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# OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

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**研究要旨**

本研究は, 化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG: Test Guideline）プログラム各国調整官作業班（WNT: Working Party of National Co-ordinators of the TGs programme）において, 1）日本で開発された種々のTGやガイダンス文書（GD: Guidance Document）, 有害性発現経路（AOP : Adverse Outcome Pathway）などの世界各国が必要とする成果物を公定化させること, 2）他国が提案する OECD 大型プロジェクトに関与し, その成果物に日本の主張を反映させること, 及び, これらから得られた成果を化学物質の審査及び製造等の規制に関する法律（化審法）や毒物及び劇物取締法（毒劇法）などの我が国の厚生労働行政に反映させること, を目的とする。

これまでの先行研究の成果として, 我が国で開発された腐食性試験代替法, 皮膚感作性試験代替法, 光毒性試験代替法, 内分泌かく乱性スクリーニング法などに関するTGや免疫毒性のAOPの公定化に寄与し, 非遺伝毒性発がんの試験の実施と評価のための戦略的統合方式（IATA: Integrated Approaches to Testing and Assessment）や皮膚感作性試験の確定方式（DASS: Defined Approach for Skin Sensitisation）の開発に関与してきたことが挙げられる。

本研究班では, これらの成果を生かし, TGに関しては, 令和3年度, 既存のTGである皮膚感作性試験代替法 ADRA（Amino acid Derivative Reactivity Assay）の適用濃度の変更を含む TG442C の改定をなすことができた。同時に DASS ガイドライン 497 が承認された。

令和 4 年度, 皮膚感作性試験代替法 ADRA の重量法の追加を含む TG442C の再改定をなすことができた。GD として, *in vitro* 免疫毒性試験の総説（DRP: Detailed Review Paper）が OECD に採択されたが, *in vitro* 生殖毒性試験の総説は論文投稿に留まった。

令和 5 年度, 既存の TG である皮膚感作性試験代替法 DPRA（Directive Peptide Reactivity Assay）の重量法の追加を含むTG442Cの再改定をなした。また, 既存のTGである皮膚感作性試験代替法IL-8 Luc assayを含むTG442Eの改定をなした。さらに, *in vitro* 免疫毒性試験 IL-2 Luc assay が TG444A として公表された。

AOP に関しては, AOP154：カルシニューリン阻害による T 細胞依存的抗体産生抑制及び AOP277：IL-1 receptor 結合阻害が採択され, i-library に収載された。また, 発がん性の Pathogenesis of chemically induced nasal cavity tumors in rodents: contribution to adverse outcome pathway が *J Toxicol Pathol.*に掲載された。

また, OECD で引き続き検討されている DASS や発達神経毒性, 非遺伝毒性発がんの IATA に関する大型プロジェクト等に参画して, その成果物に日本の意見や結果を反映させた。この目的を果たすため, TG や AOP それらに必要な補足実験データを取得するとともに, 日本から OECD に提出する資料を事前に相互確認し, また, OECD からの意見募集に適切に対応した。

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**A. 研究目的**

本研究は, 化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG：Test Guideline）プログラム各国調整官作業班（WNT: Working Party of National Co-coordinators of the TGs programme）において, 1）日本で開発された種々の TG やガイダンス文書（GD：Guidance Document）, 有害性発現経路（AOP：Adverse Outcome Pathway）や評価のための戦略的統合方式（IATA: Integrated

Approaches to Testing and Assessment）などの世界各国が必要とする成果物を公定化させること, 2）他国が提案するOECD大型プロジェクトに関与し, その成果物に日本の主張を反映させること, 及び, これらから得られた成果を化学物質の審査及び製造等の規制に関する法律（化審法）や毒物及び劇物取締法（毒劇法）などの我が国の厚生労働行政に反映させること, を目的とする。

**B. 研究方法**

B-1. AOPの開発

B-1-1. 免疫毒性のAOP

研究分担者の足利は, 日本免疫毒性学会会員をメンバーとする同学会試験法委員会AOP検討小委員会に免疫毒性AOPの開発を委託している。文献調査の結果に基づいて, MIE (Molecular Initiating Event), AO (Adverse Outcome)及びその間に介在するKE (Key Event)や KER(Key Event Relationship）を定めて, OECD に指定された外部（または scientific）reviewer 及びコーチの指摘事項に対応することで本のAOP開発を進めた。

B-1-2. 発がん性のAOP

研究分担者小川及び研究協力者西川が主体となり, ホルムアルデヒド誘発鼻腔発がん機序に関する論文に引き続き, 各種化学物質暴露による鼻腔発がん全般の AOP に関する論文作成を実施した。ラット, マウス, ハムスターに鼻腔腫瘍を誘発する化学物質について, PubMed の文献に加えて, NTP (National Toxicology Program), IARC (International Agency for Research on Cancer), 日本バイオアッセイ研究センターのデータベースを使用して情報収集した。誘発された鼻腔腫瘍について, 動物種, 投与経路, 組織型を分類し, 更には, 関連する非腫瘍性病変及び遺伝毒性のデータについても網羅的に検討し, 化学物質暴露による鼻腔発がん全般の AOP に関する論文として取りまとめた。投稿論文は, 査読後, 指摘事項への対応を行った。

B-1-3. 光毒性のAOP

研究分担者の尾上は, 開発中の光毒性AOP を専門家の意見に基づいて改変し, AOP wikiを更新した。

B-2. TG及びDRPの開発

B-2-1. 皮膚感作性試験

研究分担者の小島とともに, OECDのTGの開発プロジェクトWNTの進捗に合わせ, 班員を支援した。

1) ADRA

協力研究者の笠原とともに, 皮膚感作性試験代替法 *In Chemico* Skin Sensitisation：ADRA（Amino acid Derivative Reactivity Assay）の適用濃度の変更に関する追加バリデーション報告書及びTG442Cの改定案を令和 2 年度に作成した。令和 3 年度, ADRA に混合物が評価できる重量法を加えたTG442Cの再改定に向け, 尽力した。

2) DPRA

令和 4 年度, DPRA（Directive Peptide Reactivity Assay）の重量法も同 TG に追加するため, 協力研究者の笠原及び立花に加え, 他国の機関（P&G及びGivaudan）とともに共同研究を主導した。具体的には, コード化した10物質を4施設に配布し, 合

計20物質（分子量が大きく,バラツキが生じる可能性の高い感作性物質）を用い, 重量法の施設間再現性を確認するとともに, 既知法であるモル濃度法との比較研究を実施した結果を OECD に提出した。

3) IL-8 Luc assay

協力研究者の相場とともに, 令和 4 年度, IL-8 Luc assay TG442E の改定案を OECD に提出し, 各国からの改定要望に対処した。

4) EpiSensA

EpiSensA が TG442D に収載されることを目的に, バリデーション報告書と peer review report を令和 4 年度に OECD に提出した。令和 5 年度, 協力研究者の宮澤らとともに, TG 案を OECD に提出し, 各国からの改定要望に対処した。

5) Defined Approach for Skin Sensitisation（DASS）

令和 4 年度より, 足利とともに, DASS のプロジェクトに参加し, ガイドラインの成立に協力し, 他国の専門家と議論した。

B.2.2. 免疫毒性試験

1）DRP

令和 3 年度, 相場及び国際的な専門家とともに, *in vitro* 免疫毒性に関する DRP (Detailed Review Paper)を作成した。

2）IL-2 Luc assay

DRP の承認を待って提出することとした IL-2 を指標とした免疫毒性試験の TG 案を令和 4 年度に提出し, 各国からの改定要望に対処した。

3) IL-2 Luc LTT

令和 4 年度, IL-2 Luc assay の変法である IL-2 Luc LTT のバリデーション報告書の国際 peer review については, 足利は免疫毒性の専門家 5 名を reviewer に任命し, 対面形式も含め毎月会議を行って本試験法とバリデーション結果を review した。

B.2.3. 生殖毒性試験の DRP

令和 3 年度より, 小島は国際的な専門家とともに, *in vitro* 生殖発生毒性に関する DRP を作成した。

本分野の国内外の専門家とともに, *in vitro* 生殖毒性毒性に関する総説を作成し, Current Research Toxicology に投稿した。

B-2-3. Bhas42 細胞形質転換試験法の TG 開発

Bhas42 細胞形質転換試験法（Bhas42CTA）は, 化学物質の非遺伝毒性発がん性の検出が期待できる OECD 唯一の *in vitro* 試験法（GD231）である。分担研究者の大森は本試験法の SPSF（Standard Project Submission Form）案を作成した。

B-3. IATA 開発

B-3-1. 非遺伝毒性発がん性の IATA 開発への協力

OECD では, 非遺伝毒性発がん性検出を目的とした IATA 開発が 2016 年から行われている。専門委員会では Mode of Action（MoA）が議論され, それに基づき IATA 構築の方針が国際合意され, 2020 年は専門委員会として総説論文を公表した。MoA を構成する各 KE 及びそれらに対応した 13 の Assay Block において, 各種試験法の選出やその利用に関する考え方の作成及び評価を行った。

Step 1 では試験法毎にその利用に関する詳細な情報をとりまとめた考え方を作成

し, Step 2 では他のメンバーが試験法の利用に関する考え方の評価案を作成した。Step 2 の評価案をもとに, Assay Block のメンバー全体で協議し, 合意したものを Assay Block からの提案試験法とその評価結果としてグループ全体に提案する。

小川, 西川, 大森は, ひきつづき非遺伝毒性発がん性 IATA 開発専門委員会の web 会議に参加し, 開発方針に関する議論に参加した。当該 IATA における 13 の Assay Block の内 2 つまたは 3 つをそれぞれ分担し, そのサブグループ会議に参加し, 現存の試験法の利用に関する考え方などに関する論文化をすすめた。

B-3-2. 光毒性 IATA

令和 3 年度より, 小島は尾上及び JaCVAM 資料編纂委員会の協力を得て, 光毒性 IATA 案を作成し, OECD 専門家グループからのコメントに従って光毒性 IATA 案を修正した。

B-3-3. 発達神経毒性に起因する行動解析に関する情報収集

1）発達神経毒性評価のための行動解析に関する情報収集

これまでの国内外における発達神経毒性評価の現状について情報収集を行うとともに課題を抽出した。まず, 発達神経毒性試験における現状把握のために, 米国環境保護庁（EPA）及び OECD の発達神経毒性（Developmental neurotoxicity, DNT）テストガイドラインにおける, 行動解析に関する情報について内容を確認し, 比較した。また, 発達神経毒性評価の現状についての文献調査には, 医学・生物学分野の学術文献検索データベースである PubMed 及び MEDLINE を用いた。

＜文献検索に用いたキーワード＞

mice, rats, rodents, neurodevelopmental, developmental, neurotoxicity, test guideline

検索後, タイトル, 雑誌情報, アブストラクトを確認し, 下記（1）~（3）の内容を含む文献を選択した。

（1）げっ歯類（マウス, ラット）を用いた実験報告

（2）化学物質曝露による影響評価

（3）曝露時期, 投与期間, 用量等の実験条件や, 解析に用いた行動試験の具体的な記載

2) OECD からの意見募集への対応

JaCVAM 発達神経毒性資料編纂委員会にオブザーバーとして参画するとともに, 公定化に向けて進行中の *in vitro* DNT ガイダンス文書の提案資料に対して, OECD からの意見募集に適切な意見を返した。

B-4. AOP 及び TG の実験データ支援

B-4-1. *In vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

分担研究者の美谷島らは以下に示す研究を実施した。

B-4-1-1. 腸管由来組織における代替法の検討

1）*In vivo* モデルにおいて AOP となり得る毒性所見の検討

1-1) DSS （Dextran Sulfate, Sodium: MP Biomedicals, MW36,000～50,000 富士フィルム和光純薬（株））を 7 週齢の雄性

C57BL/6J マウスに 1.25, 2.5, 5.0%の濃度で 3 日から 2 週間飲水投与し, 小腸及び大腸の病理組織学的解析, 粘膜における炎症系ならびに細胞接着・機能に関与する遺伝子発現解析を行った。対照群として, 同様に溶媒を投与したマウスを用いて比較検討した。

1-2) 7 週齢の雄性 C57BL/6J マウスに DSS を 5.0%の濃度で 3, 5 または 7 日間飲水投与して, 解剖後, 小腸及び大腸を採取して, 病理組織学的観察並びに遺伝子発現解析を実施した。この検討により早期段階から生じる腸管の病態を探索するとともに, AOP の候補となり得る炎症関連因子をはじめ, 腸管バリア機能, セロトニン関連因子（受容体）について探索した。

2）マウス空腸由来のオルガノイドを用いた検討

2-1) 正常 C57BL/6J マウスから作製した腸管オルガノイドに炎症を惹起させ, 炎症関連因子の変動について検証した。DSS で誘発された *in vivo* 実験より得られた成果をもとに, TNFα を起炎物質として検討した。実験は, 通常のマウス由来腸管オルガノイドに TNFα を 0, 15, 30, 60 ng/mL の濃度で培地に添加し, 0, 1, 3, 6, 24 時間後に培養組織を回収し, MIP-2 (IL-8 のマウスホモログ) の発現, 細胞接着関連因子（e-Cadherin, Tight junction protein-1 (Zo-1)）への影響も解析した。

2-2) 上記 1-1)より採取した C57BL/6J マウスより対照群並びに DSS 処置を施した動物由来の空腸オルガノイドを作製し, 定法に従ってマトリゲルにて 4 日間培養した後, 各条件のオルガノイドを回収し, 遺伝子発現解析を実施した。この検討により in vivo と同様に, マウス小腸由来のオルガノイドにおいても AOP の候補となり得る因子（腸管の炎症関連因子をはじめ, 腸管バリア機能, セロトニン作用関連）について探索した

3）腸管上皮由来 Caco-2 細胞を用いた平面培養による検討

これまでの検討から, DSS を通常培地 (DMEM: Low Glu に 10% 胎仔ウシ血清（FBS）, 1% 非必須アミノ酸溶液（NEAA）, 1%ペニシリン・ストレプトマイシン添加) に 1% 濃度%(w/v)で混じ, Caco-2 に 24 時間曝露した。蛍光免疫組織学的染色による観察を行うため, Cell culture slide (4-well タイプ) の各 well に Caco-2 を 1.3 × 105 cell/mL で播種した。培地は 400 µL/well とした。2 日毎に培地交換を行い, 約 2 カ月後に DSS を 1%の濃度で曝露した。DSS 処理後に 3%パラホルムアルデヒドで固定し, 0.2% Triton-X100 で透過処理を行い, 更に 1% FBS でブロッキング後, e-Cadherin Rabbit Polyclonal Antibody (ProteinTech) を添加し一晩処理し, Donkey Anti-Rabbit IgG (H+L) Highly Cross Adsorbed Secondary Antibody, Alexa Fluor 555 (Thermo Fisher Scientific) を反応させ, 4',6-diamidino-2-phenylindole（DAPI）添加剤にて封入した試料について蛍光顕微鏡を用いて観察した。

4）腸管軸に着目した腸管傷害に起因した肝臓病態の修飾効果の検討

6 週齢の雄性 C57BL/6j マウスを用い, 1.25%DSS 水を 1 週間毎の間歇飲水投与を 3 週間実施した。食餌はコリン欠乏メチオニン低減アミノ酸－高脂肪食（CDAA-HF 食）を与えた。解剖時には, 大腸及び肝臓

を採取し, 遺伝子発現解析及び病理組織学的解析を実施した。この検討により腸管バリア機能の傷害による肝臓病態の増悪を探索し, 肝線維化の AOP の候補となり得る炎症関連因子の変動を探索した。

B-4-1-2. 肝臓における代替法に関する検討

肝線維化に対する代替法の基礎的データ支援を行う研究として, 1) 肝線維化を呈するげっ歯類モデルにおける重要な組織学的変化及び因子の探索, 2) ヒト培養肝星細胞株 LX-2 における, 筋線維芽様細胞への活性化と肝線維化げっ歯類モデルとの関連性, 3) マウス肝オルガノイド培養の基礎的検討及び肝線維化げっ歯類モデルとの関連性を検討した。

1) 肝線維化を呈するげっ歯類モデルにおける重要な組織学的変化及び因子の探索

肝線維化モデル動物として, 高度な線維化を誘発し得る非アルコール性脂肪性肝炎モデルであるコリン欠乏メチオニン低減アミノ酸食（CDAA）を用いた。雄性 F344 ラットならび C57BL/6J マウスに, それぞれに適した CDAA を 3 ヶ月与えて肝線維化を誘導した。病理組織切片を作製し, 線維化を評価するための Sirius Red 染色, 星細胞の活性化を評価するための αSMA 染色, 細胆管反応を評価するための Cytokeratin 19 染色に加え, SRY-box9（SOX9）及び Cluster of Differentiation 44（CD44）に着目して, 免疫組織化学染色と遺伝子発現解析を行った。また, CDAA を 3 ヶ月, 1 年 3 ヶ月投与したマウス肝サンプルを用いて RNA-Seq.解析を実施し, シグナルの変化を検索した。

2) ヒト培養肝星細胞株 LX-2 における, 筋線維芽様細胞への活性化と肝線維化げっ歯類モデルとの関連性

肝星細胞における線維化についての *in vitro* モデルとして, ヒト培養肝星細胞株（LX-2）を用いた検討を行った。LX-2 に TGFβ1 10ng/mL 1 時間または 48 時間刺激後, 形態学的観察, 遺伝子あるいはタンパク発現について解析し, 星細胞の活性化と, SOX9 及び CD44 の発現について検討した。また, TGFβ1 受容体阻害剤の前処置による同様の検討も行った。

3) マウス肝オルガノイド培養の基礎的検討及び肝線維化げっ歯類モデルとの関連性

マウス肝オルガノイド培養の基礎的検討として, マウスから肝臓を単離し, コラゲナーゼ処理後にマトリゲル上にて三次元培養を行い, オルガノイドを得た。得られたオルガノイドに対して, CK 19, SOX9, CD44 の免疫染色を行った。また, TGFβ1 30ng/mL 24 時間刺激を行い, 形態学的観察と, CK19, SOX9, CD44 ならびに αSMA, Collagen Type 1, Collagen Type 4, Fibronectin 遺伝子発現の変動を検索した。

B-4-2. DNA損傷・幹細胞マーカー等を指標とした免疫組織化学的検索による発がん性早期検出

研究分担者の豊田は, 6 週齢の雄 F344 ラットに, 以下の被験物質を 28 日間反復経口投与した（各群 5 匹）。

【令和 3 年度】

腎発がん物質 5 種 : 300 ppm Hexachlorobutadiene (HCBD), 10000 ppm 1-Amino-2,4-dibromoanthraquinone (ADBAQ), 500 ppm Dimethylnitrosamine (DMN), 1000

ppm *N*-Ethyl-*N*-hydroxyethylnitrosamine (EHEN)及び 40 ppm Azoxymethane (AOM), HCBD, ADBAQ, DMN は混餌, EHEN, AOM は飲水投与。ただしEHEN投与群については, 顕著な体重増加抑制が認められたため, 3 週目以降投与濃度を 500 ppm に変更した。

【令和 4 年度】

腎発がん物質 6 種：0.8% Lead (II) acetate trihydrate (LAT), 0.24% 1-Amino-2-methylanthraquinone (Disperse orange), 0.2% 3-(4-Chlorophenyl)-1,1-dimethylurea (Monuron), 0.25% Nitrofurantoin (NFT), 5% Phenolphthalein (Phph)及び 4% Quercetin, ならびに腎毒性/非発がん物質 2 種：0.04%/1% Carboxin (CBX)及び 0.04%/1% Fradiomycin sulfate (Neomycin)を混餌投与した。

【令和 5 年度】

腎発がん物質 5 種：90 mg/kg Tris(2-chloroethyl) phosphate (TCEP), 50 mg/kg 1,2,3-Trichloropropane (1,2,3-TCP), 100 mg/kg Bromodichloromethane (BDCM), 75 mg/kg 8-Methoxypsoralen (8-MOP)及び 100 mg/kg Hydroquinone (HQ)を強制経口投与した。

各物質の投与濃度は短期試験における最大耐量として設定した。投与期間終了時に解剖し, 腎臓及び肝臓の重量を測定した。腎臓の病理組織学的検索を実施するとともに, 免疫組織化学的手法による γ-H2AX 形成の定量解析を実施した。右腎横断面において皮質及び髄質外帯外層の特定部位を顕微鏡下（×400）でそれぞれ 4 か所撮影し, 尿細管上皮細胞の総数ならびに γ-H2AX 陽性細胞をカウントすることで陽性細胞率を測定した。

B-4-3. 遺伝毒性の AOP 開発

研究分担者の堀端は, 遺伝毒性初期応答反応の早期検出システムを構築するため, クロマチン免疫沈降法（Chromatin immunoprecipitation; ChIP）及び定量的 PCR を用いた DNA 損傷応答の分子生物学的解析を実施した。なお, 一般的なコーディング DNA 領域と比べて, リボソーム DNA（ribosomal DNA; rDNA）は 1 細胞あたりヒトでは数百コピーから成るクラスターを形成しており, また, DNA 代謝反応である転写の機序についての知見も豊富であることから, rDNA を, 定量的 PCR を利用した DNA 損傷応答解析の標的領域とした。

Flp-In 293 細胞を用いて, DNA 損傷の定量化が可能な紫外線照射（UV-C, 10 J/m$^2$）により DNA 損傷を誘導した。その後速やかに 1%ホルムアルデヒドにより架橋した後, ChIP に供した。

ChIP において, ポリクローナル抗体としては, rDNA 上の転写装置である RNA polymerase I （RNAPI）の構成サブユニット RPA194, DNA 損傷応答マーカーであるヒストンバリアント γH2AX, DNA 損傷応答タンパク質の一つとして知られる Ku80, または DNA 二本鎖切断修復の非相同末端結合経路中において二本鎖切断を結合する ATP 依存性 DNA リガーゼとして知られる LIG4 を認識する抗体をそれぞれ使用した。

モノクローナル抗体としては, 各 DNA 修復及び DNA 損傷応答タンパク質を認識する市販の 21 種類のモノクローナル抗体を入手し, これらを用いて紫外線 DNA 損傷を誘導した Flp-In 293 細胞での予備的な

ChIP を実施し, 各抗体の DNA 沈降量を調査することで ChIP に適用可能なモノクローナル抗体として, γH2AX, ATM, MRE11 及び BRCA1 を標的とする抗体に絞りこみ, これらの抗体を続く解析に用いた。

目的とするタンパク質と共沈する DNA 画分を ChIP によりそれぞれ調製し, これらの DNA 画分を鋳型 DNA とし, rDNA unit を転写領域及び非転写領域を含む領域に分けてそれらを標的としたプライマーセットを用いた定量的 PCR により, DNA 損傷誘導時におけるそれぞれのタンパク質の rDNA 上での位置的相対量変化を解析することで, DNA 上で直接的に生じている DNA 損傷応答の定量・定性的検出を実施した。また, 効率的に研究を進める上で, モノクローナル抗体を用いる解析では H42 のプライマーセットは使用しなかった。

B-4-4. 腎障害・線維化の分子メカニズムに関する研究

研究分担者の松下は, 以下のような研究を実施した。

【令和 3 年度】

実験 1：6 週齢の雄性 SD ラットを 3 群に配し（n=5）, 媒体である 0.5%メチルセルロースもしくはアロプリノール（APL）を 100 及び 150 mg/kg 体重（5 mL/kg 体重）の用量で 1 日 1 回, 28 日間反復強制経口投与した。体重測定を週に 1 回行い, 最新体重に基づいて投与容量を算出した。最終投与 1 日後にイソフルラン深麻酔下において腹大動脈から採血した後, 放血により安楽死させて剖検を行った。剖検時に腎臓を摘出して重量を測定した後, 一部を 10%中性緩衝ホルマリンにて固定し, 残りの組織は液体窒素にて瞬間凍結もしくは OCT コンパウンドにて凍結ブロックを作製して-80℃にて保存した。得られた血液サンプルを常温下で遠心して血清を分離し, 尿素窒素（BUN）及びクレアチニン（sCre）の値を測定した。また, 10%中性緩衝ホルマリンで固定した腎臓組織を用いて定法に従いパラフィン包埋, 薄切し, HE 染色及び膠原線維を赤色に染色するシリウスレッド染色を施して病理組織学的検索を行った。さらに免疫組織学的解析のため, 組織標本を抗原賦活化処置としてクエン酸緩衝液（pH6.0）に浸漬してオートクレーブ処置し（121℃, 15 分）, 3% $H_2O_2$/メタノールにて内因性ペルオキシダーゼを除去した。引き続き, 非特異反応を除去するため 10%正常ヤギ血清を用いてブロッキング処置を施した後, 抗 CD44 抗体（ポリクローナル, x10000, Abcam）を 4℃にて一晩インキュベートし, 二次抗体（ポリマー法：ヒストファインシンプルステイン）を室温下で 30 分インキュベートした。ジアミノベンジジンにて反応を可視化し, ヘマトキシリンにより核染色を行った。

実験 2：6 週齢の雄性 SD ラットを 3 群に配し（n=5）, 媒体である生理食塩水もしくはバンコマイシン（VAN）を 200 及び 400 mg/kg 体重（10 mL/kg 体重）の用量で 1 日 1 回, 28 日間反復腹腔内投与し, 実験 1 と同様の測定及び解析を実施した。

実験 3：6 週齢の雄性 SD ラットを 3 群に配し（n=5）, 媒体である生理食塩水もしくはピューロマイシン（PAN）を 8 及び 12 mg/kg 体重（5mL/kg 体重）の用量で 1 日 1 回, 28 日間反復静脈内投与し, 実験 1 及び 2 と同様の測定及び解析を行った。

【令和 4 年度】

1 年目に実施した APL を用いた動物実験により得られた腎臓, 血清及び尿サンプルを用いて以下の追加解析を行った。全群について 10%中性緩衝ホルマリンで固定した腎臓組織を用いて定法に従いパラフィン包埋, 薄切（4 μm）し, 免疫組織学的解析により α-smooth muscle actin（αSMA）, aquaporin1（AQP1）, N-cadherin, vimentin, collagen type IV, nidogen-1 及び fibronectin の発現を解析した。また CD44 と各種因子の局在を解析するため二重蛍光免疫染色を行った。同一宿主の 2 種類の抗体を用いる場合は, チラミドシグナル増幅法により染色を実施した。さらに fibronectin1 (*Fn1*)の mRNA の局在を *in situ* hybridization 法により解析した。

また対照群及び 30 mg/kg 群の凍結ブロックを薄切（16 μm）し, on ice で迅速 HE 染色を施した。対照群の正常尿細管及び 30 mg/kg の線維化病変内の尿細管をレーザーマイクロダイセクションにより採取した。得られたサンプルから total RNA を抽出して増幅処置を行い, マイクロアレイにより遺伝子発現を網羅的に解析した。正常尿細管と比較して 30 mg/kg 群の線維化病変内の尿細管において発現の変動していた遺伝子群を抽出し, Gene ontology（GO）解析及び Ingenuity® Pathway Analysis によるパスウェイ解析を行った。

尿中タンパクを精製及び濃縮した後, ウエスタンブロッティング法により CD44 発現を解析した。また ELISA 法により血清中 CD44 値を測定した。また瞬間凍結した腎臓組織から total RNA を抽出し, total CD44 及び CD44 standard isoform に対するプライマーを用いてリアルタイム PCR を行い, 遺伝子発現量を解析した。

【令和 5 年度】

6 週齢の雄性 SD ラットを 3 群（n=5）に配し, 既報に従い実験期間を通して低 Na 食（0.05% Na）を給餌した。実験開始 1 週間後から媒体であるオリーブオイルもしくはシクロスポリン（CyA）を 15 及び 30 mg/kg の用量で 28 日間反復皮下投与した。最終投与日の翌日にイソフルラン深麻酔下において腹大動脈から採血した後, 放血により安楽死させて剖検を行った。得られた血液サンプルを常温下で遠心して血清を分離し, ELISA 法により血清 CD44 値を測定した。

剖検時に腎臓の重量を測定した後, 組織の一部を 10%中性緩衝ホルマリンにて固定し, 残りの組織は液体窒素にて瞬間凍結もしくは OCT コンパウンドにて凍結ブロックを作製して, -80℃にて保存した。全群について 10%中性緩衝ホルマリンで固定した腎臓組織を用いて定法に従いパラフィン包埋, 薄切（4 μm）し, 1 年目及び 2 年目と同様に病理組織学的解析, 免疫組織学的解析及び *in situ* hybridization を実施した。また対照群及び 30 mg/kg 群の凍結ブロックを用い, 2 年目と同様にマイクロアレイを実施し, さらに瞬間凍結した腎臓組織から total RNA を抽出し, total CD44 及び CD44 standard isoform の遺伝子発現量をリアルタイム PCR にて解析した。

結果は平均±標準偏差で示した。統計学的解析として, 各データについて一元配置分散分析（ANOVA）を実施した後に Dunnett 法による多重検定を行った。また 2 つの因子の相関関係を解析するため

Spearman の順位相関係数を求めた。有意水準は 0.05 に設定した。

B-5. OECD に提出する資料の事前確認と OECD からの意見募集への対応

B-5-1. SPSF

小島とともに, 日本から提出した SPSF の成立に寄与した。

B-5-2. Emerging technologies in the Test Guidelines Programme に関するワークショップ

小島とともに, emerging technologies in the Test Guidelines Programme のワークショップに関与し, 今後の TG の在り方について議論した。

B-5-3. Stakeholders Workshop on Operational and Financial Aspects of Test Methods Validation

小島及び足利とともに, Stakeholders Workshop on Operational and Financial Aspects of Test Methods Validation に関与し, 今後のバリデーションの在り方について議論した。

B-6　毒性等情報収集調査

B-6-1. OECD IATA Case Studies Project の調査

OECD IATA Case Studies Project における事例研究の公開資料[*1] から, 幾つかを題材として, AOP の IATA への活用に関する調査を行った。提出されたケーススタディと, 対応するレビューコメントの内容を対象とした。

[*1]http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm

2019-5:「分岐鎖が異なるカルボン酸によるリードアクロスを用いた 2-エチル酪酸の 90 日間反復投与毒性（OECD408）予測」, 2016-5:「曝露の考慮と非動物的手法に基づく化学物質安全性評価ワークフロー」, 2020-1:「1%フェノキシエタノール配合ボディローションの IATA を用いた全身毒性評価」, 2021-8:「皮膚感作性への IATA の適用 -ゲラニオールを用いた NGRA フレームワークの実証-」, の計 4 件の AOP を用いたケーススタディを対象に, また, 比較として, 非 AOP ケーススタディ 1 件, 2017-4:「アリールアルコールアルキルカルボン酸エステルの IATA を用いた亜慢性反復投与毒性のリードアクロス」, 計 5 件の調査を実施した。

（倫理面への配慮）

本研究では, 動物実験を用いない調査研究が多い。動物実験を用いる研究においても, 動物の数は最小限にとどめ, 実験は国立医薬品食品衛生研究所及び東京農業大学の実験動物取扱い規定に基づき, 動物の苦痛を最小限とするよう配慮して行った。

**C. 研究結果**

C-1. AOP の開発

C-1-1. 免疫毒性の AOP

1)「カルシニューリン阻害による T 細胞依存的抗体産生抑制：AOP154」については, WNT/WPHA(Working Party on Hazard Assessment) に提出したのち, ドイツからコメントがあり, AOP Wiki の改訂作業を行った。ドイツからの主な指摘は, TDAR (T cell Dependent Antibody Response)アッセイの方

法をガイドラインごとに詳細に記載すること, 本 AOP のみで免疫毒性試験が免除されることはなく, 本 AOP は IATA 開発に利用されるべきであること, 本 AOP の EU 地域での規制上の重要性(regulatory significance)についても記載することなどであった。指摘事項に対応し, OECD 事務局に改訂完了の連絡と著者回答ファイルを提出したところ, OECD 事務局から, 本 AOP が WNT/WPHA で承認されたという通知があった。その後 OECD 事務局に著作権譲渡に関する著者全員の署名書類を提出し, 2021 年 10 月 15 日に OECD i-library において公開された。

2)「IL-1 receptor 結合阻害：AOP277」については, IL-1β のレセプター結合阻害により T cell の活性化が抑制され, 最終的に TDAR の阻害になるという AOP である。本 AOP はすでに OECD による外部 review に入っており, 主な指摘事項は, IL-1R シグナルを阻害するストレッサーに特異抗体だけでなく化合物/医薬品を加えること, AP-1 など NF-kB が関与しない経路も考慮すること, T cell のタイプを明確にすること, 増加する感染のタイプを明確にすることなどであった。これらの指摘に対し, AO を測定可能な指標である TDAR（AOP154 の AO と共有）に変更する, KE1 に AP-1 に関する記載を追加するなどの対応案を示したところ, 外部 reviewer に承認されたことから, AOP wiki の修正を行った。さらに, AOP wiki における AO の修正に伴い, AO986 の AO984 への置き換えと KER2928 の修正も行った。その結果, 2023 年 10 月末 OECD より, 本 AOP は承認され, AOP No.30 として i-library に収載された。

3)「TLR (Toll-like receptor) 7/8 への結合による乾癬様皮膚疾患の増悪：AOP313」は, 樹状細胞に存在する TLR7/8 への結合が, 樹状細胞の成熟と IL-23 の産生, Th17 による IL-17 の過剰発現と炎症を誘導し, 最終的に乾癬様の皮膚疾患を生じさせるという AOP である。本 AOP については, OECD により選定されたコーチの指摘事項について, 対応方針（汎用性向上のために, 関連する AOP の KE とネットワークを構築できるよう KE 及び KER の修正など）をコーチに相談し, wiki の修正を試みていた。しかしサポートする情報の少なさから外部レビューを経た OECD AOP wiki への収載を断念することとなり, Toxicology letter 又 Archives of Toxicology 誌への掲載を目指すこととして, 投稿論文形式に修正した原稿を作成した。

4)「免疫細胞に存在する ER (Estrogen Receptor)の活性化による全身性リテマトーデス(SLE)の増悪：AOP314」は, さまざまなタイプの免疫細胞に存在する ER の活性化が Th2 タイプのサイトカイン(IL-4)の過剰発現を誘導し, 自己抗体産生 B 細胞の誘導から最終的に自己免疫疾患である SLE を増悪させるという AOP である。本 AOP については, コーチから指摘された, KER をサポートする実験情報の少なさや, AO の規制上の重要性の不足から, 外部レビューを経た OECD AOP wiki への収載を断念することとなり, Toxicology letter 誌の掲載を目指すこととし, 投稿論文形式に修正した原稿を作成した。

5)「JAK3 の阻害による T 細胞依存的抗体産生抑制：AOP315」は, 非受容体型チロシンキナーゼの 1 つである JAK3 の阻害により IL-4 産生が抑制され, 最終的に TDAR の

阻害となるという AOP である。本 AOP については, コーチによる内部 review が終了しており, 外部 review の進め方についてコーチを介して OECD に確認したところ, OECD と MOU (Memorandum of understanding)を締結したジャーナルかメンバー国等による外部 review のどちらか選択すべきと回答があった。そこで OECD と MOU を締結した ALTEX (Alternatives to Animal Experimentation) 誌に投稿の意向を伝えたところ, 代替法開発につながる AOP かどうか不明という指摘があった。これに対し, TDAR のような免疫反応の抑制を in vitro 試験で置き換えるには, IL-2, IL-4 といったサイトカインの産生を指標にする試験法の組み合わせが有効であると OECD の *in vitro* 免疫毒性試験に関する DRP に記載があることから, IL-4 の抑制である本 AOP の KE3 が将来 IL-4 産生を指標とする in vitro 免疫毒性試験法の開発につなげることを主張し, 論文投稿につなげる予定である。

C-1-2. 発がん性の AOP

C.1. 発がん性の AOP 開発

網羅的に情報収集した鼻腔発がん物質のうち 40 種の吸入暴露による発がん物質（ラット 38 物質, マウス 11 物質, ハムスター5 物質）及び 38 種の非吸入暴露による発がん物質（ラット 36 物質, マウス 5 物質, ハムスター17 物質）について誘発された鼻腔腫瘍を, 国際統一毒性病理用語・診断基準(International Harmonization of Nomenclature and Diagnostic Criteria: INHAND)に基づいて分類した結果, 扁平上乳頭腫, 扁平上皮癌, 腺腫, 腺癌, 腺扁平上皮癌, 神経上皮癌, 未分化癌, 非特異的な癌, 線維肉腫, 血管腫, 血管肉腫, 粘表皮腫, 横紋筋腫, 横紋筋肉腫が報告されていた。最も高頻度の鼻腔腫瘍は呼吸上皮由来の扁平上皮癌であり, 投与経路に関係なく認められ, その前駆病変として, 扁平上皮化生及び/または扁平上皮乳頭腫と呼吸上皮過形成が示唆された。2 番目に多いのは腺癌であり, その前駆病変として主に嗅上皮過形成が示唆された。一方, 腺腫の前駆病変は呼吸上皮病変と考えられた。マウス及びハムスターのデータは限定的であったものの, これらの経路には, 明らかな種差は見られなかった。当該内容について, 論文を *J Toxicol Pathol* に投稿し，査読指摘事項への対応を経て受理された。

C-1-3. 光毒性の AOP

OECD の専門家会議で意見を求め，光毒性反応のうち光刺激性に限定した AOP 作成を推進した。体内に取り込まれた光毒性物質はまず皮膚組織に到達し，薬剤の分子内 chromophore，あるいは代謝によって獲得された chromophore が皮膚深部まで到達した光によって照射されると，基底状態の $S_0$ から励起一重項状態 $S_1$ に励起される。励起一重項状態の寿命は極めて短く，すなわち蛍光を発して直ちに基底状態 $S_0$ に戻るか，項間交差により励起三重項状態 $T_1$ に遷移する。励起三重項状態にある化合物はりん光を発して基底状態 $S_0$ に戻る。基底状態ではまったく化学反応をしない条件でも，高い光エネルギーを獲得した励起分子は，そのエネルギーを駆動力として結合の解裂や生成または組み換えなどの化学反応を起こすことができる。そのような過程を光化学過

程といい，ラジカル反応である Type I 反応と，一重項酸素反応である Type II 反応とに分けられる。酸素分子は励起エネルギーのアクセプターとして機能し，それに伴い産生された singlet oxygen や superoxide 等の活性酸素種 (Reactive oxygen species; ROS) による生体内物質の酸化反応が薬剤性光線過敏症の発症原因の一つとして考えられている。これらの光化学反応の標的が細胞膜上の各種生体成分である場合には光刺激性を誘発し，また DNA の酸化あるいは塩基修飾によって光遺伝毒性や光がん原性が発現する。励起された薬物がハプテンとなりタンパク質と光付加物を形成した際には，免疫原性を示すことになり，最終的に光アレルギー反応を惹起するものと考えられる。いずれにせよ，薬剤性光線過敏症の機序を考えるとき，最も重要なトリガーとなるのは太陽光の吸収，そしてそれに伴う化合物の励起であろう。しかし，励起された全ての化合物が一様に光毒性を惹起するわけではなく，実質的な光化学的反応を引き起こす化合物が光毒性を誘発するものと考える。この観点から「太陽光の吸収しやすさ」の指標である UV/VIS 吸収特性よりも，むしろ励起エネルギーによる光化学的反応性を直接評価するアプローチがより実質的な光毒性予測に寄与できる可能性がある。すなわち，光吸収に伴う化学物質の光反応が光毒性のトリガーであるとのコンセプトに基づき，UV/VIS 吸収とそれに伴った励起を pre-MIE として定義した。またそれに伴う MIE は励起化合物からの ROS 産生とし，次いで 細胞障害を KE，最終的な Tissue response を 炎症 とした。

C-2. TG 及び DRP の開発

C-2-1. 皮膚感作性試験

1) *ADRA*

令和 2 年度から検討を続けてきた *In Chemico* Skin Sensitisation, ADRA に関しては, ADRA の国内施設の協力を得て適用濃度を 1mM から 4mM に引き上げたプロトコルを用いた追加バリデーションの報告書及び TG442C の最終改定案を作成し, 令和 2 年 7 月に OECD に提出した。11 月の専門家会議を経て, 改訂 TG 案を OECD に提出した。令和 3 年 4 月の WNT 承認を経て, 7 月に改定 TG が公表された。さらに, ADRA の中に混合物が評価できる重量法を加える TG442C の再改定案を作成し, WNT で議論された結果, 重量法を加えた TG442C の再改定が令和 4 年 9 月に公表された。

2)DPRA

OECD から要請を受け, 令和 4 年, TG442C に追加する DPRA 重量法に関する共同研究を主導した。その結果, 20 物質すべてでモル濃度法と重量法が一致した結果となることを確認した。令和 5 年 4 月に報告書を OECD に提出した。この結果が, 4 月の WNT 会議で承認され, DPRA 重量法を含む改定 TG442C が令和 5 年 7 月に公表された。

3) IL-8 Luc assay

IL-8 Luc assay　TG442E の改定案を作成し, 令和 3 年 7 月に OECD に提出した。11 月に peer review panel からコメントを受けた。令和 4 年度, 再協議を行うため, 7 月に改定案を OECD に提出し, 各国の専門家と

議論した。その結果, 令和 5 年 4 月の WNT 会議で承認され, 7 月に公表された。

4)EpiSensA

EpiSensA のバリデーション報告書と peer review report が令和 4 年度に採択された。TG 案を令和 4 年 6 月に OECD に提出し, 専門家グループ及び WNT の意見を受けて改定した。

5)DASS

研究分担者の足利とともに, 昨年度から専門家委員会で検討を続けてきた DASS の承認に寄与した結果, 令和 3 年 6 月にガイドライン 497 が承認された。

本ガイドラインは新しいタイプの OECD ガイドラインである。DA では化学物質の物性及び *in vitro* 試験データで検証された OECD の組み合わせを使用している。その一つとして, DA では, 化学物質規制に *in silico* データを受け入れることを可能にした最初の事例である。

このガイドラインには以下に示す画期的な点が複数含まれている。

(1) 初めて試験法の結果を組み合わせて評価する手法が公定化された。
(2) 初めて *in silico* の利用が組み合わせ評価に利用された。
(3) ヒトの感作性が予測できる初のガイドラインである。

令和 4 年から, DASS の改定に参画し, 日本の方法である ADRA と IL-8 Luc assay をガイドライン 497 に加えるべく, 協力研究者の解析結果を OECD に送付した。

C-2-2. 免疫毒性試験

1) DRP

令和 2 年度より, 相場及び国際的な専門家とともに, *in vitro* 免疫毒性に関する DRP を作成した。令和 3 年度, OECD が集めた意見に対応する改定版を 9 月に提出したところ, 2 次募集において追加意見が集まった。国際的な専門家の協力を得て DRP を改定し, 2 月に OECD に提出し, 令和 4 年 9 月に DRP は採択された。

2) IL-2 Luc assay

IL-2 を指標とした免疫毒性試験の TG 案を作成し, 令和 4 年 3 月に OECD に提出した。WNT 意見募集を受けて改定した結果, 令和 5 年 7 月に TG444A が公表された。

3) IL-2 Luc LTT

令和 4 年度と令和 5 度で計 6 回の会議を開催し, メールベースのやりとりも含め, IL-2 Luc LTT の validation report の最終化と peer review report の作成を行った。両 report を令和 5 年 7 月に OECD に提出し, WNT 意見募集を受けて改訂した。

