Unclassified English - Or. English

20 September 2022

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

VALIDATION STUDY REPORT OF THE AMINO ACID DERIVATIVE REACTIVITY ASSAY (ADRA)

Series on Testing and Assessment
No. 304
Second Edition

JT03502839

OECD Environment, Health and Safety Publications
Series on Testing & Assessment
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A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

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Paris 2022

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FOREWORD

This document is the second edition of the report of the Amino acid Derivative Reactivity Assay (ADRA) validation study, a JaCVAM co-ordinated study programme addressing the validation status of the ADRA for the prospective identification of skin sensitising chemicals. It supports the development of this test method for inclusion in Test Guideline (TG) 442C for *in chemico* skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on covalent binding to proteins.

It has been developed in two phases. PART I of the document describes the first validation study, completed in 2018. This study was peer reviewed by an independent peer review panel in 2018 and published in 2019 as the first edition of this publication, following endorsement by the Working Party of the National Coordinators of the Test Guidelines Programme (WNT) and declassification by the Chemicals and Biotechnology Committee (CBC).

The document was updated in 2022 with the inclusion of PART II. This second part contains an ADRA Ring Study Report that supports the updates of the ADRA adopted in 2022 in TG 442C, i.e. (i) the use of a test chemical concentration of 4 mM instead of 1mM and (ii) the use of fluorescence as an additional detection method. The Ring Study Report was reviewed in November 2021 by a review panel made of a few experts from the Expert Group on alternative methods for skin sensitisation. The WNT endorsed PART II of the ADRA Validation report at its 34th meeting in April 2022.

This second edition of the report is published under the responsibility of the Chemicals and Biotechnology Committee.

PART I

Amino acid Derivative Reactivity Assay (ADRA)
JaCVAM Validation Study Report

Version 1.3

May, 2019

Index

Summary

This report presents the results of a study of the Amino acid Derivative Reactivity Assay (ADRA) test method that was carried out at four participating laboratories to validate within- and between-laboratory reproducibility, to evaluate ADRA's predictive capacity relative to the Local lymph node assay (LLNA), and to compare and contrast ADRA's performance to that of the DPRA test method.

The assessment of skin sensitization potential is an important part of the overall safety assessment of both existing and newly developed test chemicals. Historically, animal tests such as the Guinea Pig Maximization Test (GPMT) or the murine Local Lymph Node Assay (LLNA) have been the most common methods of testing for skin sensitization potential. But the limitations of these animal-based methods as well as increased awareness of animal welfare issues has in recent years prompted development, validation, use, and regulatory acceptance of non-animal test methods based upon a knowledge of adverse outcome pathways. Validated non-animal methods for the assessment of skin sensitization potential include the Direct Peptide Reactivity Assay (DPRA, issued as OECD TG 442C in 2015), the ARE-Nrf2 Luciferase Test Method (KeratinoSens, issued as OECD TG 442D in 2015), and the human Cell Line Activation Test (h-CLAT, issued as OECD TG 442E in 2016).

One of these scientifically validated non-animal methods, the DPRA, is an *in chemico* test that predicts skin sensitization potential based on protein reactivity following exposure to a test chemical, an event which is recognized as a key molecular initiating event leading to skin sensitization (OECD Series on Adverse Outcome Pathways No.1, 2016). Compared with cell-based *in vitro* test methods, DPRA is an easy-to-use test method and exhibits excellent predictive capacity. Nevertheless, the DPRA test method has significant limitations:

- One of the nucleophilic reagents, cysteine peptide, is easily oxidized rendering some test results invalid, unreliable or difficult to interpret.
- It requires relatively high concentrations of the test chemical, making it unsuitable for evaluating poorly soluble chemicals.
- It requires a relatively large quantity of both test chemicals and nucleophilic reagents to perform.
- Hydrophobic test chemicals tend to precipitate easily in the reaction solution.

ADRA, an *in chemico* test which is also based on protein reactivity, does not have these limitations thanks to the use of highly sensitive nucleophilic reagents, which allows reagent solutions to be prepared at test chemical concentrations just 1% of those required in DPRA.

Following a modular approach applying the ECVAM principles on test validity (OECD Series on Testing and Assessment, Number 34, 2005, Hartung *et al.* 2004), the Validation Management Team (VMT) empirically evaluated modules one through four (test definition, within-laboratory reproducibility, transferability, and between-laboratory reproducibility), and used these results also to evaluate modules five and six (predictive capacity and applicability domain).

