精製水中の銅イオンの増加が関わっていることが明らかになった。それゆえ、金属キレートであるエチレンジアミン四酢酸（EthyleneDiamineteTraacetic Acid : EDTA）が 0.25 μM 含まれる条件は、0.01 ppm 銅イオンによる NAC の二量体化を抑制し、EDTA 自体が ADRA の反応に影響を与えないことが確認された[11]。この条件（被験物質濃度；1 mM、0.25 μM EDTA）がバリデーション研究に採用され、2019 年に OECD TG に収載された。

2019 年に発行された TG 442C（段落 3、Appendix 1（DPRA））には、DPRA で混合物の感作性評価を可能にするためには、重量濃度で調製した被験物質溶液による試験法の開発が必要であると記載されていた。そのため、ADRA において、重量濃度で調製した被験物質溶液を用いた重量濃度法を確立するために、0.05、0.1、0.2、0.5 mg/mL の濃度で被験物質溶液を調製し、上記 82 化合物を用いて ADRA を実施し、DPRA と ADRA（1 mM）の結果を比較した[19]、被験物質の濃度を 0.05 mg/mL から 0.5 mg/mL に上げるにしたがって、偽陰性の頻度が減少し（偽陽性の頻度は若干増加）、感作性予測精度は、被験物質溶液の濃度が 0.5 mg/mL の時に ADRA（1 mM）よりも数%高く、DPRA と同程度であった[19]。これにより、ADRA を重量濃度で実施する場合の被験物質溶液の最適濃度は、0.5 mg/mL であることが示された。しかし、この検討結果は、すでに TG 化された ADRA（1 mM）の被験物質濃度が最適ではないという疑念を生じさせた。その後実施した、プレ/プロハプテンを含む 136 種類の化学物質を含むデータセットでは、ADRA（1 mM）の予測精度は DPRA よりも低く[19]、その原因を解析したところ、NAC/NAL の減少率が、閾値よりわずかに低く、偽陰性と判定される化学物質が多いことが、原因であることが判明した。ADRA（0.5 mg/mL）の結果からも分かるように、ADRA を実施する被験物質溶液の濃度は、1 mM より高い方が、偽陰性化合物の数が減少し、予測精度も高くなると予想された。したがって、ADRA の被験物質溶液の最適モル

濃度を決定するために、ADRA（1 mM）で偽陰性と誤って判定された 8 化合物について、2、3、4 および 5 mM の被験物質溶液を用いて、ADRA を実施したところ、4 mM 以上ですべての化合物が正しく陽性と判定された。そこで、4 mM に調製した被験物質溶液を用いて、136 化合物について感作性評価を実施したところ、予測精度が向上することが確認された [20]。また、ADRA は従来の UV 検出の他に FL 検出が可能であり、NAC/NAL を特異的に検出することができることから、被験物質由来の成分と共溶出することがほとんどない [14,15]。これらの結果から、分子量既知の単一化合物については 4 mM の被験物質溶液を用い、分子量不明の単一物質および混合物については、0.5 mg/mL に調製した被験物質溶液および ADRA-FL 法を用いることが提案され、2019 年から 10 種類の習熟度確認物質について、リード施設を含む 5 施設で、施設内・施設間の再現性を評価するバリデーション研究が実施された。このバリデーション結果については、OECD の感作性専門家会議で審査され、コメンティングラウンドを経て、2022 年 4 月に、2 つの濃度の被験物質溶液（4 mM および 0.5 mg/mL）および FL 検出器を用いた ADRA-FL 法を含む改定案が、OECD 会議において議論され、6 月に承認・公表された。

Annex 2.　試験手順／判定 [28)]

2-1.　使用する求核試薬および陽性対照の調製

・NAC 含有リン酸緩衝溶液（pH 8.0）

NAC（純度：98%以上）は、リン酸緩衝液（pH 8.0）に 6.667 μM の濃度になるように溶解させる。

・NAL 含有リン酸緩衝溶液（pH 10.2）

NAL（純度：98%以上）は、リン酸緩衝液（pH 10.2）に 6.667 μM の濃度になるように溶解させる。

・陽性対照：

Phenylacetaldehyde（PADH、CAS No:122-78-1、純度≧ 90%）

PADH を、アセトニトリルに 4 mM の濃度で溶解させる。

Scuaric acid diethyl ester（SADE、CAS No:5231-87-8、純度≧95%）

SADE を、アセトニトリルに 4 mM の濃度で溶解させる。

※陽性対照物質は、PADH と SADE のどちらかを実施すればよい。ただし、PADH はアルデヒドのため化合物の安定性が良くない。そのため、NAC/NAL の減少率の値が管理幅に入らなくなることがあるので、注意が必要である。被験物質を重量濃度で調製する場合も、陽性対照物質は 4 mM に調製して使用する。

2-2.　各反応液の調製および反応手順

各被験物質、陽性対照物質、および各種クオリティチェック用コントロールは、下記のように調製する。

NAC：被験物質 or 陽性対照物質=1:200 5 μM NAC，4 mM 被験物質（or 0.5 mg/mL） ※or 4 mM 陽性対照物質	NAL：被験物質 or 陽性対照物質=1:200 5 μM NAL，4 mM 被験物質（or 0.5 mg/mL） ※or 4 mM 陽性対照物質
■150 μL NAC 溶液 ■50 μL 被験物質溶液 or 陽性対照物質溶液	■150 μL NAL 溶液 ■50 μL 被験物質溶液 or 陽性対照物質溶液

※反応液中の被験物質の濃度；　ADRA （4 mM）: 1 mM、ADRA （0.5 mg/mL）: 0.125 mg/mL

クオリティチェック用コントロール

【共溶出コントロール】

NAC 用	NAL 用
■150 μL リン酸緩衝液（pH 8.0） ■50 μL 被験物質溶液	■150 μL リン酸緩衝液（pH 10.2） ■50 μL 被験物質溶液

【参照コントロール A および B】

NAC 用	NAL 用
■150 μL NAC 溶液 ■50 μL アセトニトリル	■150 μL NAL 溶液 ■50 μL アセトニトリル

【参照コントロール C】

NAC 用	NAL 用
■150 µL NAC 溶液 ■50 µL アセトニトリルおよび被験物質の溶解に用いた溶媒*	■150 µL NAL 溶液 ■50 µL アセトニトリルおよび被験物質の溶解に用いた溶媒*

*：被験物質の溶解に用いた溶媒がアセトニトリルの場合は、アセトニトリルのみで良い。

以下のフローチャートに従い、実施する。

1. 被験物質溶液の調製
【分子量が既知の被験物質】
以下のいずれかの溶媒を使用して、80 mMの濃度に溶解させる[a)]
【混合物、ポリマー、分子量不明の被験物質】
以下のいずれかの溶媒を使用して、10 mg/mLの濃度に溶解させる[a)]
液体の混合液およびポリマーは、混合物とそれ以外の溶媒量から混合物の重量濃度を算出する。
溶媒：Water, Acetonitrile, Acetone, 5% DMSO/Acetonitrile[b)]（これら以外の溶媒を使用する場合は、溶媒が反応に影響を及ぼさないことを確認しておくこと）

2. 80 mMあるいは10 mg/mLの被験物質溶液を溶媒で20倍希釈する（4 mM or 0.5mg/mL）。液体の混合液は、総混合物とそれ以外の溶媒成分から混合物の重量濃度を算出し、適切な溶媒で、0.5 mg/mLに調製する。

3. 被験物質溶液とNAC or NAL含有リン酸バッファーを1:3の割合で混合（n=3）
・Sample：被験物質溶液（4 mM or 0.5mg/mL）+ 6.67 µM NAC or NAL含有リン酸バッファー[c)]
・Co-elusion Control：被験物質溶液 + リン酸バッファー[c)]
・Reference Control：溶媒 + 6.67 µM NAC or NAL含有リン酸バッファー[c)]

4. 各混合液を24 ± 1時間インキュベートする（暗所、25±1℃）

5. 吸光検出器および蛍光検出器を装備したHPLCで、NAC or NALを分析する（反応後、72時間以内に測定）
吸光度と蛍光強度を同時測定することが推奨される（吸光度値を優先的に採用）

6. 未反応のNACあるいはNALのピーク面積から、NACあるいはNALの減少率（depletion）を算出する。

$$\text{NACあるいはNALの減少率} = \left[1 - \left[\frac{\text{反復注入におけるNACあるいはNALのピーク面積}}{\text{基準対照CにおけるNACあるいはNALのピーク面積}}\right]\right] \times 100$$

a) 正確に、被験物質を秤量できる場合は、直接、4mM（分子量既知化合物）あるいは0.5 mg/mL（混合物、ポリマー）を調製しても良い。
b) DMSOを用い80 mMを調製し、アセトニトリルで20倍希釈して、4 mMを調製する（DMSOの含有濃度は、5%）
c) NACのリン酸バッファーは、pH 8.0、NALのリン酸バッファーは、pH 10.2

*分析条件（推奨）
推奨カラム：コアシェルタイプシリカゲル（2.5 - 2.7 µm、3.0 mm×150 mm）等
例）Wakopak Core C18 ADRA（φ3.0 × 150 mm、富士フイルム和光純薬）
カラム温度：40℃
サンプル温度：25℃（冷却機能がある場合、4℃にすることを推奨）
検出波長： 吸光度：281 nm、 蛍光（Excitation / Emission）：284/333 nm
流速：0.3 mL/min
注入量：10～20 µL（装置によって変更可能、ピーク形に応じて変更可能）
移動相： A 液：0.1%（v/v）TFA（トリフルオロ酢酸）水溶液
B 液：0.1%（v/v）TFA 含有アセトニトリル溶液

HPLC 条件

時間	NAC 溶出条件		NAL 溶出条件	
（分）	A 液（%）	B 液（%）	A 液（%）	B 液（%）
0	70	30	80	20
9.5	45	55	55	45
10	0	100	0	100
13	0	100	0	100
13.5	70	30	80	20
20	終了		終了	

2-3. 試験成立の条件 [4)]

試験成立には、以下の条件を満たさなければならない。

1) NAC/NAL のそれぞれを用いて、0.156 ~ 5.0 μM の範囲の 6 濃度にて標準曲線の作成を行い、その寄与率が 0.990 より大きい。
2) 被験物質の NAC/NAL（n=3）の減少率の標準偏差が、10%未満である。
3) 陽性対照である PADH または SADE の NAC/NAL の n=3 の減少率の平均値および標準偏差は下記の条件を満たす必要がある。
   ・NAC 減少率（n=3 の平均値）
   PADH : 30 - 80%、SADE: 30 - 80%
   ・NAL 減少率（n=3 の平均値）
   PADH : 70 - 100%、SADE: 70 - 100%
   ・減少率の標準偏差（SD）
   PADH の NAC/NAL ともに、10%未満
   SADE の NAC/NAL ともに、10%未満
4) NAC/NAL のそれぞれに 3 種類の参照コントロール（A、B、C）を設け、以下を確認する。
   参照コントロール A（n=3）：分析前の HPLC システム適合性
   参照コントロール B（n=6）：分析時間中の参照コントロールの安定性
   参照コントロール C（n=3）：使用された溶媒が NAC/NAL の減少に影響しないこと

   ・参照コントロール A および C の NAC/NAL の平均濃度が、3.2～4.4 μM※の範囲に入っていること。
   ※溶媒に 5% DMSO/アセトニトリルを使用した場合は、DMSO による NAC の SH 基の酸化により、NAC 二量体が産生され、NAC 濃度が減少することが知られているので、参照コントロール C の NAC の濃度の平均値は、2.8 ～ 4.0 μM であること [29)]。
   ・参照コントロール B（n=6）とアセトニトリルで調製した参照コントロール C（n=3）を合わせた 9 回について、NAC/NAL の平均ピーク面積、SD および CV（変動係数）を計算し、CV は、10%未満であること。

・各溶媒における参照コントロール C(n=3)の NAC/NAL ピーク面積の CV は、10%未満であること。

2-4. 評価

被験物質の反応性は、測定ごとの NAC/NAL の減少率を算出し、NAC/NAL の減少率の平均値が、閾値である 4.9%を基準に感作性および非感作性を判定する(表 S2-1)。基本的には、試験成立基準を満たしており、信頼性に影響を与える事象がなければ、1 回の測定で最終判定をすることができる。ただし、NAC/NAL の減少率の平均値が、閾値に近い場合は、判定結果が正しくない可能性があることから、確認試験を実施して判定結果の是非を確認する。つまり、NAC/NAL の減少率の平均値が、「3% ≤NAC/NAL の減少率の平均値 ≤10%」となった場合は、2 回目の試験を実施する。1 回目と 2 回目の判定結果が同じ場合は、その判定が最終判定となる。しかし、1 回目と 2 回目の判定結果が異なった場合は、さらに 3 回目の試験を実施し、3 回の判定結果の多数決により判定する。

ADRA は、検出波長が 281 nm のため、DPRA と比較して NAC あるいは NAL と被験物質の溶出時間が重なる共溶出が起こりにくい。また、FL 検出器により、NAC および NAL の特異的な検出ができることから、共溶出が起こることは極めて稀である。しかし、NAC の共溶出が、UV 検出器および FL 検出器の両方で認められた場合は感作性を評価できないが、NAL のみで共溶出が認められた場合は、NAC 単独モデルで評価が可能である。NAC 単独モデルは、NAC の減少率が、NAC 単独モデルの閾値である 5.6 を基準に感作性および非感作性を判定する(表 S2-2)。ただし、NAC の減少率の平均値が、閾値に近い場合は、判定結果が正しくない可能性があることから、確認試験を実施して判定結果の是非を確認する。つまり、NAC の減少率の平均値が、「4% ≤NAC の減少値 ≤11%」に適合する場合は、2 回目の試験を実施する。1 回目と 2 回目の判定結果が同じ場合は、その判定が最終判定となる。しかし、1 回目と 2 回目の判定結果が異なった場合は、3 回目の試験を実施し、3 回の判定結果の多数決により判定する。

表 S2-1　ADRA 感作性予測モデル

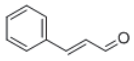
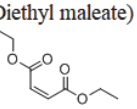
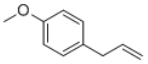
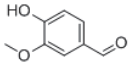
NAC/NAL の減少率の平均	**判定**
< 4.9%	非感作性物質
4.9% ≦	感作性物質

表 S2-2　ADRA NAC 感作性単独予測モデル

NAC の 減少率	**判定**
< 5.6%	非感作性物質
5.6% ≦	感作性物質

Annex 3.　バリデーション結果

表 S3-1　ADRA（1 mM）のプレトレーニング試験の試験計画の概要

試験期間	2016年6月				
化合物	PT-001 (*p*-Benzoquinone) 	PT-002 (Cinnamaldehyde) 	PT-003 (Diethyl maleate) 	PT-004 (4-Allylanisole) 	PT-005 (Vanilline) 
LLNA potency	Strong	Moderate	Moderate	Weak	Non-sensitizer
試験数	2回（各試験　N=3）				
目　標	富士フイルムの予測結果と一致すること（施設内再現性：100%，施設間再現性：100%）				

■：Sensitizer　　□：Non-sensitizer

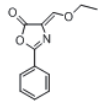
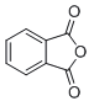
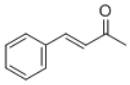
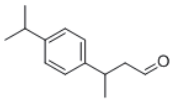
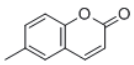
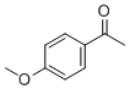
表 S3-2　ADRA（1 mM）のプレトレーニング試験の試験結果

コード	化合物	LLNA potency	予測結果（カッコ内は減少率のAverage scoreを示す）														
			A			B			C			D			富士フイルム		
			1回目	2回目	平均値	1回目	2回目	平均値	1回目	2回目	平均値	1回目	2回目	平均値	1回目	2回目	平均値
PT-001	*p*-Benzoquinone	Strong	S (89.2)	S (89.0)	**S (89.1)**	S (84.3)	S (84.2)	**S (84.3)**	S (85.9)	S (86.3)	**S (86.1)**	S (86.5)	S (89.7)	**S (88.1)**	S (86.7)	S (88.0)	**S (87.3)**
PT-002	Cinnamaldehyde	Moderate	S (27.3)	S (49.2)	**S (38.2)**	S (26.6)	S (21.5)	**S (24.0)**	S (49.5)	S (49.2)	**S (49.4)**	S (28.4)	S (52.5)	**S (40.4)**	S (13.9)	S (15.6)	**S (14.7)**
PT-003	Diethyl maleate	Moderate	S (16.9)	S (17.9)	**S (17.4)**	S (11.7)	S (12.8)	**S (12.2)**	S (18.5)	S (14.4)	**S (16.4)**	S (16.7)	S (15.7)	**S (16.2)**	S (10.5)	S (11.4)	**S (10.9)**
PT-004	4-Allylanisole	Weak	S (12.8)	S (17.0)	**S (14.9)**	S (6.6)	S (8.1)	**S (7.3)**	S (10.0)	S (9.3)	**S (9.6)**	S (7.3)	S (7.0)	**S (7.2)**	S (7.0)	S (8.5)	**S (7.8)**
PT-005	Vanilline	Non-sensitizer	NS (1.4)	NS (3.5)	**NS (2.4)**	NS (0.0)	NS (0.0)	**NS (0.0)**	NS (4.8)	NS (4.0)	**NS (4.4)**	NS (4.4)	NS (2.5)	**NS (3.4)**	NS (0.6)	NS (0.3)	**NS (0.4)**
施設内再現性 (%)			100			100			100			100			100		
施設間再現性 (%)			100														

減少率：感作性物質　≧5.05　　非感作性物質　＜5.05（プレトレーニング試験およびトレーニング試験では閾値として 5.05 が用いられた。表 S3-5 以降のバリデーション試験では閾値が 4.9 に変更された。）