C-2-3. 生殖毒性試験の DRP

令和 3 年度より, 国際的な専門家とともに, *in vitro* 生殖発生毒性に関する DRP の作成をこの一年継続して実施した。令和 4 年 2 月に Current Research Toxicology に投稿し, 3 月に改訂の指示を受けた。5 月に Current Research Toxicology に受理された。ただし, この論文をもとに OECD で DRP を作成することは断念した。

C-2-4. Bhas42 細胞形質転換試験法の TG 開発

非遺伝毒性発がん IATA の提案を受け, 令和 5 年度は Bhas 42 CTA の OECD TG 申請の SPSF 原案を作成した。Bhas 42 CTA の SPSF 原案では, Bhas 42 CTA 機序として,

我々が令和3年度に論文公表した形質転換過程の経時的なトランスクリプトーム解析の論文及びその他エピジェネティクスなどの各種論文が公表されていることを提示し，各国においてもBhas 42 CTAの機序が検討されていることを示した。また，形質転換過程の経時的な他点でのトランスクリプトーム解析の論文から，AOPの一例を導き出しSPSF原案に示した。Bhas 42 CTAの発がん性予測率については，当初の原案では既報（Sakai et al., Mutat Res., 702, 100-122, 2010）をもとに，Concordance 78%，Sensitivity 73%，Specify 84%と示したが，各国からのコメントを参考に評価対象の化合物の見直しを行った結果，Concordance 83%及びSensitivity 83%に向上した。さらに，厚生労働省の労働安全衛生法における「職場で使用される化学物質の発がん性評価の加速化」として用いられた系統樹により遺伝毒性試験であるAmes試験で陰性の場合にBhas 42 CTAを実施するバッテリー系統樹で発がん性予測率を算出したところ，Concordance 88%及びSensitivity 91%と高値になった。また，遺伝毒性試験として，Ames試験だけでなく，マウスリンフォーマ試験，染色体異常試験，小核試験を含めてのBhas 42 CTAとのバッテリーでの予測率を算出したところ，Concordance 89%及びSensitivity 93%とさらに高値となった。したがって，我が国の労働安全衛生法における発がん性のスクリーニングの仕組みとして実施された，遺伝毒性試験とBhas 42 CTAのバッテリー系統樹は，発がん性予測の系統樹として優れていることをSPSF原案において各国に示すことができた。一方で，Bhas 42 CTAのSPSF原案の提出には，同意国（組織）が多数であったが，NGTxC・IATAにおける採用アッセイのTG推薦及びNGTxC・IATAのGDとしての承認申請等，NGTxC・IATAにおける今後の計画との調整がBhas 42 CTAのTG化に向けて更なる検討事項であると考えられた。

C-3. IATAの開発

C-3-1. 非遺伝毒性発がん性IATA（NGTxC・IATA）開発への協力

OECDで進められている非遺伝毒性発がん性のIATA開発に協力した。平成30年6月の会議において, 非遺伝毒性発がん性に係る試験・検査のパラメータを優先順位に関して4つのカテゴリーに分けることになった。また, 候補となるアッセイを13のブロックに分けて, 分担してレビューしている。

IATA開発について, 小川研究分担者はcell proliferation及びresistance to apoptotic cell deathのサブグループに, 西川研究協力者はcell transformation, indicator of oxidative stress及びresistance to apoptosis cell deathのサブグループに参画し, アッセイブロックの評価を行った。

Cell proliferationのサブグループにおいては, 細胞増殖の評価に関する *in vitro* / short term *in vivo* アッセイの適応・留意点・限界などに関する議論に参加した。また, *in vivo* 評価法の留意点について分担執筆し, web会議を重ねて最終案を投稿し, 査読指摘事項対応を経て, 受理された。また, resistance to apoptotic cell deathのサブグループにおいて, アポトーシス関連バイオ

マーカーの非遺伝毒性発がん性評価について, 正常部位と前がん病変では異なる意義を持つ可能性等について議論を重ね, 論文化をすすめた。

令和 3 年度, Block 3 の“Cell Transformation”では，SHE Cell Transformation Assay (SHE CTA)，Bhas 42 CTA，Balb 3T3 CTA，Balb c/3T3 transformics assay が評価対象となった。SHE CTA 及び Bhas 42 CTA のみが，NGTxC・IATA の全ての Assay 候補の中で OECD のガイダンスドキュメントとして掲載済みの Assay である。Bhas 42 CTA 及び SHE CTA は，各種性能及び再現性等の検証データに基づき Assay description が作成され，評価案の担当者により各評価項目において A 評価を得た後，Block 3 メンバーの全会一致でランク A の合意を得た。一方，Balb 3T3 CTA については，2 step assay と 1 step assay が開発されていることから，いずれのプロトコルを Assay description に記載し評価を行うかについて議論した。その結果，かつて European Centre for the validation of Alternative Methods (ECVAM)から OECD に提案され評価が中断された 1 step assay が評価プロトコルとなった。同プロトコルに基づくトランスクリプトミクス解析法の Balb c/3T3 transformics assay も，当初は Balb 3T3 CTA とは別に Assay description が作成されたが，検証等の完成度が低いことから最終的には Balb 3T3 CTA の optional assay として Balb 3T3 CTA の Assay description に組み込まれ，Balb 3T3 CTA として評価が行われた。Balb 3T3 CTA では，NGTxC 対象化合物でのデータ，性能評価及び再現性検証の化合物数も限定的であることなどから，各評価項目は A と B が混在し，ランキング評価についても Block メンバー内での議論を要したが，最終的には Balb 3T3 CTA（Balb c/3T3 transformics assay 含む）はランク A で合意された。したがって，3 種の Cell transformation assay は全てランク A の評価となり，10 月に開催された Expert working group の全体会議での報告に至った。

Block 4 の“Gap Junction”では，提案した Dye transfer assay と Inhibition of metabolic co-operation Assay について，該当する 3 種の Assay（Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)，Gap junction dye transfer assay – combined，Gap junction multiparametric scrape loading-dye transfer assay (mSLDT)）を他のメンバーと共に選出した。Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)は，467 化合物で結果と 166 化合物での性能評価について総説（ATLA）が我が国から報告されており，それをもとに Assay description の作成を行った。他の 2 種の Assay は，他の 2 名のメンバーが 1 種ずつ Assay description を作成した。それらの評価結果は，Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)については，感度（49%）及び特異性（63%）が十分でなく施設間再現性評価の結果が未投稿のためランク B となった。Gap junction dye transfer assay – combined は，ECVAM のプロトコルではあるが，17 化合物のデータのみで検証データが無いためランク C となった。mSLDT は 328 化合物での結果と IARC グループ 1 及び 2 の 72 化合物での性能評価が報告されており，感度は 75%以上であるが特異性が 45%と低

値であることに加え，施設間再現性評価が未実施であることからランクBとなった。これらBlock 4の3種Gap junction assayの評価結果についても，10月の専門家グループの全体会議での報告に至った。

令和3年度末には, Bhas 42 CTAの細胞形質転換過程における経時的なトランスクリプトーム解析結果を論文発表し, Bhas 42 CTAの陽性対照化合物のTPAによる処理後の経時的な4点でのRNA解析において，陰性対照群と比較し有意に発現増大または減少したそれぞれ2000を超えた遺伝子のパスウェイ解析を実施した結果，Hallmark of Cancerに関連した多くの因子に発現変動がみられた。よって，Bhas 42 CTAの細胞形質転換過程において，がんの特徴的な事象と共通の因子が発現変動していることとなる。また, 本論文は，令和4年度に公表されたNGTxCのSpecial IssueにArticleとして収載された。加えて，令和4年度には, Block 3でランクAの評価済みである3種のCTA（SHE CTA，Bhas 42 CTA，Balb 3T3 CTA）関して，レビューを共著し公表した。Bhas 42 CTAについては，陽性検出が報告されている22種のNGTxCを提示し，そのうち8化合物については既にBhas 42 CTAにおける機序の報告も論文公表されていることも示した。本レビューの結論においては，CTAは腫瘍形質転換のエンドポイントを提供する唯一の *in vitro* モデルであり，オミックス解析によるCTAの *in vitro* 腫瘍形質転換につながる多段階プロセスの機序は，人間での多段階発がんプロセスで説明されている機序と一貫性及び一致性のある重要な事象である証拠を提供するものであることを示した。

令和4年度に公表したCTAのBlock 3でランクAの評価を得た3種CTA（SHE CTA，Bhas 42 CTA，Balb 3T3 CTA）のレビュー論文において，NGTxCの機序解明及びNGTxC・IATA構築におけるCTAの貢献を担うAssayとしても重要な位置づけであることが示された。

C-3-2. 光毒性IATA

作成したAOPを基盤として，その枠組みのなかで情報源を網羅的にマッピングした。光毒性に関与する因子としては，(i) Exposure consideration, (ii) Chemical descriptors, (iii) Skin penetration, (iv) Photoexcitation, (v) Oxidative stress, (vi) Cell injuryを定義し，それらに関わる情報源をリスト化した。具体的な情報源としては既にガイドライン化されているものはもちろんのこと，バリデーション研究が実施されていないアッセイ系でも光安全性保障におけるその有用性を考慮して記述した。

これまでに臨床における薬剤性光線過敏症診断としてはPhotopatch testや皮膚のbiopsy等が従来実施され，特に前者は *in vivo* 光感作試験として幅広く利用されている。一方，創薬においては，簡便に光安全性を評価する方法として *in vitro* 3T3 NRU phototoxicity test（PT）が代替試験法として利用されている。しかしながら創薬初期過程ではスクリーニングの更なる高効率化や異なる機序の光線過敏症リスク評価が求められ，これまでにも数多くの評価系が開発・応用されている。これらは，*in silico* スクリーニングツールや，光化学的特性を中心とした分子物性評価，あるいは各種毒

性反応に特異的な光生物化学的アッセイ方法などが含まれている。これらのアッセイ手法は創薬のステージによってその利用方法や位置づけが異なっており，例えばDEREK や HOMO-LUMO Gap 等の *in silico* システムでは創薬支援として化合物が実際に合成される前段階で新規化合物の光安全性予測を行うことを目的で利用されている。一方，光化学的特性評価ツールは実際に合成された新規化合物の分子物性を指標とした光感受性分析を主体とし，必ずしも光安全性を直接的に評価可能なわけではないが，光毒性反応の誘発に寄与し得る光化学的反応の有無について高いスループットで示唆することが出来る。また，光刺激性評価のためのツールはこれまでにも数多く開発されており，枯草菌，白血球や赤血球を用いたアッセイ系やヒト三次元培養表皮モデルを利用した評価系が *in vitro* 光毒性試験法として有用である。これらの *in vitro* 試験，*in vivo* 試験のみならず，*in silico* や QSAR モデルも information sources に含めることとし，多様な光安全性評価を可能とした。

先に我々は，皮膚内動態情報と光反応性情報を統合することによって光安全性評価の予測精度を向上させることを明らかにしているが，皮膚内動態については被験物質の物理化学的特性からも部分的に予測可能であり，ROS assay データによる光反応性情報との組み合わせによって動物実験に依存しない光安全性保障システムの構築が可能になるものと考える。この観点から薬物動態に関係する各種因子についても網羅的に記述した。また，OECD 専門家会議によるレビューの結果，各 情報源 について validation status と weight of importance を加えるよう要望があり，これに基づいて当該情報を追記した。

また，OECD 専門家会議における有識者の助言に従い，情報源に対して詳細な記述を加えることとし，具体的には (1) Regulatory use, (2) Validation & regulatory acceptance status, (3) Potential role in the IATA, (4) Description, (5) Scientific basis including MoA, (6) Protocol available, (7) Strengths and weakness, (8) Applicability domain and limitations, (9) Predictive capacity, (10) Reliability を各種文献情報やガイドラインを交えつつ追記した。全ての情報源に対してこれらの情報を入手するのは容易ではなく，それ故，情報が比較的入手しやすく，なおかつ重要度の高いガイドライン化された光安全性評価系に焦点をあてて記載を試みた。　また，これらの情報源を組み合わせた包括的光安全性評価に関して decision tree を新たに提案し，複数の評価系を組み合わせた光安全性評価の一例として IATA に記述した。

決定樹 は主に 3 つのカスケードによって 構 築 さ れ，(i) Initial assessment of phototoxic potential, (ii) Experimental evaluation of phototoxicity, (iii) Pharmacokinetic characterization からなる。最初の光安全性評価は光化学的特性を指標としたものであり，主として UV/VIS 吸収特性や ROS assay から構築される。光安全性評価の初期段階でこれら光化学的特性を明らかにすることは ICH S10 とも矛盾せず，高いスループットによって迅速に光毒性リスクを示唆することができる。これらが陰性であった場合にはそれ以降は特に追加での光安全性評価を必要とし

ないが，仮に光毒性リスクが疑われる結果であった場合にはフォローアップ試験として光生物学的アッセイ系によって更なる評価を行うことができる。この段階での光毒性リスク評価には 3T3 NRU PT あるいは 再構築ヒト表皮モデル（RhE） PT が用いられ，これらはより直接的な光毒性反応を示唆するものである。どちらの評価系を使用するかは特に規定しないが，被験物質の物理化学的特性や性状を考慮したうえで試験者が適切に選択することができる。このフォローアップ試験で陰性の場合は光安全性の懸念が特にないものとして判断することができる。一方，光毒性リスクが疑われる場合においては，更なるフォローアップ試験として薬物動態試験を実施し，経皮適用の場合には皮膚透過性・滞留性や蓄積性を，そして全身投与の場合には皮膚移行性や皮膚滞留性等を精査することによって，実質的な光毒性リスクを検証することができる。ただし，体内動態に関する一定の閾値を設定することは難しく，被験物質毎に光反応性，光生物学的特性や投与量を考慮した上で適切な判断が求められ，さらに評価の妥当性については科学的合理性が許容される種々の実験データをもって評価者自身によって証明される必要がある。薬物動態試験についてはその性質上 “Optional” 試験として取り扱うこととした。

### C-3-3. 発達神経毒性に起因する行動解析に関する情報収集

１）発達神経毒性評価のための行動解析に関する情報収集

まず, EPA 及び OECD の発達神経毒性に関するテストガイドラインを確認し, げっ歯類に対する行動解析に関する内容を中心に比較を行った。発達神経毒性に関連する OECD　TG426 は, 全般に EPA ガイドライン（OPPTS 876.6300）に沿った内容であり, EPA のガイドラインに比べ詳細に記載されていた。試験項目の内容に関しては, 大きく分けて「行動発生」,「自発運動量」,「運動及び感覚機能」,「学習及び記憶」における推奨検査項目や実験条件について述べられていた。しかしながら, 実施する行動評価の選択については具体的な記載はなく, 明記されているのは評価の対象となる機能及び試験を行う際の推奨日齢のみであった。また, 脳高次機能に関する試験項目としては「学習及び記憶」が該当するが, 試験方法の記述は曖昧な表現にとどまっていた。さらに, 行動試験を行うにあたり, 解析環境の記載については, 具体的な説明が乏しいことや, 特定の試験を組み合わせた評価系を構築するなど, 標準化（統一化）されたプロトコルではないことも明らかとなった。

PubMed を用い, TG426 が制定された 2007 年から, 2021 年までの範囲で文献数調査を行ったところ, 検索キーワード「“developmental neurotoxicity”」の検索条件では, 検索時点での総文献数が 1001 件であり, その報告数は年々増加していた。一方で,「"developmental neurotoxicity" AND "test guideline"」の検索条件では 8 件と, ほとんど該当しないことが分かった。

文献検索の過程で, Mundy らの Review（Expanding the test set: Chemicals with potential to disrupt mammalian brain development, *Neurotoxicol. Teratol.*, 2015）を

もとに, 発達神経毒性に関する文献における行動解析の利用頻度を把握するため, その内容をまとめた。その結果, 神経発達への影響を示すデータのある化学物質では, 形態学的解析, あるいは神経化学的解析とともに行動解析を採用し, それらの組み合わせにより評価していた。論文数に対する利用頻度は行動解析が最も多く（全 220 報, 97 種類の化学物質の文献リストのうち 145 報）, 行動解析がエンドポイントとして強力なツールであることがうかがえた。

これまでガイドラインに準拠した化学物質の評価状況については, EPA 又は TG 426 に準拠して発達神経毒性試験が実施された農薬のうち, その試験成績が審査当局に提出されたものは, 米国における 2008 年段階での承認農薬数は約 1150 有効成分, 欧州においては 2020 年段階で 479 有効成分であるという情報を得た（発達神経毒性の欧米での評価状況及び *in vitro* 発達神経毒性試験の検討状況調査 独)農林水産消費安全技術センター 農薬検査部 2020 より）。

上記情報に加え, 発達神経毒性評価に係る情報として収集した文献については, 曝露時期の情報, 被験物質, 解析に用いた行動試験, ガイドラインへの準拠等についてリスト化を行った。行動解析の項目については, 認知機能への影響評価（受動回避試験, モリス水迷路等）を用いた文献が最も多く, 次いで運動及び感覚機能（オープンフィールド試験, ロータロッド試験等）を用いたものが多かった。上記 2 項目と比較すると数は少ないが, 社会性（超音波発声, ホームケージ, 3 チャンバーテスト等）の行動解析を取り入れている文献も存在し, これら解析項目を取り入れている文献は, 主に農薬の曝露影響を評価したものであった。

2) OECD からの意見募集への対応：

JaCVAM 発達神経毒性資料編纂委員会のオブザーバーとして委員会に参画するとともに, *in vitro* DNT ガイダンス文書（Guidance on the Interpretation of Data from the Developmental Neurotoxicity (DNT) *In-Vitro* Testing Assays for Use in Integrated Approaches for Testing and Assessment (IATA)）及び Case Study に関して, OECD 事務局に提出するコメント募集に応じた。神経行動毒性の評価系, 特に *in vivo* 試験を行っている立場から, 本ガイダンスの改善点・懸念点について, 以下に示すようにその適用限界を含めて複数回コメント対応した。

(1) 提案されている *in vitro* 試験バッテリー（Developmental Neurotoxicity *In Vitro* Battery (DNT IVB)）により *in vivo* 発達神経毒性を予測するにあたって, 化学物質影響の種差（中枢移行性等）, 性差に関する言及がないこと。

(2) *in vivo* 試験で観察される神経回路の機能変化や, 顕在化する行動影響に関して, *in vitro* 試験において得られたデータはどこまで対応しうるのか, あるいは妥当性があるのか, 具体的な言及を追加すること。

(3) 被験物質の複合曝露による, 相加・相乗的影響についての懸念。

(4) げっ歯類の代替としてのゼブラフィッシュによる解析について, 検出系の施設間差, 試験プロトコルの統一性（個体数や分析法）を議論すること。

(5) 今後, DNT IVB で種々の化学物質について検討する際に, *in vitro* 試験で確認されな

かった影響が *in vivo* 試験のみで確認された場合の解釈について。

(6) 行動毒性においては, *in vitro* 試験の複数のアッセイを組み合わせたとしても予測が困難な部分があり, *in vivo* 脳高次機能影響（学習や記憶）の評価は現状難しいと考えられるが, その際に, 最終的に *in vivo* アッセイで補強すべき役割について議論を追加すること。

C-4. AOP 及び TG の実験データ支援

C-4-1. *In vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

C-4-1-1. 腸管由来組織における代替法の検討

DSS 投与により, 試験期間中に, 一般状態の悪化を呈する個体は安楽殺を施した。解剖時には腸管を摘出し, 遺伝子発現解析並びに病理組織学的解析を行った。DSS 1.25%及び 2.5%群では, 2 週間の試験期間中に瀕死個体や死亡個体が見られた。一方で, 大腸には明らかな病変を示さない個体も観察され, 病態にばらつきが生じた。DSS 5%群では, 投与 3 日で炎症パラメータの安定的な上昇が示され, 投与 7 日によって大腸における明らかな炎症像を呈し, 安定した病態発現が観察された。TNFα や IL1β は, 投与 3 日で顕著な発現上昇を示し, 投与 7 日では投与 3 日と比較して減少傾向を示した。その一方で, MIP-2 や HMGB1 は投与 3 日から顕著な発現上昇を示し, 投与 7 日でさらに増強した。以上のことから DSS 5%飲水投与が早期から安定した病態発現を誘導し, さらに炎症の増強に関与する因子が明らかとなった。明らかな病理組織学的に変化は見られなかったが, 炎症関連因子として, MIP-2 (CXCR2) の遺伝子発現が顕著に増加することが見出された。

1) *In vivo* モデルにおいて AOP となり得る毒性所見の検討

DSS 飲水投与により, 5.0%は 7 日間の投与で全例に粘膜のびらん・潰瘍, F4/80 陽性マクロファージの増加, 炎症性細胞浸潤領域が認められた。一方, 同条件の小腸においては, 明らかな組織学的変化は認めなれなかった。しかし, 小腸の遺伝子発現においては, 5.0%は投与 3 ないし 5 日目には炎症関連遺伝子に加え, 粘膜バリア機構, 酸化ストレス関連, セロトニン作用に関連する受容体発現への影響が見られた。これより, 小腸においては, 明らかな組織学的変化が生じるのに先立ち, より早期の段階から種々の遺伝子発現に影響が生じていることが明らかとなった。

2) マウス空腸由来のオルガノイドを用いた検討

TNFα 添加後 1 時間から MIP-2 の遺伝子発現は濃度依存的に増加した。DSS のいずれの濃度においても MIP-2 発現は TNFα 添加後 1 時間をピークとして 24 時間まで徐々に減弱した。さらに, TNFα 添加後の e-Cadherin の免疫組織化学染色において, 粘膜における染色性の低下が濃度依存的に認められた。

正常マウス並びに DSS 処置マウス由来の空腸オルガノイドにおける遺伝子発現を検討した。特にセロトニン作用関連因子に注目して観察したところ, より短時間の 5.0%DSS 3 日間投与から 5–HT3 受容体関連遺伝子が増加傾向を示した。他方, 細胞接着並びに粘膜保護関連遺伝子については

明らかな影響は認められなかった。

3）腸管上皮由来 Caco-2 細胞を用いた平面培養による検討

2 週間 Pre 培養し 24 時間 DSS を培地に添加したところ, 1%では細胞培養の状態に Control と明らかな差は見られなかったが, e-Cadherin の免疫組織化学染色では, 細胞間の陽性部位における染色性の減弱が認められた。

4) 腸管軸に着目した腸管傷害に起因した肝臓病態の修飾効果の検討

DSS 投与群の大腸において, 炎症関連遺伝子発現の上昇及び病理組織学的な炎症が観察され, 大腸炎の誘発が確認された。さらに, 同群の肝臓においても炎症関連遺伝子発現が上昇傾向を示し, 大腸炎の波及が示唆された。CDAA-HF 給餌群の肝臓において, 炎症・線維化関連遺伝子発現の上昇及び病理組織学的な肝細胞の脂肪化並びに炎症が観察され, 非アルコール性脂肪肝炎病態の惹起が確認された。併用群では, 肝臓において TLR4 を含む一部の炎症関連遺伝子発現が増強し, 病理組織学的に CXCL16 陽性細胞が増加した。さらに, 線維化関連遺伝子の発現が増強傾向を示した。腸管においては IL6 の遺伝子発現が増強し, 病理組織学的に杯細胞と陰窩の拡張が観察された。

C-4-1-2. 肝臓における代替法の検討

1) ラット, マウスともにそれぞれに適した CDAA を 3 ヶ月投与すると, Sirius Red 面積の増加, αSMA 陽性面積の増加, CK19 陽性面積の増加を示し, 肝線維化, 星細胞の活性化, 細胆管反応の増加が示された。SOX9 の発現を検討したところ, ラット及びマウスの線維上に SOX9 が著明に発現し, CK19 との一致を認めたことから, SOX9 は線維化に関与し, 細胆管反応と一致して増加することが示唆された。CD44 の発現は, ラットにおいて, 肝線維化が進むにつれて, 発現が著明に増加した。CD44 はラットの増生胆管上皮細胞に発現がみられ, その周囲にヒアルロン酸結合タンパクが存在していたことより, 胆管上皮細胞を介した肝線維化に寄与していると考えられた。一方で, マウスにおいてはマクロファージ様の細胞に発現がみられたため, 同じげっ歯類においても, ラットとマウスでは発現様式が異なる可能性が示唆された。マウスにおける肝サンプルでは, 1 年 3 ヶ月の長期投与により胆管線維症の発生が増加し, 胆管への分化増強が示唆された。RNA-Seq. 解析の結果より, 線維化部位においては, Rho-family GTPase や Estrogen receptor signaling の活性化をはじめとした種々の変化を見出した。

2) LX-2 を用いた検討では, TGFβ1 の刺激により, 網目状の進展と紡錘形に形態学な変化を示し, αSMA 蛋白陽性, Smad2, 3 のリン酸化, Collagen Type 1 及び Type 4 の遺伝子発現増加を認めた。また, これらの変化は TGFβ1 受容体阻害剤で解除された。一方で, SOX9 と CD44 は TGFβ1 の刺激に対して発現変化はみられなかった。

3) マウス肝オルガノイドを用いた検討では, 得られたオルガノイドは CK19 が陽性であり, 胆管の特徴を有していた。さらに, SOX9, CD44 が陽性であり, in vivo で観察された組織学的特徴を有した胆管上皮細胞である可能性が示唆された。TGFβ1 刺激において, CK19, SOX9, CD44 遺伝子発現は

TGFβ1 の刺激により Control と比較して有意に増加した。αSMA は Control では陰性であったのに対し陽性に転じた。Collagen Type 1, Fibronectin 発現は, Control と比較して有意に増加した。

C-4-2. DNA損傷・幹細胞マーカー等を指標とした免疫組織化学的検索による発がん性早期検出

投与期間終了時点で, HCBD, DMN, EHEN, AOM, Disperse orange, Monuron, NFT, Quercetin, 1,2,3-TCP及びBDCM投与群において有意な体重増加抑制が認められた。摂餌量は HCBD, DMN, EHEN, AOM, Disperse orange 及び NFT 投与群, 飲水量は EHEN, AOM 投与群で低値の傾向を示した。

HCBD, ADBAQ, 1,2,3-TCP, 8-MOP 投与群では腎絶対/相対重量の増加, 0.2% CBX, LAT, Monuron, NFT, Quercetin, TCEP, BDCM 投与群では腎相対重量の増加が, 統計学的有意差をもって認められた。また, DMN, EHEN, AOM 投与群では腎絶対重量の低下及び相対重量の増加, Disperse orange 投与群では腎絶対重量の低下, 0.04% CBX, 1% Neomycin 投与群では腎相対重量の低下が認められた。肝臓については, ADBAQ, Disperse orange, TCEP, 1,2,3-TCP, 8-MOP 投与群で絶対/相対重量増加, HCBD, Monuron, Phph, Quercetin, BDCM, HQ 投与群で相対重量の増加が観察された。DMN, EHEN, AOM, NFT 投与群では肝絶対重量の低下, 1% Neomycin 投与群では肝絶対/相対重量の低下が観察された。

HCBD, ADBAQ, DMN, Disperse orange, 0.04%/0.2% CBX, 8-MOP 投与群では再生尿細管, DMN, AOM 投与群では間質における炎症性細胞浸潤, HCBD, EHEN, AOM, Monuron, NFT, 0.2% CBX, 8-MOP 投与群では尿細管上皮細胞の変性/壊死, HCBD, ADBAQ, NFT 投与群では尿細管上皮への好酸性顆粒沈着増加, LAT, Monuron 投与群では尿細管上皮細胞における核の大型化が, 発生頻度の有意な増加を示した。Phph, Quercetin, TCEP, BDCM, HQ 投与群でもこれらの所見が散発的に観察されたが, 統計学的有意差はみられなかった。1% Neomycin 及び 1,2,3-TCP 投与群では, 明らかな腎病変は観察されなかった。

各群の腎尿細管上皮細胞における γ-H2AX 形成を免疫組織化学的に検討した結果, 対照群では陽性細胞は稀であったのに対し, HCBD, ADBAQ, DMN, EHEN, AOM, Disperse orange, Monuron, NFT, Phph, Quercetin, LAT, 1,2,3-TCP, BDCM, 8-MOP 投与群では皮質または髄質外帯外層における γ-H2AX 陽性率の有意な増加が認められた。腎毒性/非発がん物質投与群では, 0.2% CBX 投与群の髄質外帯外層において γ-H2AX 形成の有意な増加がみられたのに対し, 0.04% CBX 投与群では対照群と同じレベルにとどまった。Neomycin, TCEP, HQ 投与群では, γ-H2AX 形成の誘導は認められなかった。

C-4-3. 遺伝毒性の AOP 開発

遺伝毒性初期応答反応の早期検出システム構築に用いた rDNA unit 上のプライマーセット H1～H42.9 の内, H1～H13 は転写領域, H18～H42.9 は非転写領域を検出することができる。

ポリクローナル抗体を用いた解析では, DNA 損傷を誘発しない条件において, RNAPI 共沈 DNA 中には H1～H13 の転写領

域の DNA のみが均一に存在する一方で, 紫外線 DNA 損傷誘発時の RNAPI 共沈 DNA 中には, 転写領域の中でも H1 と H4 の DNA 領域が特に多く存在することが示された。また, DNA 損傷を誘発しない条件において, γH2AX, Ku80 及び LIG4 共沈 DNA 中には rDNA unit 全領域の H1～H42.9 が均一に存在しているが, 紫外線 DNA 損傷誘発時の γH2AX, Ku80 及び LIG4 共沈 DNA 中には H1～H42 がさらに多く存在し, その中でも特に H18 及び H27 の DNA 領域が多く存在することが示された。

モノクローナル抗体を用いた解析では, γH2AX と共沈する rDNA 量は紫外線照射により増大し, 特に, H18 及び H27 の DNA 領域で高いことが示された。同様に, ATM と共沈する rDNA 量は紫外線照射により増大し, 特に, H18 及び H27 の DNA 領域が高いことが示された。その一方で, MRE11 及び BRCA1 と共沈する rDNA について, 紫外線の照射と未照射に関わらず rDNA 上での位置的局在に大きな差は見られなかったが, 紫外線照射により共沈する rDNA 量が全体的に顕著に減少することが示された。

C-4-4. 腎障害・線維化の分子メカニズムに関する研究

【令和 3 年度】

実験 1：APL 投与群では実験期間を通して体重増加抑制が認められ, APL 100 mg/kg 群では実験開始 1 から 2 週後に, APL 150 mg/kg 群では実験開始 1 から 4 週後にかけて対照群と比較して有意な低値を示した。血清生化学的検査では, BUN 及び sCre ともに APL 100 mg/kg 群から増加傾向を示し, APL 150 mg/kg 群では有意な増加を認めた。腎重量の測定では, APL 100 及び 150 mg/kg 群ともに絶対及び相対重量が用量依存性を伴って有意に増加した。病理組織標本を用いたシリウスレッド染色による線維化の評価では, APL 100 及び 150 mg/kg 群ともに間質の膠原線維の明らかな増加を認めた。膠原線維の増加した領域では尿細管は拡張あるいは萎縮しており, これらの尿細管は免疫染色にて CD44 陽性を示した。

実験 2：体重測定において VAN 投与の影響は認められなかった。血清生化学的検査では, BUN の有意な高値が VAN 200 及び 400 mg/kg 群に, sCre の有意な高値が VAN 400 mg/kg 群に認められた。腎重量測定では, VAN 400 mg/kg 群において絶対及び相対重量の有意な増加が認められた。シリウスレッド染色では VAN 200 及び 400 mg/kg 群ともに軽度な膠原線維の増加が認められ, 膠原線維の増加した領域における尿細管は拡張あるいは萎縮しており, 免疫染色にて CD44 陽性を示した。

実験 3：体重測定において PAN 投与の影響は認められなかった。血清生化学的検査では, PAN 12 mg/kg 群において BUN の有意な低値が認められた。腎重量測定では, PAN 12 mg/kg 群において相対重量の有意な増加が認められた。PAN 8 及び 12 mg/kg 群の腎臓では糸球体及び尿細管障害を示唆する変化がみられたものの, シリウスレッドによる線維化の評価において間質の膠原線維の明らかな増加は認められず, 拡張/萎縮尿細管も観察されなかった。また CD44 の免疫染色においても, 陽性を示す尿細管は認められなかった。

【令和 4 年度】

シリウスレッド染色及び αSMA 免疫染

色では, それぞれの陽性面積が APL 投与群では対照群と比較して有意に増加していた。HE 染色標本を用いた病理組織学的解析において, APL 投与群における線維化病変内の尿細管は拡張あるいは萎縮していた。免疫組織学的解析ではこれらの尿細管は CD44 に陽性を示し, APL 投与群の CD44 陽性尿細管は対照群と比較して有意に増加していた。また, CD44 陽性尿細管の数はシリウスレッド陽性面積及び αSMA 陽性面積と正の相関を示した。蛍光二重免疫染色では, APL 投与群において CD44 陽性尿細管の周囲に αSMA 陽性反応が確認された。

マイクロアレイデータを用いた GO 解析では, 拡張/萎縮尿細管において細胞外基質に関連する遺伝子群の発現が上昇しており, トランスポーター及び代謝といった尿細管の分化に関わる遺伝子群の発現が低下していた。パスウェイ解析では, CD44 は fibronectin の産生に関わる *Fn1* を含む線維化関連遺伝子群の発現を誘導していることが示された。

免疫染色により尿細管の分化マーカーの発現を検索した結果, 拡張/萎縮尿細管では AQP1 及び N-cadherin の発現が減弱あるいは消失しており, これらの因子に陽性を示す尿細管の数は APL 投与群において対照群と比較して有意に減少していた。また AQP1 及び N-cadherin に陽性を示す尿細管の数は CD44 陽性尿細管の数と負の相関を示した。蛍光二重免疫染色では, CD44 は AQP1 及び N-cadherin と排他的な発現を示した。間葉系マーカーの検索では, 拡張/萎縮尿細管は vimentin 及び αSMA に陽性を示し, それぞれの陽性尿細管の数は APL 投与群において対照群と比較して有意に増加していた。また vimentin 及び αSMA に陽性を示す尿細管の数は CD44 陽性尿細管の数と正の相関を示した。蛍光二重免疫染色では, CD44 は vimentin 及び αSMA と同一の尿細管において発現していた。一方, 基底膜マーカーである collagen type IV 及び nidogen-1 の免疫染色では, CD44 陽性を示す拡張/萎縮尿細管の周囲に軽度に肥厚した基底膜を認めた。

Fibronectin の免疫染色では拡張/萎縮尿細管の周囲間質に陽性反応が認められた。Fibronectin 陽性面積は APL 投与群において対照群と比較して有意に増加しており, CD44 陽性尿細管の数と正の相関を示した。蛍光二重免疫染色では, CD44 陽性尿細管の周囲に fibronectin の陽性反応が認められた。一方 *in situ* hybridization において, *Fn1* mRNA の発現は間質の線維芽細胞に加えて APL 投与群の拡張/萎縮尿細管においても認められた。

ウエスタンブロッティング法による CD44 の発現解析では, 腎臓組織中及び尿中に CD44 特異的なバンドが確認された。APL 投与群では対照群と比較して尿量の有意な増加を認めたことから, 尿の濃縮は生じていないと考えられた。また ELISA 法による解析では, 血清中 CD44 値が APL 投与群において対照群と比較して有意に増加しており, 血清中 CD44 値は腎臓における CD44 陽性尿細管数及びシリウスレッド陽性面積と正の相関を示した。また定量 PCR により APL 投与群では CD44 standard isoform が高発現していることが示された。

【令和 5 年度】

CyA 投与群ではシリウスレッド染色に陽性を示す線維化領域が増加していた。線維化病変内の尿細管は萎縮, 拡張あるいは肥大していた。これらの尿細管は CD44 に陽性を示し, CD44 陽性尿細管の数は線維化面積と正の相関を示した。

マイクロアレイにおける GO 解析では, 萎縮/拡張/肥大尿細管において正常尿細管と比して細胞外基質に関連する遺伝子群の発現が増加しており, トランスポーター及び代謝といった尿細管の生理機能に関連する遺伝子群の発現が減少していた。またパスウェイ解析では CD44 は fibronectin 1（*Fn1*）を含む線維化関連遺伝子の上流因子として抽出された。

免疫組織学的解析において, 線維化病変内の萎縮/拡張/肥大尿細管では近位尿細管分化マーカーの AQP1 の発現が減少しており, AQP1 陽性尿細管の数は CD44 陽性尿細管の数と負の相関を示した。またこれらの尿細管では間葉系マーカーである vimentin が発現しており, vimentin 陽性尿細管の数は CD44 陽性尿細管の数と正の相関を示した。蛍光二重免疫染色において CD44 は AQP1 と相互に排他的な発現を示し, vimentin とは共発現していた。細胞外基質の主要な構成要素の一つであるフィブロネクチンの陽性面積は CyA 投与群において有意に増加しており, CD44 陽性尿細管と正の相関を示した。基底膜マーカーである collagen type IV の免疫染色では, 線維化病変内の尿細管周囲に軽度に肥厚した基底膜が観察された。CD44 の下流因子候補でありフィブロネクチンをコードする *Fn1* の mRNA は正常尿細管と比して線維化病変内の尿細管の細胞質において増加していたが, フィブロネクチンタンパクはそれらの尿細管の周囲間質に沈着していた。

ELISA 法による血清 CD44 値の測定では, CyA 15 及び 30 mg/kg 投与群においてそれぞれ対照群と比して上昇傾向及び有意な上昇を示した。また血清 CD44 値は腎臓における CD44 陽性尿細管の数及び線維化面積と正の相関を示した。

リアルタイム PCR により CyA 投与群では CD44 standard isoform が高発現していることが示された。

C-5. OECD に提出する資料の事前確認と OECD からの意見募集への対応

C-5-1. SPSF

日本から以下の SPSF を提出した。提出にあたり, 厚生労働省とも内容を調整した。

【令和 3 年度】

1) Epidermal Sensitization Assay (EpiSensA): An *In Vitro* Method for Identifying the Skin Sensitisation Potential of Chemicals
2) Use of an interleukin-*2* luciferase lymphotoxicity test for identifying the immunotoxic potential of chemicals that is caused by anti-proliferative effects

いずれも令和 4 年の 4 月に作業計画に載った。

【令和 4 年度】

1) Proposal for α-Sens® as FBS-free test system for detecting Key Event 2 (ARE-Nrf2 activation) of skin sensitisation.
2) Proposal for TG 493 (Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity) performance and acceptability

criteria to make it realistic.