During a preliminary training phase of the ADRA validation study using ten test chemicals, the results obtained by the four participating laboratories were 100% concordant with the lead laboratory. During the subsequent transferability phase of the study, three of the four participating laboratories were 100% (10/10) concordant and the fourth laboratory was 90% (9/10) concordant with the lead laboratory. The ADRA VMT considers these results to have confirmed that the ADRA test method is easily transferred to naïve laboratories.

During Phase I of the Study Plan, each of the four participating laboratories performed three test runs of identical sets of 10 coded test chemicals to evaluate within-laboratory reproducibility. The results, based on concordance, were 100% (10/10), 100% (10/10), 100% (7/7), and 90% (9/10). When the results from the 10 test chemicals of Phase I were combined with those of Phase II, in which each participating laboratory performed one test run of identical sets of an additional 30 test chemicals, between-laboratory reproducibility for 40 test chemicals was 92%. The VMT considers this to satisfy standards for both within- and between-laboratory reproducibility for this class of test method.

Moreover, a review of predictive capacity relative to LLNA based on the results of Phases I and II yielded a sensitivity of 81%, specificity of 98%, and accuracy of 86%. The VMT therefore considers ADRA to be an *in chemico* method with sufficient specificity, sensitivity, and predictive capacity for regulatory acceptance.

Background

The formation of covalent adducts between skin sensitizers and endogenous proteins or peptides in the skin is recognized as a key initiating event of the skin sensitization process (Smith and Hotchkiss 2001). This interaction of skin sensitizers with proteins, peptides, and model nucleophiles representing proteins or peptides is predominantly by covalent bonding between electrophiles and nucleophiles (Aleksic *et al*. 2008). It should therefore be possible to predict the skin sensitization of chemicals based on *in vitro* test methods which evaluate the reactivity of test chemicals with peptides and proteins (Gerberick *et al*. 2008, Divkovic *et al.* 2005): it is on that premise that the DPRA and ADRA test methods were developed. Numerous studies have looked at methods for directly detecting and characterizing the adducts formed by peptides and test chemicals as a means of evaluating the reactivity of peptide and proteins and skin sensitization potential. For example, an analysis of adducts of peptides and test chemicals using nuclear magnetic resonance was reported by Ahlfors *et al*. (2003) and Alvarez-Sanchez *et al*. (2004). Ahlfors *et al.* (2003) and Nilsson *et al*. (2005) developed a means for analysis using liquid chromatography-mass spectrometry (LC-MS).

In addition to these tests, there is the quantitative peptide depletion assay developed by Gerberick *et al*. (2004, 2007). This assay characterizes the skin sensitization potential of chemicals based on their ability to deplete two nucleophilic synthetic heptapeptides as measured using high-performance liquid chromatography (HPLC). In another publication, Natsh *et al*. (2008) made use of a quantitative method for LC-MS. These analytical methods measure the oxidation of the heptapeptide Cor1-C420 and characterize the formation of adducts. In addition, a kinetic spectrophotometric assay for evaluating chemical reactivity with 4-nitrobenzenethiol (NBT) using stopped-flow techniques and UV spectrophotometric measurements was developed by Chinpinda *et al*. (2010).

The direct peptide reactivity assay (DPRA), first reported by Gerberick *et al*. (2007), is a simple and versatile scientifically validated *in chemico* method of assessing the skin-sensitization potential of chemicals and will be adopted as a Test Guideline (TG) by the Organization for Economic Co-operation and Development (OECD) (Test No. 442C: *In Chemico* Skin Sensitisation). However, Natsch *et al*. (2008) and others have reported some limitations of DRPA, such as the need for test chemical solutions of relatively high concentrations, particularly in the lysine-peptide-based component of the assay (25 mM), meaning that DPRA is not suitable for testing highly hydrophobic test chemicals. These high concentrations of test chemicals are required because DPRA uses peptides with a low UV absorption in the HPLC-UV analysis which forms part of the test method. In contrast to this, the concentration of test chemicals needed in the method developed by Natsch *et al*. (2008) is a mere 1/25th of that required for DPRA. However, their test method is expensive, and because the peptides are analyzed using LC-MS/MS, it lacks versatility. The stopped-flow analysis developed by Chinpinda *et al*. (2010) makes it possible to reduce test chemical concentrations to between 1/10th and 1/10,000th of the concentrations required for the standard DPRA test method. With this method, however, the reaction solution is quantified by stopped-flow analysis without separating test chemicals from peptides and reactive products, which may as a result reduce the test method's precision because of the potential for the peptides of interest to be

analyzed together with other test chemicals at the quantitative wavelength.

