Proposal dated 16 November 2021

DRAFT PROPOSAL FOR THE UPDATE OF APPENDIX II of TG 442C BASED ON PROJECT PROPOSAL 4.141 OF THE TEST GUIDELINE PROGRAMME WORKPLAN

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 cysteine and lysine derivatives 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 performed coordinated by the Japanese center for validation of alternative methods (JaCVAM) (4) (5) (6) (7) (8) (9) (10). Co-elution occurring during examination of the sample after the reaction using the conventional ultraviolet (UV) detector (11) (12) can be prevented by a combined measure using a fluorescence (FL) detector; thus, the depletion values obtained by simultaneous measurement using the 2 detectors were also verified (9) (10). Within-laboratory reproducibility (WLR) and between-laboratory reproducibility (BLR) of ADRA were 100% each measured using both the UV detector and the FL detector (9) (10). Prediction of skin sensitisation for local lymph node assay (LLNA) from other published studies indicated that ADRA identified sensitisers and non-sensitisers with an accuracy of 76 % (104/136, 103/136), a sensitivity of 76% (74/98) and/or 74% (73/98), and a specificity of 79% (30/38, 30/38) (8). In addition, the prediction of skin sensitisation for the human to 80 chemicals indicated that ADRA has an accuracy of 84% (67/80) and/or 83% (66/80), a sensitivity of 83% (48/58) and/or 81% (47/58), and a specificity of 86% (19/22, 19/22) (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 test as well as other animal tests may not fully reflect the situation in the species of interest, which is 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. 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 ADRA 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. 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). 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 paragraphs 27 and 28); it may be possible to detect and quantify any NAC dimer formed by 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).

4. The ADRA test method allows testing of poorly soluble chemicals (15). 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.

5. In general, many organic compounds absorb UV in the range of 220 nm. In the case of co-elution of the nucleophilic reagent and the test chemical, this might result in false negative prediction. This may happen with the DPRA which specifies that quantification of the peptide-based nucleophilic reagents has to be performed at 220 nm. In contrast to this, the nucleophilic reagents used in ADRA are quantified at 281 nm. 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 (16). Furthermore, NAC and *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (NAL) have FL intensity, and thus, they can be detected using a FL detector (11) (12). Since components other than nucleophiles rarely have FL intensity, it is often possible to avoid co-elution by using a FL detector.