いずれも令和 5 年の 4 月に作業計画に載った。

【令和 5 年度】

1) TG for Bhas 42 cell transformation assay
2) Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for immunotoxicity
3) Me-Too validation of the reconstructed human epidermis EpiDerm model for the EpiSensA method
4) TG455 Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists 改定

なお, 3)はフランス, 4)は英国及び米国との共同提案である。

R6 年の 4 月に作業計画に, 1）及び 2)は載らなかった。

C-4-2. Emerging technologies in the Test Guidelines Programme に関するワークショップ

Lesson and Learned for a Validation Studyという演題で令和4年8月31日に講演した。この演題を含め20以上の発表会が数か月に渡り事前に開催された。

C-4-3. Stakeholders Workshop on Operational and Financial Aspects of Test Methods Validation

令和5年12月にOECDで開かれたワークショップにおいて, 日本からの意見を足利が発表できるように平林研究代表者とともに議論を重ねた結果, 分担研究者の足利より, 日本の主張を世界に発信できた。

C-6. 毒性等情報収集調査

OECD IATA Case Studies Project では, AOP を全身毒性評価の IATA へ活用した事例の提案が増えつつある。AOP は, リードアクロスなどに毒性機序に基づく類似性仮説の構築に有用であることは, 広く認識されている。AOP を構成する MIE や各 KE を測定する *in vitro* 試験は, 毒性予測の不確実性を減少させ, 信頼性を高める上で有効であると考えられている。

本年は 4 件の AOP を用いたケーススタディと, 比較対象として 1 件の非 AOP ケーススタディについて調査した。

ケーススタディ「分岐鎖が異なるカルボン酸によるリードアクロスを用いた 2-エチル酪酸の 90 日間反復投与毒性（OECD408）予測」は, 肝脂肪症に関連する複数の AOP を統合して AOP ネットワークを構築し, そこに内包される MIE と KE を試験し, リードアクロスにより, 陽性対照に比べ毒性が低いことを示し, PBPK model (physiologically-based pharmacokinetic model) を構築し, ラット, ヒトにおける経口透過用量を算出した。機序の信頼性, MIE プロファイルの体系的な評価が高く評価された。特に, AOP ネットワークというコンセプトの提唱が興味深い。一方, 参照物質の *in vivo* データの少なさやAOPネットワークが未承認であるとの指摘があった。

3 件のケーススタディは, 動物試験を実施しない次世代リスク評価（NGRA）の IATA ワークフローを提案し, 試行, 発展させたものである。既存情報の収集による曝露の推定, 内部曝露の推定や毒性仮説の設定, 非動物手法による精緻化や不確実性評価などの

段階を経て, TTC やリードアクロスによりリスク評価を行うとしている。

「曝露の考慮と非動物的手法に基づく化学物質安全性評価ワークフロー」は, ワークフローを提案し, ピペロニルブトキシドの化粧品としての経皮暴露について, 使用シナリオに基づく曝露量に対し, 脂肪肝, 肝線維症のAOPを元に肝毒性リスクを評価した。評価の早い段階で曝露を考慮し, TTC(Threshold of Toxicological Concern ), リードアクロス, QSAR(Quantitative Structure-Activity Relationship), *in vitro* 試験, オミクスなど多様な手法を統合している点が評価され, 非動物手法に焦点を当てた新規性も注目された。一方で, 非常に複雑なワークフローのため, 専門家の判断と詳細な正当性の検討が必要となることが課題となった。

「1%フェノキシエタノール配合ボディローションの IATA を用いた全身毒性評価」はフェノキシエタノールの代謝物フェノキシ酢酸に着目し全身毒性を予測した。さまざまなAOPに関連するヒトタンパク質やヒト細胞を用いた試験群（スクリーニングキット）, トランスクリプトーム解析からPoD（Point of Departure）を導出し, 毒性の懸念が低いこと, 及びこの手法がウサギ 90 日試験に基づく PoD より安全性が高いことを示した。先進的な試みではあるものの, 試験や細胞株の種類の妥当性や, 不確実性への影響について理解不足であり, 未だ概念実証段階にあると指摘された。

2021-8:「皮膚感作性へのIATAの適用 -ゲラニオールを用いたNGRAフレームワークの実証-」は OECD にて承認されている皮膚感作AOPに基づき, 確定方式（DA）により, 皮膚感作性を予測した。DA は専門家による個別判断を介在せず, *in vitro* 試験や *in silico* ツールの結果を, あらかじめ定められたデータ解釈手順に基づき解釈することで, 誰がやっても同じ結論が得られる手法である。OECD GD256 と OECD TG497 に記載されている計 5 種類の DA を検討した。WoE（Weight of Evidence）としての使用には好意的な反応が得られたが, DA のデータセットの少なさなどの不確実性の指摘があった。

すでに動物試験の実施が禁止されている化粧品業界のニーズに基づき, NGRA ワークフローを提唱した先進性, 近年開発が進められている多様かつ先進的な非動物試験や予測ツールを統合的に用いた点, ヒト由来の試験系により種間外挿が不要となる点が興味深い。

非AOP事例である「アリールアルコールアルキルカルボン酸エステルの IATA を用いた亜慢性反復投与毒性のリードアクロス」は, 構造, *in vivo/vitro* の TK(Toxicokinetic)/TD (Toxicodynamic), 化学的, 生物学的相互作用の共通性に着目し, 標的物質群の共通代謝物アリールアルコールが毒性をもたらす主要因とした。リードアクロスにより毒性を評価した。広範なデータによるWoEが評価された一方, *in vivo* データの不足や質に関する疑問が挙げられた。AOP のような毒性機序を示さずとも, 共通代謝物に着目し側鎖長の異なる多数の物質をカテゴリー化して一括評価できる点は興味深い。

**D. 考察**

D-1. AOP の開発

D-1-1. 免疫毒性の AOP

AOP 154 については, 免疫毒性という前

例のない取り組みであったが, 比較的順調に評価が進行したと考える。この要因として, 1983 年の谷口維紹先生による IL-2 遺伝子のクローニングと（旧）藤沢薬品工業によるカルシニューリンインヒビター(CNI)である FK506（タクロリムス）の発見と臨床応用が挙げられる。分担研究者も含め, 免疫毒性学会に IL-2 及びその発現阻害機序に知見を有する研究者が多かったことが本 AOP 成立につながったことを指摘したい。

AOP277 については, 外部 review に対応して AOP wiki を大幅に修正した結果, OECD より正式に承認の運びとなった。こちらについては, IATA 確立に寄与する AOP ネットワーク構築の観点から, 他の AOP と AO や KER を共有していくことが重要と考えられた。

D-1-2.　発がん性の AOP

論文に取りまとめたように, ラット, マウス, ハムスターにおける化学物質誘発鼻腔発がんの網羅的解析の結果, 各種鼻腔腫瘍の前駆病変は, 投与経路及び遺伝毒性の有無に関係なく, 一般的に化学物質誘発性の細胞毒性を示す部位と関連している可能性があり, 分子開始イベント後の経路は, 遺伝毒性発がん物質と非遺伝毒性発がん物質の間で重複している可能性が高いと考えられた。

D-2. TG 及び DRP の開発

D-2-1.　皮膚感作性試験

既存の TG である皮膚感作性試験代替法 ADRA に関しては, 適用濃度の変更及び重量法を, DPRA に関しては重量法を含む TG442C の再改定をなした。

また, 既存の TG である皮膚感作性試験代替法 IL-8 Luc assay を含む TG442E の改定をなすことができた。

日本で開発された皮膚感作性試験の TG は多い。他の試験法の状況を k ンが見ながら, この改定も重要であると考える。

D-2-2.免疫毒性試験

免疫毒性試験などの全身毒性に関する *in vitro* TG の開発は前例がなく, これまで以上に時間を要しており, 費用も嵩んでいる。OECD は, こうした前例のない TG を開発するために, まずは DRP を作成し, 数年掛かりで免疫毒性試験の TG 作成まで進めてきた。R4 年に DRP を公表し, R5 年, *in vitro* 免疫毒性試験の IL-2 Luc assay の TG を公表できた。さらに IL-2 Luc LTT の公定化も目指しているが, いずれの試験法も単独では免疫毒性を評価できない。今後, この分野の試験法や情報を拡大していくため, IATA の開発に取り掛かる。ここまでできないとこれまでの DRP や TG が生きてこないことから, 最後の仕上げとなる。

D-2-3. B has42 細胞形質転換試験法の TG 開発

Bhas 42 CTA の機序に関する論文は年々増加しており，今後も Bhas 42 CTA の機序は，多方面からの解析により，Bhas 42 CTA における細胞形質転換過程とヒトでの発がん過程との関連が示されることで, Bhas 42 CTA の有用性がより明確になるものと思われる。

発がん性予測試験としての Bhas 42 CTA の役割は，Bhas 42 CTA の開発の目的でも

ある，遺伝毒性試験だけでは検出できない発がん物質，すなわち非遺伝毒性発がん物質(NGTxC)の検出であり，遺伝毒性試験と Bhas 42 CTA のバッテリー系統樹は，Bhas 42 CTA の開発の目的に沿ったものである。実際にその予測率は Concordance 89%及び Sensitivity 93%の高値となったことから，我が国の優れた発がん予測系統樹は，今後も Bhas 42 CTA の有用な活用例として示すことが可能であると考えられた。

Bhas 42 CTA の SPSF 原案の提出においては，同意国（組織）は多数であったが，NGTxC・IATA における採用アッセイの TG 推薦及び NGTxC・IATA のガイダンスドキュメントとしての承認申請等，NGTxC・IATA における今後の計画との調整が Bhas 42 CTA の TG 化に向けて更なる検討事項であると考えられた。

D-3. IATA の開発

D-3-1. 非遺伝毒性発がん性の IATA 開発への協力

IATA 開発においては, 新規アッセイ法の有用性について注視すると共に, アッセイ系の評価が適切になされるよう, 引き続き協力を続ける必要があると考えられた。

D-3-2. 光毒性 AOP と IATA

信頼性の高い光安全性保障システム構築を指向して AOP ならびに IATA 作成に従事した。既に wiki に入力した AOP 案をさらに推敲し，光刺激性に関する毒性カスケードに焦点を当てたものに作り直し，外部評価に資するものに結実させた。この AOP をもとに IATA をアップデートするとともに，情報源に関する情報を多く加え，そして 決定樹 を新規に設定した。改訂した IATA 案は専門家や WNT のコメントや指摘事項に対応して修正作業を行った結果, 2024 年 4 月に WNT に承認された。

D-3-3. 発達神経毒性に起因する行動解析に関する情報収集

調査結果から, 発達神経毒性, 特に行動解析を評価する上で, 考慮しなければならない課題としては,

・学習, 記憶以外の脳高次機能の評価項目の検討

・安定性, 再現性のある実験を実現するため, 環境条件によって影響受ける測定誤差の低減

・頑強性を保ちながらも, 標準化されたプロトコルの確立

等が考えられた。

OECD からの意見募集（*in vitro* DNT ガイダンス文書）に関しては, コメント募集の際に主張した本ガイダンスの改善点・懸念点について, 記載の反映や改善がなされている箇所が多数見受けられた。一方で, 現行の *in vivo* 試験との対応や, 解決すべき課題についての記載については未だ不十分な部分があると考えられた。*in vivo* 試験と *in vitro* 試験で行われる評価手法と得られる結果のブリッジングについては, さらに議論を深める必要がある。

D-4. AOP 及び TG の実験データ支援

D-4-1. *In vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

本研究では, 主に消化管組織並びに肝臓

において, *in vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援, さらに, 代替法において AOP の成立に寄与する知見を得ることを目的とした。令和 3〜5 年度を通して,

腸管上皮における毒性評価として *in vivo* と *in vitro* の試験系からえられる種々の検討に取り組んだ。腸管に関する検討では, マウス, Caco-2 細胞及びマウス空腸由来オルガノイドで, DSS の曝露によりいずれも TJP-1 (tight junction protein-1)の変動, IL-1b の遺伝子発現減少を認め, AOP の成立に寄与する可能性が示された。

肝臓における代替法の検討として, 肝線維化の AOP に作成支援のための組織学的基礎的データを得るとともに, 培養細胞において, 肝線維化関連因子の発現変動を解析した。その結果, *in vivo* においては, 星細胞の活性化とともに細胆管反応が線維化と強く相関し, SOX9 や CD44 の関与が示唆された。ヒト培養肝星細胞株において, TGFβ の刺激により筋線維芽様細胞への活性化とコラーゲンの産生がみられた。また, マウス肝オルガノイドにおいて TGFβ の刺激により筋線維芽様細胞への活性化とコラーゲンの産生に加えて, SOX9 や CD44 の発現増加がみられた。

以上, 両実験系を通して動物モデルと in vitro 試験との相関性のある結果については, 新たな毒性指標の開発に寄与し得るものと考えられた。

D-4-2. DNA損傷・幹細胞マーカー等を指標とした免疫組織化学的検索による発がん性早期検出

腎発がん物質検出指標としての γ-H2AX の感度・特異度の検証を実施した。令和 3〜5 年度にかけて, 腎発がん物質 16 種を用いたラット 28 日間反復経口投与試験を実施し, 腎臓における γ-H2AX 形成の免疫組織化学的解析を行った。腎発がん物質のうち 14 種は, 皮質または髄質外帯外層の尿細管上皮細胞における γ-H2AX 形成を有意に増加させた。これまでの検討結果を総合すると, 腎発がん物質 26 種のうち, 22 物質（感度：84.6%）が γ-H2AX 陽性細胞を有意に増加させた一方, 非腎発がん物質は検索した 9 種のうち 8 物質（特異度：88.9%）が陰性であった。以上より, γ-H2AX 免疫染色を用いることで, 腎発がん物質の早期検出が可能であることが示唆された。

D-4-3. 遺伝毒性の AOP 開発

発がん性（遺伝毒性）の AOP への組み込みを想定し, 遺伝毒性初期応答反応の早期検出システムの構築を試みるため, クロマチン免疫沈降法（Chromatin immunoprecipitation; ChIP）を応用し, 定量的 PCR を用いた DNA 損傷応答の分子生物学的解析を実施した。特に, 数百コピーから成るクラスターを形成し, 転写の機序についての知見も豊富である rDNA を DNA 損傷応答解析の標的領域とした。RPA194, γH2AX, Ku80 及び LIG4 に対するポリクローナル抗体を用いた ChIP 解析結果から, 本手法が DNA 上で直接的に生じている DNA 損傷応答を定量・定性的かつ早期に検出できることが示された。モノクローナル抗体については複数の市販品を用いて解析した結果, γH2AX, ATM, MRE11 及び BRCA1 に対するモノクローナル抗体を用いた ChIP 解析が有効であることを明らか

にした。特に γH2AX についてはポリクローナルとモノクローナルの抗体で同様の DNA 損傷応答反応を検出できること, そして, ATM は γH2AX, Ku80 及び LIG4 と類似した DNA 上の分布を示すことを明らかにした。また, 今回の実験条件では MRE11 と BRCA1 は通常時は rDNA 上にあるが, 紫外線照射後に DNA から解離することを明らかにした。

D-4-4. 腎障害・線維化の分子機序に関する研究

腎障害後に尿細管の再生に異常が生じた場合, 不可逆的な線維化を伴う慢性腎臓病へと移行する。我々はこれまで再生異常の生じた尿細管には CD44 が発現することを見出しており, 本研究では尿細管の再生異常を Key Event, CD44 を測定可能なエンドポイントとした腎障害・腎線維化の AOP 開発の可能性について検証した。

アロプリノール（APL）, シクロスポリン（CyA）及びバンコマイシンにより誘発したラット腎線維化モデルにおいて, 線維化病変内の尿細管は CD44 に陽性を示した。CD44 の腎線維化における病態生理学的役割を検証するため, APL 及び CyA 誘発ラット腎線維化モデルにおける線維化病変内の CD44 陽性尿細管をレーザーマイクロダイセクションにより採取し, マイクロアレイを実施した。Gene ontology（GO）解析では CD44 陽性尿細管は間葉系の形質を獲得していることが示唆された。免疫染色にて CD44 陽性尿細管では近位尿細管分化マーカーの発現減少及び間葉系マーカーの発現が認められたものの, 軽度に肥厚した基底膜に囲まれており周囲間質への明らかな浸潤像は観察されなかった。パスウェイ解析では CD44 は fibronectin 1 (*Fn1*)を含む線維化関連遺伝子の上流因子として抽出された。*in situ* hybridization では *Fn1* mRNA は CD44 陽性尿細管の細胞質に確認されたが, 免疫染色において fibronectin タンパクはこれらの尿細管の周囲間質に認められた。またラット腎線維化モデルでは血清中 CD44 値が高値を示し, その値は腎臓における CD44 陽性尿細管の数と正の相関を示した。

尿細管が基底膜に接着した状態で間葉系の表現型を獲得する現象を部分的上皮間葉転換（pEMT）という。本実験結果から CD44 は pEMT の生じた尿細管において細胞外基質の分泌を誘導し, 腎線維化に寄与すると考えられた。また CD44 陽性尿細管の発現に伴い血清中 CD44 値が上昇したことから, CD44 は腎線維化のバイオマーカーとなる可能性が示唆された。以上より, 本研究成果は尿細管の再異異常を Key Event, CD44 を測定可能なエンドポイントとした腎障害・腎線維化の AOP 開発の可能性を示すものと考えられた。

D-5. OECD に提出する資料の事前確認と OECD からの意見募集への対応

OECD で Emerging technologies を如何にガイドラインに取り込むかという課題に対応して workshop が開催された。一昨年の Emerging technologies in the Test Guidelines Programme に引き続き, 昨年開催された Stakeholders Workshop on Operational and Financial Aspects of Test Methods Validation への対応は, まさしく日本が直面している問題を解決するプロジェクトである。研究代表者の平林, 足利及び厚生労

働省の担当者とも連携を図り,引き続き,日本として適切な対応を心掛けていくべきと考える。

D-6. 毒性等情報収集調査

OECD IATA Case Studies Project から, AOP を用いたケーススタディを取り上げ, その優位性や課題を整理した。

この成果は, AOP の確立と AOP を用いた毒性評価の行政導入の検討に資するものであると期待している。

**E. 健康危険情報**

特になし

**F. 結論**

研究協力者とともに, 既存の TG である皮膚感作性試験代替法 ADRA に関しては, 適用濃度の変更及び重量法及び DPRA 重量法を含む TG442C 及び皮膚感作性試験代替法 IL-8 Luc assay を含む TG442E の改定をなすことができた。さらに, *in vitro* 免疫毒性試験 IL-2 Luc assay が TG444A として承認された。AOP に関しては, 「IL-1 receptor 結合阻害：AOP277」は, 10 月末 OECD に承認され, AOP No.30 として i-library に収載された。発がん性の AOP に関しては, Pathogenesis of chemically induced nasal cavity tumors in rodents: contribution to adverse outcome pathway が J Toxicol Pathol. に掲載された。

支援研究に関しては, 以下の成果が得られた。

1) 腸管並びに肝臓における *in vivo* と相関性のある *in vitro* 毒性評価系指標を見出すための検討を実施した。これにより, 肝臓・腸管由来組織における動物実験代替法の確立に向けた検討を２次元培養ないし３次元培養条件下で実施し, それぞれ, 代替法の開発に資する基礎的情報を得た。さらに, 本成果は, 本研究を emergency technology にあたる人体摸倣システム（MPS）の開発にも寄与でき得るものと考えられた。

2) ラットを用いた 28 日間反復経口投与毒性試験において, 腎発がん物質 26 種のうち, 22 物質（感度：84.6%）が γ-H2AX 陽性細胞の有意な増加を示した一方, 非腎発がん物質は検索した 9 種のうち 8 物質（特異度：88.9%）が陰性であった。以上より, γ-H2AX 免疫染色を用いることで, 腎発がん物質の早期検出が可能であることが示唆された。

3) ポリクローナル抗体を用いた RPA194, γH2AX, Ku80 及び LIG4 を標的とした解析と, モノクローナル抗体を用いた ATM 及び γH2AX を標的とした解析結果から, ChIP 及び rDNA 領域の定量的 PCR を利用した DNA 損傷応答の解析手法により DNA 上で直接的に生じている DNA 損傷応答を定量・定性的かつ早期に検出できることが示された。また, MRE11 と BRCA1 の DNA 損傷応答反応は上記のタンパク質群とは異なる挙動を示すことを明らかにした。これらを踏まえると, ChIP に用いる抗体をアレンジすることで解析の標的タンパク質を自在に設定することができるため, 汎用性が高く, この試験法の発がん性・遺伝毒性 AOP 開発に対する高い有効性を示すことができると考えられる。

4) 再生異常の生じた尿細管に発現する

CD44 の腎線維化における病態生理学的役割の一端を明らかにすることができ, さらに CD44 の腎線維化バイオマーカーとしての可能性も示された。よって本研究成果は, 尿細管の再異異常を Key Event, CD44 を測定可能なエンドポイントとした腎障害・腎線維化の AOP 開発の可能性を示すものと考えられた。

今後も, OECD プロジェクトに日本の意見や結果を反映させ, 引き続き厚生労働行政に活用できるよう調査を進めていく所存である。

## G. 研究発表

G.1. 論文発表


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229.足利太可雄: 免疫毒性に関する AOP および OECD テストガイドラインの開発状況，日本薬学会第 144 年会（2024.3.30, 横浜）

**H. 知的財産権の出願・登録状況**

（予定を含む。）

1. 特許取得
   特になし
2. 実用新案登録
   特になし
3.その他
   特になし

**I. OECD　成果物**

1. AOP No.30; Adverse Outcome Pathway on impaired interleukin-1 receptor type (IL-1R1) signaling leading to impaired T-cell dependent antibody response.
2. OECD TG 442C; In vitro skin sensitasation, in chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins
3. OECD TG 442E; In vitro skin sensitisation assays the Key Event on activation of dendric cells on the Adverse Outcome Pathway for skin sensitization.
4. OECD TG 444A; In vitro immunotoxicity, IL-2 Luc assay

OECD Series on Adverse Outcome Pathways No. 30

# Adverse Outcome Pathway on impaired interleukin-1 receptor type I (IL-1R1) signaling leading to impaired T-cell dependent antibody response

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# *Adverse Outcome Pathway on Impaired Interleukin-1 Receptor Type I (IL-1R1) signaling leading to Impaired T-Cell Dependent Antibody Response*

**Series on Adverse Outcome Pathways No. 30**

**AOP No. 277 in the AOP-Wiki platform**

# *Foreword*

This Adverse Outcome Pathway (AOP) on Impaired Interleukin-1 Receptor Type I (IL-1R1) signaling leading to Impaired T-Cell Dependent Antibody Response has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), formerly an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA).

The AOP has been reviewed internally by the EAGMST. The scientific review was conducted by experts nominated by the WNT. The WNT and the Working Party on Hazard Assessment (WPHA) endorsed the AOP on 6 July 2023.

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The OECD's Chemicals and Biotechnology Committee agreed to declassification of this AOP on 6 October 2023.

This document is being published under the responsibility of the OECD's Chemicals and Biotechnology Committee.

The outcome of the scientific review is publicly available at the following link: [scientific review] and the AOP corresponding [discussion page] of the AOP-Wiki includes follow-up comments and discussions.

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# *Abstract*

The pleiotropic cytokine IL-1 mediates its biological functions via association with the signaling receptor IL-1R1. These may include initiation of innate immunity as well as acquired immunity, which are essential for assistance of host defense against infection. The trimeric complex consists of IL-1, IL-1R1 and IL-1R3 (a coreceptor, formerly IL-1R accessory protein) allows for the approximation of the Toll-IL-1-Receptor (TIR) domains of each receptor chain. MyD88 then binds to the TIR domains. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB. In addition to the NF-κB pathway, IL-1 receptor-associated kinase (IRAK), which is one of the kinase consisting of the cascade, activates a variety of transcription factors, including Adaptor protein-1 (AP-1). The activation of NF-κB plays a principal role in the immunological function of IL-1. Namely, it stimulates innate immunity such as activation of dendritic cells and macrophages. It also stimulates T cells via activated dendritic function or directly. The activation of T cells is crucial for B cell proliferation and their antibody production. The cooperation by T cells and B cells constitutes a main part of host defense against infection. Therefore, the impaired IL-1R1 signaling either by the decreased IL-1 production or the inhibition of IL-1β binding to IL-1R1 by IL-1 receptor antagonist（IL-1Ra）or anti-IL-1β antibody) results in the blockade of the effects of the pleiotropic cytokine IL-1β leading to suppressed T cell dependent antibody response (TDAR).

In this AOP, we selected the impaired IL-1R signaling as a molecular initiating event (MIE) in T cell, and suppression of NF-κB (and/or AP-1), suppression of T cell activation, and suppression of TDAR as key events (KE).

Although the purpose of this AOP is to elucidate biological pathways that lead to immune suppression caused by impaired IL-1R signaling by chemicals, most of the stressors presented in this AOP were limited to pharmaceuticals because of the lack of information on chemicals.

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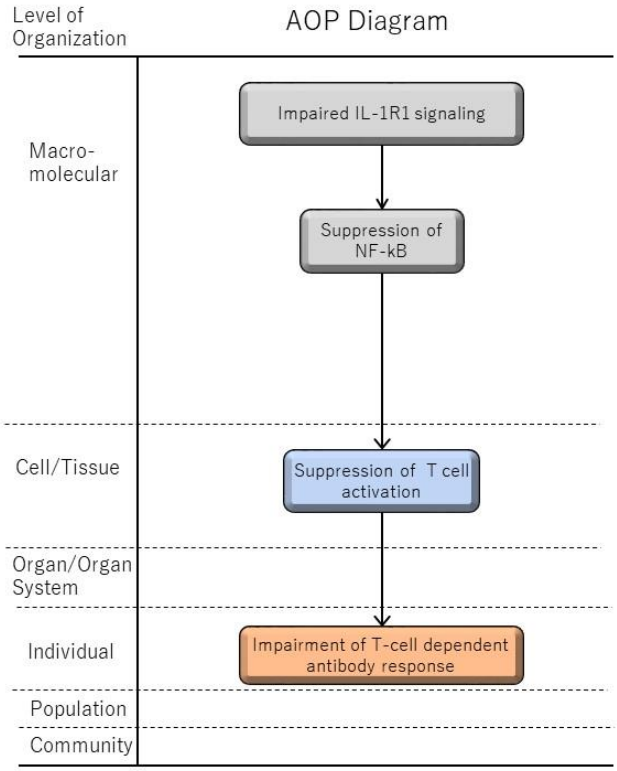
## Background

The pleiotropic cytokine IL-1 mediates its biological functions via association with the signaling receptor IL-1R1. These may include initiation of innate immunity and assistance of host defense, and sometimes, mediation of autoinflammatory, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever. The trimeric complex consists of IL-1, IL-1R1 and IL-1R3 (a coreceptor, formerly IL-1R accessory protein) allows for the approximation of the Toll-IL-1-Receptor (TIR) domains of each receptor chain. MyD88 then binds to the TIR domains. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB and/or AP-1 and fundamental inflammatory responses such as the induction of cyclooxygenase type 2, production of multiple cytokines and chemokines, increased expression of adhesion molecules, or synthesis of nitric oxide. (Dinarello, 2018; Weber et al., 2010a, b; Jain et al., 2014).

Molecules like nuclear or mitochondrial DNA, adenosine triphosphate (ATP), uridine triphosphate (UTP), uric acid and high mobility group box 1 (HMGB1) are classified as damage associated molecular patterns (DAMPs). DAMPs are secreted or produced upon cellular injury or death and induce sterile inflammation. On the other hand, bacterial products like lipopolysaccharide (LPS), peptidoglycans, lipoprotein flagellins, bacterial RNA and DNA are some of the well-characterized pathogen associated molecular patterns (PAMPs). These DAMPs and PAMPs with a few exceptions bind to pattern recognition receptors (PRRs) such as toll-like receptor (TLRs) and nucleotide oligomerization domain (NOD) like receptors (NLRs). Proinflammatory mediators such as DAMPs, PAMPs, and various inflammatory cytokines or mediators including IL-1β itself activate innate immune mechanisms in the host leading to IL-1β production (Handa et al., 2016; Newton and Dixit, 2012; Yang et al., 2017). Besides transcriptional regulation and posttranscriptional level by RNA-binding proteins, pro-IL-1β protein requires proteolytic cleavage by active caspase-1 as the effector component of stimulation-induced multi-protein inflammasomes to acquire functional activity. Altogether, these different layers of regulation allow to fine tune IL-1β production under different pathophysiological conditions (Bent et al., 2018).

Therefore, the inhibition of various targets in different layers from the stimulation of PRRs or the receptors of proinflammatory cytokines, e.g., IL-1, IL-18, or TNFa, to the activation of NF-κB and/or AP-1 or the inhibition of posttranscriptional regulation of pro-IL-1β cause impaired IL-1R1 signaling. In addition, since IL-1 also mediates autoinflammatory syndromes, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever, several inhibitors against IL-1R1 have been developed. They are IL-1 receptor antagonist（IL-1Ra）, anakinumab (anti-IL-1β antibody) and rilonacept (soluble IL-1R). Several reports described that the administration of these drugs led to increased susceptibility to infection(De Benedetti et al., 2018; Fleischmann et al., 2003; Genovese et al., 2004; Imagawa et al., 2013; Kullenberg et al., 2016; Lachmann et al., 2009; Lequerre et al., 2008; Migkos et al., 2015; Schlesinger et al., 2012; Yokota et al., 2017). In addition to these human data, the experiments using knockout mice revealed that the lack of IL-1 signaling led to bacterial, tuberculosis or viral infection(Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Tian et al., 2017; Yamada et al., 2000).

## Graphical representation



# Summary of the AOP

## Events

**Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)**

| Sequence | Type | Event ID | Title | Short name |
|---|---|---|---|---|
| 1 | MIE | 1700 | Impaired IL-1R1 signaling in T cell | Impaired IL-1R1 signaling |
| 2 | KE | 202 | Inhibition, Nuclear factor kappa B (NF-kB) | Inhibition, Nuclear factor kappa B (NF-kB) |
| 3 | KE | 1702 | Suppression of T cell activation | Suppression of T cell activation |
| 4 | AO | 984 | Impairment, T-cell dependent antibody response | Impairment, T-cell dependent antibody response |

## Key Event Relationships

| Upstream Event | Relationship Type | Downstream Event | Evidence | Quantitative Understanding |
|---|---|---|---|---|
| Impaired IL-1R1 signaling in T cell | adjacent | Inhibition, Nuclear factor kappa B (NF-kB) | High | Moderate |
| Inhibition, Nuclear factor kappa B (NF-kB) | adjacent | Suppression of T cell activation | High | Moderate |
| Suppression of T cell activation | adjacent | Impairment, T-cell dependent antibody response | High | High |

## Stressors

| Name | Evidence |
|---|---|
| IL-1 receptor antagonist（IL-1Ra）(Anakinra) | High |
| anti-IL-1b antibody (Canakinumab) | High |
| soluble IL-1R (Rilonacept) | High |
| anti-IL-1b antibody (Gevokizumab) | High |
| Dexamethasone | High |
| minocycline | High |
| Belnacasan (VX-765) | High |
| Pralnacasan (VX-740, HMR3480) | High |
| Cinnamic aldehyde | High |
| Dimethyl fumarate | High |
| curcumin | High |
| iguratimod | High |

| Name | Evidence |
| --- | --- |
| (-)-Epigallocatechin gallate | High |
| TAK-242 | High |
| IRAK4 inhibitors | High |
| Dehydroxymethylepoxyquinomicin (DHMEQ) | High |

# Overall Assessment of the AOP

## Domain of Applicability

### Life Stage Applicability

| Life Stage | Evidence |
|---|---|
| Not Otherwise Specified | High |

### Taxonomic Applicability

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

### Sex Applicability

| Sex | Evidence |
|---|---|
| Mixed | High |

Although sex differences in immune responses are well known (Klein and Flanagan, 2016), there is no reports regarding the sex difference in IL-1 production, IL-1 function or susceptibility to infection as adverse effect of IL-1 blocking agent. Again, age-dependent difference in IL-1 signaling is not known.

The IL1B gene is conserved in human, chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and frog (https://www.ncbi.nlm.nih.gov/homologene/481), and the Myd88 gene is conserved in human, chimpanzee, Rhesus monkey, dog, cow, rat, chicken, zebrafish, mosquito, and frog (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849).

The NFKB1 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, and frog.

275 organisms have orthologs with human gene NFKB1.

(https://www.ncbi.nlm.nih.gov/gene/4790)

The lower level of stress-induced IL-1b expression is demonstrated in the aged murine keratinocytes (Pilkington et al., 2018).

The IL-1b production by mouse oral mucosal leukocytes stimulated with candida albicans was reduced with aging (Bhaskaran et al., 2020).

The baseline IL-1 signaling of the upper respiratory tract lavage was reduced in murine newborn mice (Kuipers et al., 2018).

## Essentiality of the Key Events

The experiments using knockout mice revealed that the deficiency of IL-1 signaling led to bacterial, tuberculosis or viral infection (Bohrer et al., 2018; Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Labow et al., 1997; Tian, Jin and Dubin, 2017; Yamada et al., 2000).

IL-1 receptor antagonist（IL-1Ra）was purified in 1990, and the cDNA reported that same year. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (generic anakinra) is fully active in blocking the IL-1R1, and therefore, the activities of IL-1α and IL-1β. Anakinra is approved for the treatment of rheumatoid arthritis and cryopyrin-associated periodic syndrome (CAPS). Since its introduction in 2002 for the treatment of rheumatoid arthritis, anakinra has had a remarkable record of safety. However, Fleischmann et al. (Fleischmann et al., 2003) reported that serious infectious episodes were observed more frequently in the anakinra group (2.1% versus 0.4% in the placebo group) and other authors reported the increased susceptibility to bacterial or tuberculosis infection (Genovese et al., 2004; Kullenberg et al., 2016; Lequerre et al., 2008). As IL-1 signaling antagonists, two drugs went up to the market, canakinumab (anti-IL-1b antibody) and rilonacept (soluble IL-1R). Several reports described that the administration of these drugs led to immunosuppression or increased susceptibility to infection (De Benedetti et al., 2018; Imagawa et al., 2013; Lachmann et al., 2009; Schlesinger et al., 2012).

In a similar way, defect of MyD88 signaling caused by knockout of mice gene or deficiency in human patient leads to the increased susceptibility to bacterial or tuberculosis infection (von Bernuth et al., 2012).

Mice lacking NF-kB p50 are unable effectively to clear L. monocytogenes and are more susceptible to infection with S. peumoniae (Sha et al., 1995).

## Weight of Evidence Summary

The recent review of IL-1 pathway by Weber et al. (Weber, Wasiliew and Kracht, 2010a) has clearly described the intracellular signaling event from the binding of IL-1α or IL-1β to IL-1R to the activation of NF-κB through the assemble of MyD88 to the trimeric complex composed of IL-1, IL-R1, and IL-1RacP. The sequentiality and essentiality of each signaling molecule have been demonstrated by mice lacking relevant molecules (Dinarello, 2018; Weber, Wasiliew and Kracht, 2010a, b).

There were several reports that described that administration of IL-1R antagonist or neutralizing antibody led to the suppression of downstream phenomena, which included internalization of IL-1 (Dripps et al., 1991), production of $PGE_2$ (Hannum et al., 1990; Seckinger, Kaufmann and Dayer, 1990), IL-6 (Goh et al., 2014), and T cell proliferation (Seckinger, Kaufmann and Dayer, 1990).

Several reports described that the administration of IL-1 receptor antagonist（IL-1Ra）, canakinumab (anti-IL-1β antibody) and rilonacept (soluble IL-1R) led to increased susceptibility to infection (De Benedetti et al., 2018; Fleischmann et al., 2003; Genovese et al., 2004; Imagawa et al., 2013; Kullenberg et al., 2016; Lachmann et al., 2009; Lequerre

et al., 2008; Schlesinger et al., 2012; Yokota et al., 2017). In addition to these human data, the experiments using knockout mice revealed that the lack of IL-1 signaling led to bacterial, tuberculosis or viral infection (Bohrer et al., 2018; Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Labow et al., 1997; Tian, Jin and Dubin, 2017; Yamada et al., 2000). Moreover, polymorphism of IL-1b or IL-1Ra leads to the increased susceptibility to tuberculosis, severe sepsis or fungal infection (Fang et al., 1999; Motsinger-Reif et al., 2010; Wojtowicz et al., 2015).

**Biological plausibility**

- Inhibition of IL-1 binding to IL-1 receptor leads to Inhibition, Nuclear factor kappa B (NF-kB)

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. The IL-1R3 (formerly IL-1R accessory protein (IL-1RAcP)) serves as a co-receptor that is required for signal transduction of IL-1/IL-1RI complexes.

The initial step in IL-1 signal transduction is a ligand-induced conformational change in the first extracellular domain of the IL-1RI that facilitates recruitment of IL-1R3. the trimeric complex rapidly assembles two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor–activated protein kinase (IRAK) 4. This is paralleled by the (auto)phosphorylation of IRAK4, which subsequently phosphorylates IRAK1 and IRAK2, and then this is followed by the recruitment and oligomerization of tumor necrosis factor–associated factor (TRAF) 6. Activation of NF-κB by IL-1 requires the activation of inhibitor of nuclear factor B (IκB) kinase 2 (IKK2). Activated IKK phosphorylates IκBα, which promotes its K48-linked polyubiquitination and subsequent degradation by the proteasome. IκB destruction allows the release of p50 and p65 NF-κB subunits and their nuclear translocation, which is the central step in activation of NF-κB. Both NF-κBs bind to a conserved DNA motif that is found in numerous IL-1–responsive genes (Weber, Wasiliew and Kracht, 2010a, b).

- Inhibition, Nuclear factor kappa B (NF-κB) leads to Suppression of T cell activation

In T lineage cells, the temporal regulation of NF-κB controls the stepwise differentiation and antigen-dependent selection of conventional and specialized subsets of T cells in response to T cell receptor and costimulatory, cytokines and growth factor signals. Cytokines include cytokines produced from macrophage or monocyte such as IL-1β (Gerondakis et al., 2014).

- Suppression of T cell activation leads to suppression of TDAR

T cell-derived cytokines play important roles in TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation.

Th2 cells produce cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 in Th2 cells and reduces the production of

antigen specific IgE in human cell culture and mice (Yanagihara, 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of

Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one the of IL-2R. Basiliximab (Simulect) is known as anti-CD25antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Kircher, 2003).

Based on these evidences, the insufficient T cell or B cell function causes suppression of TDAR.

**Empirical support**

- Impaired IL-1R signaling.

Decreased production of IL-1 or inhibition of the binding of IL-1 to IL-1R impair IL-1R signaling.

- Decreased IL-1 production

Decreased IL-1 production by macrophages or dendritic cells can be induced by suppressed IL-1β mRNA induction or suppressed maturation of pro-IL-1β. Dexamethasone is one of the representative drugs that significantly suppress IL-1β production from monocytes (Finch-Arietta and Cochran, 1991). Other than dexamethasone, the inhibition of various targets in different layers from the stimulation of PRRs or the receptors of proinflammatory cytokines to the activation of NF-κB or the inhibition of posttranscriptional regulation of pro-IL-1β cause impaired decreased IL-1β production.

Quite a few compounds have been reported to inhibit NF-κB signaling by several different mechanisms reviewed by Fuchs (Fuchs, 2010). In fact, dimethyl fumarate inhibits the activation of NF-κB, resulting in a loss of proinflammatory cytokine production, distorted maturation and function of antigen-presenting cells, and immune deviation of T helper cells (Th) from the type 1 (Th1) and type 17 (Th17) profiles to a type 2 (Th2) phenotype (McGuire et al., 2016; Peng et al., 2012). Several studies have shown intriguing pharmacologic effects associated with curcumin, which inhibits NF-κB expression by regulating NF-κB/IκB pathway and down-regulates expression of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNFα (Wang et al., 2018). Iguratimod, a methanesulfonanilide, that is a novel disease-modifying antirheumatic drug, inhibits NF-κB but not its inhibitor, IκBα, and inhibits the production of IL-1b (Mucke, 2012). Epigalocathechin gallate (EGCG) has been reported to inhibit NF-κB activation through inhibition of p65 phosphorylation (Wheeler et al., 2004) and suppress the production of LPS-stimulated IL-1b (Wang et al., 2020). DHMEQ inhibits LPS-induced NF-κB activation by inhibiting its nuclear translocation from the cytoplasm. It also inhibits LPS-induced secretion of IL-1b (Suzuki and Umezawa, 2006).

Other than the inhibitors for NF-κB signaling, which can be stimulated by various stimulations other than TLR4 stimulation, there are signaling molecules that are specific to TLR4 signaling, such as TLR4, Mal, TRAM, Myd88, IRAK4, and IRAK1/2 (Vallabhapurapu and Karin, 2009). There are several chemicals that target some of these molecules, inhibitors of TLR4 such as TAK-242 (Matsunaga et al., 2011) and various IRAK4 inhibitors (Lee et al., 2017). IRAK4 has recently attracted attention as a therapeutic target for inflammation and tumor diseases (Chaudhary, Robinson and Romero, 2015).

Beside transcriptional regulation of IL-1b production, minocycline, and two prodrugs, pralnacasan (VX-740) and belnacasan (VX-765) that are orally absorbed and converted into the active principle, VRT-018858 and VRT-043198, respectively (Fenini, Contassot and French, 2017) suppress IL-1 signaling by the inhibition of caspase-1 activation. Caspase-1 is an essential enzyme for maturation of pro- IL-1β and the secretion of mature IL-1β (Vincent and Mohr, 2007). Recently, it has been reported that cinnamic aldehyde suppresses serum IL-1β level in endotoxin poisoning mice (Xu et al., 2017).

- Blocking of binding of IL-1 to IL-1R1

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (anakinra) is fully active in blocking the IL-1R1, and therefore, the biological activities of IL-1α and IL-1β. The binding of IL-1α and IL-1β to IL-1R1 can be suppressed by soluble IL-1R like rilonacept (Kapur and Bonk, 2009). The binding of IL-1β to IL-1R1 can be inhibited by anti-IL-1β antibody (canakinumab and gevokizumab) (Church and McDermott, 2009) (Roell et al., 2010).

Several reports described that the administration of IL-1 receptor antagonist（IL-1Ra）, canakinumab (anti-IL-1β antibody) and rilonacept (soluble IL-1R) led to increased susceptibility to infection (De Benedetti et al., 2018; Fleischmann et al., 2003; Genovese et al., 2004; Imagawa et al., 2013; Kullenberg et al., 2016; Lachmann et al., 2009; Lequerre et al., 2008; Schlesinger et al., 2012; Yokota et al., 2017).

- Immunosuppression by impaired IL-1 receptor signaling

In addition to these human data, the experiments using knockout mice revealed that the lack of IL-1 signaling either by the lack of IL-1a or IL-1b or the lack of IL-1 receptor led to bacterial, tuberculosis or viral infection (Bohrer et al., 2018; Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Labow et al., 1997; Tian, Jin and Dubin, 2017; Yamada et al., 2000). Moreover, polymorphism of IL-1b or IL-1Ra leads to the increased susceptibility to tuberculosis, severe sepsis or fungal infection (Fang et al., 1999; Motsinger-Reif et al., 2010; Wojtowicz et al., 2015).

## Quantitative Consideration

**IL-1Ra blocks IL-1 signaling:**

IL-lra alone at concentrations as high as 1 mg/mL did not induce IL-la, IL-lβ, TNFa, or IL-6 synthesis. Suppression of IL-1-induced IL-1, TNFa, or IL-6 synthesis was dose-dependent ($P \leqq .0001$). At a twofold molar excess, IL-lra inhibited IL-1-induced IL-1 or TNFa synthesis by 50% ($P < .01$); an equimolar concentration of IL-lra inhibited synthesis of these two cytokines by over 20% ($P < .05$). A 10-fold molar excess of IL-lra over IL-lβ reduced IL-lβ-induced IL-la by 95% ($P = .01$) and IL-la-induced IL-1β by 73% ($P < .01$). In elutriated monocytes, a 10-fold molar excess of IL-lra reduced IL-lβ-induced IL-la by 82% ($P < .05$), TNFa by 64% ($P = .05$), and IL-6 by 47% ($P < .05$). (Granowitz et al., 1992)

**Canakinumab (ACZ885, Ilaris):**

The antibody binds to human IL-1β with high affinity (about 40 pmol/l). The antibody was found to neutralize the bioactivity of human IL-1β on primary human fibroblasts in vitro 44.6 pmol/l ($7.1 \pm 0.56$ ng/ml; $n = 6$) of ED50. Application of Canakinumab intraperitoneally 2 hours before injecting the IL-1β producing cells completely suppressed joint swelling (0.06 mg/kg of EC50) (Alten et al., 2008).

Primary human fibroblasts are stimulated with recombinant IL-1b or conditioned medium obtained from LPS-stimulated human PBMCs in the presence of various concentrations of Cankinumab or IL-1RA ranging from 6 to 18,000 pM. Supernatant is taken after 16 h stimulation and assayed for IL-6 by ELISA. Canakinumab typically have 1 nM or less of EC50 for inhibition of IL-6 production (Canakinumab Patent Application WO02/16436.)

**Rilonacept (IL-1 Trap, Arcalyst):**

Incubation of the human MRC5 fibroblastic cell line with IL-1β induces secretion of IL-6. At a constant amount of IL-1β (4 pM), the IC50 of the IL-1 trap is ~2 pM. Another unique property of the IL-1 trap is that it not only blocks IL-1β, but also blocks IL-1α with high affinity (KD = ~3 pM; data not shown). The titration curve of IL-1 trap in the presence of 10 pM IL-1β shows an IC50 of 6.5 pM, which corresponds to a calculated KD of 1.5 pM (This affinity is 100 times higher than that of the soluble single component receptor IL-1RI (Economides et al., 2003).

## Considerations for Potential Applications of the AOP

The impaired IL-1 signaling can lead to immunosuppression. Therefore, the test guideline to detect chemicals that decrease IL-1 signaling is required to support regulatory decision-making. This AOP can promote the understanding of the usefulness of the test guideline.

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# Appendix 1 - MIE, KEs and AO

## List of MIEs in this AOP

## Event: 1700: Impaired IL-1R1 signaling in T cell

**Short Name: Impaired IL-1R1 signaling**

**AOPs Including This Key Event**

| AOP ID and Name | Event Type |
|---|---|
| Aop:277 - Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | Molecular Initiating Event |

**Stressors**

| Name |
|---|
| IL-1 receptor antagonist（IL-1Ra）(Anakinra) |
| anti-IL-1b antibody (Canakinumab) |
| soluble IL-1R (Rilonacept) |
| curcumin |
| iguratimod |
| epigallocathechin gallate |
| TAK-242 |
| IRAK4 inhibitors |
| Dehydroxymethylepoxyquinomicin (DHMEQ) |
| Dimethyl fumarate |
| anti-IL-1b antibody (Gevokizumab) |

**Biological Context**

| Level of Biological Organization |
|---|
| Molecular |

**Cell term**

| Cell term |
|---|
| T cell |

**Organ term**

| Organ term |
|---|
| immune system |

### *Evidence for Perturbation by Stressor*

**Overview for Molecular Initiating Event**

Dex inhibits IL-1β gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-κB/Rel and AP-1 activation (Jeon et al., 2000).

Dex suppresses LPS-induced gene expression of IL-1β in rat lung. (in vivo) (Qiu et al., 1997)

Dex inhibits the release of IL-1β by human leukocyte stimulated with Streptococcus pneumoniae stimulation (van Furth et al., 1995).

Treatment of peripheral blood monocytes with 2 mg/ml LPS potently increased IL-1β release (p= 0.001) and Dex ($10^{-7}$ M) significantly reduced both resting and stimulated IL-1β release (p 0.009).) (Morand, Rickard and Goulding, 1993)

Dex effectively blocks the glutamine antagonist acivicin-induced expression of IL-1β mRNA by HL-60 leukemia cells (Weinberg, Mason and Wortham, 1992).