■：Sensitizer　　□：Non-sensitizer

表 S3-3　ADRA（1 mM）のトレーニング試験の試験計画の概要

試験期間	2016年7月～8月				
評価困難ポイント	1. 陰性/陽性の閾値に極めて近い化合物を2種類（T-004，T-006）含む。 2. 陰性/陽性の閾値に比較的近い化合物を2種類（T-003，T-005）含む。 3. わずかにNACと共溶出する化合物を1種類（T-010）含む。				
化合物	T-001 (Oxazolone) 	T-002 (Phthalic anhydride) 	T-003 (Formaldehyde) 	T-004 (Glyoxal) 	T-005 (Benzylideneacetone) 
LLNA potency	Strong	Strong	Strong	Moderate	Moderate
化合物	T-006 (Cyclamen aldehyde) 	T-007 (1-Butanol) 	T-008 (6-Methylcoumarin) 	T-009 (Lactic acid) 	T-010 (4'-Methoxyacetophenone) 
LLNA potency	Weak	Non-sensitizer	Non-sensitizer	Non-sensitizer	Non-sensitizer
試験数	2回（各試験　N=3）				
目　標	施設内再現性：70%以上，施設間再現性70%以上				

：Sensitizer　　：Non-sensitizer

表 S3-4　ADRA（1 mM）トレーニング試験の施設内および施設間再現性試験の結果

コード	化合物	LLNA potency	予測結果（カッコ内は減少率のAverage scoreを示す）														
			A			B			C			D			富士フイルム		
			1回目	2回目	平均値*	1回目	2回目	平均値*	1回目	2回目	平均値*	1回目	2回目	平均値*	1回目	2回目	平均値*
T-001	Oxazolone	Strong	S (74.3)	S (73.2)	**S (73.7)**	S (75.4)	S (75.3)	**S (75.4)**	S (80.1)	S (77.0)	**S (78.6)**	S (77.1)	S (74.9)	**S (76.0)**	S (77.2)	S (77.7)	**S (77.5)**
T-002	Phthalic anhydride	Strong	S (44.2)	S (40.8)	**S (42.5)**	S (25.7)	S (29.2)	**S (27.5)**	S (43.5)	S (40.4)	**S (41.9)**	S (45.2)	S (45.9)	**S (45.4)**	S (47.4)	S (47.3)	**S (47.3)**
T-003	Formaldehyde	Strong	S (11.7)	S (9.3)	**S (10.5)**	S (15.5)	S (8.0)	**S (11.8)**	S (20.1)	S (8.4)	**S (14.2)**	S (10.0)	S (9.3)	**S (9.7)**	S (9.6)	S (9.6)	**S (9.6)**
T-004	Glyoxal	Moderate	S (6.8)	S (10.3)	**S (8.5)**	S (6.6)	S (7.9)	**S (7.2)**	S (6.7)	S (5.4)	**S (6.0)**	S (9.4)	S (15.2)	**S (12.3)**	S (5.4)	S (6.0)	**S (5.7)**
T-005	Benzylideneacetone	Moderate	S (7.7)	S (8.3)	**S (8.0)**	S (8.0)	S (7.6)	**S (7.8)**	S (9.0)	S (10.3)	**S (9.7)**	S (11.6)	S (7.4)	**S (9.5)**	S (9.3)	S (14.1)	**S (11.7)**
T-006	Cyclamen aldehyde	Weak	NS (3.5)	NS (2.5)	**NS (3.0)**	S (12.3)	S (9.4)	**S (10.9)**	S (5.7)	S (10.7)	**S (8.2)**	S (6.5)	S (6.6)	**S (6.5)**	NS (1.9)	NS (2.0)	**NS (1.9)**
T-007	1-Butanol	Non-sensitizer	NS (3.2)	NS (1.9)	**NS (2.6)**	NS (0.3)	NS (1.2)	**NS (0.7)**	NS (0.1)	NS (1.6)	**NS (0.8)**	NS (0.0)	NS (0.7)	**NS (0.3)**	NS (0.0)	NS (0.0)	**NS (0.0)**
T-008	6-Methylcoumarin	Non-sensitizer	NS (0.3)	NS (0.2)	**NS (0.2)**	NS (2.4)	NS (1.6)	**NS (2.0)**	NS (2.4)	NS (4.9)	**NS (3.7)**	NS (1.1)	NS (2.8)	**NS (1.9)**	NS (0.1)	NS (0.4)	**NS (0.2)**
T-009	Lactic acid	Non-sensitizer	NS (2.6)	NS (1.1)	**NS (1.8)**	NS (0.6)	NS (1.9)	**NS (1.3)**	NS (2.8)	NS (2.0)	**NS (2.4)**	NS (0.1)	NS (0.1)	**NS (0.1)**	NS (0.0)	NS (0.0)	**NS (0.0)**
T-010	4'-Methoxyacetophenone	Non-sensitizer	NS (3.6)	NS (2.3)	**NS (3.0)**	S (6.0)	NS (3.8)	**NS (4.9)**	S (9.7)	S (12.2)	**S (10.9)**	NS (1.2)	NS (1.9)	**NS (1.6)**	NS (0.8)	NS (0.9)	**NS (0.8)**
施設内再現性 (%)			100			90.0			100			100			100		
施設間再現性(全化合物) (%)			80.0														
施設間再現性(T-010以外) (%)			88.9														

* 平均値は、2回の結果の平均をもとに判定

減少率：感作性物質　≧5.05　　非感作性物質　＜5.05（プレトレーニング試験およびトレーニング試験では閾値として 5.05 が用いられた。表 S3-5 以降のバリデーション試験では閾値が 4.9 に変更された。）

：Sensitizer　　：Non-sensitizer

表 S3-5　ADRA（1 mM）の施設内および施設間再現性試験の結果

No.	Chemicals	LLNA Category	DPRA result	Lab. A			Lab. B			Lab. C			Lab. D		
				Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
Phase I															
1	Lauryl gallate	Strong	S	97.8	96.3	96.3	59.3	58.5	96.8	83.0	60.3	71.6	96.3	96.4	96.8
2	4-(Methylamino) phenol hemisulfate salt	Strong	S	56.6	55.9	56.5	58.0	58.1	56.0	57.9	57.7	56.2	56.2	56.5	56.6
3	Chloramine T	Strong	S	94.1	95.0	96.3	99.0	99.3	99.5	97.7	97.5	96.5	50.0	54.1	55.6
4	Cinnamaldehyde	Moderate	S	30.8	28.2	15.1	21.0	22.5	22.7	54.0	–	36.2	24.7	16.1	26.6
5	2-Mercaptobenzothiazole	Moderate	S	25.0	22.1	21.7	27.0	27.7	27.5	24.5	–	40.6	50.0	44.2	50.0
6	Ethyl acrylate	Weak	S	45.7	45.7	46.1	46.7	48.1	47.0	48.8	25.1	48.9	51.2	50.3	53.1
7	Imidazolidinylurea	Weak	S	14.9	10.1	11.5	10.2	11.5	10.2	12.3	15.1	15.1	13.0	14.7	19.8
8	Glycerol	NC	NS	0.3	0.1	0.6	0.1	0.0	0.1	0.2	2.8	2.9	0.6	1.1	4.8
9	Salicylic acid	NC	NS	1.5	1.4	1.8	2.8	2.0	4.9	0.6	–	6.1	2.8	1.3	9.0
10	Isopropanol	NC	NS	0.0	0.0	0.0	0.5	0.0	0.1	0.3	2.5	3.0	0.4	1.9	4.8
Phase II															
11	Diphenylcyclopropenone	Extreme	S	18.2			14.2			17.9			13.5		
12	p-Benzoquinone	Extreme	S	81.1			84.2			79.3			72.2		
13	Hydroquinone	Strong	S	62.7			62.9			70.6			64.4		
14	2-Methyl-2H-isothiazol-3-one	Strong	S	50.2			50.0			50.2			48.4		
15	2-Aminophenol	Strong	S	68.2			66.0			66.5			63.3		
16	Iodopropynyl butylcarbamate	Strong	S	50.3			47.2			50.2			50.0		
17	Propyl gallate	Strong	S	86.5			82.0			84.3			84.2		
18	Dihydroeugenol	Moderate	NS	4.5 / 6.5 / 5.6			2.9			5.1 / 5.2			2.2		
19	Benzylsalicylate	Moderate	NS	0.8			0.4			1.9			0.0		
20	Squaric acid diethylester	Moderate	NS	47.7			48.4			46.0			34.5		
21	citral	Moderate	S	4.6 / 6.9 / 6.5			5.1 / 2.4 / 6.3			6.1 / 0.0 / 2.8			3.4 / 8.3 / 11.9		
22	Palmitoyl Chloride	Moderate	S	46.5			27.5			29.9			47.8		
23	Resorcinol	Moderate	NS	2.8			0.6			6.9 / 3.8 /4.6			3.0 / 4.4		
24	Benzylcinnamate	Weak	NS	0.8			0.0			0.5			0.1		
25	2,3-Butanedione	Weak	S	15.2			17.8			19.5			34.5		
26	Farnesol	Weak	NS	20.2			18.6			18.9			18.9		
27	Eugenol	Weak	S	12.2			16.1			19.1			10.8		
28	Penicilln G	Weak	S	1.2			0.0			3.0 / 1.4			0.2		
29	Lilial	Weak	S	7.1 / 7.0			13.8			4.9 / 6.2			6.6 / 15.0		
30	Hydroxycitronellal	Weak	S	3.9 / 10.5 / 6.4			11.3			6.4 / 5.4			3.7 / 7.0 / 4.6		
31	Benzyl alcohol	NC	NS	0.2			2.5			2.2			0.2		
32	Dimethylisophthalate	NC	NS	0.9			0.5			0.0			0.6		
33	4-Aminobenzoic acid	NC	NS	1.1			0.0			2.3			1.3		
34	Diethyl phthalate	NC	NS	0.8			0.0			0.0			0.1		
35	Methylsalicylate	NC	NS	0.6			0.0			0.5			0.0		
36	Dextran	NC	- [a)]	5.0 / 1.4 / 4.7			1.1			7.2 / 7.5			2.9		
37	Coumarin	NC	NS	4.6 / 2.7			0.8			1.8			0.1		
38	Propyl paraben	NC	NS	1.0			0.1			0.2			0.0		
39	Sulfanilamide	NC	NS	0.9			1.7			1.6			7.4 / 2.9 / 2.3		
40	Fumaric acid	NC	S	5.3 / 3.5 / 3.1			2.4			2.4			0.6		

：Sensitizer　　：Non-sensitizer

減少率：感作性物質　≧ 4.9　　非感作性物質　＜　4.9

Phase　Ⅱにおいて、NAC/NAL の減少率の平均値が閾値付近（3.0 -10.0）となった化合物について、追加試験を実施したため、複数の値が記載されている。

Lab.C で「－」の表記は、欠測データ（陽性対照物質の減少率が試験成立条件を満たしていなかったにも関わらず、再試験を実施しなかったため。

a)　Dextran は高分子であり、100 mM の被験物質溶液が調製できないため、DPRA では評価不能

表 S3-6-1　UV 検出および FL 検出による ADRA（0.5 mg/mL）バリデーション研究（NAC 減少率）

Test chemical	Depletion of NAC (%)														
	Lab.A			Lab.B			Lab.C			Lab.D			Lab.E		
	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set
Result of UV detection															
p-Benzoquinone	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0				97.5	100.0	100.0
Diphenylcyclopropenone	50.1	49.9	55.3	47.2	49.6	48.2	62.2	56.0	60.1				53.2	51.6	52.2
2-Methyl-2H-isothiazol-3-one	100.0	100.0	100.0	100.0	100.0	100.0	94.1	93.3	91.8				93.0	92.6	92.8
Palmitoyl Chloride	9.7	14.4	20.8	0.0	5.8	6.0	12.2	11.8	19.8				14.5	12.5	13.5
Imidazolidinyl urea	31.1	38.5	46.0	23.8	25.2	22.6	36.6	39.4	33.7				37.1	33.8	35.3
Farnesal	34.2	36.8	45.6	54.0	53.5	54.6	77.5	88.8	66.2				25.1	46.8	45.7
Glycerol	1.0	2.9	0.0	0.0	0.5	1.3	2.5	0.4	0.1				1.5	0.1	0.1
Isopropanol	1.2	0.0	0.0	0.0	0.0	0.3	1.6	2.0	0.0				0.3	0.0	0.3
Dimethyl isophthalate	0.0	3.6	1.2	1.6	0.4	0.2	3.5	4.2	3.3				1.8	0.9	0.0
Propyl paraben	0.0	3.7	0.7	2.8	0.0	1.2	7.5	3.9	3.2				1.2	0.2	0.0
Result of FL detection															
p-Benzoquinone	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Diphenylcyclopropenone	50.0	51.8	56.6	50.1	49.2	47.2	61.2	54.3	58.0	99.8	99.7	50.0	52.5	51.4	52.5
2-Methyl-2H-isothiazol-3-one	98.9	98.9	100.0	98.0	97.9	97.7	100.0	100.0	100.0	98.4	98.1	98.7	99.1	99.2	99.1
Palmitoyl Chloride	25.5	19.4	24.0	4.7	4.4	4.9	10.2	10.9	28.5	3.9	15.8	16.9	16.7	13.3	14.9
Imidazolidinyl urea	31.3	31.3	44.7	18.2	27.2	39.0	25.7	27.5	28.7	19.9	31.2	24.2	29.6	27.0	26.5
Farnesal	47.3	45.8	60.1	51.7	49.4	55.5	75.6	85.7	75.5	48.5	56.9	72.9	29.1	46.0	46.4
Glycerol	0.7	0.6	2.3	0.1	1.6	4.0	1.0	0.9	1.3	0.0	2.4	0.4	6.3	0.5	0.1
Isopropanol	1.3	3.1	2.4	2.5	0.7	0.3	0.4	4.7	0.6	0.0	3.3	0.7	0.6	0.3	0.1
Dimethyl isophthalate	5.5	0.0	1.7	1.5	0.1	1.8	3.1	2.3	0.0	0.0	3.1	0.0	3.2	0.7	1.0
Propyl paraben	3.4	3.2	0.7	1.4	0.4	3.2	7.3	2.6	0.0	0.0	5.2	0.0	3.0	0.3	1.3

表 S3-6-2　UV 検出および FL 検出による ADRA（0.5 mg/mL）バリデーション研究（NAL 減少率）

Test chemical	Depletion of NAL (%)														
	Lab.A			Lab.B			Lab.C			Lab.D			Lab.E		
	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set
Result of UV detection															
p-Benzoquinone	100.0	91.4	89.6	100.0	100.0	89.6	93.1	94.2	89.9				90.8	91.3	89.7
Diphenylcyclopropenone	1.9	9.1	7.0	2.4	2.6	1.4	2.0	8.2	6.8				4.3	3.2	3.2
2-Methyl-2H-isothiazol-3-one	0.2	0.0	1.3	0.0	0.0	0.0	0.0	0.5	1.5				0.0	0.0	0.0
Palmitoyl Chloride	98.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0				100.0	100.0	100.0
Imidazolidinyl urea	2.0	1.8	2.1	2.5	0.0	0.0	0.8	0.7	2.4				6.3	1.2	1.1
Farnesal	4.4	9.8	16.0	6.8	8.5	14.9	7.3	14.6	16.1				20.6	4.9	1.9
Glycerol	0.0	0.3	0.0	0.0	2.3	0.0	0.0	0.0	1.3				0.0	0.6	0.3
Isopropanol	0.2	0.8	0.0	0.0	0.2	0.0	0.0	0.1	0.5				0.0	0.6	0.0
Dimethyl isophthalate	0.0	0.2	4.1	0.0	0.0	0.0	0.0	5.1	4.6				0.0	0.3	0.0
Propyl paraben	0.0	4.9	4.5	0.0	0.0	0.0	0.0	4.5	4.8				0.0	0.5	0.0
Result of FL detection															
p-Benzoquinone	89.1	90.5	90.3	89.8	90.0	90.6	93.1	92.9	91.6	91.5	88.7	86.3	92.4	90.4	92.1
Diphenylcyclopropenone	0.0	6.7	6.8	3.6	2.3	2.6	2.9	8.0	4.1	0.0	7.6	0.8	4.4	3.8	6.6
2-Methyl-2H-isothiazol-3-one	0.0	0.8	5.2	1.6	0.2	0.1	0.3	0.2	3.3	0.0	11.0	0.0	0.2	0.6	1.8
Palmitoyl Chloride	98.2	99.9	99.9	97.6	100.0	100.0	100.0	100.0	100.0	98.8	92.9	99.9	100.0	100.0	100.0
Imidazolidinyl urea	2.0	0.0	5.4	0.0	0.0	0.0	2.6	1.3	7.7	0.0	9.2	0.0	4.1	0.7	1.9
Farnesal	5.9	5.2	17.7	9.3	9.1	17.0	10.3	20.5	22.5	11.1	20.3	19.3	24.4	8.9	10.7
Glycerol	1.4	0.0	4.0	0.0	4.8	0.0	1.0	0.1	7.5	0.0	0.3	0.0	1.7	0.7	1.4
Isopropanol	0.0	0.6	4.0	1.6	1.0	1.3	1.7	2.0	0.8	0.0	0.3	0.0	0.4	1.0	1.3
Dimethyl isophthalate	0.0	0.0	6.5	0.0	0.0	0.0	1.7	3.2	5.2	0.1	3.5	0.0	0.0	1.2	3.1
Propyl paraben	0.5	4.4	5.7	0.0	0.0	0.0	1.3	3.1	1.7	0.0	2.3	0.0	0.0	0.9	3.2