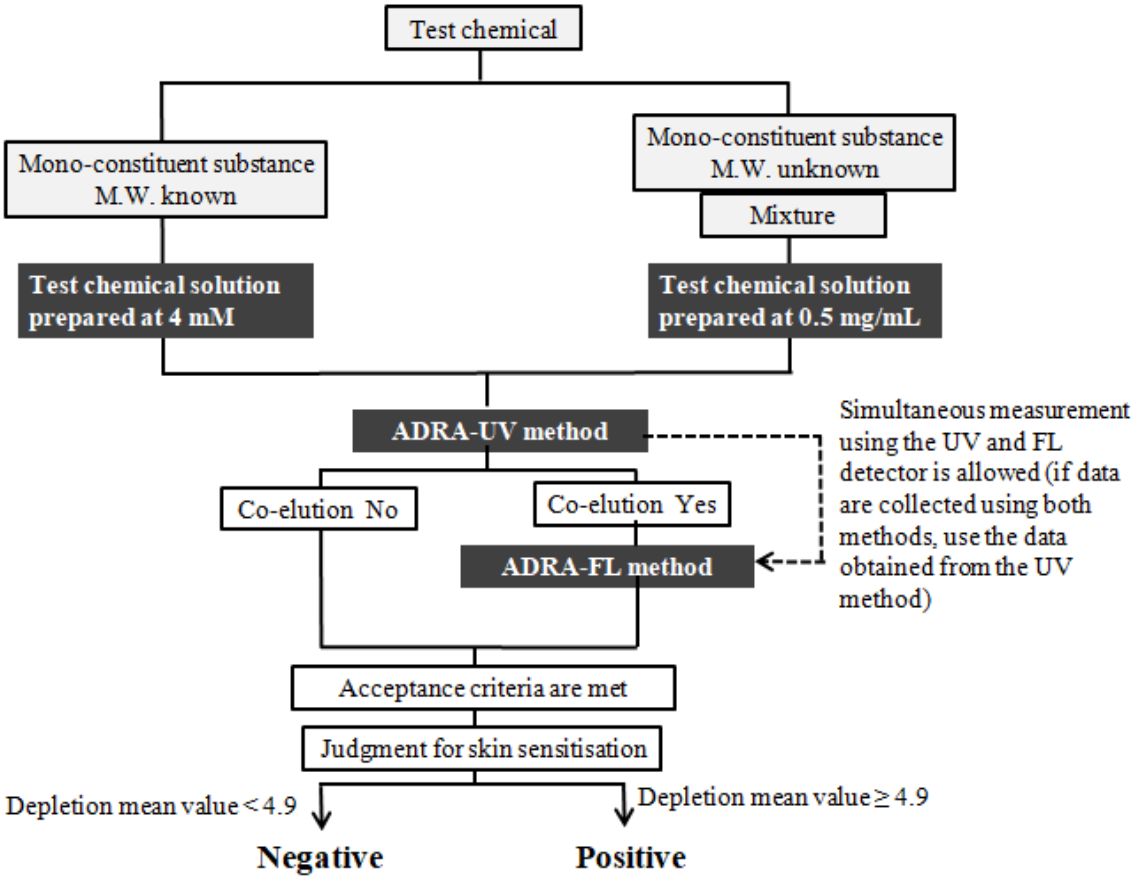
6. When assessing the sensitisation for a test chemical by using ADRA, if the test chemical is a single component with a known molecular weight, ADRA should be performed using a 4 mM chemical solution; if the test chemicals is a mixture, ADRA should be performed using a chemical solution prepared at 0.5 mg/mL regardless of whether components of the mixture are known or not (See paragraph 15). In case of co-elution, which is likely to happen with chemicals such as mixtures of unknown or variable composition, complex reaction products, or biological materials (UVCB), a combination

of conventional ultraviolet (UV) detector and fluorescence (FL) detector should be used (see Figure 1 and paragraph 18).

7. 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

8. ADRA is an *in chemico* test method that quantifies residual concentrations 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, 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 high-performance liquid chromatography (HPLC) using UV detection (optical density, 281 nm) possibly in combination with FL detection (excitation/emission [Ex/Em], 284/333 nm) with gradient elution (see paragraph 18). Percent depletion values are then calculated for both NAC and NAL and compared to a prediction model (see paragraph 26). Figure 1 provides a flowchart for assessment of sensitisation using ADRA.



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

Figure 1: Procedure for skin sensitisation using amino acid derivative reactivity assay

9. Prior to routine use of the method described in this test method, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 1 of this Appendix.

PROCEDURE

10. 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 Annex 1 of this Appendix.

Quality of NAC and NAL

11. 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. Residual levels of NAC and NAL are to be a minimum of 90% in either case (17). The residual level of NAC is calculated as a percentage of the sum of NAC and the residual level of NAC dimers.

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances given in Annex 1 and should satisfy the requirement given therein.

Preparation of the NAC and NAL stock solution

12. 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 solution is 5 μM in pH 8.0 phosphate buffer, and the final concentration of the NAL solution is 5 μM in pH 10.2 phosphate buffer.

Preparation of the test chemical solution

13 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. 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. 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. 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 solution.

14. Mono-constituent substances of unknown molecular weight should be tested in a test chemical solution at a concentration of 0.5 mg/mL rather than 4 mM (7) (See Figure1). Polymers which are well characterised should also be tested at a concentration of 0.5 mg/mL. If the results of ADRA between the monomer and the polymer are not concordant, the judgement for sensitisation of the polymer needs to be considered.

15. Mixtures comprising multiple known or unknown substances should be dissolved at 0.5 mg/mL in an appropriate solvent (See paragraph 13). For a solution of the mixture using any solvent, the concentration of the mixture should be adjusted at 0.5 mg/mL on the basis of the weight of the total components, excluding the solvent (See Figure 1). If a solvent other than the appropriate solvent for ADRA is used for the mixture, it is necessary to confirm that the solvent itself has no potential of sensitisation.