LPS treatment induced a significant upregulation of the mRNA and release of IL-1β from retinal microglia. Minocycline inhibited its releases. Thus, minocycline might exert its anti-inflammatory effect on microglia by inhibiting the expression and release of IL-1β (Wang et al., 2005).

Caspase-1 inhibition reduced the release of IL-1β in organotypic slices exposed to LPS+ATP. Administration of pralnacasan (intracerebroventricular, 50 μg) or belnacasan (intraperitoneal, 25–200 mg/kg) to rats blocked seizure-induced production of IL-1β in the hippocampus, and resulted in a twofold delay in seizure onset and 50% reduction in seizure duration (Ravizza et al., 2006).

Belnacasan, an orally active IL-1β converting enzyme/caspase-1 inhibitor, blocked IL-1β secretion with equal potency in LPS-stimulated cells from familial cold urticarial associated syndrome and control subjects (Stack et al., 2005).

In LPS-induced acute lung injury (ALI) mice model, LPS induced inflammatory cytokines such as TNF-α, IL-6, IL-13 and IL-1β were significantly decreased by cinnamaldehyde (CA) (Huang and Wang, 2017).

The suppressing capacities of six cinnamaldehyde-related compounds were evaluated and compared by using the LPS-primed and ATP-activated macrophages. At concentrations of 25~100 μM, cinnamaldehyde and 2-methoxy cinnamaldehyde dose-dependently inhibited IL-1β secretion (Ho, Chang and Chang, 2018).

In vitro, CA decreased the levels of pro-IL-1β and IL-1β in cell culture supernatants, as well as the expression of NLRP3 and IL-1β mRNA in cells. In vivo, CA decreased IL-1β production in serum. Furthermore, CA suppressed LPS-induced NLRP3, p20, Pro-IL-1β, P2X7 receptor (P2X7R) and cathepsin B protein expression in lung, as well as the expression of NLRP3 and IL-1β mRNA (Xu et al., 2017).

IL-1 is known to mediates autoinflammatory syndrome, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever. Blocking of binding of IL-1 to IL-1R1 by anakinra, canakinumab, and rilonacept have been already used to treat these autoinflammatory syndrome associated with overactivation of IL-1 signaling (Quartier, 2011).

Various inhibitors for NF-κB, such as dimethyl fumarate, curcumin, iguratimod, epigalocathechin gallate (EGCG), and DHMEQ inhibits LPS-induced NF-κB activation

and LPS-induced secretion of IL-1b (McGuire et al., 2016; Mucke, 2012; Peng et al., 2012; Suzuki and Umezawa, 2006; Wang et al., 2020; Wang et al., 2018; Wheeler et al., 2004).

Several chemicals that targe some of these molecules, an inhibitors of TLR4 such as TAK-242 (Matsunaga et al., 2011) and various IRAK4 inhibitors (Lee et al., 2017). IRAK4 has recently attracted attention as a therapeutic target for inflammation and tumor diseases.

IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (anakinra) is fully active in blocking the IL-1R1, and therefore, the biological activities of IL-1α and IL-1β. The binding of IL-1α and IL-1β to IL-1R1 can be suppressed by soluble IL-1R like rilonacept (Kapur and Bonk, 2009). The binding of IL-1β to IL-1R1 can be inhibited by anti-IL-1β antibody (canakinumab and gevokizumab) (Church and McDermott, 2009) (Roell et al., 2010).

Various IRAK4 inhibitors are currently under the investigation on the possibility of clinical use for autoimmune disorders (Chaudhary, Robinson and Romero, 2015).

### *Domain of Applicability*

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

Although sex differences in immune responses are well known (Klein and Flanagan, 2016), there is no reports regarding the sex difference in IL-1 production, IL-1 function or susceptibility to infection as adverse effect of IL-1 blocking agent. Age-dependent difference in IL-1 signaling is not known.

The IL1B gene is conserved in human, chimpanzee, rhesus monkey, dog, cow, mouse, rat, and frog (https://www.ncbi.nlm.nih.gov/homologene/481), and the Myd88 gene is conserved in chimpanzee, rhesus monkey, dog, cow, rat, chicken, zebrafish, mosquito, and frog (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849).

The lower level of stress-induced IL-1β expression is demonstrated in the aged murine keratinocytes (Pilkington et al., 2018).

The IL-1β production by mouse oral mucosal leukocytes stimulated with candida albicans was reduced with aging (Bhaskaran et al., 2020).

The baseline IL-1 signaling of the upper respiratory tract lavage was reduced in murine newborn mice (Kuipers et al., 2018).

## *Key Event Description*

### Decreased IL-1 production

Decreased IL-1 production by macrophages or dendritic cells can be induced by suppressed IL-1β mRNA induction or suppressed maturation of pro-IL-1β. Dexamethasone is one of the representative drugs that significantly suppress IL-1β production from monocytes (Finch-Arietta and Cochran, 1991). Other than dexamethasone, the inhibition of various targets in different layers from the stimulation of PRRs or the receptors of proinflammatory cytokines to the activation of NF-κB or the inhibition of posttranscriptional regulation of pro-IL-1β cause impaired IL-1R1 signaling. Among various PRRs, the signaling through TLR4 is best characterized. In addition, it is beyond the scope of this AOP to cover all signaling through each PRR. So, this AOP focuses on TLR4 signaling.

Lipopolysaccharide (LPS) from the bacteria binds to TLR4 in complex with myeloid differentiation factor-2 (MD2), and this complex initiates signalling by recruiting the adaptor proteins MyD88, TIR domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TIR-domain containing adaptor (TRAM). MYD88 associates with IL-1R-associated kinase 1 (IRAK1) and IRAK4 and recruits TNFR-associated factor 6 (TRAF6). This complex recruits TGF-β-activated kinase 1 (TAK1), leading to phosphorylation of NF-κB inhibitor (IκB), activation of nuclear factor-κB (NF-κB) and consequent transcription of a range of genes coding for pro-inflammatory cytokines, including tumour necrosis factor (TNF), IL-6, pto-IL-1b, and pro-IL-18 (Mills, 2011).

Therefore, chemicals that affect the signaling pathway leading to the activation of these transcription factors are supposed to suppress IL-1β production. Among them, the chemical substances that affect NF-κB signaling have been investigated most thoroughly. Quite a few compounds have been reported to inhibit NF-κB signaling by several different mechanisms reviewed by Fuchs (Fuchs, 2010). In fact, dimethyl fumarate inhibits the activation of NF-κB, resulting in a loss of proinflammatory cytokine production, distorted maturation and function of antigen-presenting cells, and immune deviation of T helper cells (Th) from the type 1 (Th1) and type 17 (Th17) profiles to a type 2 (Th2) phenotype (McGuire et al., 2016; Peng et al., 2012). Several studies have shown intriguing pharmacologic effects associated with curcumin, which inhibits NF-κB expression by regulating NF-κB/IκB pathway and down-regulates expression of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNFα (Wang et al., 2018). Iguratimod, a methanesulfonanilide, that is a novel disease-modifying antirheumatic drug, inhibits NF-κB but not its inhibitor, IκBα, and inhibits the production of IL-1β (Mucke, 2012). Epigalocathechin gallate (EGCG) has been reported to inhibit NF-κB activation through inhibition of p65 phosphorylation (Wheeler et al., 2004) and suppress the production of LPS-stimulated IL-1β (Wang et al., 2020). DHMEQ inhibits lLPS-induced NF-κB activation by inhibiting its nuclear translocation from the cytoplasm. It also inhibits LPS-induced secretion of IL-1β (Suzuki and Umezawa, 2006).

Other than the inhibitors for NF-κB signaling, which can be stimulated by various stimulations other than TLR4 stimulation, there are signaling molecules that are specific to TLR4 signaling, such as TLR4, Mal, TRAM, Myd88, IRAK4, and IRAK1/2 (Vallabhapurapu and Karin, 2009). There are several chemicals that targe some of these molecules, an inhibitors of TLR4 such as TAK-242 (Matsunaga et al., 2011) and various IRAK4 inhibitors (Lee et al., 2017). IRAK4 has recently attracted attention as a therapeutic target for inflammation and tumor diseases.

Beside transcriptional regulation of IL-1β production, minocycline, and two prodrugs, pralnacasan (VX-740) and belnacasan (VX-765) that are orally absorbed and converted into the active principle, VRT-018858 and VRT-043198, respectively (Fenini et al., 2017) suppress IL-1 signaling by the inhibition of caspase-1 activation. Caspase-1 is an essential enzyme for maturation of pro- IL-1β and the secretion of mature IL-1β (Vincent and Mohr, 2007). Recently, it has been reported that cinnamicaldehyde suppresses serum IL-1β level in endotoxin poisoning mice (Xu et al., 2017).

**Blocking of binding of IL-1 to IL-1R1**

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (anakinra) is fully active in blocking the IL-1R1, and therefore, the biological activities of IL-1α and IL-1β. The binding of IL-1α and IL-1β to IL-1R1 can be suppressed by soluble IL-1R like rilonacept (Kapur and Bonk, 2009). The binding of IL-1β to IL-1R1 can be inhibited by anti-IL-1β antibody (canakinumab and gevokizumab) (Church and McDermott, 2009) (Roell et al., 2010).

This AOP focus on the blocking of binding of IL-1 to IL-1R1, and an inhibition or suppression of IL-1 signaling is out of scope, because the molecular initiating event of IL-1 blocking is simple and appropriate for developing AOP. This AOP is expected to be applicable to any chemicals which bind to IL-1R, although such stressor has not been reported.

### *How it is Measured or Detected*

1. Real time polymerase chain reaction to measure IL-1α or IL-1β mRNA
2. Enzyme-linked immunosorbent assay (ELISA) to detect IL-1α or IL-1 b protein
3. Competitive inhibition binding experiments using $^{125}$I-IL-1α to type I IL-1R present on EL4 thymoma cells, 3T3 fibroblasts, hepatocytes, and Chinese hamster ovary cells expressing recombinant mouse type I IL-1R (McIntyre et al., 1991; Shuck et al., 1991).
4. Measure the ability of the reagent to neutralize the bioactivity of human IL-1β on primary human fibroblasts in vitro (Alten et al., 2008).

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## List of Key Events in the AOP

## Event: 202: Inhibition, Nuclear factor kappa B (NF-kB)

**Short Name: Inhibition, Nuclear factor kappa B (NF-kB)**

**Key Event Component**

| Process | Object | Action |
|---|---|---|
| I-kappaB kinase/NF-kappaB signaling | transcription factor NF-kappa-B subunit | decreased |

**AOPs Including This Key Event**

| AOP ID and Name | Event Type |
|---|---|
| Aop:14 - Glucocorticoid Receptor Activation Leading to Increased Disease Susceptibility | Key Event |
| Aop:278 - IKK complex inhibition leading to liver injury | Key Event |
| Aop:277 - Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | Key Event |
| Aop:447 - Kidney failure induced by inhibition of mitochondrial electron transfer chain through apoptosis, inflammation and oxidative stress pathways | Key Event |

**Stressors**

| Name |
|---|
| IL-1 receptor antagonist（IL-1Ra）(Anakinra) |
| anti-IL-1b antibody (Canakinumab) |
| soluble IL-1R (Rilonacept) |

**Biological Context**

| Level of Biological Organization |
|---|
| Molecular |

**Cell term**

| Cell term |
|---|
| T cell |

**Organ term**

| Organ term |
|---|
| immune system |

### *Domain of Applicability*

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

The binding of sex steroids to their respective steroid receptors directly influences NF-κB signaling, resulting in differential production of cytokines and chemokines (McKay and Cidlowski, 1999; Pernis, 2007). 17b-estradiol regulates pro-inflammatory responses that are transcriptionally mediated by NF-κB through a negative feedback and/or transrepressive interaction with NF-κB (Straub, 2007). Progesterone suppresses innate immune responses and NF-κB signal transduction reviewed by Klein et al. (Klein and Flanagan, 2016). Androgen-receptor signaling antagonises transcriptional factors NF-κB(McKay and Cidlowski, 1999).

### *Evidence for perturbation of this molecular initiating event by stressor*

Dex inhibits IL-1β gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-κB/Rel and AP-1 activation (Jeon et al., 2000).

Various inhibitors for NF-κB, such as dimethyl fumarate, curcumin, iguratimod, epigalocathechin gallate (EGCG), and DHMEQ inhibits lLPS-induced NF-κB activation and LPS-induced secretion of IL-1β (McGuire et al., 2016; Mucke, 2012; Peng et al., 2012; Suzuki and Umezawa, 2006; Wang et al., 2020; Wang et al., 2018; Wheeler et al., 2004).

TAK-242 (Matsunaga et al., 2011) inhibit TLR4 itself. There are several IRAK4 inhibitors (Lee et al., 2017). These molecules block the upstream signal to NF-κB activation. IRAK4 has recently attracted attention as a therapeutic target for inflammation and tumor diseases (Chaudhary et al., 2015).

LPS treatment induced a significant upregulation of the mRNA and release of IL-1β from retinal microglia. Minocycline inhibited its releases. Thus, minocycline might exert its antiinflammatory effect on microglia by inhibiting the expression and release of IL-1β (Wang et al., 2005).

Caspase-1 inhibition reduced the release of IL-1β in organotypic slices exposed to LPS+ATP. Administration of pralnacasan (intracerebroventricular, 50 μg) or belnacasan (intraperitoneal, 25–200 mg/kg) to rats blocked seizure-induced production of IL-1β in the hippocampus, and resulted in a twofold delay in seizure onset and 50% reduction in seizure duration (Ravizza et al., 2006).

Belnacasan, an orally active IL-1β converting enzyme/caspase-1 inhibitor, blocked IL-1β secretion with equal potency in LPS-stimulated cells from familial cold urticarial associated syndrome and control subjects (Stack et al., 2005).

In LPS-induced acute lung injury (ALI) mice model, LPS induced inflammatory cytokines such as TNF-α, IL-6, IL-13 and IL-1β were significantly decreased by cinnamaldehyde (CA) (Huang and Wang, 2017).

The suppressing capacities of six cinnamaldehyde-related compounds were evaluated and compared by using the LPS-primed and ATP-activated macrophages. At concentrations of 25~100 mM, cinnamaldehyde and 2-methoxy cinnamaldehyde dose-dependently inhibited IL-1β secretion (Ho et al., 2018).

In vitro, CA decreased the levels of pro-IL-1β and IL-1β in cell culture supernatants, as well as the expression of NLRP3 and IL-1β mRNA in cells. In vivo, CA decreased IL-1β production in serum. Furthermore, CA suppressed LPS-induced NLRP3, p20, Pro-IL-1β, P2X7 receptor (P2X7R) and cathepsin B protein expression in lung, as well as the expression of NLRP3 and IL-1β mRNA (Xu et al., 2017).

IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (anakinra) is fully active in blocking the IL-1R1, and therefore, the biological activities of IL-1α and IL-1β. The binding of IL-1α and IL-1β to IL-1R1 can be suppressed by soluble IL-1R like rilonacept (Kapur and Bonk, 2009). The binding of IL-1β to IL-1R1 can be inhibited by anti-IL-1β antibody (canakinumab and gevokizumab) (Church and McDermott, 2009) (Roell et al., 2010).

IL-1 is known to mediates autoinflammatory syndrome, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever. Blocking of binding of IL-1 to IL-1R1 by anakinra, canakinumab, and rilonacept have been already used to treat these autoinflammatory syndrome associated with overactivation of IL-1 signaling (Quartier, 2011).

Dex inhibits IL-1β gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-κB/Rel and AP-1 activation (Jeon et al., 2000).

Inhibition of IL-1 binding to IL-1R or the decreased production of IL-1b leads to the suppression of IL-1R signaling leading to NF-κB activation.

### *Key Event Description*

The NF-κB pathway consists of a series of events including IRAK (IL-1 receptor-associated kinase) signaling, where the transcription factors of the NF-κB family play the key role. The canonical NF-κB pathway can be activated by a range of stimuli, including TNF receptor activation by TNF-a. Upon pathway activation, the IKK complex will be phosphorylated, which in turn phosphorylates IkBa. This NF-κB inhibitor will be K48-linked ubiquitinated and degradated, allowing NF-κB to translocate to the nucleus. There, this transcription factor can express pro-inflammatory and anti-apoptotic genes. Furthermore, negative feedback genes are also transcribed and include IkBa and A20. When the NF-κB pathway is inhibited, its translocation will be delayed (or absent), resulting in less or no regulation of NF-κB target genes. This can be achieved by IKK inhibitors, proteasome inhibitors, nuclear translocation inhibitors or DNA-binding inhibitors (Gupta et al., 2010; Liu et al., 2017). Therefore, inhibition of IL-1R activation suppresses NF-κB.

In addition to the NF-kB pathway, IRAK activates a variety of transcription factors, including Interferon regulatory factor 5 (IRF5), Adaptor protein-1 (AP-1) and cAMP response element binding protein (CREB), resulting in the expression of broad array of inflammatory molecules and apoptosis-related proteins (Jain, 2014).

### *How it is Measured or Detected*

NF-κB transcriptional activity: Beta lactamase reporter gene assay (Miller et al. 2010)

NF-κB transcription: Lentiviral NF-κBGFP reporter with flow cytometry (Moujalled et al. 2012)

IκBα phosphorylation: Western blotting (Miller et al. 2010)

NF-κB p65 (Total/Phospho) ELISA :

ELISA for IL-6, IL-8, and Cox

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## Event: 1702: Suppression of T cell activation

**Short Name: Suppression of T cell activation**

**Key Event Component**

| Process | Object | Action |
|---|---|---|
| T cell activation involved in immune response | T cell | decreased |

**AOPs Including This Key Event**

| AOP ID and Name | Event Type |
|---|---|
| Aop:277 - Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | Key Event |

**Biological Context**

| Level of Biological Organization |
|---|
| Cellular |

**Cell term**

| Cell term |
|---|
| T cell |

**Organ term**

| Organ term |
|---|
| immune system |

### *Domain of Applicability*

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

Although sex differences in immune responses are well known (Klein and Flanagan, 2016), there is no reports regarding the sex difference in IL-1 production, IL-1 function or

susceptibility to infection as adverse effect of IL-1 blocking agent. Again, age-dependent difference in IL-1 signaling is not known.

The IL1B gene is conserved in human, chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and frog (https://www.ncbi.nlm.nih.gov/homologene/481), and the Myd88 gene is conserved in human, chimpanzee, Rhesus monkey, dog, cow, rat, mouse, chicken, zebrafish, mosquito, and frog (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849).

The NFKB1 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, and frog.

275 organisms have orthologs with human gene NFKB1.

(https://www.ncbi.nlm.nih.gov/gene/4790)

The lower level of stress-induced IL-1b expression is demonstrated in the aged murine keratinocytes (Pilkington et al., 2018).

The IL-1b production by mouse oral mucosal leukocytes stimulated with candida albicans was reduced with aging (Bhaskaran et al., 2020).

The baseline IL-1 signaling of the upper respiratory tract lavage was reduced in murine newborn mice (Kuipers et al., 2018).

### *Key Event Description*

T cells are key orchestrators of the response against pathogens and are also fundamental in maintaining self-tolerance. A number of clinically important conditions have been described in which T-cell functions are altered, as in AIDS or upon immunosuppression after application of various immunosuppressive drugs to treat autoimmune disorders or allogeneic graft rejection. T-cell progenitors differentiate in the thymus into immature T cells that acquire the expression of the T-cell receptor (TCR), which recognizes antigen peptides from pathogens presented along with major histocompatibility complex (MHC). In addition to the TCR, T cells are characterized by expression of the co-receptor molecules CD4 and CD8 on their cell surface. CD4+ T cells, also called T helper (Th) cells, recognize antigen/MHC-II complexes on antigen presenting cells (APCs) and coordinate the activation of other immune cells including B cells, macrophages, etc.

Therefore, CD4+ T cells are crucial for coordination of the immune response and for the elimination of invading pathogens. On the other hand, CD8+ T cells, referred to as T cytotoxic cells, recognize antigen/MHC-I complexes and are responsible for the killing of pathogen-infected cells.

T-cell activation and differentiation depends on antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages and B cells. Depending on the insult affecting a given tissue, . Different subsets of DCs can be generated that in turn are able to coordinate the differentiation of a particular Th subset. To date, the following Th subsets have been described: Th1, Th2, Th9, Th17, Th22, Tfh (follicular helper T cells), Tr1 (type 1regulatory

T cells) and Treg (regulatory T cells), each possessing a specific function in the elimination of pathogens. (reviewed by Simeoni et al. (Simeoni et al., 2016))

Although CD4 T cells are able to commit to Th1, Th2 and Th17 lineages in the absence of IL-1R signaling at steady state, these committed CD4 T cells are unable to effectively secrete their cytokines upon TCR ligation. Namely, IL-1 is indispensable for CD4 T cell effector function. (Lin et al, 2015)

Moreover, since full activation of B cells and antibody production and class switch depends on T cell help. The impaired activation of T cells leads to impaired B cell activation and antibody production (reviewed by Mok (Mok, 2010)).

### *How it is Measured or Detected*

T cell activation can be evaluated by measuring IL-2 production by ELISA or T cell proliferation by incorporation of the analysis of CFSE labeled T cells or [$^{3}$H]thymidine incorporation.

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## List of Adverse Outcomes in this AOP

## Event: 984: Impairment, T-cell dependent antibody response

**Short Name: Impairment, T-cell dependent antibody response**

**Key Event Component**

| Process | Object | Action |
|---|---|---|
| Immunosuppression | | increased |

**AOPs Including This Key Event**

| AOP ID and Name | Event Type |
|---|---|
| Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response | Adverse Outcome |
| Aop:277 - Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | Adverse Outcome |

**Stressors**

| Name |
|---|
| Tacrolimus (also FK506) |
| Cyclosporin |
| 1,2:5,6-dibenzanthracene |
| psychosocial stress |

**Biological Context**

| Level of Biological Organization |
|---|
| Individual |

### *Domain of Applicability*

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |
| Cynomolgus monkey | Macaca fascicularis | High | NCBI |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

CNIs induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). Moreover, Treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum SRBC-specific IgM and IgG (Kevin, G. et al. 2014). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell–dependent antigens (Heidt et al, 2009). Also, in SKW6.4 cells (IL-6–dependent, IgM-secreting, human B-cell line) cultures, FK506 or CsA suppressed the production of IgM antibodies in the presence of T-cell activation (Sakuma et al. 2001b). Considering that FKF506 and CsA reduce T cell-derived cytokines including IL-2 and IL-4, these findings strongly suggest that impairment of TDAR following reduced production of such cytokines occurs at least in common among humans monkey and rodents.

### *Key Event Description*

Antibody production to T-cell–dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T-cell–derived cytokines, which stimulate B cells to proliferate and differentiate. T-cell–dependent antibody response (TDAR) might be altered if any of these cell populations is affected.

Interleukin (IL)-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell–related cytokines appears to impair TDAR, as seen in the result of FK506 treatment (Heidt et al, 2009).

IL-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (Alberts et al. 2008).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) decrease in anti- keyhole limpet hemocyanine (KLH) immunoglobulin (Ig)G. (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene (DBA) exposure reduced total IgG antibody in spleen cell culture supernatants after in vitro stimulation with lipopolysaccharide (LPS) (Donna, C. et al. 2010).

Treatment with cyclosporin A (CsA) at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum sheep red blood cells (SRBC)-specific IgM and IgG (Kevin, G. et al. 2014).

After a 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41.6 and 83.2 nM) of CsA (Heidt et al, 2009).

After a 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.012 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.083 to 83.2 nM) of CsA (Sakuma et al. 2001b).

Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG was reduced at the dose level of 3 mg/kg/day (Ulrich et al. 2004).

Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

As immunosuppression-derived adverse outcomes by calcineurin inhibition, FK506 and CsA increase the frequency and/or severity of infections and allergic reactions impaired TDAR deems to be one of the causative factors for these side effects. Some clinical trials of FK506 and CsA revealed these adverse effects as follows.

- In clinical trials of renal transplantation using FK506 or CsA, opportunistic infections such as candida, cytomegalovirus and herpes simplex virus were reported (Ekberg et al. 2007).
- In recipients of liver transplants treated with FK506 or CsA, opportunistic infections such as cytomegalovirus, hepatitis C virus, hepatitis B and herpes simplex virus were reported (Fung et al. 1991).
- Cardiac transplant patients treated with cyclosporin developed pulmonary infections within the first year after surgery (Luster, M.I. et al. 1993).
- In patients of X-linked autoimmune enteropathy treated with CsA or FK506, serum levels of IgE developed extremely high during the immunosuppressive therapy (Kawamura et al. 1997).
- Renal transplant recipients treated with belatacepy/mycophenolate (MMF)/predonisone or FK506/MMF/prednisone showed significantly lower the geometric mean hemagglutination inhibition titer against influenza vaccine, hemagglutination-specific IgG and isotype IgG1 antibodies, and IgG-antibody secreting cells response (Gangappa et al. 2019).

### *How it is Measured or Detected*

TDAR could be examined in vivo and in vitro.

In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) or with a plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T-cell–dependent, antigen-specific, IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).
- Cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBC, after which the serum was examined for Anti-

SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Kevin, G. et al. 2014).

- Mice were exposed a single pharyngeal aspiration of DBA, after which supernatants of splenocytes cultured for 24 h in the presence of LPS and assayed using a mouse IgM or IgG matched pairs antibody kit (Bethyl Laboratories, Montgomery, TX) (Donna, C. et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation rather than measuring antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human peripheral blood mononuclear cells (PBMC) were co-cultured with a CNIs for nine days in the presence of polyclonal–T-cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. Treatment with FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
- SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNIs, and cytokine mRNA levels of Interferon-gamma, IL-2, IL-4, IL-5, IL-10, IL-13, and other B-cell–stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

### *Regulatory Significance of the AO*

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

It was difficult to define susceptibility to infection as an AO, so TDAR, which is recommended as an indicator of immunosuppression in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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# Appendix 2 - Key Event Relationships

## List of Key Event Relationships in the AOP

## List of Adjacent Key Event Relationships

## Relationship: 2002: Impaired IL-1R1 signaling leads to Inhibition, Nuclear factor kappa B (NF-kB)

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | adjacent | High | Moderate |

### *Evidence Supporting Applicability of this Relationship*

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

### *Key Event Relationship Description*

After binding of IL-1α or IL-1β to IL-1R, IL-1 and IL-1R1 facilitates recruitment of IL-1RacP. Then this trimeric complex rapidly assembles two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor–activated protein kinase (IRAK) 4. IL-1, IL-1RI, IL-RAcP, MYD88, and IRAK4 form a stable IL-1–induced first signaling module. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB.

### *Evidence Supporting this KER*

Mice lacking MYD88 or IRAK4 show severe defects in IL-1 signaling (Adachi et al., 1998; Suzuki et al., 2002). In the cell culture, lacking MYD88 show a block of NF-κB activation by IL-1 (Medzhitov et al., 1998). MyD88 can strongly activate an AP-1 and this activity is

inhibited by dominant-negative TRAF6; therefore, MyD88 and TRAF6 are involved in IL-1R-mediated NF-κB activation, and both activate AP-1 (Medzhitov et al., 1998). Similarly, humans with mutations in the IRAK4 gene have defects in IL-1RI and Toll-like receptor (TLR) signaling (Picard et al., 2003).

**Biological Plausibility**

The initial step in IL-1 signal transduction is a ligand-induced conformational change in the first extracellular domain of the IL-1RI that facilitates recruitment of IL-1RacP (Cavalli et al., 2015). Through conserved cytosolic regions called Toll- and IL-1R–like (TIR) domains (Radons et al., 2003), the trimeric complex rapidly assembles two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor–activated protein kinase (IRAK) 4 (Brikos et al., 2007; Li et al., 2002). IL-1, IL-1RI, IL-RAcP, MYD88, and IRAK4 form a stable IL-1–induced first signaling module. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB reviewed by (Brikos et al., 2007; Weber, Wasiliew and Kracht, 2010).

**Empirical Evidence**

**IL-1Ra blocks IL-1 signaling:**

IL-1Ra down modulation of EGF receptor (3 nM of ED50) (Dripps et al., 1991)

IL-1Ra suppression of IL-1-induced endothelial cell-leukocyte adhesion (approximately 10 ng/ml of ED50) (Dripps et al., 1991)

IL-1Ra suppresses rhIL-1a-induced mouse thymocytes proliferation (ED50 almost 3 mg/mL) (Arend et al., 1990)

IL-1Ra competed for binding of $^{125}$I-IL-1a to type I IL-1R present on EL4 thymoma cells, 3T3 fibroblasts, hepatocytes, and Chinese hamster ovary cells expressing recombinant mouse type I IL-1R. The IC50 values for IL-1ra binding (ranging from 2 to 4 ng/ml) were similar to those of IL-1a. (McIntyre et al., 1991)

Recombinant mIL-1Ra competitively inhibited $^{125}$I-labeled IL-1 alpha binding to murine type I IL-1R present on EL4 6.1 cells (Ki value of 0.21 nM) and antagonized IL-1-stimulated co-mitogenesis in murine thymocytes (0.7 x 10(6)-1.1 x 10(6) units/mg). (Shuck et al., 1991)

Peripheral blood mononuclear cells (PBMC) obtained after completion of the IL-lra infusion synthesized significantly less interleukin 6 ex vivo than PBMC from saline-injected controls. (Granowitz et al., 1992)

**Canakinumab (ACZ885, Ilaris):**

Canakinumab binds to human IL-1β with high affinity; the antibody-antigen dissociation equilibrium constant is approximately 35–40 pM(Dhimolea, 2010).

The antibody binds to human IL-1β with high affinity (about 40 pmol/l). The antibody was found to neutralize the bioactivity of human IL-1β on primary human fibroblasts in vitro 44.6 pmol/l (7.1 ± 0.56 ng/ml; n = 6) of ED50. Application of Canakinumab

intraperitoneally 2 hours before injecting the IL-1β producing cells completely suppressed joint swelling in mouse models of arthritis (0.06 mg/kg of EC50) (Alten et al., 2008).

Primary human fibroblasts are stimulated with recombinant IL-1b or conditioned medium obtained from LPS-stimulated human PBMCs in the presence of various concentrations of Canakinumab or IL-1RA ranging from 6 to 18,000 pM. Supernatant is taken after 16 h stimulation and assayed for IL-6 by ELISA. Canakinumab typically have 1 nM or less of EC50 for inhibition of IL-6 production (Canakinumab Patent Application WO02/16436.)

**Rilonacept (IL-1 Trap, Arcalyst):**

Incubation of the human MRC5 fibroblastic cell line with IL-1β induces secretion of IL-6. At a constant amount of IL-1β (4 pM), the IC50 of the IL-1 trap is ∼2 pM. Another unique property of the IL-1 trap is that it not only blocks IL-1β, but also blocks IL-1α with high affinity (KD = ∼3 pM; data not shown). The titration curve of IL-1 trap in the presence of 10 pM IL-1β shows an IC50 of 6.5 pM, which corresponds to a calculated KD of 1.5 pM (This affinity is 100 times higher than that of the soluble single component receptor IL-1RI (Economides et al., 2003).

**IRAK4 inhibitor:**

By reconstituting IRAK-4-deficient cells with wild type or kinase-inactive IRAK-4, it is demonstrated that the kinase activity of IRAK-4 is required for the optimal transduction of IL-1-induced signals, including the activation of IRAK-1, NF-κB, and JNK, and the maximal induction of inflammatory cytokines (Lye et al., 2008).

Various concentrations of kinase-active or kinase-inactive IRAK-4 were transiently overexpressed in IRAK-4-deficient cells that were also transiently transfected with an NF-κB-dependent luciferase reporter and α-galactosidase expression vector. IRAK-4 is recruited to the IL-1R-associated complex 1 min after IL-1β treatment (10 ng/mL). Transfected cells were left untreated or treated with IL-1β (10 ng/ml) for 6 h before luciferase and α-galactosidase activities were measured. The luciferase activity was divided by the α-galactosidase activity, and fold activation was calculated compared with the activity of untreated cells carrying an empty α-vector (normalized as 1). The results demonstrated that kinase-active IRAK-4 dose dependently activates IL-1-mediated NF-κB. Kinase-inactive IRAK-4 expression resulted in severely reduced IL-1 responses and defective NF-κB and JNK activation induced by IL-1 (Lye et al., 2004).

### *Quantitative Understanding of the Linkage*

See Empirical Evidence.

**Response-response relationship**
**IL-1Ra blocks IL-1 signaling:**

Suppression of IL-1-induced IL-1, TNFa, or IL-6 synthesis was dose-dependent ($P \leqq .0001$). At a twofold molar excess, IL-lra inhibited IL-1-induced IL-1 or TNFa synthesis by 50% ($P < .01$); an equimolar concentration of IL-lra inhibited synthesis of these two cytokines by over 20% ($P < .05$). A 10-fold molar excess of IL-lra over IL-lb reduced IL-

lb-induced IL-la by 95% (P = .01) and IL-la-induced IL-1b by 73% (P < .01). In elutriated monocytes, a 10-fold molar excess of IL-lra reduced IL-lb-induced IL-la by 82% (P < .05), TNFa by 64% (P = .05), and IL-6 by 47% (P < .05). (Granowitz et al., 1992)

**Rilonacept (IL-1 Trap, Arcalyst) blocks IL-1 signaling:**

The titration curve of IL-1 trap in the presence of 10 pM IL-1β shows an IC50 of 6.5 pM, which corresponds to a calculated KD of 1.5 pM (This affinity is 100 times higher than that of the soluble single component receptor IL-1RI (Economides et al., 2003).

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## Relationship: 2003: Inhibition, Nuclear factor kappa B (NF-kB) leads to Suppression of T cell activation

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | adjacent | High | Moderate |

### *Evidence Supporting Applicability of this Relationship*

#### Taxonomic Applicability

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

#### Life Stage Applicability

| Life Stage | Evidence |
|---|---|
| All life stages | High |

#### Sex Applicability

| Sex | Evidence |
|---|---|
| Unspecific | High |

### *Key Event Relationship Description*

NF-kB plays a crucial role in the activation of dendritic cells as well as T cells. In dendritic cells, the activation of the canonical NF-kB pathway in response to pro-inflammatory stimuli, such as cytokines including IL-1a or IL-1b and TLR ligands, stimulate the maturation of dendritic cells with enhanced antigen presenting function. The inhibition of NF-kB suppress antigen presenting function of dendritic cells, resulting in suppression of T cell activation (reviewed by Reinhard et al (Reinhard et al., 2012) and van Delft et al (van Delft, Huitema and Tas, 2015).

In T cells, NF-kB can be activated by several pathways of signal transduction. The engagement of the TCR by major histocompatibility complex (MHC) plus antigen initiates downstream CD3 immunotyrosine activation motif (ITAM) phosphorylation by the Src family kinases, FYN and leukocyte C-terminal src kinase (LCK). Phosphorylated CD3 activates the T cell specific tyrosine kinase, zeta-chain associated protein kinase (ZAP-70), which ultimately trigger calcium release and protein kinase (PK)C activation, respectively. Activation of a specific PKC isoform, PKCμ, connects the above described TCR proximal signaling events to distal events that ultimately lead to NF-kB activation. Importantly, PKCm activation is also driven by engagement of the T cell co-stimulatory receptor CD28 by B7 ligands on antigen presenting cells (APCs). In addition, the stimulation of T cells by IL-1 activates NF-kB as already described before. Once in the nucleus, NF-kB governs the transcription of numerous genes involved in T cell survival, proliferation, and effector functions (Paul and Schaefer, 2013).

### *Evidence Supporting this KER*

Mice lacking NF-kB p50 are unable to effectively clear L. monocytogenes and are more susceptible to infection with S. peumoniae (Sha et al., 1995).

**Biological Plausibility**

Although CD4 T cells are able to commit to Th1, Th2 and Th17 lineages in the absence of IL-1R signaling at steady state, these committed CD4 T cells are unable to effectively secrete their cytokines upon TCR ligation. Namely, IL-1 is indispensable for CD4 T cell effector function. (Lin et al, 2015)

RelB deficient mice had an impaired cellular immunity, as observed in contact sensitivity reaction (Weih et al., 1995).

Delayed-type hypersensitivity (DTH) responses were significantly suppressed in IL-1b-deficient and IL-1a/b-deficient mice. Lymph node cells derived from antigen-sensitized IL-1b-deficient and IL-1a/b-deficient mice and IL-1R type I-deficient mice, exhibited reduced proliferative responses against antigen. Antigen-specific CD4+ T cell proliferative responses were significantly reduced following co-culture with IL-1RI−/− dendritic cells (DCs) (Nambu et al., 2006).

**Empirical Evidence**

RelB deficient mice had an impaired cellular immunity, as observed in contact sensitivity reaction (Weih et al., 1995).

Quite a few NF-kB inhibitors have been reported. MG132, bortezomib, curcumin, DHMEQ(Dehydroxymethylepoxyquinomicin), naringin, sorafenib, genistein and parthenolide are some of representatives (Pordanjani and Hosseinimehr, 2016).

Interferon-γ (IFN-γ) production in response to CMV-infected fibroblasts was reduced under the influence of MG132, a proteosome inhibitor as well as a NF-kB inhibitor, in a dose-dependent manner. A marked reduction was observed at 0.5 μM. Likewise, CMV-specific cytotoxicity of CD8(+) T cells was decreased in the presence of MG132 (Wang et al., 2011).

In vivo MG132 administration to NC/Nga mice with DNFB-induced dermatitis reduced Th17 cells but maintained the level of Th1 cells, resulting in the alleviation of dermatitis lesions by decreasing both serum IgE hyperproduction and mast cell migration (Ohkusu-Tsukada et al., 2018).

Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro (Satou et al., 2004). Bortezomib inhibits T-cell function versus infective antigenic stimuli in a dose-dependent manner in vitro (Orciuolo et al., 2007).

Dehydroxymethylepoxyquinomicin (DHMEQ), a novel nuclear factor-kappaB inhibitor, induces selective depletion of alloreactive or phytohaemagglutinin-stimulated peripheral blood mononuclear cells, decreases production of T helper type 1 cytokines, and blocks maturation of dendritic cells (Nishioka et al., 2008).

Regarding the suppression of NF-kB by impaired IL-1 signaling, it was reported that delayed-type hypersensitivity (DTH) responses were significantly suppressed in IL-1β-deficient and IL-1α/β-deficient mice. Lymph node cells derived from antigen-sensitized IL-1β-deficient and IL-1α/β-deficient mice and IL-1R type I-deficient mice, exhibited reduced proliferative responses against antigen. These data suggest that IL-1β is necessary for the efficient priming of T cells. In addition, CD4+ T cell-derived IL-1 plays an important role in the activation of DCs during the elicitation phase, resulting in the production of TNF, that activate allergen-specific T cells (Nambu et al., 2006).

### *Quantitative Understanding of the Linkage*

A representative NF-kB inhibitor, MG132 that suppresses NF-kB activity at more than 10 mM (Fiedler et al. 1998) suppresses IL-2-induced activation of STAT5 at 50 mM. (Yu and Malek., 2001). However, MG-132 did not decrease the effect of TNF-α on AP-1 activation (Fiedler, Wernke-Dollries and Stark, 1998).

A representative NF-kB inhibitor, DHMEQ (1μg/mL) blocked phytohaemagglutinin (PHA-)-induced nuclear translocation of NF-kB in Jurkat cells via inhibition of degradation of IkBa. Preincubation of peripheral blood mononuclear cells and Jurkat cells with DHMEQ (1 μg/ml, 3 hr) greatly reduced PHA-stimulated expression of IFN-γ, IL-2 and TNF-α genes although DHMEQ alone without PHA-stimulation did not affect cytokine production in unstimulated PBMC. DHMEQ (0·5–3 μg/mL, 3 days) inhibited PHA-stimulated proliferation of peripheral blood mononuclear cells (PBMC) in a dose-dependent manner although did not affect the viability of resting PBMC under identical culture conditions. DHMEQ (3 μg/mL, 24 hr) induced apoptosis of PHA-stimulated PBMC. DHMEQ (0·5 μg/mL) decreased levels of TNF-α-stimulated expression of CD40 in monocyte-derived dendritic cells (DCs). Exposure of DCs to DHMEQ (0·5 or 1 μg/ml) reduced their endocytic ability (Nishioka et al., 2008).

**Response-response relationship**

Interferon-γ (IFN-γ) production in response to CMV-infected fibroblasts was reduced under the influence of MG132 in a dose-dependent manner. A marked reduction was observed at 0.5 μM. Likewise, CMV-specific cytotoxicity of CD8(+) T cells was decreased in the presence of MG132 (Wang et al., 2011).

Bortezomib (1 mg/kg) inhibits T-cell function versus infective antigenic stimuli in vitro (Orciuolo et al., 2007).

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## Relationship: 2928: Suppression of T cell activation leads to Impairment, T-cell dependent antibody response

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response | adjacent | High | High |

### *Evidence Supporting Applicability of this Relationship*

#### Taxonomic Applicability

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| Homo sapiens | Homo sapiens | High | NCBI |
| Mus musculus | Mus musculus | High | NCBI |
| Rattus norvegicus | Rattus norvegicus | High | NCBI |

#### Life Stage Applicability

| Life Stage | Evidence |
|---|---|
| All life stages | High |

#### Sex Applicability

| Sex | Evidence |
|---|---|
| Unspecific | High |

### *Key Event Relationship Description*

Normal T cell and B cell function is indispensable for host defense mechanism. T cells are activated when they recognize antigens and induce T-cell dependent antibody response (TDAR) by secreting various cytokines as described below (Abbas et al. 2020). Therefore, suppression of T cell activation leads to impairment of TDAR. Various Interleukins (ILs) such as IL-2 and IL-4 are produced and secreted by activated helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, IL-2 promotes differentiation of B cells through IL-2 receptors and stimulates the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs T cell dependent antibody production (Alberts et al. 2008).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR. Activated T cell-derived cytokines play important roles in the development of TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production.

Thus, suppressing the production of IL-2, IL-4, and other cytokines in T cells reduces stimulation of B cells including proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, suppressing the production of these B-cell-related

cytokines appears to be the main factor in impairment of TDAR by inhibitors of T-cell–dependent-antibody production.

### *Evidence Supporting this KER*

In cynomolgus monkeys, the effects of CsA on production of IL-2 and IL-4, and antigen-specific IgM and IgG in TDAR were demonstrated (Gaida K. 2015).

Suppressed IgE and antigen specific IgG1 productions by the blocking of IL-4 receptor were reported in mice using dupilumab (antiIL-4/13R antibody) (Sanofi K.K. 2018).

Suppressed antigen specific IgE production by the inhibition of IL-4 production was reported in mice using suplatast tosilate (Taiho Pharmaceutical 2013). Suppressed antigen specific IgE and IL-4 productions by the inhibition of IL-4 production were reported in human cell culture using suplatast tosilate(Taiho Pharmaceutical 2013).

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004).

The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001). The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans, monkeys and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

**Biological Plausibility**

Cyclosporin A (CsA) is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015).

It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig)E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These

findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). They suggest that the blocking of IL-2 signaling by anti-IL-2R antibody results in decreased rejection through the inhibition of the activation of antigen specific T cell with reduced antibody production.

FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cells (PBMC) treated with FK506 and CsA suppressed the production of IgM and IgG antibodies to T-cell dependent antigens (Heidt et al, 2009).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR. However, as for the suppression of humoral immunity induced by the inhibition of calcineurin (CN) phosphatase activity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2010).