Seeking to ameliorate these limitations of DPRA, Fujifilm Corporation synthesized two amino acid derivatives—*N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine (NAL)—for use as nucleophilic reagents in the Amino acid Derivative Reactivity Assay (ADRA) (Fujita *et al*. 2014, Yamamoto *et al*. 2015). In these two amino acid derivatives, naphthalene rings have been introduced to the N-termini of cysteine and lysine, making them sensitive enough to use at just 1% of the concentrations needed for the peptide reagent solutions used in DPRA. There are many other resulting advantages, including the fact that assay measurements are made at relatively long wavelengths, which virtually eliminates the problem of co-elution. NAC is a cysteine derivative, like the cysteine peptide used in DPRA, contains thiol group that is susceptible to oxidation (resulting in dimer formation). This susceptibility to oxidation has been shown to be exacerbated by the presence of minute quantities of metallic ions in the solutions, which lead Fujifilm Corporation to develop a modification that involves the addition of EDTA—a recognized metal chelating agent—to the NAC stock solution to eliminate this problem, resulting in a Standard Operating Procedure (SOP) that provides even greater accuracy and test method versatility.

Integrated Approaches to Testing and Assessment (IATA) are essential to the application of Adverse Outcome Pathways as a conceptual framework for the assessment of skin sensitization potential in that they enable in vitro testing, structure-activity relationships, in silico models, and other methodological approaches to be used in combination with physiochemical properties to evaluate bonding with proteins, activation of keratinocytes, activation of dendritic cells, and other typical phenomenon that occur in the skin sensitization process. Like DPRA, ADRA is an alternative test method that measures bonding with proteins as an index for predicting skin sensitization potential. DPRA involves the use of peptides as reagents to simulate proteins in living organisms. Cysteine and lysine are amino acids known to be involved in the bonding of test chemicals with proteins in living organisms, which is why heptapeptides like these two amino acids are used. In contrast to DPRA, ADRA involves the use of NAC and NAL, which are derived from amino acids by introducing naphthalene rings to cysteine and lysine and which, like DPRA, are intended to simulate reactions in living organisms.

Management of the Study

1. Study objectives

The ADRA validation study was initiated in 2016. The primary study objective was to evaluate the technical transferability and reliability (based on within- and between-laboratory reproducibility) of ADRA with the intention of evaluating ADRA's potential suitability for future use in combination with other alternative test methods in place of the animal, and *in vitro*, tests that are currently in use.

A secondary objective was to evaluate ADRA's potential for use in distinguishing skin sensitizers (Category 1) from non-sensitizers (No Category) under the UN Globally Harmonised System (GHS) for the classification and labelling of substances for skin sensitization.

This report presents the results of testing performed at four independent laboratories primarily in order to validate the transferability and reliability of ADRA.

2. Study Plan

The Study Plan was drafted, approved and issued by the Validation Management Team (VMT) prior to the start of testing. It is included here Appendix A.

2-1. Structure of the study

The ADRA validation study was designed in accordance with the modular approach described in *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (OECD Series on Testing and Assessment, Number 34, 2005) and "A Modular Approach to the ECVAM Principles on Test Validity" (Hartung *et al*. 2004) in order to obtain data related to modules 1 through 4 (test definition, within-laboratory reproducibility, transferability, and between-laboratory reproducibility, respectively).

Additionally, the data obtained during the validation study was to be used to evaluate module 5 (predictive capacity) and module 6 (applicability domain).

The Study Plan, selection of the test chemicals, and the SOP used in the testing were correlated by JaCVAM in consultation with the VMT. Figure 1 shows the framework through which the administration and management, test methods, participating laboratories, selection of test chemicals, coding and distribution of test chemicals, collection of data, and statistical analysis for this validation were planned, managed, conducted and coordinated.