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

16. 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. Phenylacetaldehyde should not be stored for a long time after opening since it may be polymerised and oxidised; it is recommended that

it is purchased as appropriate while paying attention to the NAC and NAL depletion. Squaric acid diethyl ester should be stored away from high temperature or humidity, since it may be hydrolysed. 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, so they can be 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 Annex 2). 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

17. Both the NAC and the NAL solutions should be incubated with the test chemical at 1:200 ratio in a 96-well microplate. 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 should be interpreted with due care (see also paragraph 4 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 as a fixing solution to stop the reaction (3).

HPLC preparation and analysis

18. The test chemical should be measured with ADRA-UV and, in case of co-elution, in combination with ADRA-FL measurement. Although a mixture of substances such as unknown or variable composition, complex reaction products, or biological materials (UVCB) substances is likely to cause co-elution with various components in the mixture, ADRA-FL using a FL detector can detect only NAC and NAL in the mixture (11). There are two options for measurement with ADRA. Successive measurement should be started with ADRA-UV and ADRA-FL was allowed to be used if inconclusive due to co-elution. Simultaneous measurement by connecting the UV and FL detector to the HPLC system may be easier to put in place and is allowed in all cases although not required in cases, co-elution is determined afterwards. If no co-elution, only UV data is used. If inconclusive due to co-elution, FL data will be used (see Figure 1).

19. 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).

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. Serial dilution of the NAC and NAL stock solutions (5.0 μM) will be used to prepare six calibration solutions in concentrations from 5.0 to 0.156 μM as well as a blank of the dilution buffer. Suitable calibration curves should have an $R^2 > 0.990$.

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 18). The appropriate column is installed in the HPLC system. The recommended HPLC set-up described in the validated protocol uses a column (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 Annex 1. 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 Annex 2.

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 have FL intensity, it cannot be detected by

using ADRA-FL. Any apparent dimerisation should be noted, since overestimation of NAC depletion could result in false-positive predictions (See paragraphs 26 and 27).

DATA AND REPORTING

Data evaluation

23. The concentration of both NAC and NAL is determined at optical density (OD), 281 nm (UV detector) and if needed Ex/Em, 284/333 nm (FL detector) (see paragraph 18) 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 Annex 2) according to the formula described below.

$$\text{Percent NAC or NAL depletion} = \left[1-\left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}}\right]\right] \text{x}100$$

Acceptance criteria

25. The following criteria should be met:

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)

Both phenylacetaldehyde and squaric acid diethyl ester: < 10%, for both NAC and NAL depletion,

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.

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.

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 3 and 5.

28. 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. 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. 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 Annex 1. 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. 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 and in accordance with the provisions of paragraphs 3 and 5.

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.

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

(1) Fujita M, 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. DOI: 10.1016/j.vascn.2014.06.001.

(2) Yamamoto Y, 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, 35:1348-60. DOI: 10.1002/jat.3139.

(3) Fujita M, 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, Journal of Applied Toxicology, 39,191-208. DOI: 10.1002/jat.3707.

(4) Fujita M, Yamamoto Y, Watanabe S, Sugawara T, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kurokawa Y, Kawakami T, Kojima K, Sozu T, Nakayama T, Kusao T, Richmond J, Kleinstreuer N, Kim BH, Kojima H, Kasahara T, Ono A, (2019), The within- and between-laboratory reproducibility and predictive capacity of the *in chemico* Amino acid Derivative Reactivity Assay (ADRA): Results of validation study implemented in four participating laboratories, Journal of Applied Toxicology, 39, 1492-1505. DOI: 10.1002/jat.3834.

(5) OECD (2019), Validation report: Amino acid Derivative Reactivity Assay (ADRA) – JaCVAM Validation Study Report. Series on testing and Assessment n° 304. Organisation for Economic Cooperation and Development, Paris.

(6) OECD (2019), Amino acid Derivative Reactivity Assay (ADRA) – Report of the Peer Review Panel. Series on testing and Assessment n° 305. Organisation for Economic Cooperation and Development, Paris.