表 S3-6-3　UV 検出および FL 検出による ADRA（0.5 mg/mL）バリデーション研究（NAC/NAL 平均値）

Test chemical	Mean depletion (%)														
	Lab.A			Lab.B			Lab.C			Lab.D			Lab.E		
	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set
Result of UV detection															
p-Benzoquinone	100.0	95.7	94.8	100.0	100.0	94.8	96.6	97.1	94.9				94.1	95.7	94.9
Diphenylcyclopropenone	26.0	29.5	31.2	24.8	26.1	24.8	32.1	32.1	33.5				28.7	27.4	27.7
2-Methyl-2H-isothiazol-3-one	50.1	50.0	50.7	50.0	50.0	50.0	47.0	46.9	46.7				46.5	46.3	46.4
Palmitoyl Chloride	54.0	57.2	60.4	50.0	52.9	53.0	56.1	55.9	59.9				57.3	56.3	56.8
Imidazolidinyl urea	16.6	20.2	24.1	13.2	12.6	11.3	18.7	20.0	18.1				21.7	17.5	18.2
Farnesal	19.3	23.3	30.8	30.4	31.0	34.8	42.4	51.7	41.2				22.9	25.9	23.8
Glycerol	0.5	1.6	0.0	0.0	1.4	0.6	1.3	0.2	0.7				0.7	0.4	0.2
Isopropanol	0.7	0.4	0.0	0.0	0.1	0.1	0.8	1.0	0.2				0.2	0.3	0.2
Dimethyl isophthalate	0.0	1.9	2.7	0.8	0.2	0.1	1.7	4.6	3.9				0.9	0.6	0.0
Propyl paraben	0.0	4.3	2.6	1.4	0.0	0.6	3.8	4.2	4.0				0.6	0.3	0.0
Result of FL detection															
p-Benzoquinone	94.6	95.3	95.2	94.9	95.0	95.0	96.6	96.5	95.8	95.8	94.4	93.1	96.2	95.2	96.0
Diphenylcyclopropenone	25.0	29.3	31.7	26.9	25.7	24.9	32.1	31.2	31.1	49.9	53.7	25.4	28.4	27.6	29.6
2-Methyl-2H-isothiazol-3-one	49.5	49.9	52.6	49.8	49.1	48.9	50.1	50.1	51.6	49.2	54.5	49.4	49.7	49.9	50.5
Palmitoyl Chloride	61.9	59.7	62.0	51.1	52.2	52.4	55.1	55.4	64.3	51.3	54.3	58.4	58.3	56.7	57.5
Imidazolidinyl urea	16.7	15.7	25.1	9.1	13.6	19.5	14.2	14.4	18.2	10.0	20.2	12.1	16.9	13.9	14.2
Farnesal	26.6	25.5	38.9	30.5	29.2	36.2	43.0	53.1	49.0	29.8	38.6	46.1	26.8	27.5	28.6
Glycerol	1.1	0.3	3.2	0.0	3.2	2.0	1.0	0.5	4.4	0.0	1.4	0.2	4.0	0.6	0.8
Isopropanol	0.7	1.9	3.2	2.0	0.8	0.8	1.0	3.4	0.7	0.0	1.8	0.3	0.5	0.6	0.7
Dimethyl isophthalate	2.8	0.0	4.1	0.8	0.0	0.9	2.4	2.7	2.6	0.0	3.3	0.0	1.6	1.0	2.1
Propyl paraben	2.0	3.8	3.2	0.7	0.2	1.6	4.3	2.8	0.8	0.0	3.8	0.0	1.5	0.6	2.2

：Sensitizer 減少率 ≧4.9　　：Non-sensitizer 減少率 ＜4.9

表 S3-7-1　UV 検出による ADRA（4 mM）バリデーション研究

No.	Test chemicals	LLNA potency	depletion	Lab.A						Lab.B						Lab.C						Lab.D						Lab.E					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
1	Diphenylcyclopropenone	Extreme	depletion (%)	65.2	2.1	70.7	4.0	68.6	5.4	68.8	3.7	69.3	2.7	64.7	6.0	70.6	4.2	75.5	5.7	73.5	7.0	68.7	5.8	71.7	5.4	72.9	6.3	69.0	7.3	69.3	5.5	68.8	6.5
			mean % depletion	33.6		37.4		37.0		36.3		36.0		35.3		37.4		40.6		40.2		37.3		38.6		39.6		38.2		37.4		37.6	
2	2-Methyl-2H-isothiazol-3-one	Moderate	depletion (%)	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.6	100.0	0.0	100.0	0.1	100.0	0.0	100.0	4.8	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.2
			mean % depletion	50.0		50.0		50.0		50.3		50.0		50.1		50.0		52.4		50.0		50.0		50.0		50.0		50.0		50.0		50.1	
3	Palmitoyl Chloride	Moderate	depletion (%)	8.4	82.1	7.1	87.3	8.6	82.3	7.9	89.4	10.2	94.8	9.4	100.0	11.0	100.0	20.0	100.0	36.6	100.0	10.6	100.0	17.7	100.0	19.1	100.0	2.7	100.0	7.5	100.0	5.6	97.9
			mean % depletion	45.3		47.2		45.4		48.7		52.5		54.7		55.5		60.0		68.3		55.3		58.8		59.6		51.4		53.8		51.8	
4	Imidazolidinyl urea	Weak	depletion (%)	58.6	3.6	58.9	3.6	60.2	6.8	64.7	4.0	65.0	7.0	58.4	8.0	61.2	4.7	65.3	11.2	61.2	6.8	59.2	2.4	61.6	3.2	60.3	3.6	61.6	6.3	62.2	7.3	61.3	8.4
			mean % depletion	31.1		31.3		33.5		34.3		36.0		33.2		33.0		38.3		34.0		30.8		32.4		31.9		34.0		34.7		34.8	
5	Farnesal	Weak	depletion (%)	86.0	16.7	85.1	21.6	81.6	23.8	89.6	11.3	94.2	11.4	92.2	18.8	97.1	14.6	100.0	24.2	100.0	30.2	33.7	18.9	64.4	16.9	30.0	17.2	81.8	12.9	83.6	14.5	89.9	15.9
			mean % depletion	51.3		53.3		52.7		50.4		52.8		55.5		55.8		62.1		65.1		26.3		40.6		23.6		47.4		49.0		52.9	
10	Isopropanol	Non-sensitizer	depletion (%)	0.0	0.0	0.4	1.2	0.2	0.2	0.4	0.5	2.5	0.1	0.0	1.1	0.7	0.0	2.9	3.5	0.0	0.3	0.0	0.0	0.2	0.0	0.0	0.0	0.7	0.0	0.0	0.4	0.0	0.4
			mean % depletion	0.0		0.8		0.2		0.5		1.3		0.6		0.3		3.2		0.2		0.0		0.1		0.0		0.4		0.2		0.2	
11	Dimethyl isophthalate	Non-sensitizer	depletion (%)	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.2	0.0	1.6	0.0	0.0	1.4	0.4	2.2	1.5	0.7	0.5	1.2	0.0	0.0	0.0	0.0	0.4	0.1	0.1	0.1	0.0	0.0	0.0
			mean % depletion	0.0		0.0		0.3		0.1		0.8		0.0		0.9		1.8		0.6		0.6		0.0		0.2		0.1		0.1		0.0	
12	Propyl paraben	Non-sensitizer	depletion (%)	0.0	0.1	0.2	0.4	0.0	0.7	0.0	0.3	0.0	1.5	0.0	0.0	1.5	0.3	2.1	1.7	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.3	0.0	0.0	0.2
			mean % depletion	0.0		0.3		0.4		0.2		0.8		0.0		0.9		1.9		0.7		0.0		0.0		0.1		0.1		0.2		0.1	

：Sensitizer：減少率 ≧4.9　　：Non-sensitizer：減少率 ＜4.9　　：co-elution

表 S3-7-2　FL 検出による ADRA（4 mM）バリデーション研究

No.	Test chemicals	LLNA potency	depletion	Lab.A						Lab.B						Lab.C						Lab.D						Lab.E					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
1	Diphenylcyclopropenone	Extreme	depletion (%)	64.1	2.6	70.0	3.5	68.1	5.2	69.9	4.1	69.6	3.3	65.9	6.5	70.5	4.2	75.2	5.1	73.5	8.0	69.5	5.5	72.1	6.1	72.5	6.4	68.2	7.4	68.8	5.2	68.4	6.3
			mean % depletion	33.3		36.7		36.7		37.0		36.5		36.2		37.4		40.2		40.8		37.5		39.1		39.5		37.8		37.0		37.4	
2	2-Methyl-2H-isothiazol-3-one	Moderate	depletion (%)	99.2	0.1	99.2	0.1	99.3	0.3	100.0	1.2	100.0	0.3	100.0	1.4	99.2	0.1	99.2	4.5	99.1	0.9	99.3	0.0	99.2	0.0	99.2	0.0	100.0	0.0	99.5	0.6	99.6	0.9
			mean % depletion	49.6		49.7		49.8		50.6		50.1		50.7		49.7		51.9		50.0		49.7		49.6		49.6		50.0		50.0		50.3	
3	Palmitoyl Chloride	Moderate	depletion (%)	8.7	81.9	7.5	86.9	8.7	82.5	8.7	89.5	10.9	94.6	9.6	99.3	11.0	100.0	20.6	100.0	37.1	100.0	11.4	100.0	16.1	99.9	19.1	100.0	2.8	100.0	7.6	99.3	5.3	97.9
			mean % depletion	45.3		47.2		45.6		49.1		52.7		54.4		55.5		60.3		68.5		55.7		58.0		59.5		51.4		53.5		51.6	
4	Imidazolidinyl urea	Weak	depletion (%)	57.9	2.8	58.3	1.8	59.1	5.6	66.1	2.0	65.3	4.0	59.1	3.9	61.4	3.7	65.4	10.7	61.5	6.2	59.3	1.4	60.6	2.6	60.0	2.4	60.9	4.6	62.4	5.4	60.8	5.5
			mean % depletion	30.3		30.1		32.4		34.0		34.7		31.5		32.5		38.1		33.8		30.3		31.6		31.2		32.7		33.9		33.2	
5	Farnesal	Weak	depletion (%)	97.9	17.7	91.9	19.9	94.8	22.9	95.7	11.8	97.9	12.4	95.5	21.0	96.6	16.3	98.9	25.3	100.0	30.3	83.5	19.1	83.7	18.0	81.1	17.9	80.7	13.5	82.9	13.3	88.1	14.9
			mean % depletion	57.8		55.9		58.9		53.8		55.1		58.3		56.5		62.1		65.1		51.3		50.8		49.5		47.1		48.1		51.5	
10	Isopropanol	Non-sensitizer	depletion (%)	0.0	0.1	0.6	1.4	0.0	0.3	1.5	1.0	3.6	0.0	0.8	2.3	0.7	0.0	2.8	3.3	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.3	0.0	0.4
			mean % depletion	0.1		1.0		0.2		1.2		1.8		1.5		0.4		3.0		0.3		0.0		0.0		0.0		0.2		0.2		0.2	
11	Dimethyl isophthalate	Non-sensitizer	depletion (%)	0.0	0.3	0.1	0.0	0.0	0.3	0.0	0.1	0.1	1.3	0.3	1.3	1.5	0.3	1.8	1.3	0.7	0.5	1.1	0.0	3.2	0.0	0.0	0.7	0.0	0.1	0.0	0.0	0.0	0.0
			mean % depletion	0.2		0.0		0.2		0.0		0.7		0.8		0.9		1.6		0.6		0.5		1.6		0.4		0.1		0.0		0.0	
12	Propyl paraben	Non-sensitizer	depletion (%)	0.0	0.4	0.5	0.2	0.0	0.7	0.5	0.1	0.0	1.3	0.6	0.7	1.7	0.2	2.3	1.2	1.4	0.7	0.0	0.0	0.0	0.1	0.0	0.4	0.0	0.0	0.3	0.0	0.0	0.2
			mean % depletion	0.2		0.3		0.4		0.3		0.7		0.6		0.9		1.8		1.1		0.0		0.1		0.2		0.0		0.1		0.1	

：Sensitizer：減少率≧4.9　　：Non-sensitizer：減少率 ＜4.9

表 S3-8-1　ADRA（1 mM）と ADRA（4 mM）で判定結果が異なる化合物の ADRA（4 mM）による評価（UV 検出器）

No.	Test chemicals	LLNA potency	depletion	Lab.A						Lab.B						Lab.C						Lab.D						Lab.E					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
6	m-Aminophenol	Moderate	depletion (%)	32.1	0.0	33.8	0.0	30.8	0.0	24.3	0.1	27.1	0.0	22.3	0.0	14.8	0.8	2.9	2.1	10.8	0.1	24.9	0.0	28.6	0.0	32.5	0.0	12.8	0.0	18.9	0.5	23.9	0.5
			mean % depletion	16.1		16.9		15.4		12.2		13.5		11.2		7.8		2.5		5.5		12.4		14.3		16.2		6.4		9.7		12.2	
7	3-Propylidenephthalide	Moderate	depletion (%)	34.1	50.4	42.0	59.2	42.1	52.9	35.5	64.2	24.5	72.5	23.0	72.4	31.2	69.2	20.4	80.5	26.1	71.8	13.2	53.3	21.9	56.4	25.5	62.0	16.8	59.9	23.3	67.5	18.1	58.8
			mean % depletion	42.3		50.6		47.5		49.9		48.5		47.7		50.2		50.5		49.0		33.3		39.1		43.8		38.3		45.4		38.4	
8	Ethyleneglycol dimethacrylate	Weak	depletion (%)	16.6	1.0	21.0	0.3	17.6	2.4	21.6	1.0	18.2	1.5	16.5	0.3	20.3	1.5	18.7	3.8	29.1	3.0	14.0	0.2	20.1	1.3	19.9	1.5	19.0	1.6	19.3	1.2	19.4	0.7
			mean % depletion	8.8		10.7		10.0		11.3		9.9		8.4		10.9		11.2		16.1		7.1		10.7		10.7		10.3		10.2		10.1	
9	n-Butyl glycidyl ether	Weak	depletion (%)	24.6	1.2	23.2	0.0	25.7	2.1	32.3	3.2	30.7	1.6	28.0	4.8	23.6	1.0	27.2	4.8	37.3	3.8	26.2	0.1	31.9	0.7	33.7	0.0	21.0	2.5	26.0	2.9	25.7	3.1
			mean % depletion	12.9		11.6		13.9		17.7		16.1		16.4		12.3		16.0		20.6		13.2		16.3		16.8		11.7		14.4		14.4	

：Sensitizer：減少率≧4.9　　：Non-sensitizer：減少率 ＜4.9

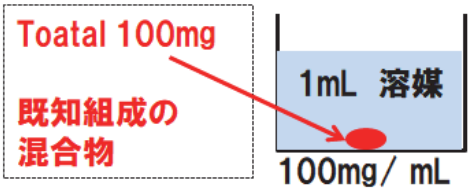
表 S3-8-2　ADRA（1 mM）と ADRA（4 mM）で判定結果が異なる化合物の ADRA（4 mM）による評価（FL 検出器）

No.	Test chemicals	LLNA potency	depletion	Lab.A						Lab.B						Lab.C						Lab.D						Lab.E					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
6	m-Aminophenol	Moderate	depletion (%)	31.5	0.4	32.9	0.0	30.7	0.3	24.0	1.8	26.4	0.3	23.2	1.8	14.1	0.6	2.9	1.6	11.2	0.2	24.6	0.0	29.5	0.0	32.8	0.0	12.6	0.1	18.6	0.6	23.8	1.0
			mean % depletion	15.9		16.5		15.5		12.9		13.4		12.5		7.4		2.2		5.7		12.3		14.7		16.4		6.4		9.6		12.4	
7	3-Propylidenephthalide	Moderate	depletion (%)	33.6	50.8	41.5	59.4	41.7	53.3	33.2	64.1	25.3	72.6	24.4	73.4	31.4	70.0	20.2	81.2	25.9	71.7	17.7	52.9	22.9	56.2	26.9	61.8	16.6	60.1	23.9	66.7	18.4	58.0
			mean % depletion	42.2		50.4		47.5		48.7		48.9		48.9		50.7		50.7		48.8		35.3		39.6		44.4		38.4		45.3		38.2	
8	Ethyleneglycol dimethacrylate	Weak	depletion (%)	15.7	1.6	20.5	0.3	17.6	2.5	23.2	1.4	19.1	2.1	18.3	2.1	20.7	1.5	18.8	3.5	29.7	2.7	13.9	0.6	20.8	2.9	20.4	3.2	18.9	4.2	19.5	1.3	19.0	0.8
			mean % depletion	8.6		10.4		10.0		12.3		10.6		10.2		11.1		11.2		16.2		7.2		11.8		11.8		11.6		10.4		9.9	
9	n-Butyl glycidyl ether	Weak	depletion (%)	24.0	1.3	23.2	0.3	25.7	2.5	33.3	3.0	31.0	2.8	29.5	4.5	23.7	1.3	26.9	5.6	37.2	4.0	26.4	0.0	31.6	1.1	34.0	0.0	20.3	2.4	25.7	3.0	25.4	3.2
			mean % depletion	12.7		11.7		14.1		18.2		16.9		17.0		12.5		16.3		20.6		13.2		16.4		17.0		11.4		14.4		14.3	

：Sensitizer：減少率≧4.9　　：Non-sensitizer：減少率 ＜4.9

Annex 4.　構成成分情報が既知の混合物溶液の見かけの分子量の計算方法

例）構成成分情報が既知の10% 混合物溶液



構成成分（Total 100mg）
A 40mg（MW. 50）
B 30mg（MW. 100）
C 20mg（MW. 200）
D 10mg（MW. 400）

見かけの分子量（A single aggregated molecular weight）

＝（50×40/100）+（100×30/100）+（200×20/100）+（400×10/100）

＝130

Annex 5.　ADRA と DPRA の予測性

ADRA は、136 種類の LLNA のデータセットおよび 81 種類のヒトのデータセットに対して、ADRA（1 mM）、ADRA（4 mM）および ADRA（0.5 mg/mL）が実施され、それぞれの試験法の感度、特異度、正確度および平均正確度（Balanced accuracy）が計算された。なお、平均正確度は、感度と特異度の平均値であり、この指標は、*in vivo* 陽性と *in vivo* 陰性の化学物質の数が異なる場合に、有効である。また、参考までに DPRA のデータも掲載した。136 種類の LLNA のデータセットに対して ADRA は、被験物質濃度を 1 mM から 4 mM にすることで、11 種類の偽陰性化合物が、感作性物質と判定された一方、4 種類の非感作性物質が偽陽性と判定されたが、総合的に LLNA と結果が一致する化合物が 7 種類増加した。同様に、被験物質濃度を 1 mM から 0.5 mg/mL にすることで、10 種類の偽陰性化合物が、感作性物質と判定される一方、4 種類の非感作性物質が偽陽性と判定されたが、総合的に LLNA の結果と一致する化合物が 6 種類増加した。81 種類のヒトのデータセットに対して、ADRA は、被験物質濃度を 1 mM から 4 mM にすることで、7 種類の偽陰性化合物が、感作性物質と判定される一方、非感作性物質が偽陽性と判定された化合物はなく、総合的にヒトと結果が一致する化合物が 7 種類増加した。同様に、被験物質濃度を 1 mM から 0.5 mg/mL にすることで、6 種類の偽陰性化合物が、感作性物質と判定される一方、非感作性物質が偽陽性と判定された化合物はなく、総合的にヒトの結果と一致する化合物が 6 種類増加した。このように、被験物質濃度を 4 mM や 0.5 mg/mL に上げることで、1 mM の被験物質濃度では、NAC/NAL の減少率が閾値よりわずかに低いため非感作性と判定される moderate や weak に分類される感作性物質の一部を陽性として判定できることから、その効果は明らかである。ADRA（4 mM）と ADRA（0.5 mg/mL）の平均正確度は ADRA（1 mM）と同等以上であると考えられる。