Therefore, it is concluded that decreased amounts of IL-2 and IL-4 secreted from helper T cells is the main factor for suppression of TDAR induced by CN phosphatase inhibition.

**Empirical Evidence**

Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale

- Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).
- In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018).
- In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Yanagihara, 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced

productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Yanagihara, 2013).

- In the clinical study of renal transplantation, basiliximab decreased incidence of acute rejection at 20 mg/kg (Kircher, 2003). In human T cell culture immunized with PPD, basiliximab reduced activation of antigen specific T cell at the concentration of 300 ng/mL (Kircher, 2003).
- In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001).
- Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigenspecific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).
- 1,2:5,6-dibenzanthracene single administration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG (Alessandro, B. et al. 2003).

**Uncertainties and Inconsistencies**

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR; however, there remains some possibility of additional suppression of other immune functions.

### *Quantitative Understanding of the Linkage*

Luster et al (1993) demonstrated that Concanavalin A response of splenocytes showed the linear dose-response relationship with the host resistance to Listeria monocytogenes or Streptococcus pneumoniae.

**Response-response relationship**

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In the blocking of IL-4 receptor in mice by dupilumab (anti-IL-4/13R antibody) at 25 mg/kg of twice weekly subcutaneous administration for 4weeks, IgE production was suppressed to about 1/100 and antigen specific IgG1 production was suppressed to about 1/200 (Sanofi K.K. 2018).

In the inhibition of IL-4 production in mice by suplatast tosilate at 10, 20, 50 and 100 mg/kg of oral administration for 5 days, antigen specific IgE production was suppressed from about 1/10 to 1/100 (Taiho Pharmaceutical 2013). In human T cell culture by suplatast tosilate at the concentration of 10 μg/mL, antigen specific IgE production after 10 days was suppressed from 56 to 72% and IL-4 production after 3 days was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

s for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. That is, murine small resting B cells, cultured with irradiated hapten-specific TH1 clone, were induced to enter cell cycle at 2 days and to secret antibody at 5 days. An anti-IL-2 and anti-IL-2R antibodies completely inhibited this T-cell dependent antibody production (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the m-RNA levels of T-cell cytokines at 8h post-stimulation including IL-2 and IL-4 at 1.0ng/mL (1.24nM) FK506 or 100ng/mL (90.7nM) CsA and

inhibited IgM and IgG productions after 9 days at 0.3 and 1.0ng/mL FK506 and 50 and 100ng/mL CsA (Heidt S. 2010).

**Time-scale**

In CsA-treatment for 24 days at 50 mg/kg BID, cynomolgus monkeys showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In human T cell culture, suplatast tosilate inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (Taiho Pharmaceutical 2013).

In the human T-B cell co-culture, CNIs of FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8h post-stimulation and inhibited IgM and IgG productions after 9 days (Heidt S. 2010).

**Known Feedforward/Feedback loops influencing this KER**

At present, no evidence is found.

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Section 4
Health effects

# Test Guideline No. 442C

## *In Chemico* Skin Sensitisation

*Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins*

4 July 2023

OECD Guidelines for the Testing of Chemicals

# *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).

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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

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

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

(3) United Nations (UN) (2021), Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fifth revised edition, UN New York and Geneva, 2021. Available at: https://unece.org/sites/default/files/2021-09/GHS_Rev9E_0.pdf

(4) M Fujita, Yamamoto Y, Tahara H, Kasahara T, Jimbo Y and 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.

(5) Y Yamamoto, Tahara H, Usami R, Kasahara T, Jimbo Y, Hioki T and Fujita M (2015), A novel *in chemico* method to detect skin sensitisers in highly diluted reaction conditions, Journal of Applied Toxicology, 35(11):1348-60, (doi: 10.1002/jat.3139).

(6) M Fujita, Yamamoto Y, Watanabe S, Sugawara T, Wakabayashi K, Tahara K, Horie N, Fujimoto K, Kusakari K, Kurokawa Y, Kawakami T, Kojima K, Kojima H, Ono A, Katsuoka Y, Tanabe H, Yokoyama H and Kasahara T (2019), Cause of and Countermeasures for Oxidation of the Cysteine-Derived Reagent Used in the Amino acid Derivative Reactivity Assay, J. Appl. Toxicology, Feb;39(2):191-208 (doi: 10.1002/jat.3707).

(7) FDA (Food and Drug Administration) (2018), Guidance for Industry: Bioanalytical Method Validation 41 pp. Accessible at: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

# 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 $90\% < \text{mean}^2$ 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<br>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<br>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<br>STD2<br>STD3<br>STD4<br>STD5<br>STD6<br>Dilution buffer<br>Reference control A, rep 1<br>Reference control A, rep 2<br>Reference control A, rep 3 |
| **Co-elution controls** | Co-elution control 1 for test chemical 1<br>Co-elution control 2 for test chemical 2 |
| **Reference controls** | Reference control B, rep 1<br>Reference control B, rep 2<br>Reference control B, rep 3 |
| **First set of replicates** | Reference control C, rep 1<br>Cinnamic aldehyde, rep 1<br>Sample 1, rep 1<br>Sample 2, rep 1 |
| **Second set of replicates** | Reference control C, rep 2<br>Cinnamic aldehyde, rep 2<br>Sample 1, rep 2<br>Sample 2, rep 2 |
| **Third set of replicates** | Reference control C, rep 3<br>Cinnamic aldehyde, rep 3<br>Sample 1, rep 3<br>Sample 2, rep 3 |
| **Reference controls** | Reference control B, rep 4<br>Reference control B, rep 5<br>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*

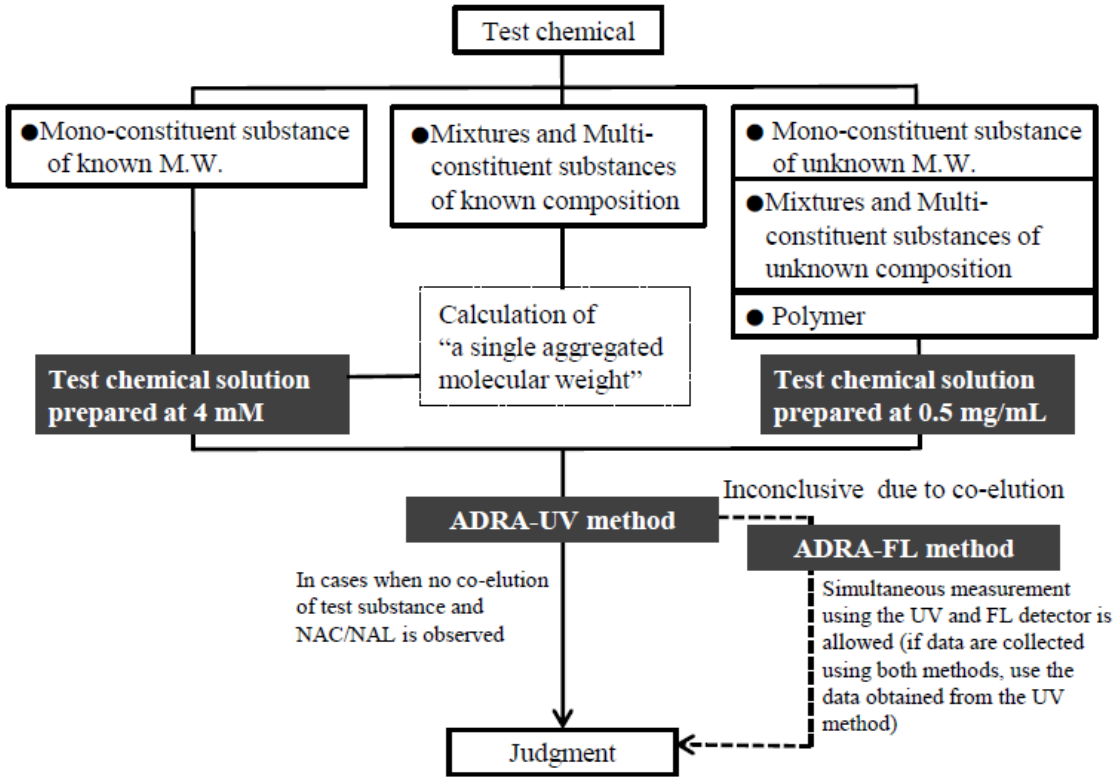
18. Both the NAC and the NAL stock solutions are incubated with the test chemical stock solution in a 3:1 ratio in a 96-well microplate. For the 4 mM test chemical stock solution this gives a final concentration of 1 mM test chemical and 5 µM NAC/NAL (17). For the 0.5 mg/ml test chemical stock solution, 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<br><br>Chemicals that become sensitisers after abiotic transformation are reported to be in some cases correctly detected by the test method | 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 | | | 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<br><br>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<br>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)<br>Therefore, ADRA may prevent erroneous judgement due to the oxidizing action of the test chemical.<br>However, since the NAC dimer does not have fluorescence, it can only be detected by ADRA-UV. | DMSO<br>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<br>STD2<br>STD3<br>STD4<br>STD5<br>STD6<br>Dilution buffer<br>Reference control A, rep 1<br>Reference control A, rep 2<br>Reference control A, rep 3 |
| Co-elution controls | Co-elution control 1 for test chemical 1<br>Co-elution control 2 for test chemical 2 |
| Reference controls | Reference control B, rep 1<br>Reference control B, rep 2<br>Reference control B, rep 3 |
| First set of replicates | Reference control C, rep 1<br>Positive control, rep 1<br>Sample 1, rep 1<br>Sample 2, rep 1 |
| Second set of replicates | Reference control C, rep 2<br>Positive control, rep 2<br>Sample 1, rep 2<br>Sample 2, rep 2 |
| Third set of replicates | Reference control C, rep 3<br>Positive control, rep 3<br>Sample 1, rep 3<br>Sample 2, rep 3 |

| | |
|---|---|
| Reference controls | Reference control B, rep 4<br>Reference control B, rep 5<br>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*

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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 |

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# 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.

Section 4
Health effects

# Test Guideline No. 442E
## *In Vitro* Skin Sensitisation

*In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation*

4 July 2023

OECD Guidelines for the Testing of Chemicals

# *OECD KEY EVENT BASED GUIDELINE FOR THE TESTING OF CHEMICALS*

## ***In Vitro* Skin Sensitisation Assays Addressing the Adverse Outcome Pathway Key Event on Activation of Dendritic Cells**

# 1. GENERAL INTRODUCTION

## Activation of dendritic cells Key Event based Test Guideline

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

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

The test methods described in this TG are:

- Human Cell Line Activation test (h-CLAT)
- U937 cell line activation Test (U-SENS™)
- Interleukin-8 Reporter Gene Assay (IL-8 Luc assay)
- Genomic Allergen Rapid Detection (GARD™) for assessment of skin sensitisers (GARD™skin)

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

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

4. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman, and the Buehler Test (TG 406) (4), assess both the induction and elicitation phases of skin

sensitisation. The murine tests, the LLNA (TG 429) (3) and its two non-radioactive modifications, LLNA: DA (TG 442 A) (5) and LLNA: BrdU-ELISA (TG 442 B) (6), all assess the induction response exclusively, and have also gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

5. Mechanistically-based *in chemico* and *in vitro* test methods addressing the first key event (OECD TG 442C (7)), and second key event (OECD TG 442D (8)) of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals.

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

7. However, as DC activation represents only one key event of the skin sensitisation AOP (2) (13), information generated with test methods measuring markers of DC activation alone may not be sufficient 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 support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers when used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information, e.g. derived from in vitro assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including *in silico* modelling and read-across from chemical analogues (13). Examples of the use of data generated with these methods within Defined Approaches, i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to the data to derive predictions, have been published (13) and are implemented in an OECD TG on defined approaches for skin sensitisation (14).

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

9. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the test methods to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. Limited information is currently available on the applicability of the test methods to multi-constituent substances/mixtures (15) (16). The test methods are nevertheless technically applicable to the testing of multi-constituent substances and mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses.

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# ANNEX 1: IN VITRO SKIN SENSITISATION: HUMAN CELL LINE ACTIVATION TEST (H-CLAT)

## INITIAL CONSIDERATIONS AND LIMITATIONS

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

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

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

4. On the basis of the data currently available, the h-CLAT method was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physicochemical properties (3) (14) (15). The h-CLAT

method is applicable to test chemicals soluble or that form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent/vehicle into different phases) in an appropriate solvent/vehicle (see paragraph 14). Test chemicals with a Log Kow greater than 3.5 tend to produce false negative results (14). Therefore negative results with test chemicals with a Log Kow greater than 3.5 should not be considered. However, positive results obtained with test chemicals with a Log Kow greater than 3.5 could still be used to support the identification of the test chemical as a skin sensitiser. Furthermore, because of the limited metabolic capability of the cell line used (16) and because of the experimental conditions, pro-haptens (i.e. substances requiring enzymatic activation for example via P450 enzymes) and pre-haptens (i.e. substances activated by oxidation) in particular with a slow oxidation rate may also provide negative results in the h-CLAT (15). Fluorescent test chemicals can be assessed with the h-CLAT (17), nevertheless, strong fluorescent test chemicals emitting at the same wavelength as fluorescein isothiocyanate (FITC) or as propidium iodide (PI), will interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies or PI. In such a case, other fluorochrome-tagged antibodies or other cytotoxicity markers, respectively, can be used as long as it can be shown they provide similar results as the FITC-tagged antibodies (see paragraph 24) or PI (see paragraph 18) e.g. by testing the proficiency substances in Appendix II. In the light of the above, negative results should be interpreted in the context of the stated limitations and together with other information sources within the framework of IATA. In cases where there is evidence demonstrating the non-applicability of the h-CLAT method to other specific categories of test chemicals, it should not be used for those specific categories.

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

6. Definitions are provided in Appendix I.

## PRINCIPLE OF THE TEST

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

## DEMONSTRATION OF PROFICIENCY

8. Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Appendix II. Moreover, test method users should maintain an historical database of data generated with the reactivity

checks (see paragraph 11) and with the positive and solvent/vehicle controls (see paragraphs 20-22), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

## PROCEDURE

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

### *Preparation of cells*

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

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

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

### *Dose finding assay*

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

*Preparation of test chemicals and control substances*

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

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

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

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

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

*Application of test chemicals and control substances*

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

*Propidium iodide (PI) staining*

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

alternative stains can be shown to provide similar results as PI, for example by testing the proficiency substances in Appendix II.

*Cytotoxicity measurement by flow cytometry and estimation of CV75 value*

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

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

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

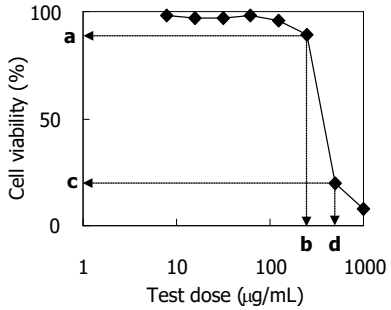
$$\textbf{Log CV75} = \frac{(75 - c) \times \text{Log}(b) - (75 - a) \times \text{Log}(d)}{a - c}$$

Where:

a is the minimum value of cell viability over 75%

c is the maximum value of cell viability below 75%

b and d are the concentrations showing the value of cell viability a and c respectively




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

### *CD86/CD54 expression measurement*

#### *Preparation of the test chemicals and control substances*

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

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

#### *Application of test chemicals and control substances*

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

*Cell staining and analysis*

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

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

## DATA AND REPORTING

### *Data evaluation*

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

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

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

### *Prediction model*

26. For *CD86/CD54 expression measurement*, each test chemical is tested in at least two independent runs to derive a single prediction (POSITIVE or NEGATIVE). An h-CLAT prediction is considered

POSITIVE if at least one of the following conditions is met in 2 of 2 or in at least 2 of 3 independent runs, otherwise the h-CLAT prediction is considered NEGATIVE (Figure 1):

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

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

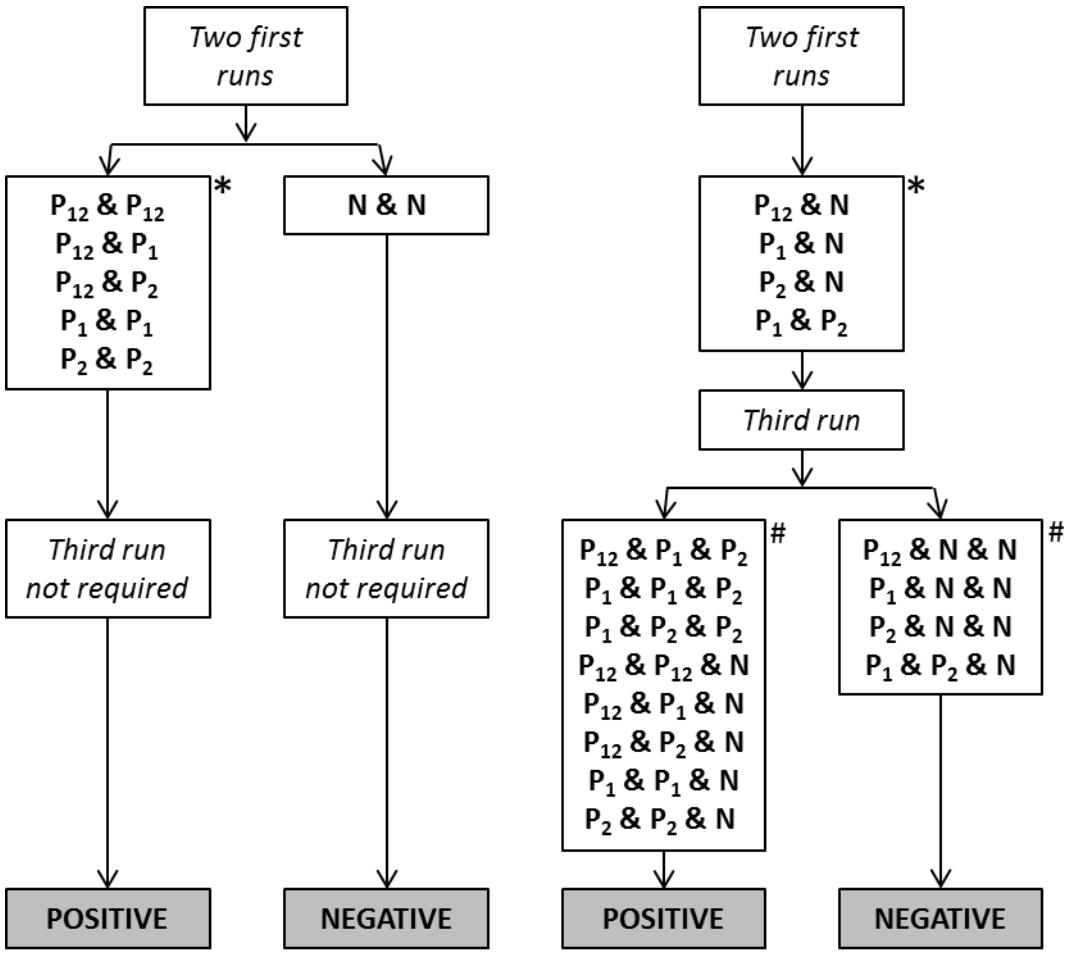



**Figure 1: Prediction model used in the h-CLAT test method.**

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

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

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

where

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

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

$A_{RFI}$ is the RFI at the lowest concentration with RFI > 150 (CD86) or 200 (CD54)

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

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

### *Acceptance criteria*

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

- The cell viabilities of medium and solvent/vehicle controls should be higher than 90%.
- In the solvent/vehicle control, RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI ≥ 150% and CD54 RFI ≥ 200%). RFI values of the solvent/vehicle control are calculated by using the formula described in paragraph 25 ("MFI of chemical" should be replaced with "MFI of solvent/vehicle", and "MFI of solvent/vehicle" should be replaced with "MFI of (medium) control").
- For both medium and solvent/vehicle controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%.
- In the positive control (DNCB), RFI values of both CD86 and CD54 should meet the positive criteria (CD86 RFI ≥ 150 and CD54 RFI ≥ 200) and cell viability should be more than 50%.
- For the test chemical, the cell viability should be more than 50% in at least four tested concentrations in each run.

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

### *Test report*

31. The test report should include the following information.

Test chemical

- Mono-constituent substance
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, Log Kow, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical.
- Multi-constituent substance, UVCB and mixture
  - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
  - Physical appearance, water solubility, DMSO solubility and additional relevant physicochemical properties, to the extent available;
  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical.

Controls

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

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

Test method conditions

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

Test acceptance criteria

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

Test procedure

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

Results

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

Discussion of the results

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

Conclusions

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## APPENDIX I

## DEFINITIONS

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

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

**CV75:** The estimated concentration showing 75% cell viability.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## PROFICIENCY SUBSTANCES

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

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

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

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1] The *in vivo* hazard and (potency) prediction is based on LLNA data (3) (14). The *in vivo* potency is derived using the criteria proposed by ECETOC (24).
[2] Based on historical observed values (13) (25).

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

# ANNEX 2: IN VITRO SKIN SENSITISATION: U937 CELL LINE ACTIVATION TEST (U-SENS™)

## INITIAL CONSIDERATIONS AND LIMITATIONS

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

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

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

4. On the basis of the data currently available, the U-SENS™ method was shown to be applicable to test chemicals (including cosmetics ingredients e.g. preservatives, surfactants, actives, dyes) covering a

variety of organic functional groups, of physicochemical properties, skin sensitisation potency (as determined in in vivo studies) and the spectrum of reaction mechanisms known to be associated with skin sensitisation (i.e. Michael acceptor, Schiff base formation, acyl transfer agent, substitution nucleophilic bi-molecular [SN2], or nucleophilic aromatic substitution [SNAr]) (1) (8) (9) (10). The U-SENS™ method is applicable to test chemicals that are soluble or that form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent/vehicle into different phases) in an appropriate solvent/vehicle (see paragraph 13). Chemicals in the dataset reported to be pre-haptens (i.e. substances activated by oxidation) or pro-haptens (i.e. substances requiring enzymatic activation for example via P450 enzymes) were correctly predicted by the U-SENS™ (1) (10). Membrane disrupting substances can lead to false positive results due to a non-specific increase of CD86 expression, as 3 out of 7 false positives relative to the *in vivo* reference classification were surfactants (1). As such positive results with surfactants should be considered with caution whereas negative results with surfactants could still be used to support the identification of the test chemical as a non-sensitiser. Fluorescent test chemicals can be assessed with the U-SENS™ (1), nevertheless, strong fluorescent test chemicals emitting at the same wavelength as fluorescein isothiocyanate (FITC) or as propidium iodide (PI), will interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies (potential false negative) or PI (viability not measurable). In such a case, other fluorochrome-tagged antibodies or other cytotoxicity markers, respectively, can be used as long as it can be shown they provide similar results as the FITC-tagged antibodies or PI (see paragraph 18) e.g. by testing the proficiency substances in Appendix II. In the light of the above, positive results with surfactants and negative results with strong fluorescent test chemicals should be interpreted in the context of the stated limitations and together with other information sources within the framework of IATA. In cases where there is evidence demonstrating the non-applicability of the U-SENS™ method to other specific categories of test chemicals, it should not be used for those specific categories.

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

6. Definitions are provided in Appendix I.

## PRINCIPLE OF THE TEST

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

## DEMONSTRATION OF PROFICIENCY

8. Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Appendix II in compliance with the Good *in vitro* Method Practices (11). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 11) and with the positive and solvent/vehicle controls (see paragraphs 15-16), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

## PROCEDURE

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

### *Preparation of cells*

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

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

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

### *Preparation of test chemicals and control substances*

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

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

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

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

### *Application of test chemicals and control substances*

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

### *Cell staining*

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

### *Flow cytometry analysis*

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

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

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

surface expression of CD86 is analysed in a FL1 / SSC dot plot gated on viable cells (R2).

For the complete medium / IgG1 wells, the analysis marker is set close to the main population so that the complete medium controls have IgG1 within the target zone of 0.6 to 0.9%.

Colour interference is defined as a shift of the FITC-labelled IgG1 dot-plot (IgG1 FL1 Geo Mean S.I. ≥ 150%).

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

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

% of IgG1$^{+}$ untreated control cells: referred to as percentage of FL1-positive IgG1 cells defined with the analysis marker (accepted range of ≥ 0.6% and < 1.5%, see paragraph 22) among the viable untreated cells.

% of IgG1$^{+}$/CD86$^{+}$ control/treated cells: referred to as percentage of FL1-positive IgG1/CD86 cells measured without moving the analysis marker among the viable control/treated cells.

## DATA AND REPORTING

### *Data evaluation*

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

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

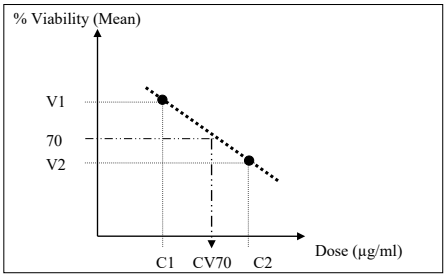
$$CV70 = C1 + [(V1 - 70) / (V1 – V2) * (C2 – C1)]$$

Where:

V1 is the minimum value of cell viability over 70%

V2 is the maximum value of cell viability below 70%

C1 and C2 are the concentrations showing the value of cell viability V1 and V2 respectively.



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

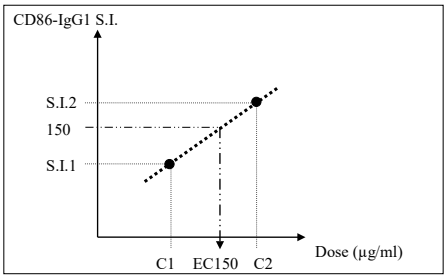
EC150 is calculated by log-linear interpolation using the following equation:

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

Where:

C1 is the highest concentration in µg/mL with a CD86 S.I. < 150% (S.I. 1)

C2 is the lowest concentration in µg/mL with a CD86 S.I. ≥ 150% (S.I. 2).




The EC150 and CV70 values are calculated

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

### *Prediction model*

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

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

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

- An U-SENS™ prediction is considered POSITIVE if at least two independent runs are positive (P) (Figure 1). If the first two runs are both positive (P), the U-SENS™ prediction is considered POSITIVE and a third run does not need to be conducted.
- Because a dose finding assay is not conducted, there is an exception if, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only. The run is then considered to be NOT CONCLUSIVE (NC), and additional concentrations (between the highest non cytotoxicity concentration and the lowest cytotoxicity concentration - see paragraph 20) should be tested in additional runs. In case a run is identified as NC, at least 2 additional runs should be conducted, and a fourth run in case runs 2 and 3 are not concordant (N and/or P independently) (Figure 1). Follow up runs will be considered positive even if only one non cytotoxic concentration gives a CD86 equal or above 150%, since the concentration setting has been adjusted for the specific test chemical. The final prediction will be based on the majority result of the three or four individual runs (i.e. 2 out of 3 or 2 out of 4) (Figure 1).

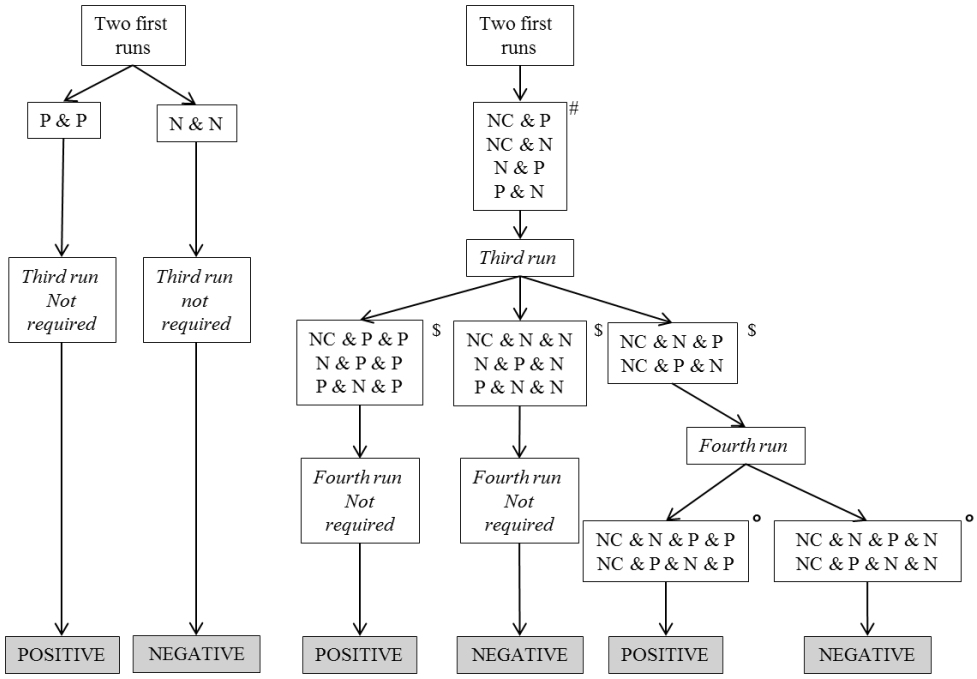



**Figure 1: Prediction model used in the U-SENS™ test method. An U-SENS™ prediction should be considered in the framework of an IATA and in accordance with the provision of paragraph 4 and of the General introduction paragraphs 7, 8 and 9.**

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

### *Acceptance criteria*

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

- At the end of the 45±3 hours exposure period, the mean viability of the triplicate untreated U937 cells had to be > 90% and no drift in CD86 expression is observed. The CD86 basal expression of untreated U937 cells had to be comprised within the range of ≥ 2% and ≤ 25%.
- When DMSO is used as a solvent, the validity of the DMSO vehicle control is assessed by calculating a DMSO S.I. compared to untreated cells, and the mean viability of the triplicate cells had to be > 90%. The DMSO vehicle control is valid if the mean value of its triplicate CD86 S.I. was smaller than 250% of the mean of the triplicate CD86 S.I. of untreated U937 cells.
- The runs are considered valid if at least two out of three IgG1 values of untreated U937 cells fell within the range of ≥ 0.6% and < 1.5%.
- The concurrent tested negative control (lactic acid) is considered valid if at least two out of the three replicates were negative (CD86 S.I. < 150%) and non-cytotoxic (cell viability ≥ 70%).
- The positive control (TNBS) was considered as valid if at least two out of the three replicates were positive (CD86 S.I. ≥ 150%) and non-cytotoxic (cell viability ≥ 70%).

### *Test report*

23. The test report should include the following information.

Test Chemical

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

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

Controls

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

Test method Conditions

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

Test Acceptance Criteria

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

Test procedure

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

Results

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

Discussion of the Results

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

Conclusions

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## APPENDIX I

## DEFINITIONS

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

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

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

**CV70:** The estimated concentration showing 70% cell viability.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## APPENDIX II

## PROFICIENCY SUBSTANCES

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

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

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

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

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

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

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

# ANNEX 3 : IN VITRO SKIN SENSITISATION: INTERLEUKIN-8 REPORTER GENE ASSAY (IL-8 LUC ASSAY)

## INITIAL CONSIDERATIONS AND LIMITATIONS

1. In contrast to assays analysing the expression of cell surface markers, the IL8-Luc assay quantifies changes in IL-8 expression, a cytokine associated with the activation of dendritic cells (DC) (1) (2). In the THP-1-derived IL-8 reporter cell line (THP-G8, established from the human acute monocytic leukemia cell line THP-1), IL-8 expression is measured following exposure to sensitisers (2) (3) (4). The expression of luciferase is then used to aid discrimination between skin sensitisers and non-sensitisers. In 2023, the test method was updated with a revised prediction model enhancing the applicability of the method to some poorly soluble chemicals, based on the evaluation of cytotoxicity using an inherent viability marker (5).

2. The IL-8 Luc method has been evaluated in a validation study (5) conducted by the Japanese Centre for the Validation of Alternatives Methods (JaCVAM), the Ministry of Economy, Trade and Industry (METI), and the Japanese Society for Alternatives to Animal Experiments (JSAAE) and subsequently subjected to independent peer review (6) under the auspices of JaCVAM and the Ministry of Health, Labour and Welfare (MHLW) with the support of the International Cooperation on Alternative Test Methods (ICATM). Considering all available evidence and input from regulators and stakeholders, the IL-8 Luc assay is considered useful as part of Integrated Approaches to Testing and Assessment (IATA) to discriminate sensitisers from non-sensitisers for the purpose of hazard classification and labelling. Examples of the use of IL-8 Luc assay data in combination with other information are reported in the literature (3) (7).

3. The IL-8 Luc assay proved to be transferable to laboratories experienced in cell culture and luciferase measurement. Within and between laboratory reproducibilities were 87.7% and 87.5%, respectively (5). Data generated in the validation study (5) and other published papers (3) (4) (8) presented the performance of the IL-8 Luc assay compared with the LLNA. In these studies, the IL-8 Luc assay judged 130 out of 143 chemicals as positive or negative and judged 13 chemicals as inconclusive. The accuracy of the IL-8 Luc assay in distinguishing skin sensitisers (UN GHS Cat. 1) from non-sensitisers (UN GHS No Cat.) is 83.6% (109/130) with a sensitivity of 92.0% (92/100) and a specificity of 56.7% (17/30). Excluding substances outside the applicability domain, such as surfactants, anhydrides and chemicals interfering with luciferase, described below (paragraph 6), the performance of the IL-8 Luc assay was 93.9% (92/98) for sensitivity, 68.0% (17/25) for specificity, and 88.6% (109/123) for accuracy. Using human data cited in Urbisch et al. (7), the IL-8 Luc assay judged 84 out of 90 chemicals as positive or negative and judged 6 chemicals as inconclusive. The performance was 89.7% (52/58) for sensitivity, 50.0% (13/26) for specificity, and 77.4% (65/84) for accuracy. Excluding substances outside the applicability domain, the IL-8 Luc assay judged 78 out of 84 chemicals

as positive or negative and judged 6 chemicals as inconclusive and the accuracy is 82.1% (64/78) with a sensitivity of 89.7% (52/58) and a specificity of 60.0% (12/20). The difference in the performance of the IL-8 Luc assays for chemicals with Log$K_{ow}$ <3.5 and those with Log$K_{ow}$ ≥3.5 was examined and it was demonstrated that Log$K_{ow}$ ≥3.5 did not reduce the sensitivity of the IL-8 Luc assay (8).

4. False negative predictions with the IL-8 Luc assay are more likely to occur with chemicals showing low/moderate skin sensitisation potency (UN GHS subcategory 1B) than those with high potency (UN GHS subcategory 1A) (3) (4) (8). Together, the information supports a role for the IL-8 Luc assay in the identification of skin sensitisation hazards. The accuracy given for the IL-8 Luc assay as a standalone test method is only for guidance, as the method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be remembered that the LLNA and other animal tests may not fully reflect the situation in humans.

5. On the basis of the data currently available, the IL-8 Luc assay was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in in vivo studies) and physicochemical properties (3) (4) (5). The IL-8 Luc assay is also technically applicable to the testing of multi-constituent substances and mixtures. However, mixtures that do not dissolve completely at 20 mg/ml of X-VIVOTM 15 and are found to be non-sensitizers may contain hidden sensitizers due to the toxicity of other chemicals present. This should be taken into account along with the general concerns about in vitro sensitisation tests when examining mixtures described in paragraph 9 of the General Introduction.

6. A high false negative rate for anhydrides was seen in the validation study. Furthermore, because of the limited metabolic capability of the cell line (9) and the experimental conditions, pro-haptens (substances requiring metabolic activation) and pre-haptens (substances activated by air oxidation) might give negative results in the assay. However, although negative results for suspected pre/pro-haptens should be interpreted with caution, the IL-8 Luc assay correctly judged 11 out of 11 pre-haptens, 6/6 pro-haptens, and 5/8 pre/pro-haptens in the IL-8 Luc assay data set (5) (7). Based on the recent comprehensive review on three non-animal methods (the DPRA, the KeratinoSens™ and the h-CLAT) to detect pre and pro-haptens (10), and based on the fact that THP-G8 cells used in the IL-8 Luc assay is a cell line derived from THP-1 that is used in the h-CLAT, the IL-8 Luc assay may also contribute to increase the sensitivity of non-animal methods to detect pre and pro-haptens in the combination of other methods. Surfactants tested so far gave (false) positive results irrespective of their type (e.g. cationic, anionic or non-ionic). Finally, chemicals that interfere with luciferase can confound its activity/measurement, causing apparent inhibition or increased luminescence (11). For example, phytoestrogen concentrations higher than 1μM were reported to interfere with luminescence signals in other luciferase-based reporter gene assays due to over-activation of the luciferase reporter gene. Consequently, luciferase expression obtained at high concentrations of phytoestrogens or compounds suspected of producing phytoestrogen-like activation of the luciferase reporter gene needs to be examined carefully (12). Based on the above, surfactants, anhydrides and chemicals interfering with luciferase are outside the applicability domain of this assay. In cases where there is evidence demonstrating the non-applicability of the IL-8 Luc assay to other specific categories of test chemicals, the method should not be used for those specific categories.

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

8. Definitions and abbreviations are provided in Table 1 and Appendix I.

## PRINCIPLE OF THE TEST

9. The IL-8 Luc assay makes use of a human monocytic leukemia cell line THP-1 that was obtained from the American Type Culture Collection (Manassas, VA, USA). Using this cell line, the Dept. of Dermatology, Tohoku University School of Medicine, established a THP-1-derived IL-8 reporter cell line, THP-G8, that harbours the Stable Luciferase Orange (SLO) and Stable Luciferase Red (SLR) luciferase genes under the control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively (2). This allows quantitative measurement of luciferase gene induction by detecting luminescence from well-established light producing luciferase substrates as an indicator of the activity of the IL-8 and GAPDH in cells following exposure to sensitising chemicals. The dual-colour assay system comprises an orange-emitting luciferase (SLO from *Rhagophthalmus ohbai*; λmax = 580 nm) (13) for the gene expression of the IL-8 promoter as well as a red-emitting luciferase (SLR from *Phrixothrix hirtus*; λmax = 630 nm) (14) for the gene expression of the internal control promoter, GAPDH. The two luciferases emit different colours upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (15) (Appendix II). In addition, GAPDH mRNA is ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real time polymerase chain reaction in some experimental systems, because its expression is constant at different times and after experimental manipulation (16) (17) (18). The inhibition of GAPLA (Inh-GAPLA) has proven to be a good marker of cell viability, with a strong correlation to propidium iodide (PI)-exclusion cells, a marker commonly used to determine cell viability by flow cytometry. Inh-GAPLA below 0.8 indicates cytotoxicity of the test chemical, which in turn suggests that the chemical dissolved in the culture medium. Therefore, the assay can be used to verify exposure with poorly soluble chemicals and to reduce the number of inconclusive results (8).

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

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

| Abbreviations | Definition |
|---|---|
| GAPLA | SLR luciferase activity reflecting GAPDH promoter activity |
| IL8LA | SLO luciferase activity reflecting IL-8 promoter activity |
| nIL8LA | IL8LA / GAPLA |
| Ind-IL8LA | nIL8LA of THP-G8 cells treated with chemicals / nIL8LA of untreated cells |
| Inh-GAPLA | GAPLA of THP-G8 treated with chemicals / GAPLA of untreated cells |
| CV05 | The lowest concentration of the chemical at which Inh-GAPLA becomes <0.05. |
| PI reduction | % of PI-excluding cells among THP-G8 cells treated with chemicals/ % of PI-excluding cells among THP-G8 cells without chemical treatment. |

## DEMONSTRATION OF PROFICIENCY

11. Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 9 Proficiency Substances listed in Appendix III in compliance with the Good in vitro Method Practices (19). Moreover, test method users should maintain a historical database of data generated with the reactivity checks and with the positive and solvent/vehicle controls, and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

## PROCEDURE

12. The Standard Operating Procedure (SOP) for the IL-8 Luc assay is available and should be employed when performing the test (5). Laboratories willing to perform the test can obtain the recombinant THP-G8 cell line from Tottori Bioscience Promotion Organization, Tottori, Japan, upon signing a Material Transfer Agreement (MTA) in line with the conditions of the OECD template. The following paragraphs provide a description of the main components and procedures of the assay.

### *Preparation of cells*

13. The THP-G8 cell line should be used for performing the IL-8 Luc assay (see paragraphs 9 and 12). On receipt, cells are propagated (2-4 passages) and stored frozen as a homogeneous stock. Cells from this stock can be propagated up to a maximum of 12 passages or a maximum of 6 weeks. The medium used for propagation is the RPMI-1640 culture medium containing 10% foetal bovine serum (FBS), antibiotic/antimycotic solution (100U/mL of penicillin G, 100µg/mL of streptomycin and 0.25µg/mL of amphotericin B in 0.85% saline) (e.g. GIBCO Cat#15240-062), 0.15µg/mL Puromycin (e.g. CAS:58-58-2) and 300µg/mL G418 (e.g. CAS:108321-42-2).

14. Prior to use for testing, the cells should be qualified by conducting a reactivity check. This check should be performed 1-2 weeks or 2-4 passages after thawing, using the positive

control, 4-nitrobenzyl bromide (4-NBB) (CAS:100-11-8, ≥ 99% purity) and the negative control, lactic acid (CAS:50-21-5, ≥85% purity). 4-NBB should produce a positive response to Ind-IL8LA (≥1.4), while lactic acid should produce a negative response to Ind-IL8LA (<1.4). If this condition cannot be met, it is recommended to use a new frozen stock vial and recheck the concentration of 4-NBB. Only cells that pass the reactivity check are used for the assay. The check should be performed according to the procedures described in paragraphs 21-23.

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

### *Preparation of the test chemical and control substances*

16. The test chemical and control substances are prepared on the day of testing. For the IL-8 Luc assay, test chemicals are dissolved in X-VIVO™ 15, a commercially available serum-free medium (Lonza[2], 04-418Q), to the final concentration of 20 mg/mL. X-VIVO™ 15 is added to 20 mg of test chemical (regardless of the chemical's solubility) in a microcentrifuge tube and brought to a volume of 1mL and then vortexed vigorously and shaken on a rotor at a maximum speed of 8 rpm for 30 min at an ambient temperature of about 20°C. Furthermore, if solid chemicals are still insoluble, the tube is sonicated until the chemical is dissolved completely or stably dispersed. For test chemicals soluble in X-VIVO™ 15, the solution is diluted by a factor of 5 with X-VIVO™ 15 and used as an X-VIVO™ 15 stock solution of the test chemical (4 mg/mL). For test chemicals not fully soluble at 20 mg/mL in X-VIVO™ 15, the mixture is rotated again for at least 30 min, then centrifuged at 15,000 rpm (≈20,000g) for 5 min; the resulting supernatant is used as an X-VIVO™ 15 stock solution of the test chemical. A scientific rationale should be provided for the use of other solvents, such as DMSO, water, or the culture medium. The detailed procedure for dissolving chemicals is shown in Appendix V. The X-VIVO™ 15 solutions described in paragraphs 17-22 are mixed 1:1 (v/v) with the cell suspensions prepared in a 96-well flat-bottom black plate (see paragraph 15).