(7) Yamamoto Y, Fujita M, Wanibuchi S, Katsuoka Y, Ono A, Kasahara T.. (2019), Expanding the applicability of the Amino acid Derivative Reactivity Assay (ADRA): Determining a weight concentration for preparation of test chemical solutions that yields a predictive capacity identical to the conventional method using molar concentration and demonstrating the capacity to detect sensitizers in liquid mixtures. Journal of Pharmacological and Toxicological Methods. 97, 67-79. DOI: 10.1016/j.vascn.2019.01.001

(8) Imamura M, Wanibuchi S, Yamamoto Y, Kojima H, Ono A, Kasahara T, Fujita M. (2021) Improving predictive capacity of the Amino acid Derivative Reactivity Assay test method for skin sensitisation potential with an optimal molar concentration of test chemical solution. Journal of Applied Toxicology, 41, 303-329. doi: 10.1002/jat.4082.

(9)Yamamoto Y, Watanabe S, Yamaga H, Yoshida K, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Takeuchi K, Kamiya K, Kojima K, Kawakami T, Sozu T, Wanibuchi S, Fujita M. Kasahara T, Ono, A, Kojima H (2021). The within- and between-laboratory

reproducibility and predictive capacity of the in chemico ADRA (0.5 mg/mL): Results of validation study implemented in five participating laboratories. (Submitting)

(10) Fujita M, Yamamoto Y, Watanabe S, Yamaga H, Yoshida K, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Takeuchi K, Kamiya K, Kojima K, Kawakami T, Sozu T, Wanibuchi S, Kasahara T, Ono, A, Kojima H (2021). The within- and between-laboratory reproducibility and predictive capacity of the in chemico amino acid derivative reactivity assay: Results of validation study implemented in five participating laboratories. (Submitting)

(11) Fujita M, Yamamoto Y, Wanibuchi S, Katsuoka Y, Kasahara T A Newly Developed Means of HPLC-Fluorescence Analysis for Predicting the Skin Sensitisation Potential of Multi-Constituent Substances Using ADRA. Toxicology In Vitro, 59, 161-178. DOI: 10.1016/j.tiv.2019.04.014.

(12) Wanibuchi S, Yamamoto Y, Sato A, Kasahara T, Fujita M. (2019) The amino acid derivative reactivity assay with fluorescence detection and its application to multi-constituent substances. Journal of Toxicological Sciences, 44, 821-832. doi: 10.2131/jts.44.821.

(13) Yamamoto Y, Fujita M, Wanibuchi S, Sato A, Akimoto M, Katsuoka Y, Ono A, Kasahara T (2019) Applicability of amino acid derivative reactivity assay for prediction of skin sensitisation by combining multiple alternative methods to evaluate key events. Journal of Toxicological Sciences, 44, 585-600. doi: 10.2131/jts.44.585.

(14) Imamura M, Yamamoto Y, Fujita M, Wanibuchi S, Kojima H, Ono A, Kasahara T, Fujita M. (2021) Applicability of ADRA (4mM) for prediction of skin sensitisation by combining multiple alternative methods to evaluate Key events. Journal of Applied Toxicology. (revising)

(15) Yamamoto Y, Wanibuchi S, Sato A, Kasahara T, Fujita M. (2019) Precipitation of test chemicals in reaction solutions used in the amino acid derivative reactivity assay and the direct peptide reactivity assay. Journal of Pharmacological and Toxicological Methods, 100, 106624. doi: 10.1016/j.vascn.2019.106624.

(16) Fujita M, Yamamoto Y, Wanibuchi S, Katsuoka Y, Kasahara T. (2019) The underlying factors that explain why nucleophilic reagents rarely co-elute with test chemicals in the ADRA. Journal of Pharmacological and Toxicological Methods. 96, 95-105. doi: 10.1016/j.vascn.2019.02.004.

(17) ADRA protocol: JaCVAM Statements. Available at: http://www.jacvam.jp/en_effort/effort02.html

(18) James P. Tam JP, Cui Rong Wu CR, Wen Liu W, Jing Wen Zhang JW. (1991), Disulfide bond formation in peptides by dimethyl sulfoxide. Scope and applications. Journal of the American Chemical Society, 113, 6657–6662. DOI: 10.1021/ja00017a044

(19) Akimoto M, Yamamoto Y, Watanabe S, Yamaga H, Yoshida K, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kamiya K, Kojima K, Kawakami T, Kojima H, Ono A, Kasahara T, Fujita M (2020) Oxidation of a cysteine-derived nucleophilic reagent by dimethyl sulfoxide in the amino acid derivative reactivity assay. J Appl Toxicol., 40, 843-854. DOI: 10.1002/jat.3948.