表 S5-1　ADRA および DPRA の感作性予測結果のまとめ [15)]

	感度 (%) (Sensitivity)	特異度(%) (Specificity)	正確度 (%) (Accuracy)	平均正確度 (%) (Balanced accuracy)
vs LLNA data				
ADRA (4mM)	76 (74 / 98)	79 (30 / 38)	76 (104 / 136)	77
ADRA (1 mM)	65 (64 / 98)	89 (34 / 38)	72 (98 / 136)	77
ADRA (0.5mg/mL)	74 (73 / 98)	79 (30 / 38)	76 (103 / 136)	77
DPRA	72 (71 / 98)	76 (29 / 38)	74 (100 / 136)	74
vs human data				
ADRA (4mM)	83 (48 / 58)	86 (19 / 22)	84 (67 / 80)	85
ADRA (1 mM)	71 (41 / 58)	86 (19 / 22)	75 (60 / 80)	79
ADRA (0.5mg/mL)	81 (47 / 58)	86 (19 / 22)	83 (66 / 80)	84
DPRA	78 (45 / 58)	86 (19 / 22)	80 (64 / 80)	82
LLNA	97 (56 / 58)	73 (16 / 22)	90 (72 / 80)	85

表 S5-2　ADRA の感作性予測結果 [17]　(1/3)

№	Test chemicals	LLNA EC3(%)	Human category	ADRA (4mM)	ADRA (1mM)	ADRA (0.5mg/mL)	DPRA
	Extreme sensitizers						
1	Diphenylcyclopropenone	0.00030		Pos	Pos	Pos	Pos
2	Oxazolone	0.0030	Pos	Pos	Pos	Pos	Pos
3	Chlorothalonil	0.0040		Pos	Pos	Pos	Pos
4	MCI/MI	0.0050	Pos	Pos	Pos	Pos	Pos
5	*p* -Benzoquinone	0.0099	Pos	Pos	Pos	Pos	Pos
6	Tetrachlorosalicylanilide	0.040	Pos	Pos	Pos	Pos	Pos
7	Bandrowski's base	0.040		Pos	Pos	Pos	Pos
8	4-Nitrobenzyl bromide	0.050		Pos	Pos	Pos	Pos
	Strong sensitizers						
9	Glutaraldehyde	0.10	Pos	Pos	Pos	Pos	Pos
10	Hydroquinone	0.11	Pos	Pos	Pos	Pos	Pos
11	Phthalic anhydride	0.16	Neg	Pos	Pos	Pos	Pos
12	Maleic anhydride	0.16		Pos	Pos	Pos	Pos
13	1,4-Phenylenediamine	0.16	Pos	Pos	Pos	Pos	Pos
14	Hexyl salicylate	0.18	Pos	Neg	Neg	Neg	Neg
15	Benzyl bromide	0.20		Pos	Pos	Pos	Pos
16	Benzoyl peroxide	0.22	Pos	Pos	Pos	Pos	Pos
17	Lauryl gallate	0.30	Pos	Pos	Pos	Pos	Pos
18	Propyl gallate	0.32	Pos	Pos	Pos	Pos	Pos
19	2,5-Diaminotoluene sulfate	0.40	Pos	Pos	Pos	Pos	Pos
20	2-Aminophenol	0.40	Pos	Pos	Pos	Pos	Pos
21	2-Nitro-1,4-phenylendiamine	0.50	Pos	Pos	Pos	Pos	Pos
22	Chloramine-T dihydrate	0.60		Pos	Pos	Pos	Pos
23	CD-3	0.60		Pos	Pos	Pos	Pos
24	Formaldehyde	0.61	Pos	Pos	Pos	Pos	Pos
25	Iodopropynyl butylcarbamate	0.90	Pos	Pos	Pos	Pos	Pos
26	1,2-Dibromo-2,4-dicyanobutane	0.90	Pos	Pos	Pos	Pos	Pos
	Moderate sensitizers						
27	Isoeugenol	1.2	Pos	Pos	Pos	Pos	Pos
28	1-Naphthol	1.3		Pos	Pos	Pos	Pos
29	1-Phenyl-1,2-propanedione	1.3		Pos	Pos	Pos	Pos
30	2-Hydroxyethyl acrylate	1.4	Pos	Pos	Pos	Pos	Pos
31	Glyoxal	1.4	Pos	Pos	Pos	Pos	Pos
32	Bisphenol A diglycidyl ether	1.5	Pos	Pos	Pos	Pos	Pos
33	Vinyl pyridine	1.6		Pos	Pos	Pos	Pos
34	2-Mercaptobenzothiazole	1.7	Pos	Pos	Pos	Pos	Pos
35	2-Methyl-2H-isothiazol-3-one (MI)	1.9	Pos	Pos	Pos	Pos	Pos
36	3-Dimethylamino propylamine	2.2	Pos	Pos	Neg	Pos	Neg
37	Ethylenediamine	2.2	Pos	Neg	Neg	Neg	Neg
38	1,2-Benzisothiazolin-3-one	2.3	Pos	Pos	Pos	Pos	Pos
39	Methyl pyruvate	2.4		Neg	Neg	Neg	Neg
40	Methyl 2-nonynoate	2.5	Pos	Pos	Pos	Pos	Pos
41	Benzyl salicylate	2.9	Neg	Neg	Neg	Neg	Neg
42	Phenylacetaldehyde	3.0	Pos	Pos	Pos	Pos	Pos
43	Cinnamic aldehyde	3.0	Pos	Pos	Pos	Pos	Pos
44	m-Aminophenol	3.2		Pos	Neg	Pos	Neg
45	Diethyl sulfate	3.3		Neg	Neg	Pos	Pos
46	3-Propylidenephthalide	3.7	Pos	Pos	Neg	Pos	Neg
47	Benzylideneacetone	3.7	Pos	Pos	Pos	Pos	Pos
48	2,4-Heptadienal	4.0		Pos	Pos	Pos	Pos
49	Tropolone	4.3		Neg	Neg	Neg	Neg
50	α-Methylcinnamaldehyde	4.5		Pos	Pos	Pos	Pos

表 S5-2　ADRA の感作性予測結果 [17)]　(2/3)

№	Test chemicals	LLNA EC3(%)	Human category	ADRA (4mM)	ADRA (1mM)	ADRA (0.5mg/mL)	DPRA
51	Nickel (II) sulfate	4.8	Pos	Neg	Neg	Neg	Neg
52	Tetramethylthiuram disulfide	5.2	Pos	Pos	Pos	Pos	Pos
53	*trans* -2-Hexenal	5.5	Pos	Pos	Pos	Pos	Pos
54	Resorcinol	5.5	Pos	Pos	Neg	Pos	Pos
55	Diethyl maleate	5.8	Pos	Pos	Pos	Pos	Pos
56	2-Methoxy-4-methyl-phenol	5.8		Pos	Neg	Pos	Pos
57	Diethylenetriamine	5.8	Pos	Pos	Neg	Pos	Pos
58	2-Phenylpropionaldehyde	6.3	Pos	Pos	Pos	Pos	Pos
59	4-Chloroaniline	6.5		Neg	Neg	Neg	Neg
60	10-Undecenal	6.8		Neg	Neg	Neg	Neg
61	12-Bromo-1-dodecanol	6.9		Neg	Neg	Neg	Pos
62	*dl* -α-Tocopherol	7.4	Neg	Neg	Neg	Neg	Neg
63	Methyl methanesulfonate	8.1		Pos	Pos	Pos	Pos
64	Perillaldehyde	8.1		Pos	Pos	Pos	Pos
65	5-Methyl-2-phenyl-4H-pyrazol-3-one	8.5		Pos	Neg	Neg	Neg
66	Trimellitic anhydride	9.2		Pos	Pos	Pos	Neg
	Weak sensitizers						
67	2-Ethylhexyl acrylate	10		Pos	Pos	Pos	Pos
68	1-Bromohexane	10		Pos	Neg	Neg	Neg
69	α-Hexylcinnamaldehyde	11	Neg	Neg	Neg	Neg	Neg
70	α-Amylcinnamaldehyde	11	Pos	Neg	Neg	Neg	Neg
71	2,3-butanedione	11		Pos	Pos	Pos	Pos
72	N-Butyl acrylate	11		Pos	Pos	Pos	Pos
73	R-Carvone	13	Pos	Pos	Pos	Pos	Pos
74	Citral	13	Pos	Pos	Pos	Pos	Pos
75	Eugenol	13	Pos	Pos	Pos	Pos	Pos
76	Abietic acid	15	Pos	Pos	Pos	Pos	Pos
77	Oxalic acid	15		Neg	Neg	Neg	Neg
78	Benzyl benzoate	17	Neg	Neg	Neg	Neg	Neg
79	Lyral	17	Pos	Pos	Pos	Pos	Pos
80	4-Allylanisole	18		Pos	Pos	Pos	Pos
81	Benzyl Cinnamate	18		Neg	Neg	Neg	Neg
82	Lilial	19	Pos	Pos	Pos	Pos	Neg
83	N,N-Dibutylaniline	20		Neg	Neg	Neg	Neg
84	Phenyl benzoate	20	Pos	Neg	Neg	Neg	Pos
85	Cinnamyl alcohol	21	Pos	Neg	Neg	Neg	Neg
86	Cyclamen aldehyde	22		Pos	Neg	Pos	Pos
87	Benzocaine	22	Pos	Neg	Neg	Neg	Pos
88	Imidazolidinyl urea	24	Pos	Pos	Pos	Pos	Pos
89	Geraniol	26	Pos	Pos	Pos	Pos	Neg
90	5-Methyl-2,3-hexanedione	26	Pos	Pos	Pos	Pos	Pos
91	Ethyleneglycol dimethacrylate	28	Pos	Pos	Neg	Pos	Pos
92	Linalool	30	Pos	Neg	Neg	Neg	Neg
93	N-Butyl glycidyl ether	31	Pos	Pos	Neg	Pos	Pos
94	Hydroxycitronellal	33	Pos	Pos	Pos	Pos	Pos
95	*d,l* -Citronellol	44	Neg	Neg	Neg	Neg	Neg
96	Bisphenol A Glycidyl Methacrylate	45		Pos	Pos	Pos	Pos
97	2-Ethylbutyraldehyde	76		Pos	Pos	Pos	Pos
98	Aniline	89	Pos	Neg	Neg	Neg	Neg

表 S5-2　ADRA の感作性予測結果 [17)]　(3/3)

№	Test chemicals	LLNA EC3(%)	Human category	ADRA (4mM)	ADRA (1mM)	ADRA (0.5mg/mL)	DPRA
	Non-sensitizers						
99	Dibutyl phthalate	NC		Neg	Neg	Neg	Neg
100	1-Bromobutane	NC		Neg	Neg	Neg	Neg
101	1-Butanol	NC	Neg	Neg	Neg	Neg	Neg
102	1-Iodohexane	NC		Neg	Neg	Neg	Pos
103	2-Acetylcyclohexanone	NC		Pos	Neg	Pos	Pos
104	2-Fluoro-5-nitroaniline	NC		Neg	Neg	Neg	Neg
105	2-Hydroxypropyl methacrylate	NC		Pos	Neg	Pos	Pos
106	3-Phenoxypropiononitrile	NC		Neg	Neg	Neg	Pos
107	4-Hydroxybenzoic acid	NC	Neg	Neg	Neg	Neg	Neg
108	4'-Methoxyacetophenone	NC	Neg	Neg	Neg	Neg	Neg
109	6-Methylcoumarin	NC	Pos/Neg	Neg	Neg	Neg	Neg
110	Benzalkonium chloride	NC	Neg	Pos	Pos	Pos	Neg
111	Benzaldehyde	NC	Neg	Pos	Pos	Pos	Neg
112	1-Methoxy-4-methyl-2-nitrobenzene	NC		Neg	Neg	Neg	Neg
113	Benzyl butyl phthalate	NC		Pos	Pos	Pos	Neg
114	Chlorobenzene	NC		Neg	Neg	Neg	Neg
115	Clofibrate	NC		Neg	Neg	Neg	Neg
116	Coumarin	NC	Pos	Neg	Neg	Neg	Neg
117	Diethyl phthalate	NC	Neg	Neg	Neg	Neg	Neg
118	*N,N*-Diethyl-*m*-toluamide	NC		Pos	Neg	Pos	Neg
119	*N,N*-Dimethylformamide	NC		Neg	Neg	Neg	Neg
120	Ethyl benzoylacetate	NC		Neg	Neg	Pos	Neg
121	Ethyl vanillin	NC		Neg	Neg	Neg	Pos
122	Glycerol	NC	Neg	Neg	Neg	Neg	Neg
123	Isopropanol	NC	Neg	Neg	Neg	Neg	Neg
124	Lactic acid	NC	Neg	Neg	Neg	Neg	Neg
125	Methyl salicylate	NC	Neg	Neg	Neg	Neg	Neg
126	Octanoic acid	NC	Neg	Neg	Neg	Neg	Neg
127	Methyl 3-bromopropanoate	NC		Pos	Pos	Pos	Pos
128	Propylparaben	NC	Neg	Neg	Neg	Neg	Neg
129	Propylene glycol	NC	Neg	Neg	Neg	Neg	Neg
130	Saccharin	NC		Neg	Neg	Neg	Pos
131	Streptomycin sulfate	NC	Pos	Pos	Neg	Neg	Neg
132	Sulfanilamide	NC	Neg	Neg	Neg	Neg	Neg
133	Sulfanilic acid	NC		Neg	Neg	Neg	Neg
134	Vanillin	NC	Neg	Neg	Neg	Neg	Pos
135	Nonanoic acid	21 (false pos)		Neg	Neg	Neg	Neg
136	Sodium lauryl sulfate	14 (false pos)	Neg	Neg	Neg	Neg	Pos

NC; No Classification, Pos; Positive, Neg; Negative

■ : Sensitizer　　□ : Non-sensitizer

添付資料 3

OECD/OCDE 442C

Adopted:
4 July 2023

OECD KEY EVENT BASED GUIDELINE FOR THE TESTING OF CHEMICALS

In chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins

INTRODUCTION

Covalent binding to proteins Key Event based Test Guideline.

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP) (2) starting with a molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with amino-acid residues (i.e. cysteine or lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event), is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation.
2. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman and the Buehler Test (OECD TG 406) (11) assess both the induction and elicitation phases of skin sensitisation. The murine tests, such as the LLNA (OECD TG 429) (12) and its three non-radioactive modifications — LLNA:DA (OECD TG 442A) (13), LLNA:BrdU-ELISA, and BrdU-FCM (OECD TG 442B) (14) — all assess the induction response exclusively and have gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.
3. Mechanistically-based *in chemico* and *in vitro* test methods addressing the first three key events of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals: the present Test Guideline assesses covalent binding to proteins, addressing the first key event; the OECD TG 442D assesses keratinocyte activation (15), the second key event and the OECD TG 442E addresses the activation of dendritic cells (16), the third key event of the skin sensitisation AOP. Finally, the fourth key event representing T-cell proliferation is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (12).

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

4. This Test Guideline (TG) describes *in chemico* assays that address mechanisms described under the first key event of the AOP for skin sensitisation, namely covalent binding to proteins (2). The test methods currently included in this Test Guideline are:
   - The Direct Peptide Reactivity Assay (DPRA) (Appendix I),
   - The Amino Acid Derivative Reactivity Assay (ADRA) (Appendix II), and
   - The kinetic Direct Peptide Reactivity Assay (kDPRA) (Appendix III).
5. The test methods are based on *in chemico* covalent binding to proteins and are considered to be scientifically valid. 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) (3) (4) (5). The ADRA underwent a validation study coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6) (7) (8) (9) followed by an independent peer-review (10). The kDPRA underwent an industry-coordinated validation study followed by an independent peer-review (17).
6. The test methods included in this Test Guideline might differ with regard to the procedures used to generate the data but can each be used to address countries' requirements for test results on protein reactivity, while benefiting from the Mutual Acceptance of Data.
7. The correlation of protein reactivity with skin sensitisation potential is well established (18) (19) (20). Nevertheless, since protein reactivity represents only one key event of the skin sensitisation AOP (2) (21), information generated with test methods developed to address this specific key event may not be sufficient as stand-alone methods to conclude on the presence or absence of skin sensitisation potential of chemicals. Therefore, data generated with the test methods described in this Test Guideline are proposed to be used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information from *in vitro* assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including *in silico* modelling and read-across from chemical analogues (21). Examples on the use of data generated with these methods within Defined Approaches (DAs), i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to derive predictions, have been published (21) and are implemented in an OECD TG on defined approaches for skin sensitisation (22).
8. The DPRA and ADRA described in Appendixes I and II to this Test Guideline, respectively, support the discrimination of skin sensitisers (Category 1) from non-sensitisers. Depending on the regulatory framework, positive results generated with these methods may be used on their own to classify a chemical into UN GHS Category 1. However, these test methods do not allow on their own, the sub-categorisation of skin sensitisers into subcategories 1A and 1B (23), as defined by UN GHS (1) for authorities implementing these two optional subcategories, or potency prediction for safety assessment decisions.

9. In contrast, the kDPRA described in Appendix III of this Test Guideline, allows discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) i.e., subcategory 1B or no category (1) but does not allow to distinguish sensitisers (Category 1) from non-sensitisers. Depending on the regulatory framework, positive results generated with the kDPRA may be used on their own to classify a chemical into UN GHS subcategory 1A.

10. Definitions are provided in the Annex. Performance Standards for the assessment of proposed similar or modified *in vitro* skin sensitisation DPRA and ADRA test methods have been developed (24).

Literature for introduction

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Annex 1.A. 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 (1). The formula used to derive accuracy is shown under "Calculation" of predictive capacity.