17. The first test run is aimed to determine the cytotoxic concentration and to examine the skin sensitising potential of chemicals. Using X-VIVO™ 15, serial dilutions of the X-VIVO™ 15 stock solutions of the test chemicals are made at a dilution factor of two (see Appendix V) using a 96-well assay block (e.g. Costar Cat#EW-01729-03). Next, 50 µL/well of diluted solution is added to 50 µL of the cell suspension in a 96-well flat-bottom black plate. Thus, for test chemicals that are soluble in X-VIVO™ 15, the final concentrations of the test chemicals range from 0.002 to 2 mg/mL (Appendix V). For test chemicals that are not soluble in X-VIVO™ 15 at 20 mg/mL, only dilution factors that range from 2 to 210, are determined, although the actual final concentrations of the test chemicals remain uncertain and are dependent on the saturated concentration of the test chemicals in the X-VIVO™ 15 stock solution.

18. In subsequent test runs (i.e. the second, third, and fourth replicates), the X-VIVO™ 15

[2] https://bioscience.lonza.com/lonza_bs/IT/en/Culture-Media-and-Reagents/p/0000000000000217685/X-VIVO-15-Serum-free-Hematopoietic-Cell-Medium

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

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

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

21. The recommended positive control is 4-NBB. 20 mg of 4-NBB is prepared in a 1.5-mL microfuge tube, to which X-VIVOTM 15 is added up to 1 mL. Since 4-NBB is not fully soluble at 20 mg/mL in X-VIVO 15 (see paragraph 16), the tube is vortexed vigorously and shaken on a rotor at a maximum speed of 8 rpm for at least 30 min. After centrifugation at 20,000g for 5 min, the supernatant is diluted by a factor of 4 with X-VIVOTM 15, and 500 µL of the diluted supernatant is transferred to a well in a 96-well assay block. The diluted supernatant is further diluted with X-VIVO™ 15 at factors of 2 and 4, and 50 µL of the solution is added to 50 µL of THP-G8 cell suspension in the wells of a 96-well flat-bottom black plate (Appendix VI). Each concentration of the positive control should be tested in 4 wells. The plate is agitated on a plateshaker, and incubated in a $CO_2$ incubator for 16 hours (37°C, 5% $CO_2$), after which the luciferase activity is measured as described in paragraph 28.

22. The recommended negative control is lactic acid. 20 mg of lactic acid prepared in a 1.5-mL microfuge tube, to which X-VIVO™ 15 is added up to 1 mL (20 mg/ mL). Twenty mg/mL of lactic acid solution is diluted by a factor of 5 with X-VIVO™ 15 (4 mg/mL); 500 µL of this 4 mg/mL lactic acid solution is transferred to a well of a 96-well assay block. This solution is diluted by a factor of 2 with X-VIVO™ 15 and then diluted again by a factor of 2 to produce 2 mg/mL and 1 mg/mL solutions. 50 µL of these 3 solutions and vehicle control (X-VIVO™ 15) are added to 50 µl of THP-G8 cell suspension in the wells of a 96-well flat-bottom black plate. Each concentration of the negative control is tested in 4 wells. The plate is agitated on a plate shaker and incubated in a $CO_2$ incubator for 16 hours (37°C, 5% $CO_2$), after which the luciferase activity is measured as described in paragraph 28.

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

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

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

### *Luciferase activity measurements*

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

27. 100µL of pre-warmed Tripluc® Luciferase assay reagent (Tripluc) is transferred to each well of the plate containing the cell suspension treated with or without chemical. The plate is shaken for 10 min at an ambient temperature of about 20°C. The plate is placed in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. Justification should be provided for the use of alternative settings, e.g. depending on the model of luminometer used.

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

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

## DATA AND REPORTING

### *Data evaluation*

30. The criteria for a positive/negative decision in each run in the IL-8 Luc assay are the following:

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

### *Prediction model*

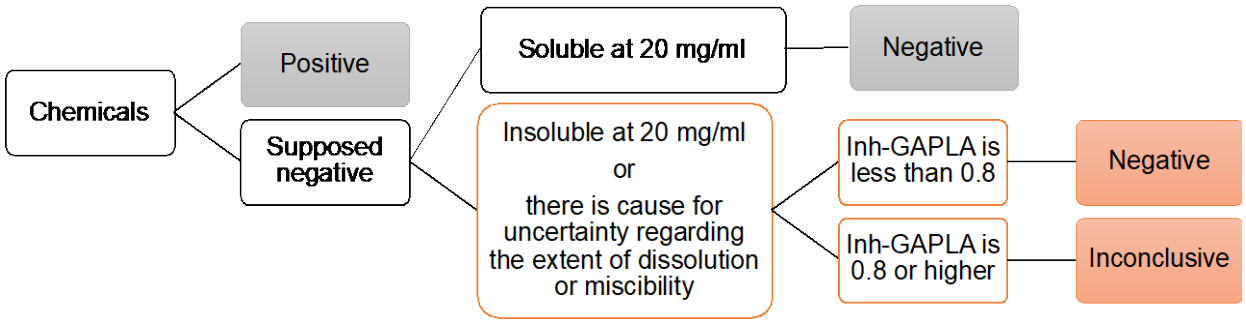
31. Test chemicals that provide two positive results from among the 1st, 2nd, 3rd. and 4th

runs are identified as positives whereas those that give three negative results from among the 1st, 2nd, 3rd and 4th runs are identified as supposed negative (Table 2). Among supposed negative chemicals, if chemicals are dissolved at 20 mg/ml in X-VIVO™ 15, they are judged as negative. If chemicals are not dissolved at 20 mg/ml in X-VIVO™ 15, or there is cause for uncertainty regarding the extent of dissolution or miscibility, chemicals that give less than 0.8 of Inh-GAPLA are judged as negative, while those that give 0.8 or higher of Inh-GAPLA are judged as inconclusive (Figure 1).

**Table 2. Criteria for identifying positive and supposed negative**

| 1st run | 2nd run | 3rd run | 4th run | Judge |
|---|---|---|---|---|
| Positive | Positive | - | - | Positive |
| Positive | Negative | Positive | - | Positive |
| Positive | Negative | Negative | Positive | Positive |
| Positive | Negative | Negative | Negative | Supposed negative |
| Negative | Positive | Positive | - | Positive |
| Negative | Positive | Negative | Positive | Positive |
| Negative | Positive | Negative | Negative | Supposed negative |
| Negative | Negative | Positive | Positive | Positive |
| Negative | Negative | Positive | Negative | Supposed negative |
| Negative | Negative | Negative | - | Supposed negative |

**Figure 1. Prediction model for final judgment**




***Acceptance criteria***

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

- Ind-IL8LA should be more than 5.0 at least in one concentration of the positive control, 4-NBB, in each run.
- Ind-IL8LA should be less than 1.4 at any concentration of the negative control, lactic acid, in each run.
- Data from plates where GAPLA in control wells containing cells and Tripluc but no chemicals is less than five times GAPLA in wells containing only test medium and Tripluc should be rejected.
- Data from plates with Inh-GAPLA of less than 0.05 for all concentrations of test chemical should be rejected. In this case, the initial test should be repeated so that the highest final concentration of the repeated test is the lowest final concentration of the previous test.

## TEST REPORT

33. The test report should include the following information:

*Test chemicals*

- Mono-constituent substance:

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Solubility in X-VIVO™ 15. For chemicals that are insoluble in X-VIVO™ 15, whether precipitation or flotation are observed after centrifugation;
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical if X-VIVO™ 15 has not been used.

- Multi-constituent substance, UVCB and mixture:

  - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
  - Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available;
  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;

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

*Controls*

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

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

*Test method conditions*

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions, and source (e.g. the facility from which it was obtained);
- Lot number and origin of FBC, supplier name, lot number of 96-well flat-bottom black plate, and lot number of Tripluc reagent;

- Passage number and cell density used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure homogeneous cell number distribution;
- Luminometer used (e.g. model), including instrument settings, luciferase substrate used, and demonstration of appropriate luminescence measurements based on the control test described in Appendix II;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

*Test procedure*

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

*Results*

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

*Discussion of the results*

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

*Conclusion*

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# APPENDIX I

## DEFINITIONS

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

**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.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

**Propidium-iodide (PI) exclusion cells**: The fluorescent marker propidium-iodide (PI) is not taken up by living cells. Therefore, in fluorescence staining and flow cytometry, cells that are not stained with PI are considered live cells and cells that are stained are considered dead cells.

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

**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.

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

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

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

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

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

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

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

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

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

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

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

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

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

## APPENDIX II

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

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

(1) Measurement of two-colour luciferases with an optical filter.

This is an example using Phelios AB-2350 (ATTO). This luminometer is equipped with a 600 nm long pass filter (R60 HOYA Co.), 600 nm LP, Filter 1) for splitting SLO ($\lambda$max = 580 nm) and SLR ($\lambda$max = 630 nm) luminescence.

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

| Transmission coefficients | | Abbreviation | Definition |
|---|---|---|---|
| SLO | Filter 1 Transmission coefficients | $\kappa O_{R60}$ | The filter's transmission coefficient for the SLO |
| SLR | Filter 1 Transmission coefficients | $\kappa R_{R60}$ | The filter's transmission coefficient for the SLR |

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

$$F0=O+R$$

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

These formulas can be rephrased as follows:

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

Then using calculated transmittance factors ($\kappa O_{R60}$ and $\kappa R_{R60}$) and measured F0 and F1, you can calculate O and R-value as follows:

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

### *Materials and methods for determining transmission coefficients*

(1) Reagents

· Single purified luciferase enzymes:

Lyophilised purified SLO enzyme

Lyophilised purified SLR enzyme

(which for the validation work were obtained from Tottori Bioscience Promotion Organization, Tottori, Japan with THP-G8 cell line)

· Assay reagent:

Tripluc® Luciferase assay reagent (for example from TOYOBO Cat#MRA-301)

· Medium: for luciferase assay (30 ml, stored at 2 – 8°C)

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

(2) Preparation of enzyme solution

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

(3) Bioluminescence measurement

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to

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

Transmission coefficient of the optical filter was calculated as follows:

Transmission coefficient (SLO ($\kappa O_{R60}$))= (#B1 of F1+ #B2 of F1+ #B3 of F1) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLR ($\kappa R_{R60}$))= (#D1 of F1+ #D2 of F1+ #D3 of F1) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

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

### *Quality control of equipment*

The procedures described in the IL-8 Luc protocol should be used (5)

**APPENDIX III**

## PROFICIENCY SUBSTANCES

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

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

| Proficiency substances | CAS no. | State | Solubility in X-VIVO15 at 20 mg/mL | *In vivo* prediction[1] | IL-8 Luc prediction[2] | Reference range (µg/mL)[3] | |
|---|---|---|---|---|---|---|---|
| | | | | | | CV05[4] | IL-8 Luc MIT[5] |
| 2,4-Dinitrochlorobenzene | 97-00-7 | Solid | Insoluble[6] | Sensitiser (Extreme) | Positive | 2.3-3.9 | 0.5-2.3 |
| Formaldehyde | 50-00-0 | Liquid | Soluble | Sensitiser (Strong) | Positive | 9-30 | 4-9 |
| 2-Mercaptobenzothiazole | 149-30-4 | Solid | Insoluble[6] | Sensitiser (Moderate) | Positive | 250-290 | 60-250 |
| Ethylenediamine | 107-15-3 | Liquid | Soluble | Sensitiser (Moderate) | Positive | 500-700 | 0.1-0.4 |
| Ethyleneglycol dimethacrylate | 97-90-5 | Liquid | Insoluble[6] | Sensitiser (Weak) | Positive | >2000 | 0.04-0.1 |
| Citral | 5392-40-5 | Liquid | Insoluble[6] | Sensitiser (Weak) | Positive | 12-30 | 4-12 |
| Streptomycin sulphate | 3810-74-0 | Solid | Soluble | Non-sensitiser | Negative | >2000 | >2000 |
| Glycerol | 56-81-5 | Liquid | Soluble | Non-sensitiser | Negative | >2000 | >2000 |
| Isopropanol | 67-63-0 | Liquid | Soluble | Non-sensitiser | Negative | >2000 | >2000 |

Abbreviations: CAS no. = Chemical Abstracts Service Registry Number

[1] The *in vivo* potency is derived using the criteria proposed by ECETOC (20).

[2] Based on historical observed values (2) (9).

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

[4] CV05: the minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA.[5] MIT: the lowest concentrations at which a chemical satisfies the positive criteria.

[6] Insoluble or not fully soluble

**APPENDIX IV**

## INDEXES AND JUDGMENT CRITERIA

### *nIL8LA (nSLO-LA)*

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

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

This is the basic unit of measurement in this assay.

### *Ind-IL8LA (FInSLO-LA)*

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

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

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

**95% confidence interval (95% CI)**

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

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

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

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

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

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

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

Inh-GAPLA (II-SLR-LA)

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

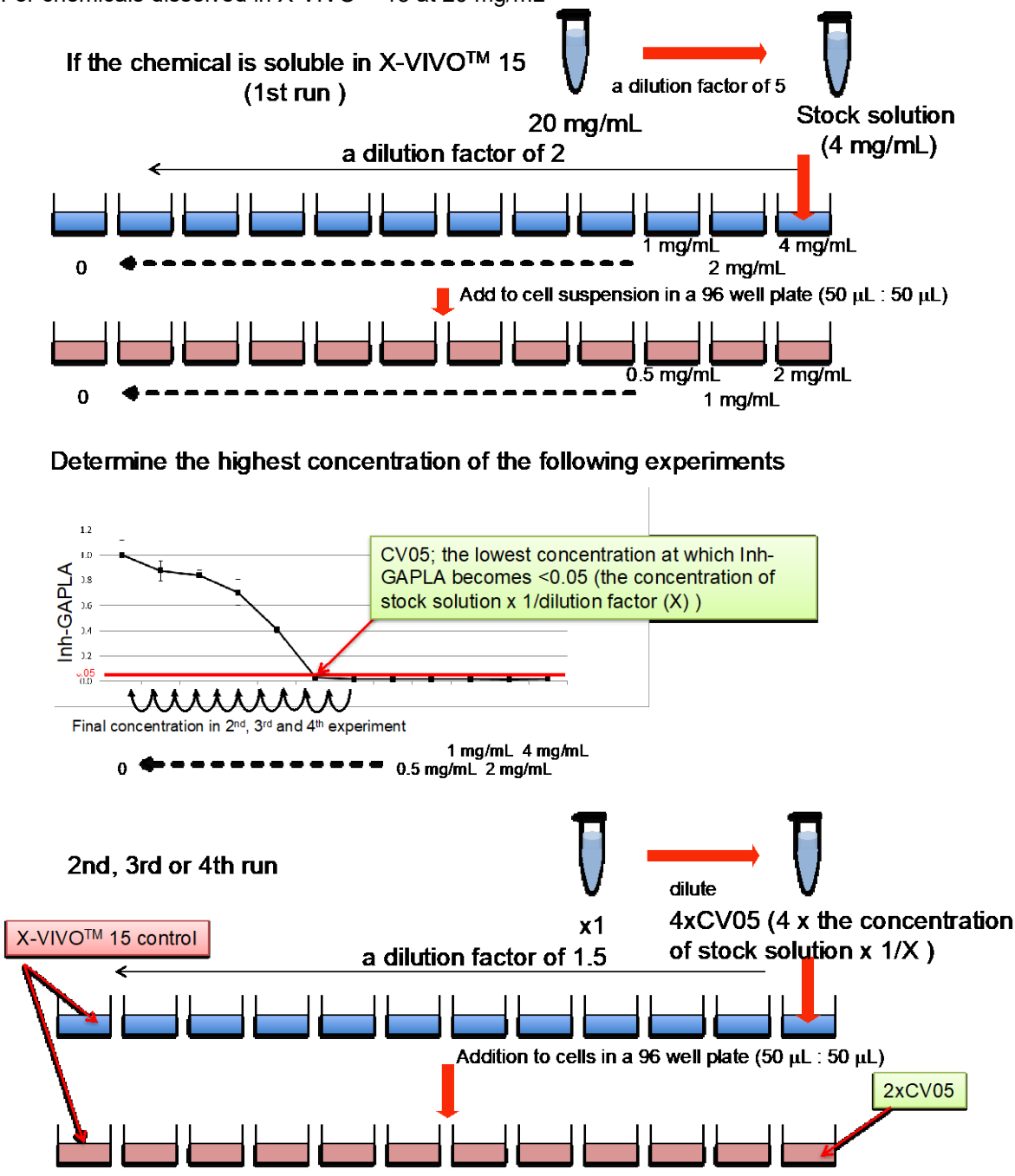
Inh-GAPLA $_i$ = $\{(1/4)\times\sum_j GAPLA_{ij}\}/\{(1/4)\times\sum_j GAPLA_{0j}\}$.

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

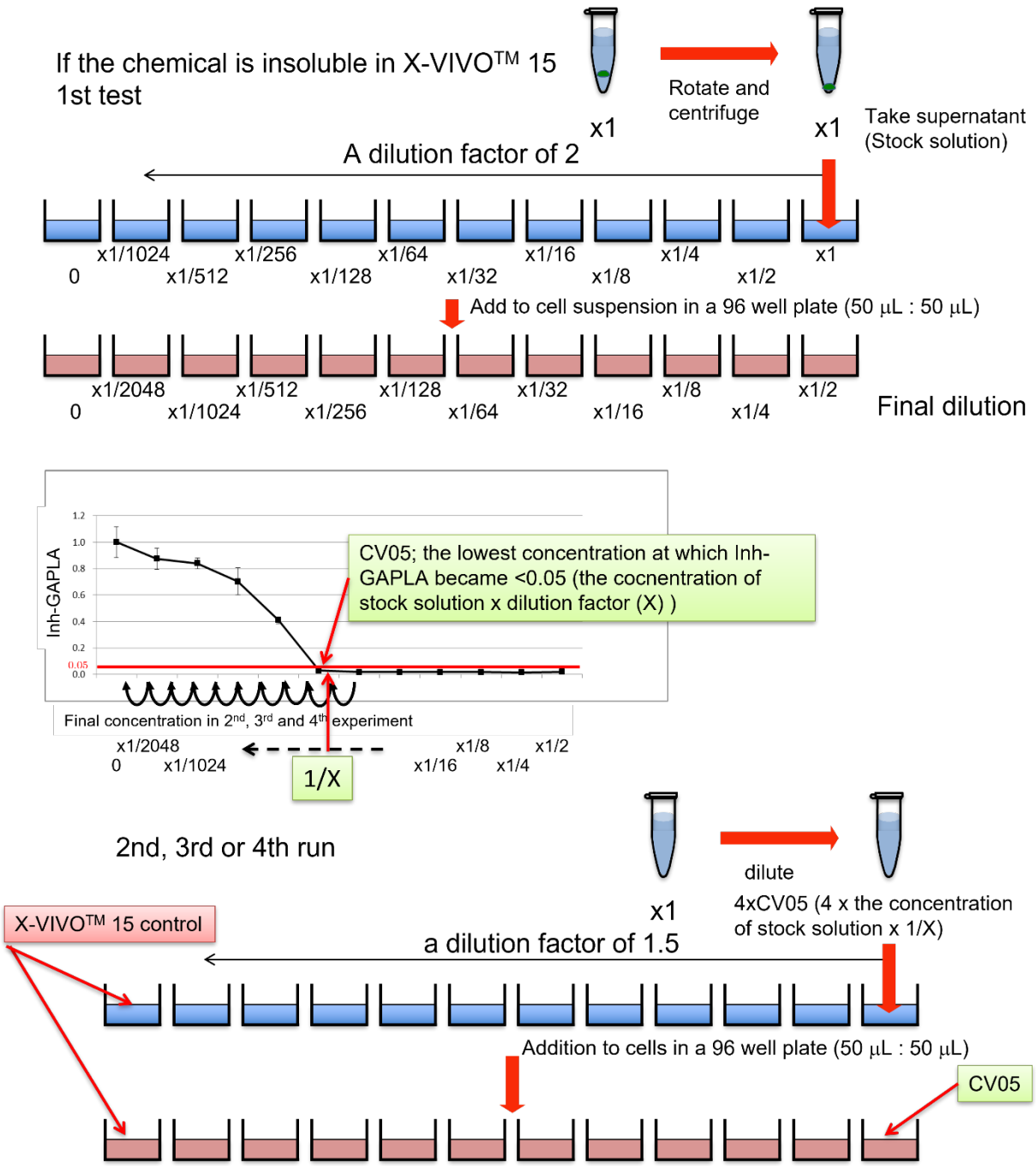
**APPENDIX V**

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

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

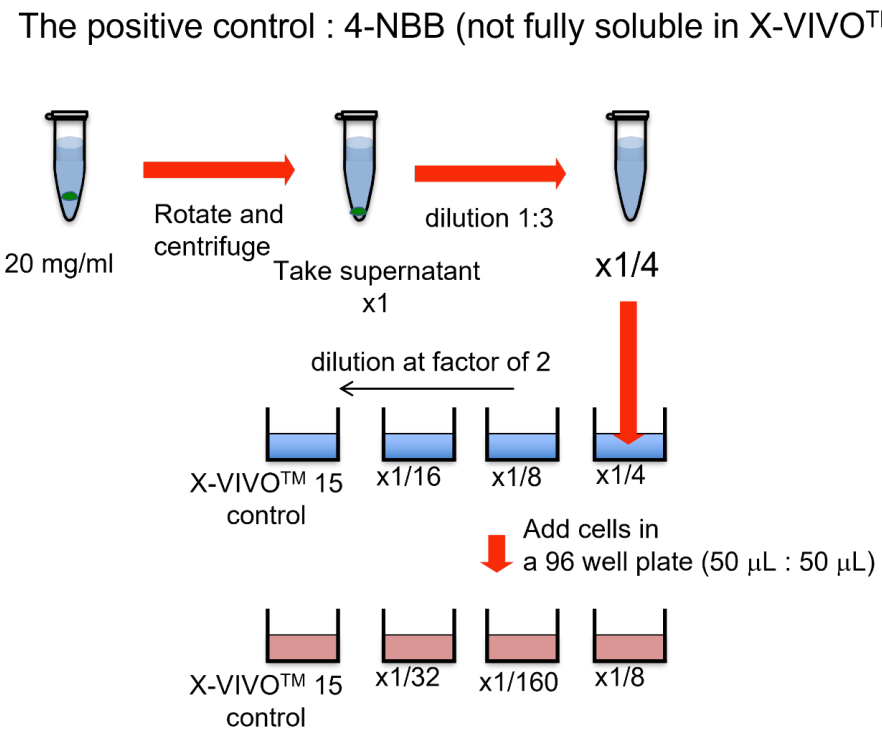


b) For chemicals insoluble in X-VIVO™ 15 at 20 mg/mL



**APPENDIX VI**

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



# ANNEX 4: *IN VITRO* SKIN SENSITISATION: GENOMIC ALLERGEN RAPID DETECTION (GARD™) FOR ASSESSMENT OF SKIN SENSITISERS (GARD™skin)

## INITIAL CONSIDERATIONS AND LIMITATIONS

1. The GARD™skin method provides binary hazard identification of skin sensitisers (i.e. UN GHS Category 1 versus non-sensitisers). The method evaluates the transcriptional patterns of an endpoint-specific genomic biomarker signature, referred to as the GARDskin Genomic Prediction Signature (GPS), in the SenzaCell™ cell line (1) (2), a subclone of the myeloid leukaemia cell line MUTZ-3 (3) (4) (5), exposed to test chemicals.

2. The GARDskin GPS (N genes = 196) was identified by genome-wide data-driven analysis of a discovery dataset based on the human surrogate DC-like SenzaCell cell line exposed to a panel of well-characterised skin sensitisers (UN GHS Category 1) (N=20) and non-sensitisers (N=20) (6). The GPS monitors mechanistic events associated with xenobiotic recognition, generation of immunological danger signals and DC activation, as described by KE3 of the OECD AOP. Of note, certain mechanistic events associated with the GARDskin GPS may also be associated with other KE:s, albeit monitored in a DC cell line. For further details on the origin and the biological functions of the GPS, please refer to the Supporting document to the Test Guideline for the GARDskin test method (7). The potential utilisation of the GARDskin GPS in a predictive assay was proposed (8), and the functionality was demonstrated in a GARDskin application based on the GeneChip® microarray platform (9). Following the evaluation of alternative technological platforms for targeted gene expression analysis (10), GARDskin was transferred to a NanoString nCounter® system (11) format, on which it was demonstrated to exhibit retained predictive performance, as well as improved resource effectiveness (12).

3. The GARDskin method has been evaluated in a validation study (13) (14) coordinated by SenzaGen AB and subsequently independently peer reviewed by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) Scientific Advisory Committee (ESAC) (15). Considering all available evidence, the GARDskin was recommended to be used as part of an IATA to support the discrimination between skin sensitisers and non-sensitisers for the purpose of hazard classification and labelling.

4. In addition to a review of data produced within the context of the ring trial, the ESAC also performed an in-depth review of the complete GARDskin analysis pipeline and its bioinformatical components, as hosted in the GARD Data Analysis Application (GDAA) and described in the Supporting document to the Test Guideline for the GARDskin test method (7). The ESAC was able to reproduce the prediction

algorithm from the training dataset and verify and reproduce all steps from raw data to final classifications of test chemicals (15).

5. GARDskin was demonstrated to be transferable to naïve laboratories experienced in routine cell culture and molecular biology techniques, including flow cytometry and isolation of RNA (test chemicals N=28). The levels of within-laboratory reproducibility (WLR) for GARDskin obtained in the validating ring trial ranged between 78.6-89.2% when considering also the concordance of missing data points[3] and 82.1-88.9% when excluding missing data points. Similarly, an estimation of between-laboratory reproducibility (BLR) was calculated to 82.1% when considering also the concordance of missing data points and 92.0% when excluding missing data points (13) (14) (15).

6. Results generated in the validation study (13) (14) overall indicated that, when compared with an expert judgement-based classification reference data set, using weight of evidence incorporating human (16) and LLNA (17) data sources as summarised and presented by the ESAC (15), the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers was 91.7% (N=28) with a sensitivity of 92.4% (N=19) and a specificity of 90.1% (N=9). The balanced accuracy was 91.2%. Omitting test chemicals which overlap with the GARDskin training dataset, thereby only considering truly naïve test chemicals, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers was 95.4% (N=17) with a sensitivity of 96.6% (N=13) and a specificity of 91.7% (N=4). The balanced accuracy was 94.1%.

7. Following the submission of the GARDskin method, OECD published the guideline on Defined Approaches for Skin Sensitisation (18). With this publication, an extended dataset of chemicals with curated LLNA and human reference data became public (19). Taking this curated reference data into account, the predictive performance of GARDskin was calculated, using GARDskin data generated in the validation study (13) (14) or available in other published studies (20). Calculations were performed both when including and excluding test chemicals overlapping with chemicals used during method development, as summarised in Table 1A and 1B, respectively. As these figures are based on imbalanced datasets, the measure of specificity should be regarded as uncertain.

**Table 1A. Performance of GARDskin in comparison to LLNA or human reference data (19). Calculations include test chemicals overlapping with chemicals used during method development.**

| | | LLNA | | Human | |
|---|---|---|---|---|---|
| | | NS (N=11) | S (N=64) | NS (N=9) | S (N=27) |
| GARDskin[1] | NS | 9.89 | 8.16 | 4.94 | 3.68 |
| | S | 1.11 | 55.9 | 4.06 | 23.3 |
| Accuracy | | 87.6% | | 78.5% | |
| Sensitivity | | 87.2% | | 86.4% | |
| Specificity | | 89.9% | | 54.9% | |
| Balanced accuracy | | 88.6% | | 70.7% | |
| N | | 75 | | 36 | |

[1] Confusion matrices are based on weighted calculations of GARDskin results, as implemented by the ESAC (15).

[3] Chemicals that failed to generate a valid result due to failed acceptance criteria, as described in section procedures.

**Table 1B. Performance of GARDskin in comparison to LLNA or human reference data (19). Calculations exclude test chemicals overlapping with chemicals used during method development.**

| | | LLNA | | Human | |
|---|---|---|---|---|---|
| | | NS (N=5) | S (N=47) | NS (N=6) | S (N=18) |
| GARDskin[1] | NS | 4.33 | 4.92 | 2.50 | 2.89 |
| | S | 0.667 | 42.1 | 3.50 | 15.1 |
| Accuracy | | | 89.3% | | 73.4% |
| Sensitivity | | | 89.5% | | 84.0% |
| Specificity | | | 86.7% | | 41.7% |
| Balanced accuracy | | | 88.1% | | 62.8% |
| N | | | 52 | | 24 |

[1] Confusion matrices are based on weighted calculations of GARDskin results, as implemented by the ESAC (15).

8. Taken together, this information indicates the usefulness of the GARDskin method to contribute to the identification of skin sensitisation hazards. However, the test method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction.

9. Known limitations of the method are mainly associated with solubility issues and compatibility with vehicles as well as the aqueous cell system. In addition, autoflourescent test chemicals may interfere with flow cytometry-based cytotoxicity assessments. The known limitations are listed in Appendix II, together with potential circumventions. The GARDskin method has been shown to be applicable to test chemicals covering a wide variety of organic functional groups, reaction mechanisms, skin sensitisation potencies and physicochemical properties (12) (14) (20). On the basis of currently available data, there are no specific classes and/or types of chemistries excluded from the applicability domain. Similar conclusions were drawn from an independent expert/expert systems review of reported data, which was also included in the data package submitted for ESAC peer-review (21). Of particular note, method applicability and predictive performance are maintained in certain chemical space subsets that are otherwise considered inherently difficult to accurately assess. This includes e.g. lipophilic compounds (logP > 3.5) (20), indirectly acting haptens (20) and metal compounds (22).

10. The GARDskin method was validated for assessment of mono-constituent chemical substances. Although not evaluated in the validation studies, the test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures (20) (23). Definitions are provided in Appendix I.

## DEMONSTRATION OF LABORATORY PROFICIENCY

11. Prior to routine use of GARDskin, laboratories should demonstrate technical proficiency in conducting the test method. Proficiency is demonstrated by testing of a specified set of proficiency chemicals with known sensitising properties, as listed in Appendix III. This testing will also confirm the responsiveness of the test system. Testing of the proficiency chemicals should be carried out in full adherence to the herein described procedure, and the results should be consistent with the listed classifications in Appendix III. A historical database of data generated with the proficiency chemicals shall be maintained at the test facility to confirm the reproducibility of the test method over time.

## PRINCIPLE OF THE TEST METHOD

12. The GARDskin method utilises the SenzaCell cell line, a subclone of the myeloid leukaemia cell line MUTZ-3, as an *in vitro* surrogate model of DC. Following test chemical exposure, at test chemical-specific exposure concentrations for 24 h, the quantifiable readout of the assay is the gene expression levels of the GARDskin GPS, obtained from measurements of isolated total RNA from exposed cell cultures, and assessed by the NanoString nCounter® system.

13. The high-dimensional data is analysed using the GDAA, hosting a Support Vector Machine (SVM) prediction algorithm (24), appropriately trained and frozen during assay development (12). Based on obtained gene expression levels in cell cultures exposed to test chemicals, the output from the GARDskin prediction algorithm predicts each test chemical as being a skin sensitiser (UN GHS Category 1) or a non-sensitiser.

## CLOUD-BASED SOFTWARE

14. The GARDskin data analysis pipeline is based on a cloud-based and version-controlled software referred to as the GDAA, which facilitates the entire data analysis-workflow, from raw-data preprocessing to final classification of test chemicals. The GDAA is designed to ensure data integrity in accordance with published guidance (25) (26).

15. Test facilities should periodically and/or before use (based on a risk assessment), check all functions of the cloud-based GDAA software (25) (26). A historic reference test dataset should therefore be uploaded to the cloud-based system and processed/analysed by the software. The test chemicals of this reference test dataset are specified by the test facility, but each test chemical should generate exact and reproducible test results over time, in terms of generated decision values and Message-Digest algorithm 5 (MD5) checksums (27). For further details and explanations of decision values and MD5 checksums, see section *Data Analysis and Reporting* of this TG. The results from this periodic and before use testing of the software shall be documented, tracking the stability of the (computerised) system over time.

## PROCEDURE

16. The *GARDskin Assay Protocol* is publicly available in the Tracking System for Alternative methods towards Regulatory acceptance (TSAR) (28). The protocol should be used when implementing the GARDskin method in the laboratory. The following paragraphs provide a description of the main components and procedures of the GARDskin test method and a graphical outline of the consecutive steps of the GARDskin method is presented in Figure 1.

17. In the GARDskin method, chemical exposures are performed in two subsequent types of experiments. In a first step, cytotoxicity assessment experiments are performed to identify a suitable and test chemical-specific exposure concentration derived from the cytotoxic properties of the test chemical, referred to as the GARD input concentration. In a second step, main stimulation experiments are performed using the previously defined GARD input concentration in order to harvest RNA for downstream analysis.

18. Three independent and biologically replicate main stimulations shall be performed. Within the context of this description of the GARDskin procedure, independent and biologically replicate experiments are defined as identical experiments being performed using i) separate cell cultures (i.e. *cell batches*), and ii) separate and independent preparations of test chemicals and controls.

19. The endpoint measurement of GARDskin, i.e. the quantification of the GARDskin GPS mRNA transcripts, is performed using the NanoString nCounter analysis system, using a CodeSet comprising probes corresponding to the genes of the GARDskin GPS. Generated raw data of gene expression levels are analysed using the GARD Data Analysis Application (GDAA), and each test chemical is classified by the GARDskin prediction model as either a sensitiser or a non-sensitiser.

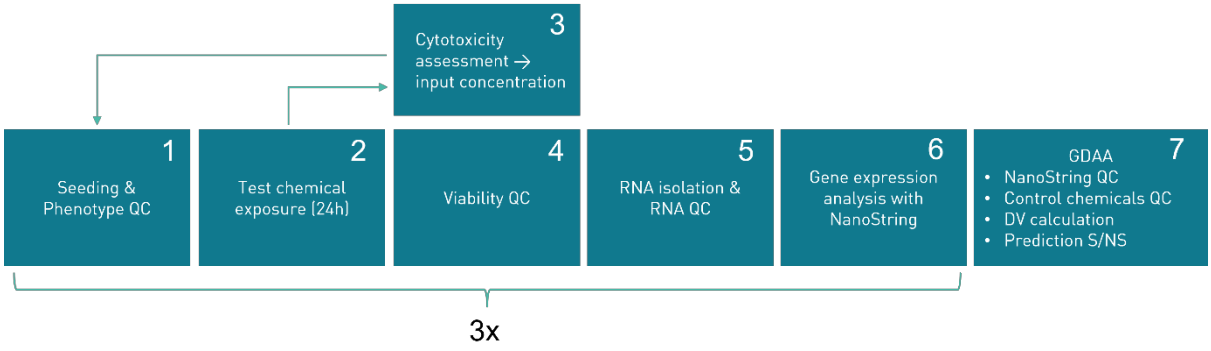



**Figure 1. Graphical outline of the consecutive steps of the GARDskin procedure. A cytotoxicity assessment experiment consists of the sequential combination of element 1-4, whereas a main stimulation experiment consists of the sequential combination of element 1, 2, 4-6. Following the completion of three independent main stimulation experiments, all data analysis is performed using the GDAA, as outlined in element 7. QC: Quality Control. DV: Decision Value.**

### *Cells*

20. The human myeloid leukemia-derived cell line, SenzaCell, should be used in the GARDskin method. The SenzaCell cell line is made available from SenzaGen AB[4], following appropriate licensing of the GARD technology. The SenzaCell cell line is provided on dry ice and should be stored at <-136°C in accordance with the Guidance Document on Good In Vitro Method Practices (GIVIMP) (29). The SenzaCell cell line should be expanded and frozen in liquid nitrogen at a concentration of $7x10^6$ cells / mL in cell medium supplemented with 10% v/v DMSO (molecular biology grade, ≥99%).

21. Cell work should be performed under sterile conditions, free of antibiotics. Centrifugations with the SenzaCell cell line should be performed at 300-315xg, 5 min, 2-8°C. Incubation of the SenzaCell cell line should be performed at 37°C under 5% $CO_2$ and humidified atmosphere. The SenzaCell cell line should be grown in cell culture flasks for maintenance and expansion, or cell culture plates for chemical exposure. The SenzaCell cell line should be grown in MEM/Alpha medium (with L-Glutamine, with Ribo- and Deoxyribonucleosides) supplemented with 20% (v/v) Fetal Bovine Serum (FBS) and 40 ng/mL GM-CSF (Premium grade, purity >97%, endotoxin level <0.1 EU/µg cytokine, and activity of $≥5x10^6$ IU/mg). Cell cultures should be counted and split to a concentration of $0.2x10^6$ cells / mL every 3-4 days for maximum 16 cell passages after thawing. The cells should be seeded for test chemical exposure directly following a

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cell split, i.e. test chemical exposure experiments should be scheduled to coincide with routine cell culture maintenance. Cells are seeded in flat-bottomed 12-well or 24-well plates, with a final total well volume of 4 ml and 2 ml, respectively. Other types and plate sizes may be used if equivalent and reproducible results can be demonstrated. Cytotoxicity assessment experiments should be performed at passage number 4 to 16 and main stimulation experiments at passage number 6 to 12.

### *Procedures for Phenotypic Quality Control and acceptance criteria*

22. The same day as performing a chemical exposure experiment, the phenotype of the untreated cells should be evaluated. This is done to ensure that cells are maintained in an inactivated state and to detect phenotypic drift.

23. All washing steps in the flow cytometer analysis should be performed in wash buffer, i.e. PBS with 0.5-1% (w/w) BSA (Cohn fraction V), 0.2 µm filter sterilisation is required. Cells should be stained with labeled monoclonal antibodies towards human antigens CD1a, CD14, CD34, CD54, CD80, CD86 and HLA-DR, as well as with relevant polyclonal isotype controls. Recommended antibodies include the following fluorescein isothiocyanate (FITC)-labelled antibodies: anti-CD86 (BD Biosciences, #555657), anti-HLA-DR (BD Biosciences, #347400), anti-CD34 (BD Biosciences, #555821), anti-CD1a (Agilent Dako, #F714101-2) and mouse polyclonal anti-IgG1-FITC (BD Biosciences, #555748). Furthermore, recommended antibodies include the following phycoerythrin (PE)-labelled antibodies: anti-CD54 (BD Biosciences, #555511), anti-CD14 (Agilent Dako, #R086401-2), anti-CD80 (BD Biosciences, #340294), and mouse polyclonal anti-IgG1-PE (BD Biosciences, #555749). In addition, Propidium Iodide, 50 µg/mL (PI) (BD Biosciences, #556463) is used for cell viability analysis. However, equivalent antibodies and viability markers may be used, provided their functional similarities can be demonstrated and documented. Note that each new lot of antibodies requires titration using the SenzaCell cell line to determine antibody concentration giving saturation. The staining of the antibodies may preferably be done by pairwise staining, using one FITC-labelled and one PE-labelled antibody per sample. In each staining sample, ~ 0.2 $x10^6$ cells are washed twice before staining. After incubation, the stained cells are washed again and resuspended in wash buffer.

24. The samples are analysed with a flow cytometer (with capability to detect PE and FITC, as applicable based on choice of antibodies) and a minimum of 10 000 events should be recorded. Gating analysis can be performed on the flow cytometer software or other related analysis software, according to instructions by the provider. For details on gating procedures and quantification of cell surface expression of phenotypic biomarkers, please refer to the GARDskin assay protocol (28).

25. Generated results shall meet the acceptance criteria listed in Table 2. If any biomarker is out of the specified ranges, the cell batch should not be used for the purpose of chemical exposure experiments and the properties of the used antibodies may need to be verified in separate assessments.

**Table 2. Acceptance criteria of the viability and phenotypic quality control**

| Parameter | Acceptance criteria (%)[1] |
|---|---|
| *Phenotypic Biomarker* | |
| CD86 | 10-40 |
| CD54 | + (>90) |
| HLA-DR | + (>60) |
| CD80 | <10 |
| CD34 | + (35-70) |
| CD14 | + (5-50) |
| CD1a | + (10-60) |
| *Viability stain* | |
| PI negative cells (absolute viability) | ≥84.5 |

[1] "+" indicates the presence of positive cells (>0 %). An entirely positive cell population is not required. Numbers given in parentheses are expected ranges based on historical data of the developing laboratory but is not part of the acceptance criteria. As the SenzaCell cell line is known to be heterogenous, variations are expected.

### *Acceptance criteria of GARD Controls*

26. With each GARDskin assessment, a set of controls should be analysed. The unstimulated control (i.e., cell culture medium) and the negative control (i.e., test chemical solvent) should be analysed in each cytotoxicity assessment experiment. The unstimulated control, the negative control and the positive control (i.e., *p*-Phenylenediamine, PPD, CAS# 106-50-3) should be analysed in each of the three replicate main stimulation experiments.

27. The unstimulated control is used for determination of absolute cell viability of cell batches, calculations of the relative cell viability in cytotoxicity assessment experiments and the main stimulation experiments and for normalisation purposes in the Data analysis workflow, as further described in the Supporting document to the Test Guideline for the GARDskin test method (7) and in section *Analysis of Data* below.

28. The negative control, should have a Relative viability ≥95.5 % in the cytotoxicity assessment experiment(s) and main stimulation experiments and be classified as a non-sensitiser by the GARDskin prediction model, as defined in paragraph 76, to verify that cells have not become activated in any steps of the method's experimental procedures.

29. The positive control (PPD) should have a Relative viability 84.5 - 95.4 % in the main stimulation experiments and be classified as a sensitiser by the GARDskin prediction model, as defined in paragraph 76, to demonstrate that the cells used during an experiment are responsive and can become activated upon exposure of a sensitiser.

### *Preparation of the test chemicals and control substances*

30. The test chemical and control substances should be stored according to instructions from the sponsor or supplier to ensure stability. Preparation of the test chemical and control substance(s) should be performed on the day of cellular exposure experiments.

31. Test chemicals should be dissolved in a compatible solvent as appropriate stocks of target in-well concentration. Compatible solvents used during method validation are listed in Table 3, together with maximum target in-well concentrations for which a non-detectable impact on genome-wide gene expression levels have been confirmed. Corresponding in-well concentrations should be used for the negative control. The positive control is preferably dissolved in DMSO at 1000x of the target in-well concentration. Other solvents than those listed in Table 3 or direct solution in cell media may be used if method compatibility can be demonstrated and sufficient scientific rationale is provided. The solvent should not cause cell cytotoxicity and should be classified as a non-sensitiser at the proposed in-well concentration.

**Table 3. List of GARDskin compatible solvents used during validation of the method.**

| Solvent | CASRN | Maximum in-well concentration (%) |
|---|---|---|
| DMSO ≥99% | 67-68-5 | 0.1 |
| Water[1] | - | 0.1 |

[1] Cell culture grade.

32. The maximum target in-well concentration of any test chemical is 500 µM. For a test chemical which has no defined molecular weight, a maximum in-well concentration of 100 µg/mL is defined as default, as derived from empirical studies (23) unless a rationale for otherwise preferred concentrations can be given.

33. Solubility of the test chemical in both the selected solvent and all downstream dilutions in cell media should be ensured by a visual inspection of the solution. If required, extensive vortexing and heat (37°C) can be applied to achieve complete dissolution, as long as it can be ensured that the stability of the test chemical is not compromised by doing so. If the test chemical is not soluble to the maximum in-well concentration of 500 µM, the solvent that generates the highest test chemical in-well concentration should be used.