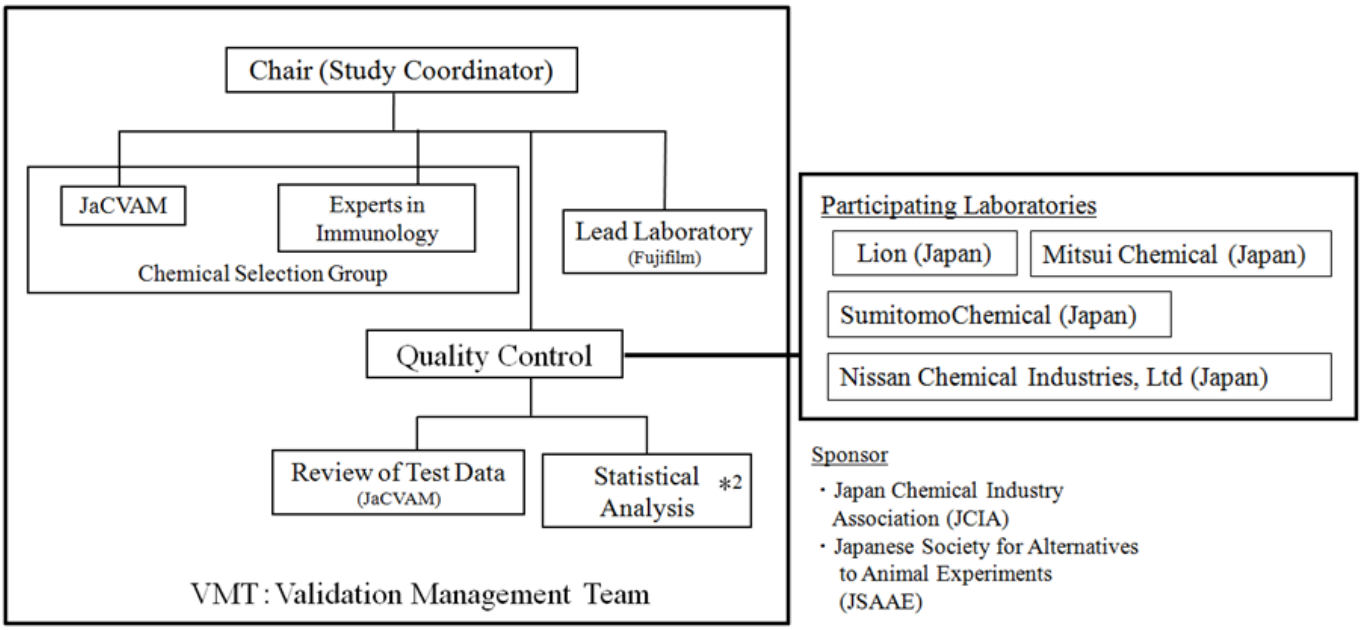


Figure1. ADRA Validation Study Structure and Organization

2-2. Validation Management Team

It was the responsibility of the VMT to specify the study objectives, design, and minimum required performance criteria of the validation study, and to administer and to oversee the testing and the evaluation and presentation of the test results.

Professor Atsushi Ono, who has experience as a trial coordinator for the preparation of OECD test guidelines, served as chair of the VMT. Other members of the VMT are shown below.

Validation Management Team

Chair (Trial coordinator)	Atsushi Ono (Okayama University)
Chair of the Chemical Selection Group	Hajime Kojima (JaCVAM)
Statistician	Takashi Sozu (Tokyo University of Science)
	Takuto Nakayama (Tokyo University of Science)
	Takeru Kusao (Tokyo University of Science)
JaCVAM representative	Hajime Kojima
Internal expert	Tsuyoshi Kawakami (National Institute of Health Sciences)
	Kohichi Kojima (Food and Drug Safety Center)
External expert	Jon Richmond (dr.jonrichmond: Advice & Consultancy)
	Nicole Kleinstreuer (NICEATM)
	Bae-Hwa Kim (College of Natural Sciences Keimyung University)
Lead laboratory	Toshihiko Kasahara (FUJIFILM Corporation)
	Masaharu Fujita (FUJIFILM Corporation)
	Yusuke Yamamoto (FUJIFILM Corporation)

*NICEATM: NTP Interagency Center for the Evaluation of Alternative Toxicological Methods

2-3. Laboratories

The following four laboratories participated in the chemical testing for the ADRA Validation Study.

2-3-1. Why the Study was conducted at four participating laboratories

Validation study testing is normally performed at a minimum of three participating laboratories. There are circumstances, however, in which not all of the participating laboratories that successfully completed the technology transfer phase are able to continue participating to the end of the validation study. To guard against such an eventuality, we allowed all of the four participating laboratories that volunteered to participate in the technology transfer to do so. And since all four participating laboratories not only successfully completed the technology transfer phase (See Module 3: Transferability) but also wished to continue to participate to the end of the validation study, validation study testing was conducted at four participating laboratories.