ADRA: Amino acid Derivative Reactivity Assay.

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).

Balanced accuracy: The average of sensitivity and specificity. This metric is particularly useful when a different number of in vivo positive and in vivo negative chemicals were tested. It is an important consideration in assessing the relevance of a test method. The formula used to derive balanced accuracy is shown under "Calculation" of predictive capacity.

Calculation

Calculating predictive capacity

Sensitivity, specificity, accuracy, and balanced accuracy are calculated based on the true positive (TP), true negative (TN), false negative (FN), and false positive (FP) values as follows:

$$\text{Sensitivity} = \frac{\text{Number of true positives (TP)}}{\text{Number of all positive chemicals (TP+FN)}} \text{ x } 100$$

$$\text{Specificity} = \frac{\text{Number of true negatives (TN)}}{\text{Number of all negative chemicals (TN+FP)}} \text{ x } 100$$

$$\text{Accuracy} = \frac{\text{Number of correct predictions (TP+TN))}}{\text{Number of all chemicals (TP+FN+TN+FP)}} \text{ x } 100$$

$$\text{Balanced accuracy} = \frac{\text{Sensitivity} + \text{Specificity}}{2}$$

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.

Defined Approach (DA): a DA consists of a fixed data interpretation procedure (e.g. statistical, mathematical models) applied to data (e.g. *in silico* predictions, *in chemico*, *in vitro* data) generated with a defined set of information sources to derive a prediction.

DPRA: Direct Peptide Reactivity Assay.

EDTA: Ethylenediaminetetraacetic acid.

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing.

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 hazards, risks, and the need for further targeted and therefore minimal testing.

JaCVAM: Japanese Center for the Validation of Alternative Methods.

kDPRA: kinetic Direct Peptide Reactivity Assay.

$\mathbf{k_{max}}$: is the maximum rate constant (in $s^{-1}M^{-1}$) determined from the reaction kinetics for a tested substance in the kDPRA (see Appendix III, paragraph 24).

LLNA: murine Local Lymph Node Assay issued as OECD TG 429 in 2010.

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 solid or liquid comprising two or more substances which do not react chemically (3).

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent comprises at least 80% (w/w) of the whole.

Multi-constituent substance: A substance, defined by its quantitative composition, in which two or more main constituents are present in concentrations ≥ 10% (w/w) and < 80% (w/w). Multi-constituent substances are the result of a manufacturing process. The difference between a mixture and a multi-constituent substance is that a mixture comprises two or more substances which do not react chemically, whereas a multi-constituent substance comprises two or more substances that do react chemically.

NAC: N-(2-(1-naphthyl) acetyl)-L-cysteine (4) (5) (6).

NAL: α-N-(2-(1-naphthyl) acetyl)-L-lysine (4) (5) (6).

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

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

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

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 (1).

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 (1).

Reproducibility: The concordance of results obtained from testing the same substance using the same test protocol (see reliability). (1)

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 (1). The formula used to derive sensitivity is shown under ”Calculation” of predictive capacity.

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 (1). The formula used to derive specificity is shown under “Calculation” of predictive capacity.

Substance: Chemical elements and their compounds in the natural state or resulting from a manufacturing process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process, but excluding solvents that may be separated without affecting the stability of the substance or changing its composition (3).

System suitability: Determination of instrument performance (e.g., sensitivity) by analysis of reference standards prior to running the analytical run (7).

Test chemical: The term test chemical is used to refer to the substance being tested.

TFA: Trifluoroacetic acid.

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 (3).

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 (1).

Literature for definitions

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APPENDIX I

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

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. 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 (1). 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 (2).

2. 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 (3). Results generated in the validation study (4) and published studies (5) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Category 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) (4) (5). 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 or a DA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in 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 (1) (2) (3) (5). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

3. 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 (see a summary of the known limitations of the DPRA in Annex 1 of this Appendix). This test method is 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 paragraphs 10 and 11). 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 (4) (5). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraphs 4 and 11). When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Appendix of the Test Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. In cases where evidence can be demonstrated on the non-applicability of the test method to specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

4. The test method described in this Appendix of the 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 most cases correctly detected by the test method (4) (9) (10). 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 or a DA. 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 assignment to a higher reactivity class (see paragraphs 23 and 24).

5. 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 (6) (11) when used in integrated approaches such as IATA or DA (12). However further

[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.

work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

6. 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 22.5-30°C. 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 23) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

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

PROCEDURE

8. This test method is based on the DPRA DB-ALM protocol n° 154 (7) 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

9. 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 > 90%, 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 method, 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

10. 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 (7). 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 have an 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). If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to peptide dimerisation and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to first solubilise the test chemical in 300 µL of DMSO and dilute the resulting solution with 2700 µL of acetonitrile. 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.

11. This molecular weight approach should apply if the test chemical is a mono-constituent substance with a known molecular weight or a mixture or multi-constituent substance of known composition. For mixtures and multi-constituent substances of known composition, a single aggregated purity value should be determined by the sum of the proportion of its constituents (excluding water), and a single aggregated 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 aggregated 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.

12. For mixtures and multi-constituent substances of unknown composition (i.e. UVCB substances of unknown or variable composition, complex reaction products or biological materials), the test solution can be prepared with a gravimetric approach to a concentration of 20 mg/mL on the basis of the weight of the total components (excluding solvent) in an appropriate solvent. This value is based on a default molecular weight of 200 g/mol. If the mixture to be investigated is known to contain a chemical class with a typical molecular weight which is significantly higher, this default

molecular weight and the test solution concentration should be adjusted accordingly (see e.g. approach for agrochemical formulations (13)). In addition, this gravimetric approach should only be applied as a last resort if no molecular weight is available and no aggregated molecular weight can be determined.

Preparation of the positive control, reference controls and coelution controls

13. 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 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 20). 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

14. 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, 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 10 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 22.5-30°C 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

15. 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

16. 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.

17. 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

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

to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 23 and 24).

18. The HPLC 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 method, up to 26 analysis samples can be accommodated in a single HPLC run (see also paragraph 9). An example of HPLC analysis sequence is provided in Annex 3.

DATA AND REPORTING

Data evaluation

19. 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.

20. 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.

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

Acceptance criteria

21. 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 (for other positive controls a reference range needs to be established) 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.

22. 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

23. 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 Table 1, 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 or DA. 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 or DA.

Table 1: 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).
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

24. 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, or with the cysteine peptide only, 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.

Table 2: 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 2 and 4.

25. 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.

26. 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. mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model), additional testing is recommended. In particular, in case of negative results in these ranges (i.e. 3% to 6.38% for the cysteine 1:10/lysine 1:50 prediction model or 9% to 13.89% for the cysteine 1:10 prediction model), a second run should be conducted, as well as a third one in case of discordant results between the first two runs.

Test report

27. The test report should include the following information

Test chemical and Controls (positive control and solvent/vehicle)

- Mono-constituent substance (test and control chemicals)
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physicochemical properties such as physical state, 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.
- Additional information for positive control

- Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

- Additional information for solvent/vehicle control
  - Solvent/vehicle used and ratio of its constituents, if applicable;
  - Justification for choice of solvent for each test chemical;
  - For acetonitrile, results of test of impact on peptide stability.

Peptides

- Supplier, lot, purity

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

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals.

Discussion of the results

- Description of any unintended modifications to the test procedure.
- Discussion of the results obtained with the DPRA test method and if it is within the ranges described in paragraph 26.

Conclusion

LITERATURE FOR APPENDIX I

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APPENDIX I, ANNEX 1

KNOWN LIMITATIONS OF THE DIRECT PEPTIDE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the DPRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential; cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. 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 or a DA	Diethylenetriamine; 111-40-0 (1A chez l'homme, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in most cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals absorbing significantly at 220 nm and having the same retention time of the peptides (co-elution)	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, or with the cysteine peptide only, then the analysis should be reported as "inconclusive" and alternative HPLC set up should be considered (see paragraph 22). 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.	Salicylic acid: 69-72-7
Complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	The molecular weight approach cannot apply - See paragraph 12 for conditions of aplication of the gravimetric approach	See paragraph 12	UVCBs, chemical emissions, products or formulations with variable or not fully known composition
Test chemicals which cannot be dissolved in an appropriate solvent at a final	Not sure if sufficient exposure can be achieved	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 be used to support the identification of the test	n/a

concentration of 100 mM		chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	
Chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved	A conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result	Isopropyl myristate CAS: 110-27-0
Test chemicals that do not covalently bind to the cysteine-peptide but promote its oxidation (i.e. cysteine dimerisation)	Could lead to a potential over-estimation of cysteine-peptide depletion, resulting in possible false positive predictions.		DMSO Oxidant
Test chemicals that are only soluble in DMSO	DMSO causes excessive peptide depletion due to cysteine dimerization resulting in high background cysteine depletion.	May lead to false negative results	n/a

APPENDIX I, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay

Prior to routine use of the test method described in this test method, 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	≤ 24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	≤ 7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	≤ 25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	≤ 7	≤ 5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	≤ 7	≤ 5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	≤ 7	≤ 5.5

4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	≤ 7	≤ 5.5

[1]The *in vivo* hazard and (potency) predictions are based on LLNA data (5). The *in vivo* potency is derived using the criteria proposed by ECETOC (8).
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.
[3] Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

APPENDIX I, 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.

APPENDIX II

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The ADRA 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 amino acid derivatives containing either lysine or cysteine (1) (2) (3). Depletion values of the cysteine derivative N-(2-(1-naphthyl)acetyl)-L-cysteine (CAS. 32668-00-1), which is known as NAC, and the lysine derivative α-N-(2-(1-naphthyl)acetyl)-L-lysine (CAS. 397841-92-8), known as NAL are then used to support the discrimination between skin sensitisers and non-sensitisers (1) (2) (3).

2. The reproducibility and transferability of the ADRA protocol were confirmed using validation studies coordinated by the Japanese Center for validation of alternative methods (JaCVAM) (4) (5) (6) (7) (8) (9) (10). There are two detection types of ADRA: ultraviolet (UV) detection and fluorescence (FL) detection (11) (12). Within-laboratory reproducibility (WLR) and between-laboratory reproducibility (BLR) of ADRA were 100% each determined using both the UV detection and fluorescence detection (9) (10). Prediction of skin sensitisation potential based on local lymph node assay (LLNA) data indicated that ADRA with UV-detection identified sensitisers and non-sensitisers with an accuracy of 76 % (104/136), a sensitivity of 76% (74/98), a specificity of 79% (30/38) and a balanced accuracy of 77% (8). In addition, the prediction of the skin sensitisation potential based on human data indicated that ADRA with UV detection has an accuracy of 84% (67/80), a sensitivity of 83% (48/58), a specificity of 86% (19/22) and a balanced accuracy of 84% (8). However, the accuracy values given here for ADRA as a stand-alone test method are for reference only, since it is recommended that the test method be used in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA as well as other animal tests may not fully reflect the situation in humans. On the basis of the overall data available, ADRA's

applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in in vivo studies), and physicochemical properties (1) (2) (3) (4). Following an independent peer review, the ADRA validation studies were considered to demonstrate that this method should be acceptable as part of an integrated testing strategy for the predictive identification of skin sensitisation hazard (6) (13) (14).

3. Co-elution occurs when the test chemical (the substance or one or several of the constituents of a multi-constituent substance or a mixture) was detected significantly at an OD of 281 nm (UV detector) or Ex/Em 284/333 nm (FL detector) and has the same retention time as NAC or NAL (15). Co-elution of UV absorbing-compounds using with the nucleophiles NAC and NAL can lead to inconclusive results when using conventional ultraviolet (UV) detection (11) (12). This problem can be prevented by an alternative or parallel measurement using a fluorescence (FL) detector; thus, the depletion values obtained by simultaneous measurement using both detectors were also collected in the validation studies (9) (10) and equivalent results to those obtained with UV-detection were obtained, indicating that both detection methods are valid, but FL-detection may lead to fewer inconclusive results. Known limitations of the ADRA are tabulated in Appendix II, Annex 1.

4. The term "test chemical" is used in this Test Guideline to refer to what is being tested[3]. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. The test method described in this Appendix of the 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 (1) (2) (3) (4) (7) (8). 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 promote the oxidation of the N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) reagent (i.e. cysteine dimerisation) could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions (see paragraph 22 and Appendix II, Annex 1); it may be possible to detect and quantify any NAC dimer formed by high-performance liquid chromatography (HPLC) using a UV detector, thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s).

[3] 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.

5. The ADRA test method allows testing of poorly soluble chemicals (16). To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 4 mM (see paragraph 14). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations. In such cases, a positive result could still be used to support 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.

6. The nucleophilic reagents used in ADRA are quantified at 281 nm (1) (2). In the case of co-elution of the nucleophilic reagent and the UV-absorbing test chemical, this might result in inconclusive predictions. However, substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for inconclusive results due to co-elution of UV-absorbing components (15). Furthermore, NAC and NAL are fluorescent and thus, they can be detected using a FL detector (11) (12). Since test chemicals rarely have fluorescence at the specific excitation/emission wavelengths, it is possible to further reduce frequency of inconclusive results by using a FL detector. This is particularly useful in the case of multi-constituent substances with UV absorbance.

7. When assessing the sensitisation potential of a test chemical by using ADRA, there are two options for the preparation of the stock solution (see Figure 1 and paragraphs 15-16): a) If the test chemical is a mono-constituent substance with a known molecular weight or a mixture or multi-constituent substance of known composition, ADRA should be performed using a stock solution prepared at a concentration of 4 mM (8); b) If the test chemical is a mono-constituent substance of unknown molecular weight or a mixture and there is no defined molecular weight (mixtures of unknown or variable composition, complex reaction products, or biological materials (UVCB)), ADRA should be performed using a gravimetric approach based on a stock solution prepared at 0.5 mg/mL. In addition, the gravimetric approach with ADRA (0.5 mg/mL) can also be used for polymers. Assessment of the predictive capacity of ADRA conducted with this gravimetric approach indicated that ADRA (0.5 mg/mL) identified sensitisers and non-sensitisers with an accuracy of 76 % (103/136), a sensitivity of 74% (73/98), a specificity of 79% (30/38) and a balanced accuracy of 77% when compared to LLNA data (8). In addition, the predictive capacity for human data indicated that the gravimetric ADRA (0.5 mg/mL) has an accuracy of 83% (66/80), a sensitivity of 81% (47/58), and a specificity of 86% (19/22) (8). The molecular weight range of the test chemicals used in the validation study of ADRA (0.5 mg/mL) was 60.10 - 388.29, and the ratio of nucleophilic reagent to test chemical in the reaction solution at that time was 1:416 - 1:64 (9).

8. ADRA can be used to support the discrimination between skin sensitisers and non-sensitisers. Further work, preferably based on human data, is necessary to determine whether ADRA results can contribute to potency assessment when considered in combination with other information sources (13) (14).

PRINCIPLE OF THE TEST

9. ADRA is an in chemico test method that quantifies residual concentrations of the NAC and NAL, following a 24±1 hour incubation at 25±1ºC in the presence of a test chemical. Both these derivatives include a naphthalene ring that is introduced to their N-terminal in order to facilitate UV detection and FL detection. The relative concentrations of NAC and NAL are measured by HPLC using UV detection (optical density, 281 nm), optionally in combination with FL detection (excitation/emission [Ex/Em], 284/333 nm) and with gradient elution (see paragraph 19). To ultimately support the discrimination between skin sensitisers and non-sensitisers, percent depletion values are then calculated for both NAC and NAL and compared to a prediction model (see paragraph 27).

10. Prior to routine use of the method described in this test method, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Appendix II, Annex 2.

PROCEDURE

11. This test method is based on the protocol (17) used for the JaCVAM-coordinated ADRA validation study and is recommended for use when implementing ADRA at a laboratory. The main components and procedures for the ADRA are described below. Before using an alternative HPLC set-up, its equivalence to the validated set-up described in the protocol should be demonstrated, preferably by testing the proficiency substances in Appendix II, Annex 2.

Quality of NAC and NAL

12. The Nucleophilic Reagents can be obtained as an ADRA Kit for Skin Sensitisation Test, from FUJIFILM Wako Pure Chemical Corporation, Catalogue No. 296-80901. The use of NAC/NAL as reagent for detecting sensitisation is patented in Japan only, by Fujifilm Corporation. Therefore, in other countries, NAC/NAL can be used without permission. In case other manufacturer's NAC/NAL are used, these should satisfy three quality criteria described below. Quality checks can be obviated and ADRA testing can be performed without delay by purchasing NAC and NAL that have been manufactured specifically to satisfy these quality criteria.

Quality required for NAC and NAL:

1) Purity: Both NAC and NAL are to be at least 98% pure.

2) Stability: Using NAC and NAL stock solution, prepare a reference control free of any test chemical and quantify the residual levels of NAC and NAL both immediately after preparation (0 hours) and after a 24 hour incubation. The residual level of NAC and NAL is calculated as follows:

$$\text{Residual levels of NAC} = \frac{\text{Peak area of NAC}}{\text{Total peak area of NAC and NAC dimer}} \times 100$$

$$\text{Residual levels of NAL} = \frac{\text{Peak area of NAL at 24 hour}}{\text{Peak area of NAL at 0 hour}} \times 100$$

The main cause of NAC stability degradation is dimerisation, which may affect reactivity with the test chemical and test reproducibility (3). Therefore, the residual level of NAC should be calculated with respect to the total amount of NAC and dimer. Since the dimers may be formed over time or may have already been formed during the preparation of the stock solution, residual level of NAC is calculated at the time of stock solution preparation and after 24 hours. Residual levels of NAC (both of 0 hour and 24 hour) and NAL (24 hour) should be a minimum of 90% in either case (17).

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances given in Appendix II, Annex 2 and should satisfy the requirement given therein.

Preparation of the NAC and NAL stock solution

13. The solubility of individual NAC and NAL batches should be verified prior to use. NAC stock solution should be prepared to a concentration of 2 mM in 100 mM of pH 8.0 phosphate buffer, including 0.333 µM of EDTA, as well as NAL stock solution to a concentration of 2 mM in 100 mM of pH 10.2 phosphate buffer. These two stock solutions are then diluted in buffer to prepare 6.667 µM stock solutions. Both NAC and NAL stock solutions should be used as soon as possible after preparation (3). In the event that they are to be stored, these stock solutions may be frozen and stored for up to twelve months time at less than -75°C prior to use. The final concentration of the NAC in the incubation mixture is 5 µM in pH 8.0 phosphate buffer, and the final concentration of the NAL in the incubation mixture is 5 µM in pH 10.2 phosphate buffer.