34. Stock solutions with an appropriate concentration should be prepared in the selected solvent, considering dilution effects and target in-well concentrations of both test chemicals and solvents. Typically, considering the solvents listed in Table 3, such a stock concentration may be prepared at 1000x target in-well concentrations. In examples below, such a stock solution is referred to as Stock A. A Stock A of a test chemical may preferably be further diluted in medium (in examples below referred to as Stock B) before adding the test chemical to the cell culture.

35. If the Stock A is poorly soluble in Stock B (typically identified as a precipitation in medium), the highest soluble concentration in Stock B is used.

36. Note that if scientifically justified and motivated by practical benefits (e.g. increased observed solubility of certain test chemicals), the dilution scheme involving Stock A and B described above may be omitted, as long as compliance with the herein described in-well cell concentration and the maximum in-well concentrations of both test chemicals and used solvents is maintained. In such instances, a direct dilution from Stock A into the well may constitute an acceptable alternative. Similarly, if the use of an alternative solvent with different limitations in regard to maximum in-well concentration is scientifically justified and proven compatible, the concentrations of both Stock A and B may differ.

### *Cytotoxicity assessment experiment*

37. The goal of a cytotoxicity assessment experiment is to define a test chemical-specific exposure concentration, referred to as a GARD input concentration, to be used in downstream main stimulations. The GARD input concentration is defined based on solubility and cytotoxic properties of the test chemical, both of which are investigated in the herein described procedure.

38. For a schematic example of a typical cytotoxicity assessment experiment, see Figure 2. A serial dilution of the test chemical is performed in the selected solvent, from the default maximum in-well concentration of 500 µM (or the otherwise highest soluble concentration below 500 µM), to get a range of Stock A concentrations. Mixing and vortexing between each dilution step is recommended. From Stock A, a range of Stock B concentrations should be prepared by adding appropriate volume of Stock A to medium. Extensive vortexing and heat (37°C) can be applied as required to optimise dissolution. In addition, a Stock B concentration of the utilised solvent (negative control) in medium should be prepared to achieve the corresponding in-well concentration of the solvent.

39. Cells are seeded for chemical exposure directly following a cell split, at an appropriate cell concentration with regards to the dilution that occurs upon addition of stock solution(s) of test chemical and/or controls. The final in-well cell concentration, after addition of test chemical, should be $0.2x10^6$ cells/mL.

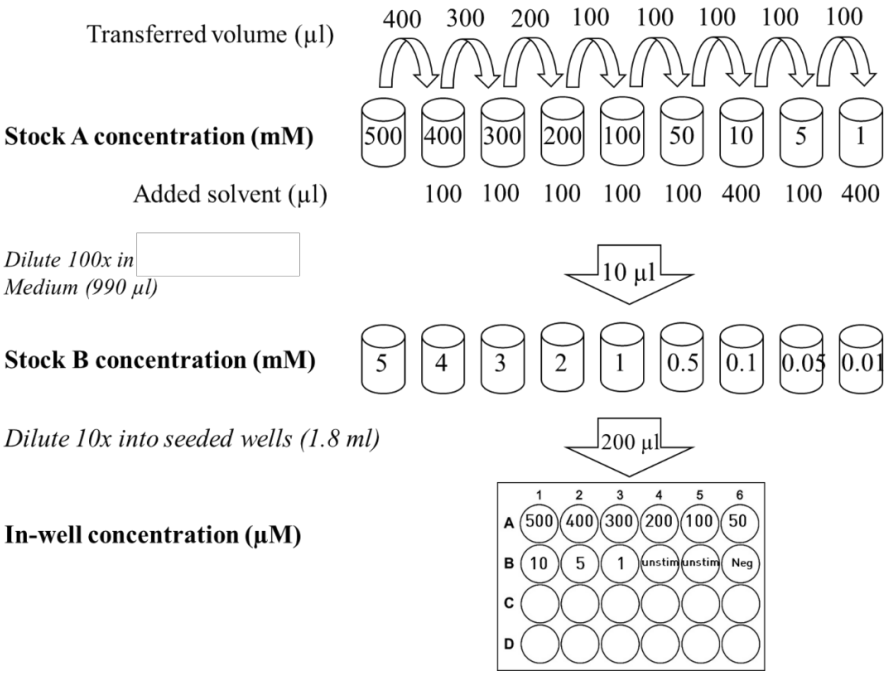


**Figure 2. A schematic example of chemical preparations and seeding of a typical cytotoxicity assessment experiment in a 24-well plate, illustrating the serial dilution of Stock A, the conversion of Stock A:s into Stock B:s by dilution in medium and a typical plate layout following cell seeding and addition of test chemical. Note the inclusion of unstimulated and negative controls in each experiment.**

40. The plate(s) with cell cultures exposed to test chemical(s) and controls are covered with plastic plate-lid(s) and incubated for 24 h at 37°C under 5% $CO_2$ and humidified atmosphere.

41. Following 24 h of incubation, the relative viability of exposed cell cultures (as compared to the absolute viability of unexposed cell cultures) are to be investigated. If a PI assay based on flow cytometry is used, the following procedure is recommended.

42. Each well suspension is split into duplicate flow cytometry samples. Staining and washing steps for the flow cytometry analysis are performed in wash buffer, as described in paragraph 23. Wash the cells twice and stain each sample with wash buffer and PI, 50:1, as described in paragraph 23. The samples are incubated in the dark at 2-8°C for ~15 min. The cells are washed once with ~1 mL and resuspended in an appropriate volume of wash buffer.

43. Duplicate sets of unstimulated cell cultures are required, in order to obtain technical duplicate flow cytometry samples of i) unstained unstimulated control samples and ii) stained unstimulated control samples. The unstained unstimulated control samples are used to set gates during analysis, while the stained unstimulated control samples are used for calculations of relative viability, as described below. The inclusion of duplicate unstimulated cell cultures is illustrated in Figure 2.

44. Prepared samples are analysed with a flow cytometer as described in paragraph 24.

45. Analysis of PI-stained samples should be done without any exclusion of dead cells and debris.

46. The unstimulated unstained sample is used to set a gate for PI-positive and -negative cells, by outlining the contours of the cell population in a PE/FITC scatter plot. The PI-positive and -negative gates are then applied in the analysis of all PI-stained samples in a PE/FITC scatter plot. The percentage of PI-negative cells are recorded for each sample, representing an estimation of absolute viability. The relative viability of each sample is calculated according to Equation 1. For each test chemical concentration of the dilution range and for each control, calculate the mean value of the duplicate samples.

$$Rv = \frac{V_S}{V_C} \cdot 100 \quad (1)$$

where

Rv is the relative viability of the sample in %.
VS is the absolute viability of the sample in %.
VC is the mean absolute viability of the two PI-stained unstimulated control samples in %.

47. The controls must pass the following criteria; unstimulated control: mean absolute viability ≥84.5% and negative control: mean relative viability ≥95.5%. (Note that these criteria are also included as part of the viability control acceptance criteria following main stimulation experiments, as further detailed in section *Main stimulations* and Table 4).

48. The GARD input concentration used for main stimulations of a test chemical should be selected as follows:

i) A test chemical that induces cytotoxicity should be used at the concentration that induces 84.5%-95.4% mean relative viability. This concentration ensures bioavailability of the test chemical, while not impairing immunological responses. If multiple concentrations fulfill the acceptance criterion, the concentration that yields the Relative viability closest to 90% is chosen as the GARD input concentration. If the Relative viability decreases from ≥95.5% to <84.5% between two data points within the dilution range, repeated cytotoxicity assessment experiment(s) with additional concentrations within the critical concentration range is needed. Interpolation between data points is not recommended, as linearity cannot be assumed.

ii) A test chemical that is not cytotoxic (Relative viability ≥95.5%) should be used at a concentration of 500 µM, or at the highest soluble concentration.

iii) A test chemical that has solubility issues in Stock A or Stock B and is not cytotoxic should be re-evaluated to control if any other solubility method, including e.g. application of heat or change of vehicle, can be used to increase the in-well concentration to get closer to the maximum in-well concentration of 500 µM.

### *Main stimulations*

49. Once the input concentration for a test chemical is established, main stimulations are repeated in three valid independent experiments with independent preparations of the test chemical and controls (unstimulated control, negative control and positive control) and independent cell cultures originating from separate batches of cells to achieve three valid biological replicate samples. The three main stimulations can either be run in parallel or sequentially, but always with independent stock solutions of both test chemical and controls. If several test chemicals are analysed in the same experiment, the same set of controls should be used, independently of number of plates, provided that all test chemicals are dissolved in the same vehicle. If different vehicles are used for different test chemicals within the same experiment, additional negative controls are required corresponding to each vehicle utilised in the experiment. In Figure 3, a schematic example of three main stimulation experiments with eight test chemicals and three controls are visualised, including one extra well with unstimulated controls. In this example, it is assumed that all test chemicals are dissolved in the same vehicle, therefore, one negative control is included.

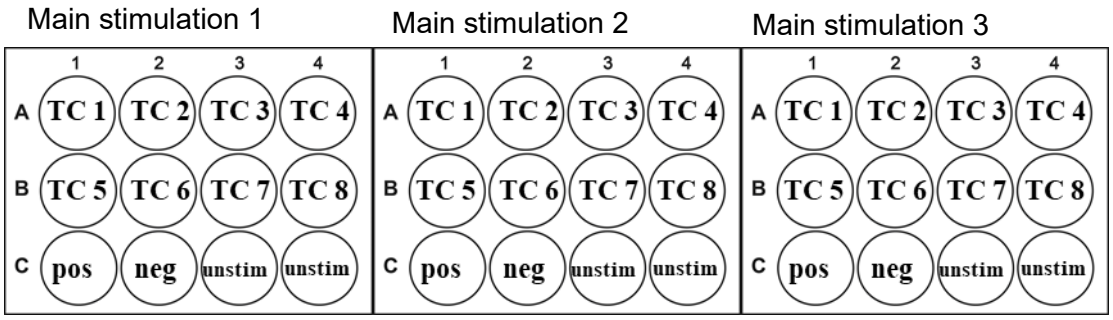


**Figure 3. A schematic example of eight test chemicals and controls stimulated in the three replicate main stimulations using 12-well plates. TC; Test chemical.**

50. The seeding procedures of main stimulations are typically identical with those described for cytotoxicity assessment experiments, as described in paragraphs 38-39, with the exception that only one concentration is investigated for each test chemical. A brief summary of a typical procedure is provided below.

51. Appropriate volume of Stock A of the test chemical is prepared in appropriate solvent as established in the preparation of the test chemical. Appropriate measures, e.g. vortexing and heat (37°C) should be applied if necessary, to achieve complete dissolution.

52. The Stock B concentration is prepared by adding appropriate volume of Stock A to cell medium (depending on the maximum target in-well concentration of solvent). Appropriate measures, e.g. vortexing and heat (37°C) may be applied if necessary, to achieve complete dissolution.

53. In addition, the positive and negative controls should be prepared to achieve appropriate in-well concentrations.

54. Cells are seeded for chemical exposure directly following a cell split, at an appropriate cell concentration with regards to the dilution that occurs upon addition of stock solution(s) of test chemical and/or controls. The final in-well cell concentration, after addition of test chemical, should be $0.2x10^6$ cells/mL.

55. Note that if justified and motivated, the same option to omit the Stock B dilution step as described in section *Cytotoxicity assessment experiment* above, applies to main stimulation experiments as well. Alternative test chemical dilution schemes, not based on the herein described A and B stock solutions, are acceptable provided the target in-well test chemical concentration, cell concentration and maximum solvent concentration are met.

56. The plate(s) are covered with plastic plate-lid(s) and incubated for 24 h at 37°C under 5% $CO_2$ and humidified atmosphere.

57. After 24 h of incubation, the cell culture is mixed by carefully pipetting up and down and each cell culture from separate wells is divided into RNase-free micro tubes and duplicate flow cytometry samples.

58. Samples in micro tubes will be used for RNA isolation. For this purpose, cell pellets are lysed using an appropriate and fit-for-purpose reagent, e.g. TRIzol reagent (Ambion, #15596018), according to instructions provided by the supplier. Cell lysate samples may be stored at ≤-70°C up to a year.

59. For each test chemical and control, several cell lysate samples may be generated from each of the three main stimulations. However, only one cell lysate sample from each of the three main stimulations is required for RNA isolation and further analysed using the NanoString nCounter system. Any remaining cell lysate replicates may be stored (≤-70°C) as backup samples due to the possibility of having insufficient RNA concentration or RNA quality in the primary cell lysate sample.

60. For the flow cytometry samples, the same washing, staining and analysis procedures as described in paragraphs 42-46 for the cytotoxicity assessment experiment should be followed.

### *Acceptance criteria of the Viability Quality Control*

61. The PI-stained samples are used as Quality Control of the viability to ensure that the test chemical and controls show a Relative or Absolute viability within the Quality Control criteria described in Table 4. If a test chemical fails the described acceptance criteria, it should not be used for downstream analysis. If any control sample fails the described acceptance criteria, all samples from the main stimulation experiment from which they originate are to be disregarded and not used for downstream analysis.

**Table 4. Viability control acceptance criteria**

| Test chemical or control | Acceptance criteria[1] |
|---|---|
| Unstimulated control | Absolute viability of ≥84.5% |
| Negative control | Relative viability of ≥95.5% |
| Positive control | Relative viability 84.5% - 95.4% |
| Test chemical with expected cytotoxicity | Relative viability 84.5% - 95.4% |
| Test chemical assayed at 500 µM or highest soluble concentration | Relative viability ≥84.5% |

[1] Listed acceptance criteria for unstimulated and negative controls apply to both cytotoxicity assessment experiments and main stimulation experiments, while criteria for the positive control and test chemical are only applicable in main stimulation experiments.

### *RNA isolation*

62. Total RNA, including mRNA, is isolated from the lysed cell samples using commercially available kit and reagents, e.g. Direct-zol RNA MiniPrep (Zymo Research, # R2052) was used during test method development and validation.

63. Quantify the RNA concentration and analyze the RNA quality from each sample using an RNA analysis equipment, e.g. with an Agilent Bioanalyzer 2100, or an equivalent instrument (i.e. an instrument measuring RNA quality and RNA concentration in the range ~5-500 ng/µL). Follow protocols provided by the instrument supplier. RNA concentration and quality should correspond to NanoString recommendations. During test method development and validation, a sample with an RNA Integrity Number (RIN) of 8.0 and above, as derived from the Agilent Bioanalyzer 2100, was considered a sample of high quality. Corresponding or otherwise equivalent RNA quality metrics may be used to assure high quality RNA.

### *Endpoint measurement; gene expression analysis using the NanoString nCounter® system*

64. The endpoint measurement of the GARDskin assay is the mRNA quantification of the endpoint-specific GPS, using the NanoString nCounter system. The NanoString nCounter protocols starts with manual processing including a hybridisation step using a thermal cycler, i.e. the nCounter XT CodeSet gene expression assay. A custom made CodeSet (i.e. sets of oligonucleotide probes representing the genes of the GARDskin GPS, the individual genes of which are presented in the Supporting document to the Test Guideline for the GARDskin test method (7), is provided by NanoString under a license agreement with SenzaGen AB. Manufacturer's instructions for the nCounter XT CodeSet gene expression assay should be followed.

65. The nCounter XT CodeSet gene expression assay is followed by automated sample processing, immobilising the probe/target on the nCounter Cartridge, and digital data acquisition, counting the color codes on the probe/targets immobilised on the cartridge, using the nCounter® instrument. Corresponding instructions for the nCounter instrument should be followed. The highest possible resolution and sensitivity mode in the nCounter instrument should be used.

66. For each RNA sample analysed in the NanoString nCounter system, a NanoString raw data file, a Reporter Code Count (RCC)-file with tabulated counts of each target molecule, is generated.

## DATA ANALYSIS AND REPORTING

### *GARD Data Analysis Application*

67. Following generation of the RCC-files, all downstream data preprocessing, normalisation and analysis are performed in the GDAA software as summarised below. For an in-depth review of all such steps, refer to the Supporting document to the Test Guideline for the GARDskin test method (7). Both the GARDskin analysis pipeline and the GDAA were extensively evaluated by the ESAC during method review, who concluded that the software was fit-for-purpose as well as user-friendly.

68. GDAA is a cloud-based application (Shinyapps on Amazon Web Services) requiring an internet connected computer with an installed web browser, e.g. Google Chrome, Mozilla Firefox or Microsoft Edge. Access to the GDAA requires a service level agreement and valid login credentials, both acquired from SenzaGen AB (www.senzagen.com)[5].

69. GDAA performs all data analysis required for generating predictions using the GARDskin method. The functionality of GDAA includes the reading of RCC-files, checking the NanoString nCounter quality control of each uploaded file, normalising the read file's gene expression values by stepwise application of a counts-per-total counts (CPTC) (12) algorithm followed by Batch Adjustment by Reference Alignment (BARA) (30). Lastly, individual samples are evaluated with the GARDskin prediction algorithm, allowing for the final classification of the test chemical by the GARDskin prediction model. For a schematic of the processes performed by the GDAA and how they relate to other steps of the procedure, see Figure 4.

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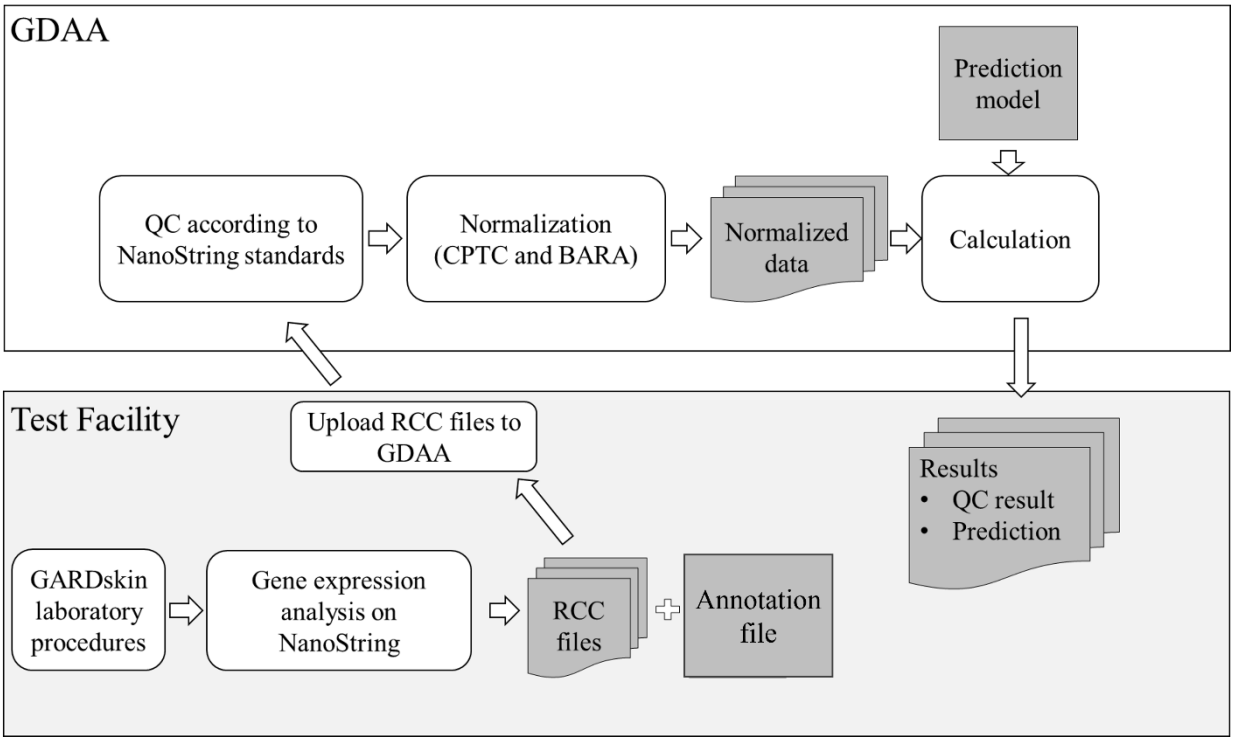


**Figure 4. A schematic of the GDAA workflow, including input and output files. RCC; Reporter Code Count (raw gene expression data), CPTC; Counts-per-total-counts (RNA content normalisation), BARA; Batch Adjustment by Reference Alignment (batch adjustment normalisation)**

70. The analysis with the GDAA requires upload of two different types of files: the RCC files (containing raw data of gene expression levels) and an Annotation file (containing sample information used to map each control and test chemical to specific RCC files). Note that the RCC files for each test chemical must be analysed together with the RCC-files of the unstimulated, positive and negative controls from the same main stimulation experiments, in order to enable both the BARA normalisation process (as further described in the Supporting document to the Test Guideline for the GARDskin test method (7), as well as the classification of negative and positive controls, in order to evaluate if acceptance criteria are met (as defined in section *Summary of acceptance criteria*).

71. After uploading the files, each RCC file is automatically quality checked in GDAA. The quality criteria listed in Table 5 are adapted from the recommended (default) acceptance criteria of the instrument supplier. Details of each quality metric listed in Table 5 are provided in the Supporting document to the Test Guideline for the GARDskin test method (7). Samples that fail any of the below described quality control criteria are not used for further analysis in the GARD data analysis and GDAA automatically rejects samples that fail the NanoString nCounter® Quality Control acceptance criteria.

**Table 5. Summary of the NanoString nCounter® quality control acceptance criteria.[1]**

| Quality Metric | | Acceptance criteria |
|---|---|---|
| Imaging Quality | Imaging quality describes the fraction of successfully processed sections of the NanoString cartridge. | > 0.75 |
| Linearity | Linearity is expressed as an R2 value that is estimated using the positive spike-in controls. | > 0.95 |
| Limit of Detection | The limit of detection is evaluated by comparing the acquired counts of the positive spike-in probe POS_E to the counts of the negative control probes. Probe POS_E is the positive spike-in probe with the lowest concentration that is expected to be observed above noise levels. | < POS_E |
| Binding Density | Binding density reports on the level of image saturation that was observed during cartridge processing. The value is dependent on the amount of sample that was loaded and the effectiveness of the NanoString hybridisation. | 0.05 - 2.25 |

[1] For details, refer to the Supporting document to the Test Guideline for the GARDskin test method (7).

72. The last steps of a GARDskin analysis includes the application of a prediction algorithm, which in turn provides input to the GARDskin prediction model, as described in section *Prediction model* below.

73. In addition to facilitating the complete GARDskin analysis pipeline, the GDAA functionality includes verification of integrity of transferred data using algorithms for calculating MD5 checksums. The MD5 algorithm takes input data of arbitrary length and calculates a 128-bit fingerprint. It will always produce the same fingerprint for a specific input, and it is highly unlikely that two different data inputs would generate the same output values. These properties of the algorithm make it useful for verifying integrity of data. For example, the integrity of a transferred file can be ensured by comparing the 128-bit fingerprints calculated prior to the transfer with a fingerprint calculated following transfer. These MD5 checksums are evaluated as part of the periodic and before use testing of the computerised system, as described in section *Periodic testing of cloud-based software*.

### *Prediction model*

74. The GARDskin prediction algorithm is a SVM hosted in the GDAA, appropriately trained and frozen during method development. The output of the prediction algorithm is referred to as a decision value (DV). Unique DVs are calculated for each replicate sample generated by test chemicals and controls, as described by equation 2.

$$DV = b + \sum_{i=1}^{n} w_i x_i \qquad (2)$$

where $n$ is the number of variables (genes, i.e., 196 for GARDskin), $b$ is a constant (i.e., the SVM's intercept), $w_i$ the weight for variable $i$, and $x_i$ the normalised gene expression value for variable $i$. For an in-depth review of how the prediction model was defined, please refer to the Supporting document to the Test Guideline for the GARDskin test method (7).

75. The DVs of the three individual replicate samples are then used as input to the GARDskin prediction model. The GARDskin prediction model is defined as follows:

76. Any test chemical with a calculated mean DV ≥ 0 is classified as a sensitiser (UN GHS category 1), whereas any test chemical assigned a mean DV < 0 is classified as a non-sensitiser. A schematic representation of the GARDskin prediction model is provided in Figure 5.

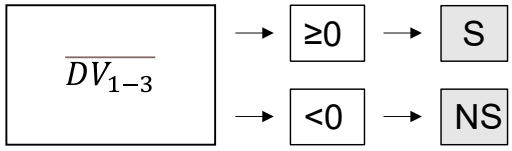


**Figure 5. Schematic of the GARDskin prediction model. Test chemicals are classified by the sign of the mean of three biological replicate samples, originating from three independent experiments. S: Sensitiser. NS: Non-sensitiser.**

### *Summary of Acceptance criteria*

77. Below is a summary of the acceptance criteria that are specified for the GARDskin method.

i) All cell exposure experiments should have been performed with a batch of SenzaCell cells that passed the acceptance criteria of the Phenotypic Quality Control (Table 2). This applies to both cytotoxicity assessment and main stimulation experiments.

ii) All generated RNA samples should originate from cellular experiments, which have passed the acceptance criteria of the Viability Quality Control (Table 4). This applies to all test chemical and positive, negative and unstimulated control samples, from the three included (i.e., valid) main stimulation experiments. Similarly, cytotoxicity assessment experiment(s) from which a GARDskin input concentration is derived should fulfill all applicable Viability Quality Control criteria (Table 4).

iii) All generated RNA samples should pass the Acceptance criteria of the NanoString nCounter® Quality Control (Table 5). This applies to all test chemical and positive, negative and unstimulated control samples, from the three included (i.e., valid) main stimulation experiments.

iv) The final classification should be made using three valid biological replicates which have all passed acceptance criteria i-iii.

v) The positive control and negative control should be accurately classified as a sensitiser and a non-sensitiser, respectively, by the GARDskin prediction model.

### *Test Report*

78. The following information should be reported. The results should be tabulated and include, when applicable, individual test results for each performed experiment as well as the overall results from all three experiments.

*General information*

- Name and address of sponsor, test facility and study director.
- Reference and description of the test method used.

*Demonstration of proficiency*

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

*Demonstration of GDAA system stability over time*

- Statement that periodic and/or before use testing of the GDAA have been performed using a historic dataset and have passed required criteria.

*Test chemical and controls*

- Source, batch/lot number, expiry date. Chemical identification, such as IUPAC name, CAS registry number, SMILES or InChI code, structural formula, and/or other identifiers like batch/lot number and expiry date.
- Physical appearance, solvent solubility as applicable, molecular weight, and additional physical chemical properties to the extent available.
- Statement on (in)solubility or stable dispersion in exposure media.
- Purity, chemical identity of impurities as appropriate and practically feasible.
- Procedure(s) used to dissolve test chemical(s).
- Storage conditions and stability to the extent available.
- Justification for choice of solvent/vehicle for each test chemical.
- Solvent (including source) used for each test chemical and control.

*Test method conditions*

- Cell line used, cell culture ID, its storage conditions and source.
- Cell media components (including source) used in the study.
- Flow cytometry equipment used.
- Antibodies and viability markers (including sources) used in the study.
- RNA isolation kit, RNA qualification kit and NanoString nCounter GARDskin CodeSets (including sources) used in the study.

*Test acceptance criteria results*

- Phenotypic Quality Control data from each experiment (percentage of positive cells for each phenotypic biomarker, as well as absolute viability of cells).
- Cell viability Quality Control data of the test chemical and negative, positive and unstimulated controls.
- NanoString nCounter Quality control data (imaging quality, linearity, limit of detection and binding density) of the test chemical as well as negative and positive controls.
- Classifications of negative and positive controls.

*Cytotoxicity assessment results*

- Test concentrations with justifications.
- Relative viability for each test chemical concentration
- Justification for selected GARD input concentration.

*Main stimulation results*

- Measured RNA-quality of test chemical and control RNA-samples
- Gene expression levels (content of RCC files) for test chemical and controls, in a format compliant with available guidance (31) (32).
- GDAA version number used within the study.
- Statement on matching MD5 checksums of uploaded RCC-files and MD5 checksums from downloaded GDAA report.
- Decision Values (individual samples as well as mean) obtained for the test chemical and positive and negative controls.
- Classification of the test chemical.
- Description of any other relevant observations, if applicable.

*Discussion of the results*

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## APPENDIX I - DEFINITIONS AND ABBREVIATIONS.

Abbreviations that are defined by the test method developers and/or specific for certain instrumentation utilised by the GARD methods are *defined in italic font*.

| | |
|---|---|
| ARE | Antioxidant Response Element |
| BARA | Batch Adjustment by Reference Alignment |
| | *An algorithm for removal of batch-effects observed between datasets.* |
| BLR | Between-Laboratory Reproducibility |
| BSA | Bovine Serum Albumin |
| CD | Cluster of Differentiation |
| | Cell Batch |
| | *Within the context of this TG, a unique cell batch is defined as:*<br>▪ *cells originating from different frozen vials, or…*<br>▪ *cells originating from the same frozen vial, which have been cultivated separately. A division of cell cultures for the purpose of achieving separate cell batches should be done no sooner than passage 3 after thawing, and no later than at least 2 passages prior to exposure experiments.* |
| CPTC | Counts-Per-Total-Counts |
| | *An algorithm for RNA content normalisation.* |
| DB-ALM | DataBase service on ALternative Methods to animal experimentation |
| DC | Dendritic Cells |
| DMSO | Dimethyl Sulfoxide |
| DV | Decision Value |
| | *A quantifiable output from a Support Vector Machine.* |
| ESAC | ECVAM Scientific Advisory Committee |
| EURL ECVAM | European Union Reference Laboratory for alternatives to animal testing |
| FBS | Fetal Bovine Serum |
| FITC | Fluorescein IsoThioCyanate |
| GARD | Genomic Allergen Rapid Detection |
| | *A series of predictive assays for immunotoxicological endpoints, the main subject of this* |

*Test Guideline.*

GARDskin GARD Test Method for Skin Sensitisation

*The specific subject of this TG. A method used for hazard assessment of skin sensitisers*

GARDskin prediction algorithm

*An algorithm that, based on raw gene expression data, provides DVs as output. The output of the prediction algorithm is in turn used as input in the prediction model. The GARDskin prediction algorithm is an SVM. See e.g., DV, GARDskin prediction model, SVM.*

GARDskin prediction model

*A heuristic that, based on triplicate DVs from a test chemical or control, provides a GARDskin classification of the same.*

GDAA GARD Data Analysis Application

*A cloud-based software for fit-for-purpose and automated data processing and analysis of all raw data generated with the GARD methods.*

GHS Globally Harmonized System

GM-CSF Granulocyte Macrophage Colony Stimulating Factor

GPS Genomic Prediction Signature

*A set of gene identities that collectively compose the set of predictors, the gene expression values of which are used as the input in the GARD prediction models, i.e., the endpoint-specific Support Vector Machine(s), each appropriately trained and frozen during test method development. Each GARD method (e.g. GARDskin) for different endpoints utilises a different GPS.*

LLNA Local Lymph Node Assay

LOD Limit of Detection

*A parameter of the NanoString instrumentation.*

MD5 Message-Digest algorithm 5

*A function that creates digital fingerprints of input data. Within the context of the GARDskin method, such fingerprints are used to verify integrity of data.*

OECD Organisation for Economic Co-operation and Development

PBS Phosphate Buffered Saline

PE Phycoerythrin

PI Propidium Iodide

RCC Reporter Code Count

*A filetype created by the NanoString instrumentation. Stores raw gene expression data.*

RIN RNA Integrity Number

*An RNA quality parameter utilised by the Agilent instrumentation.*

SVM Support Vector Machine

*Supervised prediction models with associated learning algorithms that analyze data for classification and regression analysis*

TG Test Guideline

WLR Within-Lab Reproducibility

## APPENDIX II - KNOWN LIMITATIONS OF THE GARDSKIN METHOD.

### Table AII.1. A summary of known limitations of the GARDskin method and possible adaptations.

| Substance class / interference | Possible consequence of interference | Possible adaptations | Example substance |
|---|---|---|---|
| Test chemicals absorbing and/or autoflourescing light at the wavelengths of PI-detection. | May influence cytotoxicity assessment results and may lead to inappropriately defined GARD input concentrations. | May be circumvented by use of alternative reagents for assessment of cytotoxicity, if demonstrated to generate equivalent results to those of the herein proposed methods. | Citral (CAS #5392-40-5) |
| Substances with unknown precise molecular weight. A Test chemical is preferably defined by a known molecular weight, as appropriate GARD input concentrations are defined by molar concentrations. | May lead to inappropriate GARD input concentrations, which may in turn lead to misclassifications. | May be circumvented by<br>-Use of weight-based concentrations (e.g. ppm) (1). A vast majority of skin sensitisers are detected <100 ppm (1).<br>-Approximation of an apparent molecular weight of the complex mixture, | UVCBs, chemical emissions, products or formulations with variable or not fully known composition, natural extracts. |
| Test chemicals which cannot be dissolved in an appropriate solvent at a final in-well concentration of 500 µM and do not exhibit cytotoxic properties at investigated max concentration. | A sufficient exposure concentration for detection of weak skin sensitisers may not be guaranteed. May cause false negatives. However, available data suggest that a vast majority of sensitisers are detected <100 µM (1). | Test chemicals that do not induce cytotoxicity with a maximum soluble concentration below 500 µM may be further analysed according to downstream GARDskin procedures and positive results from such testing can be used to support the identification of the test chemical as a skin sensitiser. | n/a |
| Test chemicals Incompatible with vehicles. | Insolubility or reactive interference with Test chemical, which may in turn lead to inappropriate GARD input concentrations and possible misclassifications, or complete incompatibility with the method. | If a scientific rationale is available, alternative and otherwise compatible vehicles may be used (2) (3). Compatibility of such alternative vehicles should be confirmed by inclusion of the blank vehicle as a negative control, at identical exposure concentrations. If a Test chemical remains insoluble, see handling of not sufficiently dissolved Test chemicals above. | n/a |
| Test chemicals that hydrolyse rapidly in cell system. | A sufficient in-well concentration of Test chemicals may not be guaranteed. May cause false negatives | | Hydrazine (CAS #2644-70-4) |

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## APPENDIX III - PROFICIENCY SUBSTANCES.

### Table AIII.1. Substances for demonstrating technical proficiency with GARDskin.

| Chemical ID | CASRN | Physical state | Reference classification[1] | | Expected GARDskin values (reference range) | | |
|---|---|---|---|---|---|---|---|
| | | | LLNA | Human | Classification | (DV) | (Input concentration, µM) |
| 4-nitrobenzyl bromide | 100-11-8 | Solid | S (extreme) | *NA* | S | (0 – 10) | <25 |
| Propyl gallate | 121-79-9 | Solid | S (strong) | *NA* | S | (2 – 13) | 25 - 400 |
| Isoeugenol | 97-54-1 | Liquid | S (moderate) | S | S | (2 – 13) | >100 |
| 3-(Dimethylamino)-1-propylamine | 109-55-7 | Liquid | S (moderate) | *NA* | S | (0 – 11) | >250 |
| Eugenol | 97-53-0 | Solid | S (weak) | S | S | (0 – 10) | >100 |
| Ethylene glycol dimethacrylate | 97-90-5 | Liquid | S (weak) | *NA* | S | (2 – 20) | >25 |
| Glycerol | 56-81-5 | Liquid | NS | *NA* | NS | (<0) | ≥250 |
| Hexane | 110-54-3 | Liquid | NS | NS | NS | (<0) | ≥125 |
| 1-Butanol | 71-36-3 | Liquid | NS | *NA* | NS | (<0) | ≥250 |

[1] Extracted from Annex 2 of the supporting document to OECD TG 497 (1). S: Sensitiser. NS: Non-sensitiser NA: missing value.

## Literature

1. OECD (2021). Series on Testing and Assessment No. 336: Annex 2 of the Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation. Organisation for Economic Cooperation and Development, Paris. Available at: http://www.oecd.org/env/testguidelines

Section 4
Health effects

# Test Guideline No. 444A

## *In vitro* immunotoxicity

*IL-2 Luc Assay*

4 July 2023

OECD Guidelines for the Testing of Chemicals

# *OECD GUIDELINE FOR THE TESTING OF CHEMICALS*

## In Vitro Immunotoxicity: Il-2 Luc Assay

## INITIAL CONSIDERATIONS AND LIMITATIONS

1. Immunotoxicology is defined as the study of adverse effects on any part of the immune system as the result of exposure to drugs or chemicals (1). Immunotoxicology can also include adverse effects on different organs and tissues mediated by cells and molecules of the immune system. The immune system is susceptible to toxic insults, in part because of: 1) the need to maintain the delicate balance between activation, regulation and silencing; 2) its dependence on regeneration of cells from hematopoietic stem cells in the bone marrow; 3) its requirement of clonal expansion of T cells and B cells by cellular proliferation during the adaptive response; and 4) the required maintenance of appropriate levels of lymphocytes, including effector, memory and naïve subsets.

2. Immunotoxicity by drugs or chemicals can be manifested in various ways, including dysregulation of the immune response, which could lead to immunosuppression or inappropriate immune stimulation. The latter can include exaggerated immune stimulation, sustained inflammation, hypersensitivity reactions and autoimmune diseases. With reference to chemical-induced immunotoxicity, the effect may not be exclusively in one direction and the same substance can produce immunosuppression or immune stimulation, depending on the dose and the cellular target. Thus, it may be more appropriate to define an immunotoxic substance as any agent that can alter one or more immune functions resulting in an adverse effect for the host. In this way, the focus is not on the direction of the effect, but on its consequence. For this reason, the term immunotoxicant/immunotoxicity is used in this TG, which is consistent with the Detailed Review Paper on immunosuppression that was developed by OECD (2).

3. From the OECD Adverse Outcome Pathway (AOP) development programme, AOP 154 “Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response” was approved in 2021 (3) This AOP describes calcineurin (CN) activity inhibition by binding of stressors, i.e., CN inhibitors (CNIs). CNIs bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell-derived cytokine promoters, is reduced, thereby suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 negatively affects the proliferation and differentiation of B cells to suppress the T cell–dependent antibody response (TDAR).

4. IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. IL-2 promotes Th1 differentiation by inducing IL-12Rβ2 (and IL-12Rβ1), promotes Th2 differentiation by inducing IL-4Rα, inhibits Th17 differentiation by inhibiting gp130 (and IL-6Rα), and drives Treg differentiation by inducing IL-2Rα. IL-2 also potently represses IL-7Rα, which decreases survival signals that normally promote cell survival and memory cell development (4) therefore conceivable that chemicals that affect IL-2 release by T cells can significantly impact immune function.

5. The IL-2 luciferase assay (IL-2 Luc assay) uses 2H4 cells to identify the effects of chemicals on the IL-2 and IFN-γ promoters in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) (5). 2H4, derived from Jurkat cells, contains stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the interferon (IFN)-γ promoter, and stable luciferase red (SLR) regulated by the glyceraldehyde 3-phosphate dehydrogenase **(**GAPDH) promoter. Compared to methods that directly measure IL-2 and IFN produced by Jurkat cells, the IL-2 Luc assay significantly reduce both manual lab or and assay time. Specifically, the IL-2 Luc assay requires only the manual process of diluting chemicals and dispensing chemical solution and cells. In addition, IL-2 and IFN can be quantified by ELISA, but not the amount of GAPDH.

6. The validation study of the IL-2 Luc assay was conducted by a validation management team (VMT) composed of a lead laboratory, three independent laboratories, and four international expert members coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM). This validation study met the acceptance criteria regarding within- (80.0%,4/5) and between-laboratory reproducibility (80.0%, 16/20) which could satisfy the acceptance criteria of 80% (6). To determine the predictivity, we collected immunotoxicological information and selected the reference data by classifying the chemical into immunotoxic compounds targeting T cells or others according to previously reported criteria. When compared with the reference data, the average sensitivity, specificity and predictivity in the validation study were 75.0% (36/48), 75.0% (18/24), and 75.0% (54/72), respectively (7), while the predictivity of an additional 60 chemicals was examined by the lead laboratory resulting in sensitivity, specificity and predictivity of 82.4% (28/34), 83.3% (5/6), and 82.5% (33/40), respectively. These results were reviewed by an international peer review panel. While the predictive capacity was not satisfactory for a stand-alone method, the Il-2 Luc assay is acceptable for use in an Integrated Approach to Testing and Assessment (IATA) (8). The IL-2 Luc assay provides a rapid screening tool that can be used as part of a systematic assessment of immunotoxicity when combined with other immunotoxicity tests.

7. Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterise immunosuppression and sensitization. However, animal studies have many drawbacks, such as high cost, ethical concerns, and have varying ability to predict effects on human health (9). In addition, current *in vivo* models do not always provide a mechanistic understanding of the data. Overcoming these problems requires the development of *in vitro* methods to detect immunotoxicity. A workshop hosted by the European Centre for the Validation of Alternative Methods in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (10)(11)(12) and a tiered approach was proposed. Within the tiered approach, the Multi-ImmunoTox assay (MITA) evaluates the effects of chemicals on the IL-2, IFN-γ, IL-1β, and IL-8 promoters using three stable reporter cell lines (13)(14).

8. The purpose of this Test Guideline (TG) is to describe the procedure used to evaluate the potential immunotoxic effects of chemicals on T cells. The IL-2 Luc Assay is an important method for evaluating the immunotoxic potential of chemicals as a part of a battery (JaCVAM, 2020b), because of its technical simplicity, short test duration and accuracy of the test result, based on a known mechanism of immunotoxicity. The IL-2 Luc assay is applicable to soluble test chemicals or that test chemicals that form a stable dispersion and shares the same limitations that are common to many suspension cell-based assays when testing highly hydrophobic substances. Test chemicals that interfere with luciferase can confound its activity/measurement, causing apparent inhibition or increased luminescence (15). In addition, the following limitations should be noted: (1) the method cannot detect immunotoxicity associated with inhibition of DNA synthesis and cell division (7)(13); (2) the assay cannot detect test chemicals that require metabolic activation to form an immunotoxic metabolite (7)(13).

### *Specific limitations*

9. The following limitations should be noted: 1) the use of PMA/Io as a stimulant bypasses signalling through the T cell receptor and the subsequent intracellular signalling events that precede activation of phospholipase C, and therefore precludes detection of chemicals that act on those upstream signalling molecules (16); 2) the Jurkat T cell line (from which 2H4 cells are derived) are demonstrated to be suitable for examining the molecular mechanism underlying immunotoxicity (17), they may lack several key proteins involved in the activation of normal T cells in response to TCR stimulation, and therefore may not be able to detect effects of chemicals that act on those key proteins.

10. Definitions are provided in Appendix I.

## PRINCIPLE OF THE TEST

11. The IL-2 Luc assay makes use of a human acute T lymphoblastic cell line Jurkat that was obtained from Professor Kazuo Sugamura, Department of Microbiology, Tohoku University School of Medicine. Using this cell line, Tsuruga Institute of Biotechnology, TOYOBO Co., Ltd, established a Jurkat-derived IL-2 reporter cell line, 2H4, that harbors SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ, and GAPDH promoters, respectively (5). Laboratories willing to perform the test can obtain the recombinant 2H4 cell line from Tottori Bioscience Promotion Organization, Tottori, Japan, upon signing a Material Transfer Agreement (MTA). This cell line allows quantitative measurement of luciferase gene induction by detecting luminescence from well-established light producing luciferase substrates as indicators of the activity of IL-2, IFN-γ and GAPDH in cells following exposure to immunotoxic chemicals. To simplify the assay, only the IL-2 promoter driven luciferase activity (IL2LA) and GAPDH promoter driven luciferase activity have been used based on the following observations described in the literature (14. Most of chemicals examined by 2H4 cells showed similar suppressive effects on IL2LA and IFN promoter-driven luciferase activity (IFNLA). In addition, when the lowest observed effect level (LOELs) of these chemicals were plotted against their effects on IL-2LA and those on IFNLA, they showed a significant correlation between them. Therefore, a decision was made to only use IL2LA.