(20) Natsch A, Ryan CA, Foertsch L, Emter R, Jaworska J, Gerberick F, Kern P. (2013). A dataset on 145 chemicals tested in alternative assays for skin sensitisation undergoing prevalidation. *Journal of Applied Toxicology*, **33**(11):1337-52, DOI:10.1002/jat.2868.

(21) Gerberick GF, Vassalo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin J-P, (2007), Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach, Toxicological Sciences, 97, 417-427. DOI: 10.1093/toxsci/kfm064.

(22) Basketter DA, Scholes EW, (1992), Comparison of the local lymph node assay with the guinea-pig maximization test for the detection of a range of contact allergens, Food and Chemical Toxicology, 30, 65-69.

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

APPENDIX II, ANNEX 1

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

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

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. Negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA	Diethylenetriamine; 111-40-0 (human 1A, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in some cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals that have a UV absorption (OD, 281 nm) or FL intensity (Ex/Em, 284/333 nm) intensity and have the same retention time of the 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 intensity in this range of FL are generally limited to those having a naphthalene ring. 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 27). 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 ubknown 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 test chemicals, which are prepared at a molar concentration of 0.5 mg/mL. When the mixture is a liquid, the evaluation of sensitisation using ADRA cannot be performed if the weight concentration of the mixture components is not known.	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 key events and *in vivo* results of similar substances.	n/a
Test chemicals which cannot be dissolved in an appropriate solvent at a final concentration of 4 mM (although it is unlikely to happen since test chemical solution in ADRA is prepared at a low concentration (4 mM))	Not sure if sufficient exposure can be achieved	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 (although the test chemical is hardly precipitated when the test chemical solution is added to the reaction solution since ADRA is dissolved in a solvent at a low concentration (4 mM))	Not sure if sufficient exposure can be achieved	Test chemicals that precipitate in the reaction solution even if dissolved in the solvent may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	Isopropyl myristate CAS: 110-27-0
Test chemicals that do not covalently bind to the NAC but promote its oxidation (i.e. NAC dimerisation)	Could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions.	It may be possible to detect and quantify any NAC dimer formed by HPLC (UV detector), thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and	DMSO Oxidant

		covalent bonding to the test item substance(s) Therefore, ADRA may prevent erroneous judgement due to the oxidizing action of the test chemical. However, since the NAC dimer does not have FL intensity, it cannot be detected by using HPLC (FL detector).	
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. 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. 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 _4mM prediction[2]	Range of % depletion	
							NAC[3]	NAL[3]
1	*p*-Benzoquinone	106-51-4	Solid	108.09	Sensitiser (extreme)	Positive	90-100	40-70
2	Diphenylcyclopropenone	4886-38-4	Solid	206.24	Sensitiser (strong)	Positive	15-45	≤ 10
3	2-Methyl-2H-isothiazol-3-one	2682-20-4	Solid	115.15	Sensitiser (moderate)	Positive	80-100	≤ 7
4	Palmitoyl Chloride	112-67-4	Liquid	274.87	Sensitiser (moderate)	Positive	≤ 10	50-100
5	Imidazolidinyl urea	39236-46-9	Solid	388.29	Sensitiser (weak)	Positive	10-45	≤ 10
6	Farnesal	19317-11-4	Liquid	220.35	Sensitiser (weak)	Positive	20-40	≤ 15
7	Glycerol	56-81-5	Liquid	92.09	Non-sensitiser	Negative	≤ 7	≤ 7
8	Isopropanol	67-63-0	Liquid	60.10	Non-sensitiser	Negative	≤ 7	≤ 7
9	Dimethyl isophthalate	1459-93-4	Solid	194.19	Non-sensitiser	Negative	≤ 7	≤ 7
10	Propyl paraben	94-13-3	Solid	180.20	Non-sensitiser	Negative	≤ 7	≤ 7

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

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

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

APPENDIX II, ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

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

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

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

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

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

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

Three sets of reference controls (NAC or NAL dissolved in the appropriate solvent) should be included in the analysis sequence:

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

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

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