Preparation of the test chemical solution

14. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay in accordance with the solubilisation procedure described in the ADRA JaCVAM protocol (17). An appropriate solvent should dissolve the test chemical completely. Since the ADRA protocol stipulates that either NAC or NAL are incubated in an excess volume of the test chemical, visual inspection of the clear test chemical solution is considered sufficient to confirm that the test chemical (and all its constituents, if testing a multi-constituent substance or a mixture) is dissolved (17). Suitable solvents are distilled water, acetonitrile and acetone. If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts (19). It is important to note that DMSO may lead to dimerisation of the nucleophilic reagent NAC (18) (19) and as a result, it may be more difficult to meet the acceptance criteria. If a

DMSO-acetonitrile solvent is chosen (5% DMSO in acetonitrile), the test chemical should be dissolved at 80 mM in DMSO, and then this solution should be diluted 20-fold with acetonitrile to prepare a 4 mM test chemical solution. In case the use of DMSO leads to increased dimerisation of the NAC reagent, this can be checked analytically as the NAC dimer can be detected by HPLC. If a solvent other than those already considered appropriate for the ADRA is used for the test chemical, it is necessary to confirm that the solvent itself does not lead to NAC or NAL depletion (e.g., dimerisation, oxidation) and does not degrade or disrupt the integrity of the test subtances or mixture components. The test chemical should be pre-weighed into a disposable polypropylene tube and dissolved immediately before testing in an appropriate solvent to prepare a 4 mM stock solution (See paragraph 5).

15. This molecular weight approach should apply if the test chemical is a mono-constituent substance with a known molecular weight or a mixture or multi-constituent substance of known composition (See Figure 1). For mixtures and multi-constituent substances of known composition, a single aggregated purity value should be determined by the sum of the proportion of its constituents (excluding water), and a single aggregated 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 aggregated molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 4 mM solution.

16. Mono-constituent substances of unknown molecular weight should be tested based on a test chemical stock solution at a concentration of 0.5 mg/mL rather than 4 mM (7) (See Figure1 and paragraph 7). Polymers can also be tested at a concentration of 0.5 mg/mL. For mixtures and multi-constituent substances of unknown composition (i.e. UVCB substances of unknown or variable composition, complex reaction products or biological materials), the test solution can be prepared with a gravimetric approach. The substance should then be dissolved in the stock solution at 0.5 mg/mL on the basis of the weight of the total components (excluding solvent) in an appropriate solvent (See paragraph 14 and Figure 1). This 0.5 mg/mL of test chemical concentration corresponds to a molecular weight of 125 g/mol when ADRA (4 mM) is performed. The ADRA gravimetric approach with ADRA (0.5 mg/mL) has been shown to be almost as accurate in prediction as ADRA (4 mM) for 136 chemicals in a wide molecular weight range (30.03 - 512.60) (8) (see paragraph 7). This assessment of the predictive capacity of the gravimetric approach is based on testing chemicals with defined molecular weight and not based on the testing of mixtures, as no reference data for mixtures are available. Therefore, if the mixture to be investigated is known to contain a chemical class with a typical molecular weight which is significantly higher, this default molecular weight and the test solution concentration should be adjusted accordingly [see e.g. approach for agrochemical formulations in (24)]. The gravimetric approach should only be applied as a last resort if no aggregated molecular weight can be calculated. As for any testing with mixtures, as much as possible, information should be gathered on the sensitization potential and reactivity of individual constituents.

Preparation of the positive control, reference controls and co-elution controls

17. Either phenylacetaldehyde (CAS 122-78-1, purity ≥ 90%) or squaric acid diethyl ester (CAS 5231-87-8, purity > 95%) should be used as the positive control (PC) at a concentration of 4 mM in acetonitrile (10). Phenylacetaldehyde is prone to oxidation and polymerisation and integrity of the sample has to be assured by proper storage or by using fresh samples. Squaric acid diethyl ester should be stored protected from high temperature or humidity, since it is prone to hydrolysis. Other suitable positive controls that provide mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition, reference controls comprising only NAC or only NAL 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 analysis (Reference Control A), the stability of the reference controls over time (Reference Control B), and any effects of the solvent used on depletion of NAC or NAL (Reference Control C) (See Appendix II, Annex 3). The percent NAC and NAL depletion for a test chemical is calculated using an appropriate reference control for that test chemical (see paragraph 23). Also, a co-elution control comprising only the test chemical should be included in the run sequence to detect possible co-elution of the test chemical with either the NAC or NAL.

Incubation of the test chemical with the NAC and NAL solutions

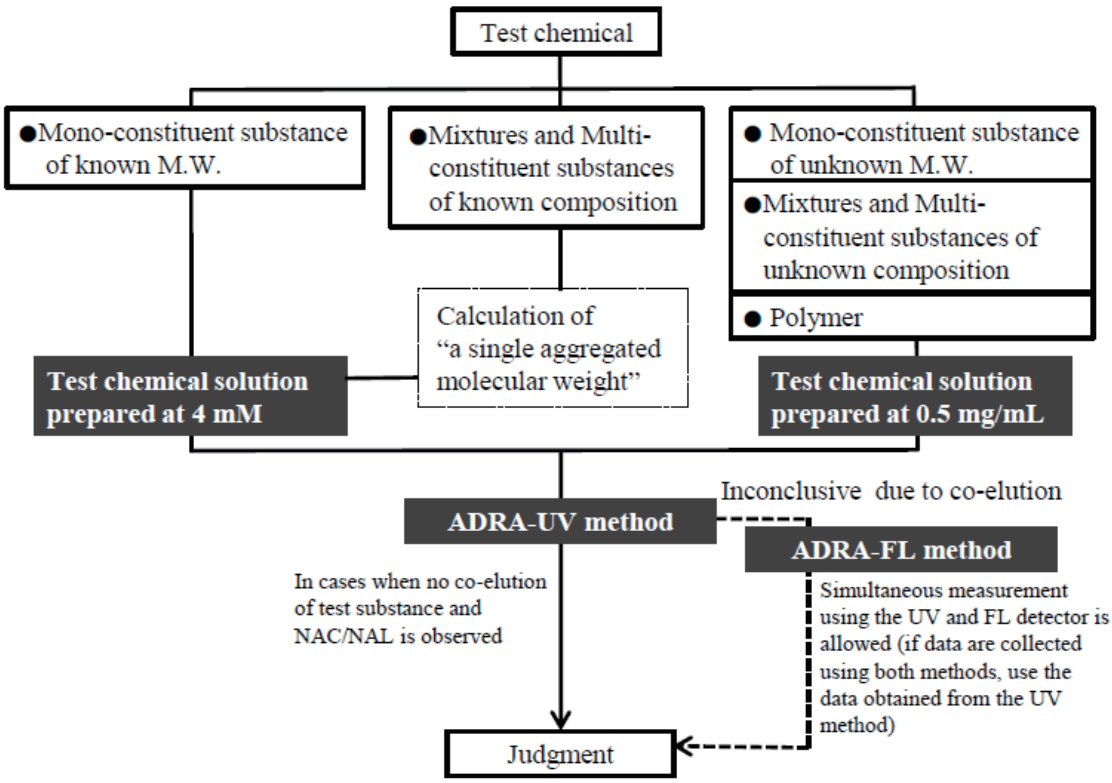
18. Both the NAC and the NAL stock solutions are incubated with the test chemical stock solution in a 3:1 ratio in a 96-well microplate. For the 4 mM test chemical stock solution this gives a final concentration of 1 mM test chemical and 5 µM NAC/NAL (17). For the 0.5 mg/ml test chemical stock solution, the final level of the test chemical is 0.125 mg/ml. The observation of precipitate immediately upon addition of the test chemical solution to the NAC and the NAL solutions is an indication of poor solubility, which means that there is no way to know exactly how much test chemical is contained in the solution. Thus, although positive results can be used with confidence, negative results are uncertain and no firm conclusion on the lack of reactivity should be drawn from a negative result (see also paragraph 5 regarding the testing of chemicals not soluble at concentrations as high as 4 mM). The reaction solution should be incubated in the dark at 25±1ºC for 24±1 hours before performing HPLC analysis. After incubation, trifluoroacetic acid (TFA) (≥ 98%) should be added to reaction solution as a fixing solution to stop the reaction (3). 2.5% (v/v) TFA aqueous solution is added to the reaction solution in a 1:4 ratio. Thus, final concentration of NAC/NAL and TFA are 4 µM and 0.5%, respectively.

HPLC preparation and analysis

19. NAC/NAL depletion is monitored by HPLC coupled with an UV-detector. In case of co-elution of NAC/NAL with an UV-absorbing component in the test chemical solution, a fluorescence detector is used (11) (12). There are two options for NAC/NAL detection: Successive measurement should be started with UV-detection and fluorescent detection is used only if inconclusive results due to co-elution are obtained. Alternatively, simultaneous measurement is performed by

connecting both the UV and FL detector to the HPLC system for parallel detection. If no co-elution of UV-absorbing components is observed, only the UV data are used. If inconclusive results due to co-elution are observed, FL data will be used (see Figure 1). In the unlikely event that a co-elution also appears in ADRA-FL, the operation should be performed according to paragraph 28. Each test chemical should be analysed in triplicate to determine percent depletion for both NAC and NAL. Although adding the fixing solution does stop the reaction, measurement of the reaction solution is to be performed as soon as possible and in any case within three days after adding the fixing solution. For example, when HPLC analysis of NAC and NAL are performed separately using two 96-well microplates, up to 34 samples may be analysed at one time, including 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 in triplicate. All of the replicates analysed in a single run should use identical batches of NAC and NAL stock solution. Test chemical and control solutions are to be visually inspected prior to HPLC analysis and may be centrifuged at low speed (100–400 × g) to force any precipitate to the bottom of the vial as a precaution against large amounts of precipitate clogging the HPLC tubing or columns. Observation of precipitation or phase separation after the incubation period is an indication that NAC and NAL depletion could be misleading, and negative results in that case are uncertain and should be interpreted with due care, as well as for any precipitate observed at the beginning of the incubation period (see above).

Figure 1: Procedure to assess NAC/NAL depletion in ADRA including a gravimetric approach for mixtures and alternative fluorescent detection in case of co-elution with UV-absorbing components.



MW, molecular weight; ADRA, amino acid derivative reactivity assay; UV, ultraviolet; FL, fluorescence

20. A standard calibration curve should be generated for both NAC and NAL. Standard solutions of both NAC and NAL should be prepared in 20% acetonitrile in buffer and containing 0.5% trifluoroacetic acid. For NAC, a phosphate buffer at pH 8.0, and for NAL, a phosphate buffer at pH 10.2 should be used. Using the NAC and NAL stock solutions (6.667 µM), six calibration solutions should be prepared in concentrations from 5.0 to 0.156 µM. A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an $R2 > 0.990$.

21. The suitability of the HPLC system should be verified before conducting the analysis. Both NAC and NAL depletion is monitored by HPLC coupled with an UV-detector (photodiode array detector or fixed wavelength absorbance detector with 281 nm signal) and a FL detector (Ex, 284 nm and Em, 333 nm) (see paragraph 19). The appropriate column is installed in the HPLC system. The recommended HPLC set-up described in the validated protocol uses a column with the

following specifications. Base particle: core-shell type silica gel, Particle size: 2.5~2.7 µm, column size: 3.0 × 150 mm as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated for at least 30 minutes at 40ºC with 50% phase A (0.1% (v/v) trifluoroacetic acid in water), 50% phase B (0.1% (v/v) trifluoroacetic acid in acetonitrile) before use. Then, the column is conditioned by running the gradient at least twice before actual use. The HPLC analysis should be performed using a flow rate of 0.30 mL/min and a linear gradient from 30% to 55% acetonitrile for NAC and from 25% to 45% acetonitrile for NAL within 10 minutes, followed by a rapid increase to 100% acetonitrile to remove other materials. Equal volumes of the standard solutions, test chemical solutions, and control solutions should be injected. The column should be re-equilibrated under initial conditions for 6.5 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 NAC and NAL, including the injection volume, which may vary according to the system used (typically in the range from 10–20 µL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated, preferably by testing the proficiency substances in Appendix II, Annex 2. Using the UV detection method, absorbance is monitored at 281 nm. If a photodiode array detector is used, absorbance at 291 nm should also be recorded. It should be noted that some batches of acetonitrile could have a negative impact on NAC and NAL stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 281 nm peak area and the 291 nm peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90% < mean area ratio of control samples < 100% would give a good indication that co-elution has not occurred. An example of HPLC analysis sequence is provided in Appendix II, Annex 3.

22. There are some test chemicals that could potentially promote oxidation of NAC. The peak of the dimerised NAC may be monitored visually in the case of ADRA-UV. However, since the NAC dimer does not exhibit fluorescence, it cannot be detected in the fluorescent detection mode. Any apparent dimerisation should be noted, since overestimation of NAC depletion could result in false-positive predictions (See paragraphs 4, 14 and Appendix II, Annex 1).

DATA AND REPORTING

Data evaluation

23. The concentration of both NAC and NAL is photometrically determined at 281 nm (UV detector) and if needed by fluorescence detection with Ex/Em, 284/333 nm (FL detector) (see paragraph 21) in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of both NAC and NAL using the linear calibration curve derived from the standards.

24. The percent depletion for both NAC and NAL 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 Appendix II, Annex 3) according to the formula described below.

$$\text{Percent NAC or NAL depletion} = \left[1-\left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}}\right]\right]x100$$

Acceptance criteria

25. The following criteria should be met for a run to be considered valid:

a) the standard calibration curve should have an $R^2 > 0.990$,

b) the mean percent NAC and NAL depletion value and the maximum standard deviation (SD) of the three replicates for the positive control (phenylacetaldehyde or squaric acid diethyl ester) should meet the following criteria:

- NAC depletion:

Phenylacetaldehyde: 30 - 80%; Squaric acid diethyl ester: 30 - 80 %

- NAL depletion:

Phenylacetaldehyde: 70 - 100%; Squaric acid diethyl ester: 70 - 100 %

- Maximum standard deviation (SD) for NAC and NAL depletion for both phenylacetaldehyde and squaric acid diethyl ester: < 10%,

c) the mean NAC and NAL concentration of both Reference Controls A and C should be 3.2–4.4 µM and the coefficient of variation (CV) of NAC and NAL peak areas for the nine Reference Controls B and C in acetonitrile should be < 10%.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated for that specific test chemical.

26. The following criteria should be satisfied for a test chemical's results to be accepted as valid:

a) the maximum standard deviation for the test chemical replicates should be < 10% for the percent depletion of both NAC and NAL,

b) the mean NAC and NAL concentration of the three Reference Controls C in the appropriate solvent should be 3.2–4.4 µM. The permissible range of the mean NAC concentration of Reference Control C when 5% DMSO in acetonitrile is used as a solvent is 2.8 to 4.0 µM (19).

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

27. The mean percent depletion of NAC and NAL is calculated for each test chemical. Negative depletion is considered to be "0" when calculating the mean. By using the NAC/NAL prediction model shown in Table 1, the threshold of 4.9% mean depletion should be used to support the discrimination between skin sensitisers and non-sensitiser in the framework of an IATA or a DA. The 4.9% of cut-off value for the mean percent depletion of NAC and NAL was set by using 2 class classification model so that the sensitizer and non-sensitizer could be predicted most appropriately.

Table 1: NAC/NAL prediction model[1]

Mean NAC and NAL percent depletion	ADRA prediction[2]
Less than 4.9%	Negative
4.9% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 13 and 14.

28. If co-elution is observed using either the UV or the FL detector, the depletion value measured using the detector in which co-elution is not observed should be used (See Figure 1). If co-elution is observed with both detectors, 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 NAC or NAL. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated, preferably by testing the proficiency substances in Appendix II, Annex 2. When co-elution occurs, it is not possible to integrate the peak of the NAC or NAL, thereby preventing calculation of the percent depletion of NAC or NAL. If co-elution of test chemicals occurs with both the NAC and NAL and separation of elution time is not feasible, then the analysis should be reported to be inconclusive. In cases where co-elution occurs only with NAL and separation of elution time is not feasible, the NAC-only prediction model (See Table 2) can be used to make a prediction. In this case, the NAC data of ADRA-UV should still be preferentially adopted than that of ADRA-FL. The 5.6% cut-off value for the percent depletion of NAC was set by using 2 class classification model so that the sensitizer and non-sensitizer could be predicted most appropriately.

Table 2: NAC-only prediction model[1]

Mean NAC percent depletion	ADRA prediction[2]
Less than 5.6%	Negative
5.6% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] An ADRA prediction should be considered in the framework of an IATA (13) (14).

29. When a result is unequivocal, a single HPLC analysis for both NAC and NAL should be sufficient for a test chemical. However, in case of results close to the threshold used to discriminate between positive and negative results (i.e. in the range of 3% to 10% for NAC/NAL prediction model or NAC percent depletion falls in the range of 4% to 11% for NAC-only prediction model), additional testing is recommended. In particular, in case of negative results in these ranges (i.e. 3% to 4.9% for NAC/NAL prediction model or 4 % to 5.6% for NAC-only prediction model), a second run should be conducted, as well as a third one in case of discordant results between the first two runs. In the above cases, the majority of the three test results is adopted.

Test report

30. The test report should include the following information:

Test chemical and Controls (positive control and solvent/vehicle)

- For all mono-constituent substance (test and control chemicals)
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers
    - Physicochemical properties such as physical state, 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 (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability to the extent available
- Multi-constituent substance, UVCB, and mixtures
    - Characterisation by 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) for mixtures or polymers of known composition, or other information relevant to the study

- Treatment prior to testing, if applicable (warming, grinding)
- Concentration(s) tested
- Storage conditions and stability, to the extent available.