12. The multicolour assay system (18)(19) comprises a green-emitting luciferase (SLG; λmax = 550 nm) (20) for the gene expression of the IL-2 promoter, an orange-emitting luciferase (SLO; λmax = 580 nm) (21) for the gene expression of the IFN-γ, promoter as well as a red-emitting luciferase (SLR; λmax = 630 nm) (22) for the gene expression of the internal control promoter, GAPDH. The three luciferases emit different colours upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using two optical filters (18) (see Appendix II). For accurate luminescence measurements, a highly sensitive luminescence meter (e.g. a luminescence meter dedicated to luminescence measurements as described in Appendix II) should be used.

13. 2H4 cells are treated for 1 hour with the test chemical, and then treated for 6 hours with PMA and Io after which SLG luciferase activity (SLG-LA) reflecting IL-2 promoter activity, SLO luciferase activity (SLO-LA) reflecting IFN-γ promoter activity and SLR luciferase activity (SLR-LA) reflecting GAPDH promoter activity are measured. To make the abbreviations easier to understand, SLG-LA, SLO-LA and SLR-LA are designated as IL2LA, IFNLA and GAPLA, respectively. Table 1 provides a description of the terms associated with luciferase activity in the IL-2 Luc assay. The measured values are used to calculate the normalised IL2LA (nIL2LA) and IFNLA (nIFNLA), which is the ratio of IL2LA and IFNLA to GAPLA, respectively, and the inhibition of GAPLA (Inh-GAPLA), which is the ratio of the arithmetic means of quadruple-measured values of the GAPLA of 2H4 cells treated with a test chemical and the values of the GAPLA of untreated 2H4 cells, and used as an indicator for cytotoxicity. The % suppression, calculated as shown in the table, indicates the effect of tested chemicals on IL-2 and IFN-γ promoter.

Table 1. Description of terms associated with the luciferase activity in the IL-2 Luc assay

| Abbreviations | Definition |
|---|---|
| IL2LA | SLG luciferase activity reflecting IL-2 promoter activity |
| IFNLA | SLO luciferase activity reflecting IFN-γ promoter activity |
| GAPLA | SLR luciferase activity reflecting GAPDH promoter activity |
| nIL2LA | IL2LA / GAPLA |
| nIFNLA | IFNLA / GAPLA |
| Inh-GAPLA | GAPLA of 2H4 treated with chemicals / GAPLA of untreated cells |
| % suppression | (1-(nIL2LA of 2H4 treated with chemicals) / (nIL2LA of non-treated 2H4)) x 100 |
| CV05 | The lowest concentration of the test chemical at which Inh-GAPLA becomes <0.05. |

14. The IL-2 Luc assay can simultaneously examine GAPLA and IL2LA. GAPDH mRNA is ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real time polymerase chain reaction in several experimental systems because its expression is constant at different times and after experimental manipulation (23)(24)(25). In addition, the lead laboratory demonstrated that Inh-GAPLA is more sensitive in detecting dying cells than the percentage of propidium iodide (PI)-excluding cells and that cells showing Inh-GAPLA $\geq$ 0.05 maintain more than 75% of the PI-excluding cells. (26). Therefore, the results were evaluated using only data obtained in the concentration at which Inh-GAPLA is $\geq$ 0.05.

## DEMONSTRATION OF PROFICIENCY

15. Prior to routine use of the test method described in Test Guideline, laboratories should demonstrate technical proficiency, using nine Proficiency Substances listed in Appendix III in compliance with the Good in vitro Method Practices (27). Moreover, test method users should maintain a historical database of data generated with the reactivity checks and with the positive and solvent/vehicle controls, and use these data to confirm that the reproducibility of the test method in their laboratory is maintained over time.

## PROCEDURE

16. The Standard Operating Procedures (SOP) for the IL-2 Luc assay are available and should be employed when performing the test (28). The following paragraphs provide a description of the main components and procedures of the assay.

### *Preparation of cells*

17. On receipt, 2H4 cells are propagated (2-4 passages) and stored frozen as a homogeneous stock. Cells from this stock can be propagated up to a maximum of 12 passages or a maximum of 6 weeks. The medium used for propagation is the RPMI-1640 culture medium containing 10% foetal bovine serum (FBS), antibiotic/antimycotic solution (100 U/mL of penicillin G, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B in 0.85% saline) (e.g. GIBCO Cat#15240-062), 0.15 µg/mL Puromycin (e.g. CAS:58-58-2), 300 µg/mL G418 (e.g. CAS:108321-42-2) and 200µg/mL hygromycin B (e.g. CAS:31282-04-9).

18. Prior to use for testing, the cells should be qualified by conducting a reactivity check. This check should be performed 1-2 weeks or 2-4 passages after thawing, using the positive controls, dexamethasone (100 μg/mL) (CAS:50-02-2, ≥ 98% purity) and cyclosporine A (100 ng/mL) (CAS:59865-13-3, ≥ 95% purity). Dexamethasone and cyclosporine A should produce a positive response to % suppression (≥35). Only cells that pass the reactivity check are used for the assay. The check should be performed according to the procedures described in paragraphs 26.

19. For testing, 2H4 cells are seeded at a density of 1 to 3 × $10^5$ cells/mL, and pre-cultured in culture flasks for 72 to 96 hours. On the day of the test, cells harvested from the culture flask are washed with RPMI-1640 containing 10% FBS without any antibiotics, and then, resuspended with RPMI-1640 containing 10% FBS without any antibiotics at 4 × $10^6$ cells/mL. Then, cells are distributed into a 96-well flat-bottom black plate (e.g. Coring Costar Cat#3603) with 50 µL (2 × $10^5$ cells/well).

### *Preparation of the test chemical and control substances*

20. The test chemical and control substances are prepared on the day of testing. For the IL-2 Luc assay, the test chemical is dissolved in distilled water or dimethyl sulfoxide (DMSO) (stock solution). The test chemical is first dissolved in distilled water.

- If the chemical is soluble at 25 mg/mL, add up to 1 ml of distilled water to 0.050 g of the test chemical in a volumetric flask. If the test chemical is not soluble at 50 mg/mL, 25 mg/mL is the highest soluble concentration. If the test chemical is soluble at 50 mg/mL, add up to 1 ml of distilled water to 0.100 g of the test chemical in a volumetric flask. If the test chemical is not soluble at 100 mg/mL, 50 mg/mL is the highest soluble concentration.
- If the test chemical is not soluble at 25 mg/ml in distilled water, the test chemical is dissolved in DMSO at 500 mg/mL. If the test chemical is not soluble at 500 mg/mL, the highest concentration is determined as the maximum dissolved concentration after dilution with DMSO at a dilution factor of 2 (see scheme in Appendix IV). Sonication and vortex may be used if needed. Centrifuge at 15,000 rpm (≈20,000 x g) for 5 minutes and confirm that it is soluble by the absence of precipitates. The test chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

21. The first test run is aimed at determining the cytotoxic concentration and examining the immunotoxic potential of chemicals. Serial dilutions of distilled water or DMSO stock solutions of the test chemicals are made at a dilution factor of 2 (see Appendix IV) using a 96-well round-bottom clear plate. When the chemical is prepared in distilled water, dilute 20 µL of the diluted stock solution further with 480 µL of medium in a 96-well assay block (e.g. Coring Costar Cat#3960) and add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate. When the chemical is prepared in DMSO, dilute 10 µL of the diluted stock solution with 90 µL of the medium in a 96-well round-bottom clear plate, then, dilute 10 µL of the diluted solution with 490 µL of the medium in a 96-well assay block and then, add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate.

22. Thus, when the chemical is prepared as 100 mg/mL distilled water solution, the final concentrations of the test chemicals range from 0.004 to 2 mg/mL, and when the chemical is prepared as 500 mg/mL DMSO solution, the final concentrations of the test chemicals range from 0.001 to 0.5 mg/mL (Appendix IV).

23. In subsequent test runs (i.e., the second, third, and fourth run or replicate), the distilled water stock solution or the DMSO stock solution is made at the concentration 100 times or 2000 times higher than the concentration of cell viability 05 (CV05; the lowest concentration at which the Inh-GAPLA becomes <0.05) in the first run, respectively. If Inh-GAPLA does not decrease below 0.05 at the any concentration in the first run, the concentration of the stock solution in subsequent test runs is same as that of the first run.

24. Each concentration of test chemical should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 6 hours at 37°C and 5% $CO_2$.

25. After 1-hour incubation with the test chemical, the cells are stimulated with 25 nM PMA and 1 µM Io for 6 hours. For example, x10 PMA/ionomycin solution is made by diluting 2 mM PMA DMSO solution and 2 mM ionomycin ethanol solution using the medium, and 10 µL of the x10 solution PMA/ionomycin is added to 90 µL of the cell suspension containing the test chemical. Subsequently, the luciferase activity is measured as described in following paragraph 30.

26. The recommended positive controls are dexamethasone and cyclosporine A. For example, add 10 µL of 100 mg/mL dexamethasone DMSO solution or 10 µL of 100 µg/mL cyclosporine A DMSO solution to 90 µL of the medium in a 96-well round-bottom clear plate, dilute 10 µL of the diluted solution with 490 µL of the medium in a 96-well assay block and then, add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate. The final concentrations of dexamethasone and cyclosporine A are 100 µg/mL and 100 ng/mL, respectively. Each concentration of the positive control should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 1 hour at 37°C and 5% $CO_2$. The cells are stimulated with 25 nM PMA and 1 µM Io for 6 hours and subsequently the luciferase activity is measured as described in paragraph 30-33.

27. The solvent and negative control is RPMI-1640 containing 10% FBS containing 2% of distilled water or 1% DMSO. Other suitable positive or negative controls may be used if historical data are available to derive comparable run acceptance criteria.

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

29. The test chemicals and solvent control require 2 to 4 runs to derive data evaluation and a prediction model (see paragraph 35 and 36). Each run is performed on a different day with fresh stock solution of test chemicals and independently harvested cells. Cells may come from the same passage.

### *Luciferase activity measurements*

30. Luminescence is measured using a 96-well microplate luminometer equipped with optical filters, e.g., Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany) or the ARVO series (PerkinElmer, Waltham, MA, USA). Examples of the optical filters are sharp-cut (long-pass or short-pass) filters or band-pass filters. The luminometer can be qualified to ensure reproducibility by light emitting diode (LED) reference light source (29).

31. Prior to testing, the transmission coefficients of the filters to discriminate each bioluminescence signal colour should be determined using recombinant green, orange and red emitting luciferases (30), per Appendix II.

32. One hundred µL of pre-warmed Tripluc® Luciferase assay reagent (Tripluc) is transferred to each well of the plate containing the cell suspension treated with or without chemical and with or without PMA/Io. The plate is shaken for 10 min at an ambient temperature of about 20°C. The plate is placed in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters. Justification should be provided for the use of alternative settings, e.g. depending on the model of luminometer used.

33. Parameters for each concentration are calculated from the measured values, e.g., IL2LA, GAPLA, nIL2LA, Inh-GAPLA, the mean ±SD of IL2LA, the mean ±SD of GAPLA, the mean ±SD of nIL2LA, the mean ±SD of Inh-GAPLA, and % suppression using the excel-based spreadsheet available for the IL-2 Luc assay (see https://www.oecd.org/env/ehs/testing/section4software.htm). Definitions and calculations of the parameters

used in this paragraph, i.e. luciferase activity and suppression index are provided in Appendices II and V, respectively.

## DATA AND REPORTING

### *Data evaluation*

34. In each run, the test chemical is judged positive (immune-suppressive or -stimulatory) when all three following criteria are fulfilled:

1. The mean of % suppression is ≥ 35 (suppressive) or ≤ -35 (stimulatory) and statistically significant. The statistical significance is judged by its 95% confidence interval.
2. The outcome shows two or more consecutive statistically significant results (increase or decrease); alternatively one statistically significant result (increase or decrease) with the same trend for at least 3 consecutive data points (i.e. dose dependent trend); in this case the trend can cross the zero line, but the data point on the other side of the 0 line does not become statistically significant for the opposite effect.
3. The results are judged using only data obtained in the concentration range at which Inh-GAPLA is ≥ 0.05.

Graphs illustrating criteria 2 are available in Appendix V.

In all other cases the test chemical is judged as not active (negative).

### *Prediction model*

35. The runs are repeated until two consistent positive (or negative) runs are obtained. A maximum of three runs is possible. The identification of an immunotoxicant is evaluated by the mean of % suppression and its 95% simultaneous confidence interval.

36. As already described in Paragraph 4, IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. Indeed, IL-2 promotes Th1 and Th2 differentiation, while it also drives Treg differentiation. Therefore, it suggests that the augmentation of IL-2 transcription can lead to either immune stimulation or immunosuppression depending on the surrounding tissue environment *in vivo*. Therefore, in this assay, a test chemical that is either stimulating or suppressing is considered positive.

### *Acceptance criteria*

37. The following test acceptance criterion applies for this test:

if fold induction of nIFNLA of PMA/Io wells without chemicals (= (nIFNLA of 2H4 cells treated with PMA/Io) / (nIFNLA of non-treated 2H4 cells)) results in a value lower than 3.0, then the results obtained from the plate containing the control wells should be rejected.

## TEST REPORT

38. The test report should include the following information:

Test Chemical

- Mono-constituent substance:
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers.
  - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc.
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Concentration(s) tested.
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical if distilled water or DMSO has not been used.

- Multi-constituent substance, UVCB and mixture:
  - Characterisation as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available.
  - Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available.
  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Concentration(s) tested.
  - Storage conditions and stability to the extent available.
  - Justification for choice of solvent/vehicle for each test chemical, if distilled water or DMSO has not been used.

Controls

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

- Treatment prior to testing, if applicable (e.g., warming, grinding);
- Justification for choice of solvent vehicle for each test chemical (if distilled water or DMSO has not been used).
- Concentration(s) tested.
- Storage conditions and stability to the extent available.
- Reference to historical positive control results demonstrating suitable acceptance criteria, if applicable.

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

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

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

Results
- Measurements of IL2LA, IFNLA and GAPLA.
- Calculations for nIL2LA, nIFNLA, Inh-GAPLA and % suppression;

- The 95% confidence interval of % suppression.
- A graph depicting dose-response curves for induction of luciferase activity and viability.
- Description of any other relevant observations, if applicable.
- Discussion of the results
- Discussion of the results obtained with the IL-2 Luc assay.
- Consideration of the assay results in the context of an IATA, if other relevant information is available.

Any modification to the Test Guideline

Conclusion

## LITERATURE


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## APPENDIX I- DEFINITIONS

**2H4:** An IL-2 reporter cell line used in IL-2 Luc assay. The human acute T lymphoblastic leukaemia cell line Jurkat was transfected the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ and GAPDH promoters, respectively.

**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.

**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.

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

**GAPLA:** Luciferase activity of stable luciferase red (SLR) (λmax = 630 nm), regulated by GAPDH promoter and demonstrates cell viability and viable cell number.

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

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

**IFNLA:** Luciferase activity of stable luciferase orange (SLO) (λmax = 580 nm), regulated by interferon (IFN)- γ promoter.

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

**IL-2 (Interleukin-2):** A cytokine derived from T lymphocytes that causes activation of T lymphocytes, B lymphocytes, monocyte and natural killer cells.

**IL2LA:** Luciferase activity of stable luciferase green (SLG) (λmax = 550 nm), regulated by IL-2 promoter.

**Inh-GAPLA:** Inhibition of GAPLA. It is obtained by dividing GAPLA of 2H4 treated with chemicals with GAPLA of non-treated 2H4 and represents cytotoxicity of chemicals.

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

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

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

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

**nIL2LA:** The SLG luciferase activity reflecting IL-2 promoter activity (IL2LA) normalised by the SLR luciferase activity reflecting GAPDH promoter activity (GALPA). It represents IL-2 promoter activity after considering cell viability or cell number.

**nSLG-LA:** Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to nIL2LA. See nIL2LA for definition.

**nSLO-LA:** Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to nIFNLA. See nIFNLA for definition.

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

**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.

**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.

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

**SLG-LA:** Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to IL2LA. See IL2LA for definition.

**SLO-LA:** Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to IFNLA. See IFNLA for definition.

**SLR-LA:** Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to GAPLA. See GAPLA for definition.

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

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

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

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

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

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

# APPENDIX II - PRINCIPLE OF MEASUREMENT OF LUCIFERASE ACTIVITY AND DETERMINATION OF THE TRANSMISSION COEFFICIENTS OF OPTICAL FILTER FOR SLG, SLO AND SLR

Multi Reporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-colour detection system, which can equip at least two kinds of optical filters (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). Examples of the optical filters used in measurement are a 560 nm long-pass filter and a 600 nm long-pass filter.

(1) Measurement of three-color luciferase with two optical filters.

This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 560 nm long-pass (LP) filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

First, using recombinant luciferase enzyme of SLG ($\lambda$max = 550 nm), SLO ($\lambda$max = 580 nm) and SLR ($\lambda$max = 630 nm), measure i) the intensity of light without filter (all optical), ii) the intensity of 560 nm LP (Filter 1) transmitted light iii) the intensity of 600 nm LP (Filter 2) transmitted light, and calculate the transmission coefficient factor listed below.

Table. Definition of the parameters in the luciferase assay

| Transmission coefficient factor | | Abbreviation | Definition |
|---|---|---|---|
| SLG | Filter 1 transmittance factor | $\kappa G_{R56}$ | The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical) |
| | Filter 2 transmittance factor | $\kappa G_{R60}$ | The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical) |
| SLO | Filter 1 transmittance factor | $\kappa O_{R56}$ | The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO |

| | | | |
|---|---|---|---|
| | | | without filter (all optical) |
| | Filter 2 transmittance factor | $\kappa O_{R60}$ | The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical) |
| SLR | Filter 1 transmittance factor | $\kappa R_{R56}$ | The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical) |
| | Filter 2 transmittance factor | $\kappa R_{R60}$ | The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical) |

When the intensity of SLG, SLO and SLR in test sample are defined as G, O and R, respectively, i) the intensity of light without filter (all optical): F0, ii) the intensity of 560 nm LP (Filter 1) transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$F0=G+O+R$

$F1=\kappa G_{R56} \times G + \kappa O_{R56} \times O + \kappa R_{R56} \times R$

$F2=\kappa G_{R60} \times G + \kappa O_{R60} \times O + \kappa R_{R60} \times R$

These formulas can be rephrased as follows.

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Then using calculated transmission coefficient factors and measured F0, F1 and F2, you can calculate G, O and R-value as follows.

$$\begin{pmatrix} G \\ O \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix}$$

*Materials and methods for determining transmittance factor*

(1) Reagents

· Single purified recombinant luciferase enzymes:

Lyophilised purified SLG enzyme

Lyophilised purified SLO enzyme

Lyophilised purified SLR enzyme

(which for the validation work were obtained from Tottori Bioscience Promotion Organization, Tottori, Japan with 2H4 cell line)

· Assay reagent:

Tripluc® Luciferase assay reagent (for example from TOYOBO Cat#MRA-301)

· Medium: RPMI-1640 with 10% FBS for luciferase assay (30 ml, stored at 2 – 8°C)

(2) Preparation of enzyme solution

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

ice to prevent deactivation.

(3) Bioluminescence measurement

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilise. Transfer 100 µL of the diluted enzyme solution to a black 96 well plate (flat bottom) (the SLG reference sample to #B1, #B2, #B3, the SLO reference sample to #D1, #D2, #D3, the SLR reference sample to #F1, #F2, #F3). Then, transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the diluted enzyme solution using a pipette. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles from the solutions in wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filter.

Transmission coefficient of the optical filter was calculated as follows:

Transmission coefficient (SLG ($\kappa G_{R56}$)) = (#B1 of F1+ #B2 of F1+ #B3 of F1) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLO ($\kappa O_{R56}$)) = (#D1 of F1+ #D2 of F1+ #D3 of F1) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

Transmission coefficient (SLR ($\kappa R_{R56}$)) = (#F1 of F1+ #F2 of F1+ #F3 of F1) / (#F1 of F0+ #F2 of F0+ #F3 of F0)

Transmission coefficient (SLG ($\kappa G_{R60}$)) = (#B1 of F2+ #B2 of F2+ #B3 of F2) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLO ($\kappa O_{R60}$)) = (#D1 of F2+ #D2 of F2+ #D3 of F2) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

Transmission coefficient (SLR ($\kappa R_{R60}$)) = (#F1 of F2+ #F2 of F2+ #F3 of F2) / (#F1 of F0+ #F2 of F0+ #F3 of F0)

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

*Quality control of equipment*

The procedures described in the IL-2 Luc protocol should be used (JaCVAM, 2020c).

# APPENDIX III - PROFICIENCY SUBSTANCES

Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the 9 Proficiency Substances listed in this Appendix in compliance with the Good in vitro Method Practices (1). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 18) and with the positive and solvent/vehicle controls (see paragraphs 26-27), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

1. OECD (2017), Draft Guidance document: Good *In Vitro Method Practices (GIVIMP) for the Development and Implementation of In Vitro €i0Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris.*

Table 1: Recommended substances for demonstrating technical proficiency with the IL-2_Luc assay

| No. | Chemical name | CAS No. | T cell targeting | Physical state | Reference range (µg/mL) CV05[1] | Reference range (µg/mL) MIT[2] |
|---|---|---|---|---|---|---|
| 1 | Dexamethasone | 50-02-2 | Yes | Solid | >2000 | 16-63 |
| 2 | Cyclosporine | 59865-13-3 | Yes | Solid | >1 | 0.002-0.006 |
| 3 | Lead(II) acetate trihydrate | 6080-56-4 | Yes | Solid | >2000 | 31-63 |
| 4 | Indomethacin | 53-86-1 | Yes | Solid | 500-2000 | 16-63 |
| 5 | Perfluorooctanoic acid | 335-67-1 | Yes | Solid | 250-1000 | 8-31 |
| 6 | Tributyltin chloride | 1461-22-9 | Yes | Liquid | 0.5-1.0 | 0.12-0.24 |
| 7 | Zinc dimethyldithiocarbamate (DMDTC) | 137-30-4 | No | Solid | 1-4 | >2000 |
| 8 | Mannitol | 69-65-8 | No | Solid | >2000 | >2000 |
| 9 | Acetonitril | 75-05-8 | No | Liquid | >2000 | >2000 |

Abbreviations: CAS no. = Chemical Abstracts Service Registry Number

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

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

# APPENDIX IV – TEST CHEMICALS DISSOLUTION IN THE IL-2 LUC ASSAY.

Fig. 1 Dissolution by vehicle

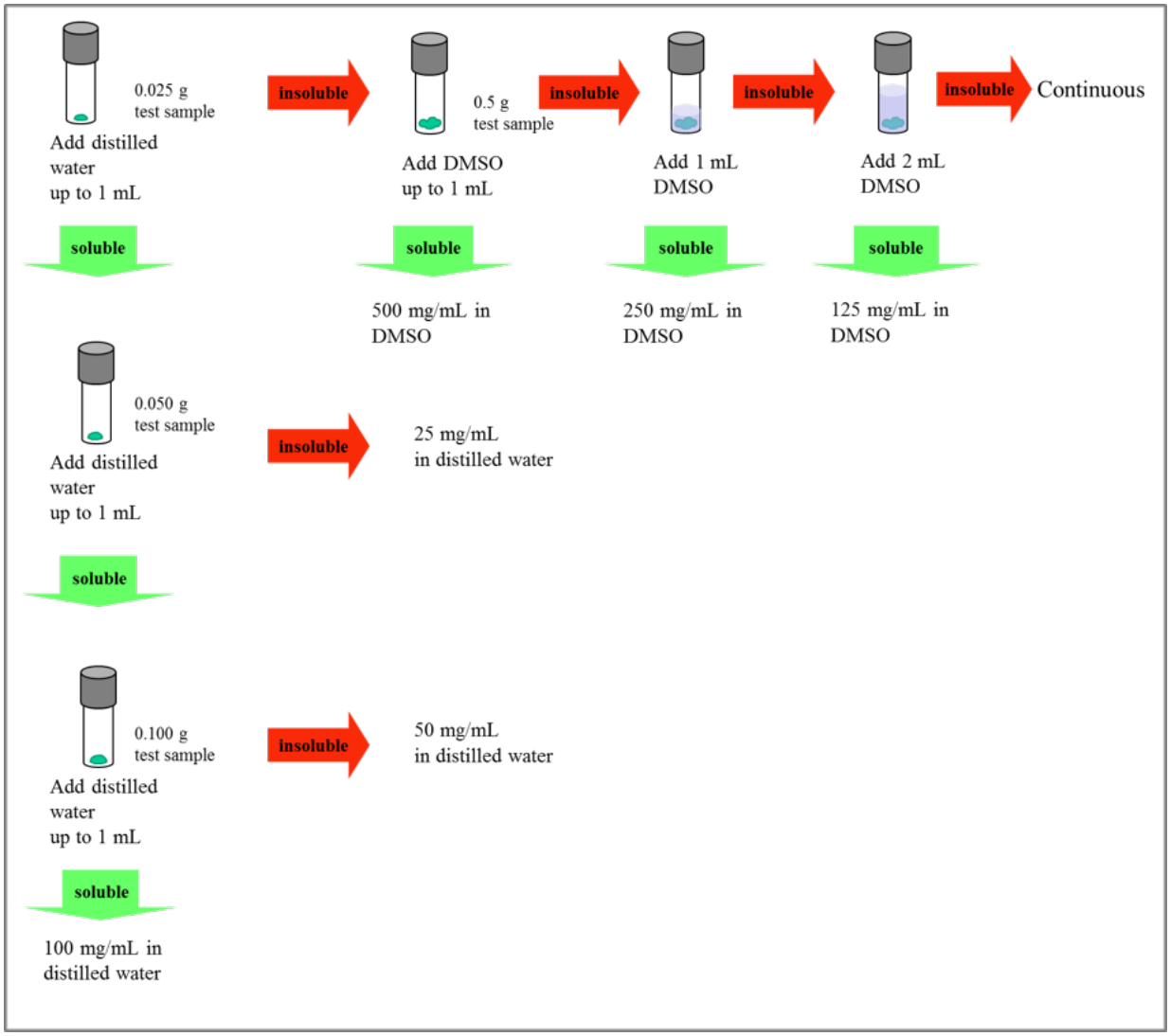


Fig. 2 The scheme of the procedure when the chemical is prepared in distilled water at 100 mg/mL.

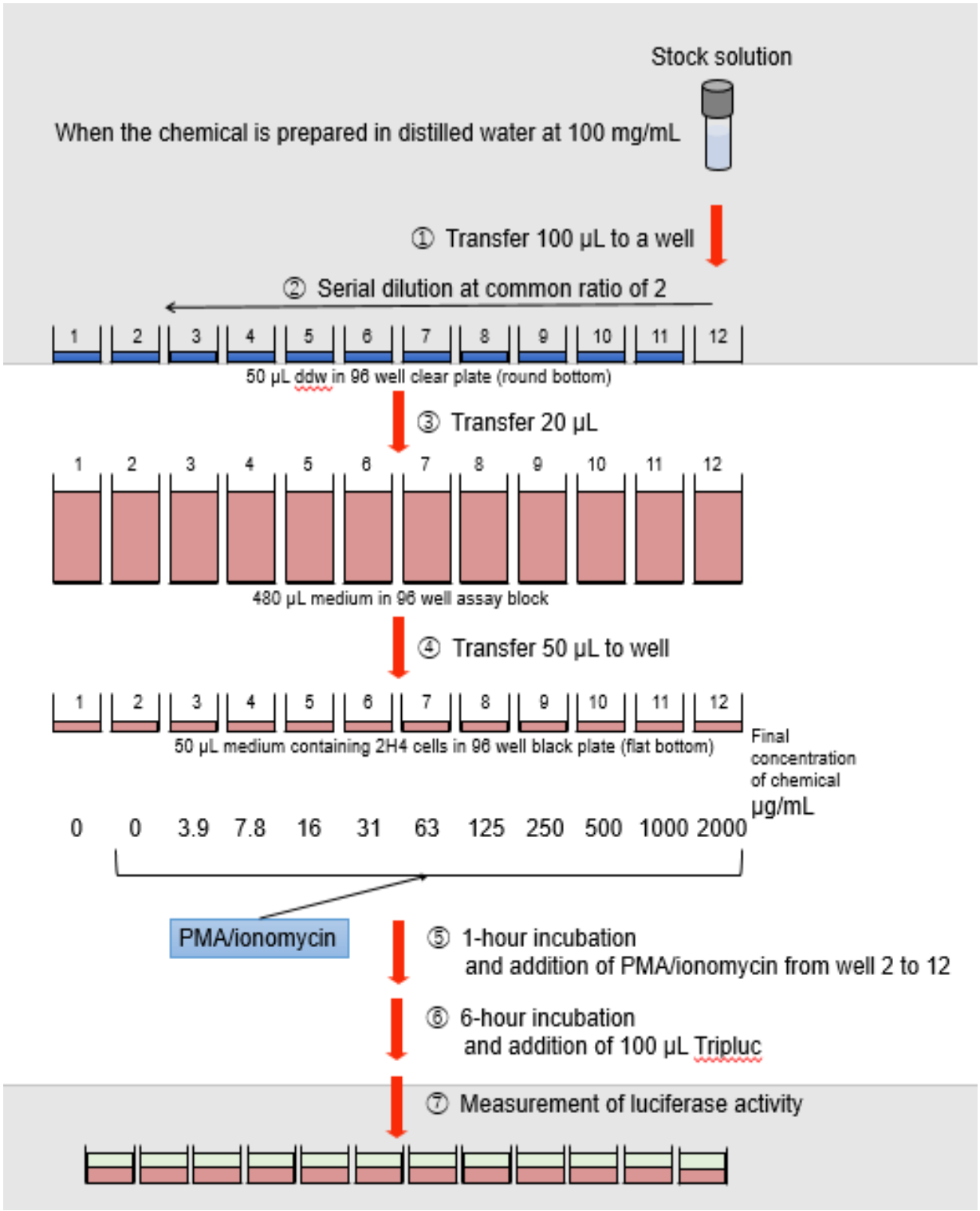


Fig. 3 The scheme of the procedure when the chemical is prepared in DMSO at 500 mg/mL.

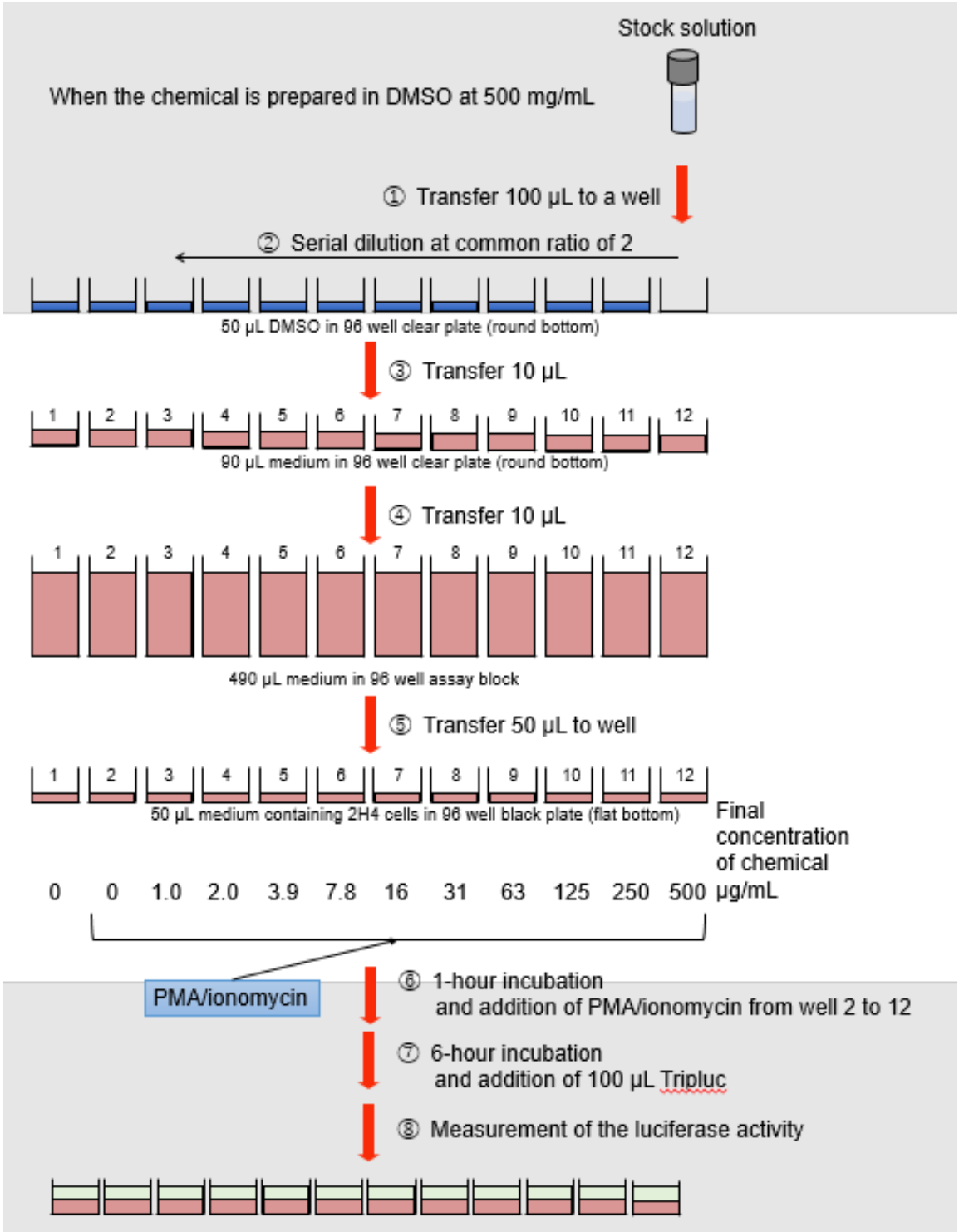
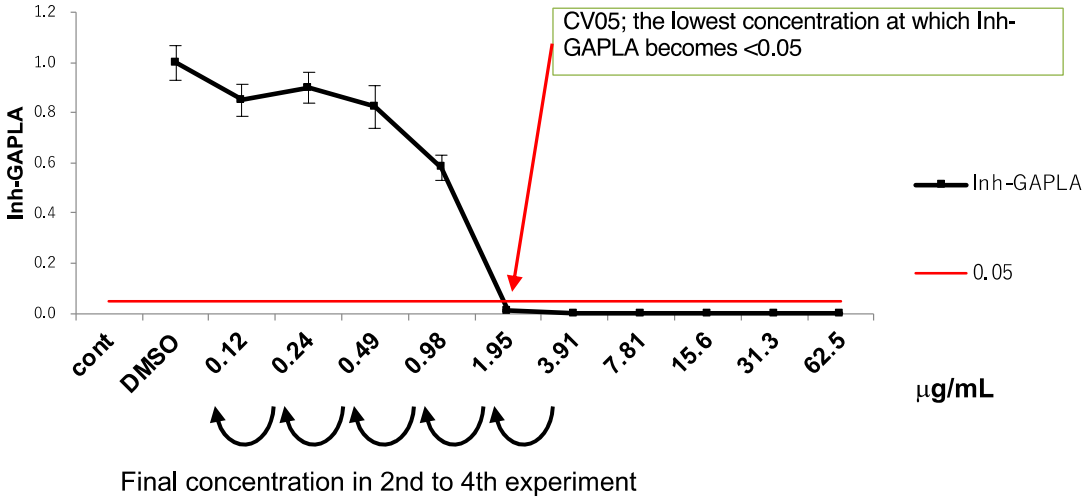


Fig. 4 Determination of the concentration of the test chemicals in subsequent test runs (i.e., the second, third, and fourth experiment)



# APPENDIX V – CALCULATION OF THE SUPPRESSION INDEXES

### *IL2LA*

The j-th repetition (j= 1 to 4) of the i-th concentration (i = 0 to 10) is measured for IL2LA and GAPLA respectively. The normalized IL2LA is referred as nIL2LA, and is defined as

$nIL2LA_{ij} = IL2LA_{ij} / GAPLA_{ij}$

This is the basic unit of measurement in this assay.

#### % suppression

The % suppression is an index for the averaged nIL2LA for the repetition on the i-th concentration compared with it on the 0 concentration, it is the primary measure of this assay. The % suppression is able to write by the following formula,

$$\%\ \text{suppression}_{\text{i}} = \left\{1 - \frac{\left(\frac{1}{4}\right)\sum_{\text{i}} \text{nIL2LA}_{\text{ij}}}{\left(\frac{1}{4}\right)\sum_{\text{i}} \text{nIL2LA}_{0\text{j}}}\right\} \times 100 \qquad (1)$$

The lead laboratory has proposed that ±35 of the value suggests suppressive and stimulatory for a tested chemical. This value is based on the investigation of the historical data of the lead laboratory. Data management team followed to use the value through all the phase of present validation study.

The primary outcome measure, % suppression, is basically the ratio of 2 arithmetic means of nIL2LA as shown in equation (1). The 95% confidence interval (95% CI) of the % suppression for the i-th concentration can be estimated.

The lower limit of the 95% CI above 0 is interpreted as that the nIL2LA with the i-th concentration is statistical-significantly greater than it with the 0-concentration, whereas the upper limit of the 95% CI blow 0 is interpreted as that the nIL2LA with the i-th concentration is statistical-significantly lesser than it with the 0-concentration.

There are several ways to construct the 95% CI. We used the method kwon as the Delta method in this study. This 95% confidence interval theorem is obtained from the following formula.

$$\%\text{suppression} \pm 100 \times \left\{ z_{0.975} \times \sqrt{\frac{\text{sd}_{\text{i}}^2}{\text{mean}_0^2} + \frac{\text{mean}_{\text{i}}^2 \times \text{sd}_0^2}{\text{mean}_0^4}} \right\},$$

where $\text{mean}_{\text{i}}$ is the mean of nIL2LA at the i-th concentration, $\text{mean}_0$ is the mean of nIL2LA at 0 concentration, $\text{sd}_{\text{i}}$ is the standard deviation of nIL2LA at the i-th concentration and $\text{sd}_0$ is the standard deviation of nIL2LA at 0 concentration. $z_{0.975}$ is 97.5 percentile of the standard normal distribution.

### *Inh-GAPLA*

The Inh-GAPLA is a ratio of the averaged GAPLA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

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

Since the GAPLA is the denominator of the nIL2LA, the extremely smaller value of this is considered to cause the large variation of the nIL2LA. Therefore, the i-th %suppression value with extremely smaller value of the Inh-GAPLA might be poor precision.

### *Judgment for "Suppressive", "Stimulatory" or "No effect" in each experiment*

Criteria to judge a positive effect (either suppressive or stimulatory) are provided in the section "Data evaluation", paragraph 34, and illustrated through Figure 1 below, extracted from Appendix 17 of the validation report.

Paragraph 34 says that an experiment is judged positive when all three following criteria are fulfilled:

1. The mean of % suppression is $\geq$ 35 (suppressive) or $\leq$ -35 (stimulatory) and statistically significant. The statistical significance is judged by its 95% confidence interval.
2. The outcome shows two or more consecutive statistically significant results (increase or decrease); alternatively one statistically significant result (increase or decrease) with the same trend for at least 3 consecutive data points (i.e. dose dependent trend); in this case the trend can cross the zero line, but the data point on the other side of the 0 line does not become statistically significant for the opposite effect.
3. The results are judged using only data obtained in the concentration range at which Inh-GAPLA is $\geq$ 0.05.

The following four representative graphs that are judged as positive (either suppressive or stimulatory).

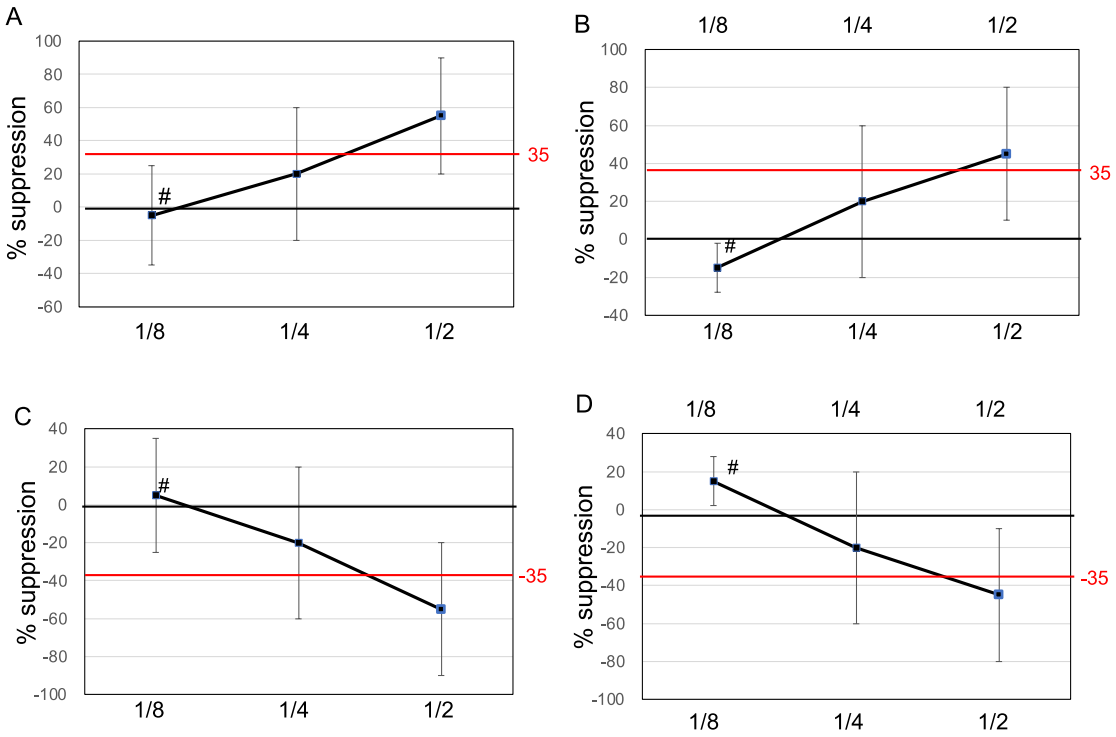



Fig. 1. Four patterns showing one positive data point (increase or decrease) with the same trend for at least 3 consecutive data points

The x-axis represents the chemical concentration and the y-axis represents % suppression. Each plot is the % suppression value from quadruple assays with 95% confidence intervals. The red lines indicate 35 and -35, respectively. All graphs show one positive data point (increase or decrease) with the same trend for at least 3 consecutive data points (i.e. concentration dependent trend) and the trends cross the zero line. A and C are judged as suppression and stimulation, respectively, because the 95% confidence interval of the data point indicating the opposite response (#) crosses 0. In contrast, B and D are judged negative because the 95% confidence interval of the data point indicating the opposite response (#) does not cross 0.