- Additional information for positive control
  - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Additional information for solvent/vehicle control
  - Solvent used and ratio of its constituents, if applicable
  - Justification for choice of solvent for each test chemical
  - Impact on NAC and NAL stability when using acetonitrile

Preparation of NAC and NAL, positive control and test chemical solution

- Characterisation of NAC and NAL solutions (supplier, lot, exact weight of NAC and NAL, volume added for the stock solution)
- Characterisation of positive control solutions (exact weight of positive control reagent, volume added for the control solution)
- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test chemical solution)

HPLC instrument setting and analysis

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

System suitability

- NAC and NAL peak area at OD 281 nm (UV detector) or Ex/Em 284/333 nm (FL detector) of each standard and reference control A replicate
- Linear calibration curve graphically represented and the R2 reported
- NAC and NAL concentration of each Reference Control A replicate
- Mean NAC and NAL concentration (µM) of the three reference controls A, SD and CV
- NAC and NAL concentration of Reference Controls A and C.

Analysis sequence

- For Reference Controls
    - NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector)of each replicate of Reference Controls B and C
    - Mean NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of reference controls over analysis time)
    - For each solvent used, the mean NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of the three appropriate Reference Controls C (for the calculation of percent NAC and NAL depletion)
    - For each solvent used, the NAC and NAL concentration (µM) of the three appropriate Reference Controls C
    - For each solvent used, the mean NAC and NAL concentration (µM) of the three appropriate Reference Controls C, SD and CV.
- For positive controls
    - NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of each replicate
    - Percent NAC and NAL depletion of each replicate
    - Mean percent NAC and NAL 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
- NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of each replicate
- Percent NAC and NAL depletion of each replicate
- Mean of percent NAC and NAL depletion of the three replicate, SD and CV
- Mean of percent NAC and percent NAL depletion values
- Prediction model used and ADRA prediction

Proficiency testing

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals

Discussion of the results

- Description of any unintended modifications to the test procedure.
- Discussion of the results obtained with the ADRA test method and if it is within the ranges described in paragraph 29.

Conclusion

Literature for Appendix II

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APPENDIX II, ANNEX 1

Known limitations of the Amino acid Derivative Reactivity Assay (ADRA)

The table below provides a summary of the known limitations of the ADRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. 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	Diethylenetriamine; 111-40-0 (human 1A, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in some cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals that have a UV absorption (OD, 281 nm) or FL (Ex/Em, 284/333 nm) and have the same retention time than NAC or NAL (co-elution)	When co-elution occurs the peak of the NAC or NAL cannot be integrated and the calculation of the percent NAC or NAL depletion is not possible.	The substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for co-elution. The substances that have a FL in this range are generally limited to polyaromatic or polyheterocyclic compounds, including naphthalene derivatives. If co-elution of such test chemicals occurs with both the NAC and the NAL or with the NAC only, then the analysis should be reported as "inconclusive" and alternative HPLC set up should be considered (see paragraph 28). In cases where co-elution occurs only with the NAL, then the NAC-only prediction model reported in Table 2 can be used."	Safranal; 116-26-7
Complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	ADRA using a 4 mM chemical solution needs for defined molar ratio of test chemical and nucleophilic reagent, but ADRA using a 0.5 mg/mL solution does not need the defined molar ratio of a test chemical and can predict sensitisation for	Since plant extract contains various polyphenols, which react with NAC, it may be judged as a sensitiser when a solution containing a high concentration of the plant extract is evaluated using ADRA. Therefore, these results should be considered with reference to results obtained using alternative methods for other	n/a

	test chemicals, which are prepared at a weight concentration of 0.5 mg/mL. When the mixture is a liquid, the evaluation of sensitisation using ADRA cannot be performed if the total weight of the mixture components dissolved in solvent (water, dissolving solution, extraction solvent, etc) is not known, since it is then impossible to prepare a 0.5 mg/mL test chemical solution.	key events and *in vivo* results of similar substances.	
Test chemicals which cannot be dissolved in an appropriate solvent at a final concentration of 4 mM	Not sure if sufficient exposure can be achieved If the mixture is liquid and the total weight of the mixed components dissolved in a solvent (e.g., water, dissolving solution, extraction solvent) is not known, it is not possible to prepare a 0.5 mg/mL test substance solution, and thus the sensitisation potential cannot be evaluated by ADRA.	The ADRA test method allows testing of poorly soluble chemicals. Test chemicals that are not soluble at this concentration though may still be tested at lower soluble concentrations. In such a case, a positive result could 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.	n/a
Chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved	Test chemicals that precipitate in the reaction solution even if dissolved in the solvent 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.	Isopropyl myristate CAS: 110-27-0
Test chemicals that do not covalently bind to the NAC but promote its -oxidation (i.e. NAC dimerisation)	Could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions.	It may be possible to detect and quantify any NAC dimer formed by HPLC (UV detector), thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s) Therefore, ADRA may prevent erroneous judgement due to the oxidizing action of the test chemical. However, since the NAC dimer does not have fluorescence, it can only be detected by ADRA-UV.	DMSO Oxidant
Test chemicals that are only soluble in DMSO	DMSO causes excessive NAC depletion due to NAC dimerization resulting in high background NAC depletion.	DMSO is allowed to be contained in the test chemical solution up to 5%. If DMSO is chosen, attempts should be made to solubilise the test chemical in a 1:20 mixture of DMSO and acetonitrile (5% DMSO in acetonitrile).	n/a

APPENDIX II, ANNEX 2

Proficiency Substances

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

Prior to routine use of the test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected ADRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining NAC and NAL depletion values that fall within the respective reference ranges for 8 out of the 10 proficiency substances. The test to demonstrate technical proficiency in ADRA is basically ADRA with 4 mM (10). If ADRA with 4 mM has been proven to be mastered by performing proficiency substances, ADRA with 0.5 mg/mL can be exempt from demonstrating the technical proficiency (9). These proficiency substances were selected to represent the full 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 ADRA data are available, and that they were used during the JaCVAM-coordinated validation study to demonstrate successful implementation.

Table 1. Recommended chemicals for demonstrating technical proficiency with ADRA_4 mM

No.	Test chemicals	CAS No.	Physical state	Molecular weight	*In vivo* Prediction[1]	ADRA 4 mM prediction[2]	Range of % depletion	
							NAC[3]	NAL[3]
1	*p*-Benzoquinone	106-51-4	Solid	108.09	Sensitiser (extreme)	Positive	90-100	70-100
2	Diphenylcyclopropenone	886-38-4	Solid	206.24	Sensitiser (extreme)	Positive	50-90	≤ 10
3	2-Methyl-2H-isothiazol-3-one	2682-20-4	Solid	115.15	Sensitiser (strong)	Positive	80-100	≤10
4	Palmitoyl Chloride	112-67-4	Liquid	274.87	Sensitiser (moderate)	Positive	≤ 40	70-100
5	Imidazolidinyl urea	39236-46-9	Solid	388.29	Sensitiser (weak)	Positive	40-70	≤ 20
6	Farnesal	19317-11-4	Liquid	220.35	Sensitiser (weak)	Positive	60-100	5-40
7	Glycerol	56-81-5	Liquid	92.09	Non-sensitiser	Negative	≤ 7	≤ 7
8	Isopropanol	67-63-0	Liquid	60.10	Non-sensitiser	Negative	≤ 7	≤ 7
9	Dimethyl isophthalate	1459-93-4	Solid	194.19	Non-sensitiser	Negative	≤ 7	≤ 7
10	Propyl paraben	94-13-3	Solid	180.20	Non-sensitiser	Negative	≤ 7	≤ 7

[1]The *in vivo* hazard (and potency) predictions are based on LLNA data (20) (21) (22). The *in vivo* potency is derived using the criteria proposed by ECETOC (23).

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 3 and 5.

[3] Ranges determined on the basis of at least 10 depletion values generated by 5 independent laboratories.

APPENDIX II, ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

Each sample of HPLC analysis should be analysed in number order below. Refer to the table showing Examples of HPLC Sample Analysis Sequences for more practical sequences about HPLC analysis.

1. Start to analyse calibration standards and Reference Control A (N = 3).

2. The co-elution Control does not need to be analysed by turns if it is analysed after analysis of standard solution and Reference Control A.

3. Reference Control B should be analysed three times (total six times) before and after the analysis of sample, Reference Control C and Positive Control.

4. The Reference Control C, Positive Control and Test chemical solutions are analysed. (After the first set of replicates of each sample is analysed, the second set of replicates of each should be analysed).

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 Positive control, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Positive control, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Positive control, 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 (NAC or NAL dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC and NAL from each calibration curve after addition of acetonitrile rather than test chemical.

Reference control B: Control for verifying stability of reaction solution under analysis. Reference Control B is used to verify variability (CV) of each three NAC/NAL peak areas in the solution after addition of acetonitrile rather than test chemical at the start of analysis and at the end of analysis.

Reference control C: Control for calculating NAC/NAL depletion of each test chemical solution. To calculate depletion of NAC/NAL, measure three Reference Controls C after addition of solvent instead of test chemical. Prepare reference Control C for all solvents used to dissolve the test chemicals.

APPENDIX III

In Chemico Skin Sensitisation: kinetic Direct Peptide Reactivity Assay (kDPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The kDPRA is proposed to address the molecular initiating event of the skin sensitisation AOP - namely, protein reactivity - by quantifying the reactivity of test chemicals towards a synthetic model peptide containing cysteine in a time- and concentration dependent manner (1) (2). Kinetic rate constants are calculated and the logarithm of the maximum rate constant (log k_{max} value in $s^{-1}M^{-1}$) for a tested substance is then used to support the discrimination of UN GHS subcategory 1A skin sensitisers (subcategory 1A) from those not categorised as subcategory 1A (non-subcategory 1A) i.e., subcategory 1B or no category according to UN GHS (3). Based on theoretical consideration, the rate constant of the reaction between a test chemical and skin proteins will determine the amount of epitope formed from a given amount of chemical or, vice-versa, determine the dose needed to form the amount of epitope needed for induction of sensitization to occur and it is thus a rate limiting and potency determining step. Based on empirical evidence when evaluating 180 chemicals, the rate constant was shown to be the strongest determinant of potency among all evaluated parameters measured in OECD 442C, 442D and 442E (3).
2. The kDPRA proved to be transferable to laboratories without hands-on training (4). For the 24 test chemicals tested during the validation study, the overall within-laboratory reproducibility of kDPRA for assigning UN GHS subcategory 1A was 96% and the average between-laboratory reproducibility was 88% (4). Results from the validation study (4) as well as from other published studies (3) encompassing 180 test chemicals that fall within kDPRA's applicability domain indicate that kDPRA allows to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) according to UN GHS with a balanced accuracy of 85%, a sensitivity of 84% (38/45), and a specificity of 86% (116/135) relative to LLNA results (3). Similar performances were obtained when comparing kDPRA outcomes with the OECD LLNA database compiled within the context of the Test Guideline on Defined

Approaches for Skin Sensitization (15)[4]. In addition, the prediction for 123 test chemicals (out of the 180) having human skin sensitisation data (5) (6) has a balanced accuracy of 76%, a sensitivity of 64% (21/33), and a specificity of 89% (80/90) (3), although the human reference data are subject to a significant uncertainty[5]. 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, which is humans. For comparison, based on a data set of 123 chemicals used to evaluate the kDPRA vs. human sensitising potential, the LLNA showed a 73% balanced accuracy, a 55% (18/33) sensitivity and a 91% (82/90) specificity for the identification of UN GHS subcategory 1A. On the basis of the overall data available, kDPRA's applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in *in vivo* studies), and physicochemical properties (3). Following an independent peer review (16), the kDPRA was considered to be scientifically valid to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as 1A (non-subcategory 1A) according to UN GHS (7). The kDPRA can therefore be used (i) as a follow-up test method for sub-categorisation of chemicals identified as UN GHS Category 1 skin sensitisers, or (ii) on its own by using positive results for direct classification of a chemical into UN GHS subcategory 1A, depending on the regulatory framework.

3. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the kDPRA to the testing of substances and/or mixtures. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. Furthermore, kDPRA only measures reactivity with the cysteine peptide, so that strong sensitisers having an exclusive lysine-reactivity, such as some acyl-halides, phenol-esters or aldehydes are outside of the applicability domain of kDPRA. However, only few UN GHS subcategory 1A skin sensitisers are known currently to react exclusively with lysine residues. In addition, considering exclusive strong Lysine-reactivity from the DPRA or ADRA in a tiered strategy may reduce this uncertainty. 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 assignment to a higher reactivity class. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Reactivity of chemicals that require enzymatic bioactivation to

[4] A balanced accuracy of 85%, a sensitivity of 82% (31/38), and a specificity of 88% (102/116) were found relative to LLNA dataset compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15).

[5] A balanced accuracy of 67%, a sensitivity of 53% (9/17), and a specificity of 81% (25/31) were found relative to human skin sensitisation dataset compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15).

exert their skin sensitisation potential (i.e. pro-haptens) cannot be reliably detected by the test method. However, the limitation for detecting pro-haptens was found to be less pronounced when identifying strong sensitisers as compared to the identification of weak sensitisers (3). The majority of chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) were reported to be correctly detected by *in chemico* test methods (8) (9). However, spontaneously rapidly oxidizing pre-haptens may be under-predicted by kDPRA (as in any *in vitro* skin sensitisation assay) due to a lag-phase for oxidation which reduces the overall reaction rate. In the light of the above, results obtained with the test method that do not lead to subcategory1A categorisation should be interpreted in the context of the currently known limitations (see also Annex 1 of this Appendix), i.e.:

- aromatic amines, catechols or hydroquinones may require further data to confirm their weak reactivity even under oxidizing conditions, and
- acyl-halides, phenol-esters or aldehydes specifically reacting with Lysine-residue according to e.g. the DPRA or ADRA, may require further data to confirm their weak reactivity.

4. To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 20 mM (see paragraphs 12-13). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations as long as a k_{max} value (i.e., the maximum rate constant (in $s^{-1}M^{-1}$) determined from the reaction kinetics for a tested substance in the kDPRA (see paragraph 24)), can be derived. In such a case, a positive result leading to a UN GHS subcategory 1A skin sensitization prediction (i.e. log $k_{max} \geq -2.0$) could still be used, but no firm conclusion should be drawn from a negative result (i.e., non-reactive or log $k_{max} < -2.0$ outcome).

5. The kDPRA uses a fluorescence readout which requires attention for potential test chemical autofluorescence, fluorescence quenching or interaction with the reagent (monobromobimane). In particular, it is important to include the respective test chemical controls as described in paragraph 16 and to assess the incubation time dependence of the determined peptide depletion. Furthermore, test chemicals with primary SH-group (thiols) cannot be tested with the kDPRA as the thiol group can interact with the monobromobimane (see paragraph 8) leading to enhanced fluorescence. Finally, chemicals decomposing under the conditions of the assay (neutral, aqueous conditions) and releasing a free SH-group will be prone to the same limitations.

6. The kDPRA is considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition, although such substances were not tested during the validation studies. In this case, a single purity may be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight may 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 can then be used to calculate the weight of test chemical necessary to prepare a 20 mM solution. Results obtained with mixtures and multi-constituent substances of known composition

can lead to a non-linear behaviour, so that the provisions described in paragraph 27(ii) should be used. Regarding mixtures and substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances), the current model cannot be used due to the need for defined molar ratios. In any case, when considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Finally, in cases where evidence can be demonstrated on the non-applicability of the test method to specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

7. The kDPRA can be used for the discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory1A) according to UN GHS (3). As for any key-event based test method, the performance of kDPRA will have to be further assessed when used in combination with other assays such as DPRA or ADRA, and within integrated approaches such as IATA or DA for a more comprehensive analysis of skin sensitisation (3) (10).

PRINCIPLE OF THE TEST

8. The kDPRA is a modification of the *in chemico* test method DPRA (described in Appendix I of this Test Guideline). The kDPRA uses the cysteine peptide (Ac-RFAA**C**AA-COOH) also used in the DPRA, while it does not use a lysine containing peptide. The final concentration of the test peptide (0.5 mM) and the reaction medium (25% acetonitrile in phosphate buffer) is identical in the kDPRA and in the DPRA. While the DPRA measures only at one concentration of the test chemical (5 mM for the cysteine peptide) and at one time point (≥ 24 h), the kDPRA performs parallel reactions at five concentrations (5, 2.5, 1.25, 0.625 and 0.3125 mM) and at six time-points (10, 30, 90, 150, 210 and 1440 min) at 25±2.5ºC. Residual concentration of the cysteine peptide after the respective reaction time is measured after stopping the reaction by the addition of monobromobimane (mBrB; CAS 74235-78-2). The highly reactive and non-fluorescent mBrB rapidly reacts with unbound cysteine moieties of the model peptide to form a fluorescent complex which is measured in order to quantify the non-depleted peptide concentration. If the depletion of the highest concentration surpasses the threshold of 13.89% (cut-off used in the DPRA for positivity in the cysteine only prediction model) and this depletion is statistically significant vs. controls with peptide only, further calculations are performed (otherwise the test chemical is considered to be non-reactive according to the prediction model shown in paragraph 28). The natural logarithm of the non-depleted peptide concentrations is plotted vs. the concentration of the test chemical at each time point. If a linear relationship is observed (correlation coefficient > 0.90), the slope of this curve is determined and divided by the incubation time to calculate the rate constant in [$min^{-1}mM^{-1}$]. This value is transformed to the rate constant in [$s^{-1}M^{-1}$] and the logarithm is calculated. The maximum value observed at any time point is taken as the log k_{max}, and this maximum rate constant is the primary read-out of the test. It gives a quantification of the maximum kinetic rate of the reaction of the test chemical with the test peptide. Kinetic reaction rates of the cysteine peptide depletion are then used to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as 1A (non-subcategory 1A) according to UN GHS. Chemicals with a log k_{max} ≥ -2.0 are predicted as UN GHS subcategory 1A. The kinetic rate constant may be further used in integrated approaches such as IATA or DA to assess the skin sensitisation potency of a test chemical in a continuous scale as needed for risk assessment (3) (10).

9. Prior to routine use of this test method, laboratories should demonstrate technical proficiency, using the nine proficiency substances listed in Annex 2 of this Appendix.

PROCEDURE

10. This test method is based on the kDPRA DB-ALM protocol no 217 (11) which represents the protocol used for the industry-coordinated validation study. It is recommended that this protocol

is used when implementing and using the method in a laboratory. The main components and procedures for the kDPRA are described below.

Preparation of the cysteine-peptide

11. The stock solution of the cysteine containing synthetic peptide (Ac-RFAA**C**AA-COOH) of purity equal to or higher than 95% should be freshly prepared just before the incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer for test chemical soluble in acetonitrile and 1.0 mM for chemicals soluble in pH 7.5 phosphate buffer.

Preparation of the test chemical

12. Solubility of the test chemical in an appropriate vehicle should be assessed before performing the assay. A non-reactive, water-miscible vehicle able to completely dissolve the test chemical should be used. Solubility is checked by visual inspection where the forming of a clear solution is considered sufficient to ascertain that the test chemical is dissolved. The preferred vehicle is acetonitrile. When a substance is not soluble in acetonitrile, solubilisation in pH 7.5 phosphate buffer should be assessed. Further vehicles have not been tested yet but may be used if it is demonstrated that the vehicle does not interfere with the assay, e.g. all controls should be prepared using the same vehicle, and the reaction rates obtained for the positive control and for the proficiency chemicals should fall within the ranges described in paragraph 26 and Annex 2 of this Appendix, respectively. It is important to note that use of DMSO as a vehicle should be avoided as it may lead to peptide dimerisation.

13. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing to prepare a 20 mM solution using the appropriate vehicle as described in paragraph 12. Test chemical dilutions are prepared by serial dilution to obtain concentrations of 20, 10, 5, 2.5 and 1.25 mM.

Preparation of controls

14. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC). It is dissolved at a concentration of 20 mM in acetonitrile immediately before testing. Serial dilutions are then prepared to obtain PC concentrations of 20, 10, 5, 2.5 and 1.25 mM. Use of other positive controls is not recommended since in this assay an exact reaction rate is measured and consistent use of the positive control allows quantitative comparison between laboratories, with validation study data and as intra-laboratory historical control.

15. A vehicle control (VC), considered as the negative control, includes the peptide dissolved in buffer and vehicle respectively but no test chemical nor PC. The peptide-depletion of test chemical or PC incubated samples is calculated relative to the respective VC.

16. The assay also includes test chemical controls at the respective test chemical concentration in the vehicle and buffer but without peptide. This set of controls is used for the identification of interference of the test chemical with the fluorescence measurement (autofluorescence and quenching) to assess e.g., interference with monobromobimane and as a background measurement.

17. A blank control (BC) is used as a background measurement and is prepared with vehicle and buffer but without test chemical, PC, or peptide.

Incubation of the test chemical with the cysteine peptide solution

18. Serial dilutions of the test chemical and PC are prepared in a 96-well microtiter plate referred to as the application plate. Further, a 96-well black assay plate for each exposure time is prepared, referred to as the assay plates, by adding the relevant reagents (i.e., peptide stock solution, vehicle and buffer solution) according to a predefined plate layout such as recommended within the kDPRA protocol (11). Each test chemical concentration should be analysed in triplicate. The reaction is started by adding the test chemical and PC dilutions from the application plates to the assay plates. 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, one cannot be sure how much test chemical remained in the solution to react with the peptide. In such a case, a positive result (i.e. log $k_{max} \geq -2.0$) could still be used, but a negative result (i.e., non-reactive or log $k_{max} < -2.0$ outcome) should be interpreted with due care (see also provisions in paragraph 4 for the testing of chemicals not soluble up to a concentration of 20 mM in the kDPRA). After adding the test chemical and PC, plates are sealed with gas-tight adhesive foil and shaken at least 200 rpm for 5 min. Assay plates solution should be incubated in the dark at 25 ± 2.5° C for several incubation (exposure) times, i.e. 10, 30, 90, 150, 210, and 1440 min before addition of mBrB solution. Incubation times may be adapted to investigate the most relevant time points for a specific chemical (e.g., shorter incubation times might be more suitable for fast reacting chemicals). However, 1440 min should always be tested, as it corresponds to the incubation time of the DPRA. The incubation (exposure) time is the time interval from the application of the test chemical and PC dilutions to the assay plate until the addition of mBrB.

Fluorescence measurement

19. When the desired incubation (exposure) time is reached, freshly prepared mBrB solution (3 mM in acetonitrile) is added rapidly to the wells of the assay plates (one per exposure time) in the dark. Plates are sealed with gas-tight adhesive foil and shaken at least 200 rpm for 5 min. Fluorescence intensity is then determined using an excitation filter of 390 nm and an emission filter of 480 nm.

DATA AND REPORTING

Data evaluation

20. An automated Excel-evaluation spreadsheet is available with the DB-ALM protocol and should be used for data evaluation. Detailed instructions are provided in the DB-ALM protocol no. 217 (11).

21. For each incubation (exposure) time 't' the following parameters are calculated:

- The arithmetic mean and standard deviation of the fluorescence intensity of the 12 blank controls (BC);
- The arithmetic mean and standard deviation of the fluorescence intensity of the 12 vehicle controls (VC);
- The mean BC value is subtracted from the VCs to obtain corrected VC values.
- For each test chemical and PC concentration, the respective test chemical control value is subtracted from their obtained values to calculate corrected test chemical or PC values.

22. To determine the relative peptide depletion in % for each test chemical concentration per exposure time, the following calculation is performed:

$$relative\ peptide\ depletion\ [\%] \quad = \left[1 - \left(\frac{corrected\ test\ chemical\ or\ PC\ value}{mean\ of\ corrected\ VC}\right)\right] x100\%$$

23. For each test chemical concentration, the arithmetic mean and standard deviation of the three replicates is calculated (per exposure time). A student's t-test is performed to test whether the peptide concentrations measured in the three replicates is statistically significantly lower as compared to the concentration in the 12 VC wells.

24. In the kDPRA, reaction kinetic rate constants are determined as explained below if (i) a peptide depletion of ≥ 13.89% is observed at the highest test chemical concentration (final test chemical concentration 5 mM) at a given time and if (ii) the difference is statistically different from the VC. This 'positivity criterion' is based on the 'positive' criterion for peptide reactivity in the cysteine only prediction model of the DPRA described in Appendix I of this test guideline. If the positive criterion is not met, the test chemical is considered to be non-reactive according to the prediction model shown in paragraph 28.

The natural logarithm of the non-depleted peptide concentrations (100-relative peptide depletion (%)) is plotted vs. the concentration of the test chemical at each time point. If a linear relationship is observed (correlation coefficient > 0.90), the slope of this curve is determined. The absolute value of this negative slope corresponds to the observed reaction kinetic constant (pseudo first order rate constants $k_{observed}$ in mM^{-1}). From the $k_{observed}$ value for each exposure time, the

reaction kinetic constant (k_t) per concentration and incubation (exposure) time 't' is calculated as follows:

$$k_t\ [M^{-1}s^{-1}] = k_{observed} \cdot \frac{1000}{60 \cdot t}$$

with 't' being the exposure time in minutes. If no linear relationship is observed (i.e., correlation coefficient < 0.90), the recommendations within paragraph 27.ii should be followed.

25. For each exposure time 't' with a correlation > 0.90, the decimal logarithm (log k_t) is calculated and the highest value is determined as log k_{max}.

Acceptance criteria

26. The following criteria should be met for a run to be considered valid. If one or more of these criteria is not met the run should be repeated.

    a. PC: the log k of the PC at 90 min (log $k_{90\ min}$) should be within the following range: -1.75 to -1.40 $M^{-1}s^{-1}$. If no log $k_{90\ min}$ is obtained in case of e.g., reactivity is not yet statistically significant, the value at 150 min (log $k_{150\ min}$) can be taken into account and should lie in the following range: -1.90 to -1.45 $M^{-1}s^{-1}$.

    b. VC: The coefficient of variance of the 12 VC values of a plate should be < 12.5% for at least 5 of the 6 exposure times.

27. The data obtained for the test chemical are further assessed to check for possible conditions which may affect results:

    (i) Interrupted time-course: If significant peptide depletion is observed at early time-points but not at following time points, there is either an intrinsic non-linear reaction for the test chemical or an experimental variation. In such cases the run is repeated. If the same pattern is reproducible, a non-linear kinetic is proven and the rate-constant observed at early time points is accepted.

    (ii) Non-linear concentration-response: There are few cases where the concentration-response is not linear, but clear depletion is noted. In such cases no rate constant is calculated by the slope method, as regression coefficient is R^2 < 0.90. Alternatively, rate constants can also be calculated based on individual depletion values according to the formula:

    $$k = [\ln\,(100/(100 - dp))]/(E \times t)$$

    Where 'dp' is depletion in %, 'E' is the concentration of test chemical and 't' is the incubation (exposure) time. Rate constants according to this formula are calculated at each time point 't' and at each concentration 'E' with depletion values above the threshold of 13.89%. For

each time point 't' the average of the values for the different concentrations is taken, and then again the log k_{max} for the highest rate at any given time point is reported.

In such a case a repetition should be performed to check whether this non-linear behaviour is intrinsic to the test chemical, or whether an experimental variation is the cause. If the non-linearity is reproducible, this alternative rate calculation based on the individual depletion values is used for the final rating.

(iii) Fluorescence interference, namely autofluorescence or fluorescence quenching: Based on the control wells with test chemical only in absence of the test peptide, incidences of autofluorescence and fluorescence quenching by the test chemical can be detected. As the values are corrected for the autofluorescence recorded in the test chemical control wells, this shall not be a problem for low autofluorescence, but with a high autofluorescence, the fluorescence of the peptide-adduct and the autofluorescence may not be fully additive, and subtraction of autofluorescence may lead to apparent depletion, which is not due to loss of peptide signal but to this non-additivity. Thus, one should check whether the observed depletion is time dependent. If this is not the case and autofluorescence is observed, then depletion from autofluorescence is assumed to occur. Fluorescence quenching can also lead to 'pseudo-depletion', but this would happen immediately and resulting depletion would not increase with time. If both conditions are met, it is assumed that depletion from quenching occurs. These cases are rare. If this is not clear from the results a run may be repeated, but if the effect is clear-cut no repetition is needed. In such a case, the test chemical cannot be assessed in the kDPRA (technical limitation) unless the reaction can be measured with an alternative fluorescent probe not leading to autofluorescence or quenching (see Section II of the Annex 1 to DB-ALM protocol (11)).

(iv) All above cases are detailed in the DB-ALM protocol and automatic alerts appear in the Excel template provided with the DB-ALM protocol when evaluating the data.

Prediction model

28. The kDPRA uses kinetic rates of cysteine peptide depletion for discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) according to UN GHS (3). Results obtained with the test method that do not lead to subcategory 1A categorisation should be interpreted in the context of the limitations stated in paragraph 3 and Annex 1 of this appendix.

Table 1: kDPRA prediction model

Reaction rate	kDPRA Prediction
log k_{max} ≥ -2.0	UN GHS subcategory 1A
Non-reactive or log k_{max} < -2.0	Not categorised as UN GHS subcategory 1A* (non-subcategory 1A)

* Further information is needed to discriminate UN GHS subcategory 1B from UN GHS No Category. Depending on the context (e.g. IATA, DA) this information can be generated prior to or after performing the kDPRA.

29. In cases of a log k_{max} result close to the -2.0 threshold falling in the borderline range calculated for kDPRA (i.e., between -1.93 and -2.06 (12)), no conclusive prediction can be made. In this case, re-testing and/or additional data/information is needed before a conclusive prediction can be made.

30. The kinetic rate constant may be further used in integrated approaches such as IATA or DA to assess the skin sensitisation potency of a test chemical in a continuous scale as needed for risk assessment (3) (10).

Test report

31. The test report should include the following information

Test chemical and Controls (positive control and solvent/vehicle)

For all mono-constituent substance (test and control chemicals)

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

Physicochemical properties such as physical state, 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.

Additional information for positive control

Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

Additional information for solvent/vehicle control

Solvent/vehicle used and ratio of its constituents, if applicable;

Justification for choice of other solvent than acetonitrile and experimental assessment of the solvent effect on peptide stability.

Peptide

Supplier, lot, purity

Fluorescence analysis

Fluorimeter used (e.g., model and type), including wavelengths settings

Proficiency testing

Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals.

Discussion of the results

Description of any unintended modifications to the test procedure.

Discussion of the results obtained with the kDPRA test method and if it is within the ranges described in paragraph 29.

Description of any relevant observations made, such as appearance of precipitate in the reaction mixture at the end of the incubation time, if precipitate was resolubilised or centrifuged.

Conclusion

LITERATURE FOR APPENDIX III

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(2) Roberts, D. W.and Natsch, A., (2009) High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of michael acceptor electrophiles. Chem. Res. Toxicol. 22, 592-603.

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APPENDIX III, ANNEX 1

KNOWN LIMITATIONS OF THE KINETIC DIRECT PEPTIDE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the kDPRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Hydroquinones, catechols and aromatic amines	Lag time of oxidation may reduce apparent reaction rate	Results with log k_{max} < - 2.0 can only be accepted if low reactivity can be confirmed after oxidation	Para-phenylenediamine; 106-50-3; Human and LLNA 1A
Thiols or thiol-releasers	Test chemicals with primary SH-groups and those decomposing under the conditions of the assay can react with the detection probe	Test chemical cannot be tested in the kDPRA with derivatisation by thiol reactive probes: other kinetic data with the test peptide e.g. by HPLC may need to be generated (not part of this guideline)	Thioglycerol; 96-27-5; LLNA UN GHS category 1B; Human n/a
Test chemicals having an exclusive lysine-reactivity as observed in DPRA or ADRA	kDPRA only measures reactivity with the cysteine peptide	Results with log k_{max} < - 2.0 for chemicals which specifically deplete NH_2-groups, but not SH-groups in DPRA or ADRA are not conclusive	Some acyl-halides, phenol-esters or aldehydes, Dihydrocoumarin, 119-84-6; LLNA UN GHS category 1B; Human n/a, Glutaric aldehyde; 111-30-8; Human and LLNA UN GHS category 1A
Pro-haptens	Test chemicals for which there is evidence that they strictly require enzymatic bioactivation to exert their skin sensitizing potential	Strict pro-haptens may be underestimated. However chemicals which are i) strict pro-haptens (i.e. test chemicals not also acting as direct haptens or prehaptens, too) and ii) strong allergens were found to be rare	Diethylenetriamine; 111-40-0 (human 1A, LLNA UN GHS category 1)
Fluorescent chemicals with excitation in the range of the fluorescent probe	If fluorescence of test chemicals and of the mBrB-peptide adduct is not additive, pseudo-depletion is observed	Follow the considerations in the DB-ALM Protocol n° 217 to evaluate assay interference	Tetrachlorosalicylanilide; 1154-59-; Human and LLNA UN GHS category 1A
Test chemicals absorbing in	If test chemical quenches	Follow the considerations in the DB-ALM Protocol n° 217 to	Vanillin, 121-33-5;

the emission range of the probe	fluorescence emission of the mBrB-peptide adduct, pseudo-depletion is observed	evaluate assay interference	LLNA NC; Human n/a
Mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	no information on applicability of kDPRA is available in the published literature	n/a	UVCBs, chemical emissions, products or formulations with variable or not fully known composition
Test chemicals which cannot be dissolved in water or acetonitrile or a compatible water-miscible solvent	Not sure if sufficient exposure can be achieved	In such cases, a log k_{max} > -2.0 could still be used to support the identification of the test chemical as a UN GHS subcategory 1A skin sensitiser but no firm conclusion should be drawn in case log k_{max} is < -2.0. Alternative vehicle may be used according to the prescriptions given in paragraph 12.	n/a
Test chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved: 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, one cannot be sure how much test chemical remained in the solution to react with the peptide.	In such a case, a positive result (i.e. log kmax ≥ -2.0) could still be used, but a negative result (i.e., non-reactive or log kmax < -2.0 outcome) should be interpreted with due care (see also provisions in paragraph 4 for the testing of chemicals not soluble up to a concentration of 20 mM in the kDPRA).	Methyl-2-nonynoate[6]; 111-80-8; LLNA NC
Test chemicals promoting cysteine-peptide oxidation		May lead to a potential over estimation of peptide reactivity.	DMSO

[6] Roberts, D.W. and A. Natsch, *High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of michael acceptor electrophiles.* Chem. Res. Toxicol., 2009. **22**(3): p. 592-603

APPENDIX III, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: kinetic Direct Peptide Reactivity Assay (kDPRA)

Prior to routine use of the test method described in this appendix, laboratories should demonstrate technical proficiency by correctly obtaining the expected kDPRA prediction for at least 8 of the 9 proficiency substances recommended in Table 1 and by obtaining cysteine rate constants log k_{max} that fall within the respective reference range for 7 out of the 9 proficiency substances. These proficiency substances were selected to represent the range of responses for skin sensitisation hazard and potency. 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 kDPRA are available, and that they were used in the industry-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Proficiency substances	CASRN	Physical state	*In vivo* prediction[1]	UN GHS Category LLNA	UN GHS Category human	kDPRA prediction [2]	Range of log k_{max} [2]
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	1A	1A	1A	(-0.8) – (-0.4)
Methylisothiazolinone	2682-20-4	Solid	Sensitiser (extreme)	1A	1A	1A	(-0.5) – (-0.1)
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	1A	No data	1A	(-0.3) – (0.0)
Methyl-2-octynoate	111-12-6	Liquid	Sensitiser (strong)	1A	1A	1A	(-1.6) – (-1.2)
Isoeugenol	97-54-1	Liquid	Sensitiser (moderate)	1A	1A	1A	(-1.4) - (-1.1)
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	1B	No data	non-1A (1B or NC)	(-3.2) – (-2.1)
Ethylene glycol dimethacrylate (EGDMA)	97-90-5	Liquid	Sensitiser (weak)	1B	1B	non-1A (1B or NC)	(-2.8) – (-2.1)
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	No Cat.[3]	No Cat.[3]	non-1A (1B or NC)	Not reactive
Chlorobenzene	108-90-7	Liquid	Non-sensitiser	No Cat.[3]	No Cat.[3]	non-1A (1B or NC)	Not reactive

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

[1]The *in vivo* hazard and (potency) predictions are based on LLNA data (13). The *in vivo* potency is derived using the criteria proposed by ECETOC (14).
[2] Rounded ranges determined on the basis of at least 14 log k_{max} determinations generated by 7 independent laboratories.
[3] Non sensitisers according to the UN GHS.