The more laboratories that participate in a validation study, the easier it becomes to identify issues that arise from differences in ambient conditions at each laboratory, which we feel is an effective means for establishing as quickly as possible a test method suitable for universal use. In fact, as will be discussed in detail later, major issues that had not been experienced at the lead laboratory, Fujifilm, were identified during the transferability stage, when ADRA was performed for the first time at the four participating laboratories. Thanks to having identified these issues and implementing countermeasures at an early stage, we were able to proceed to the reliability stage in a timely manner. It is true that considerations of time and cost often result in validation study testing being performed at just three participating laboratories, which is the minimum number necessary to ensure a valid statistical analysis. For example, according to the DPRA Validation Study Report, testing was conducted at just three participating laboratories, one of which was the lead laboratory, Proctor & Gamble. Although ADRA validation study testing was conducted at four participating laboratories, calculations of within-laboratory reproducibility, between-laboratory reproducibility, and predictive capacity were based on results from sets of three participating laboratories (See 7. Statistical Analysis of Test Data, 7-1. Data Analysis.) to enable these results to be compared directly to those of DPRA and other test methods.

2-3-2. Explanation of why testing was performed at the lead laboratory under the same conditions

The objective of the testing performed during this validation study is to demonstrate that the participating laboratories are able to replicate to a high degree of certainty the same accurate test results obtained and published by the lead laboratory. Given this objective and the fact that the lead laboratory is experienced in this technology, out of fairness alone, the lead laboratory itself would ordinarily not participate in validation testing. In the event, however, that the lead laboratory were to participate in validation testing under the same conditions as the participating laboratories, test results and comments from the lead laboratory would be useful in reviewing the results of the participating laboratories, and in cases where problems were encountered during the validation, could be considered helpful in finding solutions. Thus, Fujifilm, the lead laboratory, mirrored the ADRA validation, testing the encoded chemicals and undergoing QC checks under the same conditions as the other participating laboratories. Furthermore, test results obtained by the lead laboratory were handled as reference data and used in the

evaluation of the validation testing. The results of this testing are shown in Appendix 6. Results of Phase-1 and Phase-2 Performed by Fujifilm.

Laboratory 1
Lion Corporation
Human & Environmental Safety Evaluation Center
Kanagawa, Japan
Shinichi Watanabe, study director

Laboratory 2
Mitsui Chemicals, Inc.
Chemical Safety Department
Responsible Care & Quality Assurance Division
Chiba, Japan
Koji Wakabayashi, study director

Laboratory 3
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Osaka, Japan
Keiichi Fujimoto, study director

Laboratory 4
Nissan Chemical Industries, Ltd.
Toxicology & Environmental Science Department
Biological Research Laboratories
Saitama, Japan
Kei Kusakari, study director

3. Test Design

Reference document: Study plan (Appendix 1)

Study stages

The validation study comprised and was implemented in two distinct stages:

Transferability: Pre-training to provide the participating laboratories with the technology and the expertise required to perform the test method, and to confirm the transferability of the test method.

Reliability: Evaluation of test performance based on the results of testing performed with coded chemicals at the participating laboratories. There were two phases to this stage of the study (see below).

The physical properties and quantities of the test chemicals used during the Transferability Stage were determined by the lead laboratory. Initial pre-training was conducted with five uncoded test chemicals that are considered to be relatively easy to identify as either sensitizers or non-sensitizers. Four sensitizers and one non-sensitizer were used. A review of the results of the pre-training having confirmed that the participating laboratories were proficient with the method, transferability was then evaluated using an additional ten uncoded test chemicals that included some which are known to be difficult by conventional test methods to characterize as either sensitizers or non-sensitizers. The results were evaluated by the lead laboratory and VMT before the participating laboratories were deemed to be ready to proceed to the Reliability Stage of the study.

The number and nature of the test chemicals used during the Reliability Stage was determined by the VMT, taking into account testing performed during the DPRA validation study and the minimum criteria established for evaluating within- and between-laboratory reproducibility. As a result, Phase I Reliability testing to evaluate within-laboratory reproducibility was conducted using three replicate sets of coded ten test chemicals, and Phase II Reliability testing to evaluate within- and between laboratory reproducibility was conducted using one set of thirty coded test chemicals.

A breakdown of the test chemicals used in Phases I and II is as follows:

Phase I (within- and between-laboratory reproducibility): seven sensitizers, three non-sensitizers
Phase II (between-laboratory reproducibility): twenty sensitizers, ten non-sensitizers

4. Selection of Test Chemicals

Reference document: Chemical selection (Appendix 4)

The selection of test chemicals by the VMT Chemical Selection Committee (CSC) took account of published papers on *in vivo* skin sensitization tests and previous validation studies for *in vitro* alternative

methods for predicting skin sensitization potential.

Specific consideration was given to the following aspects.

Information on mode or site of action
Coverage of a range of relevant chemical and product classes
Quality and quantity of reference data from *in vivo* and *in vitro* testing
The availability of high-quality data derived from animal and human studies,
Information on interspecies variations (e.g., variability in uptake of chemicals, metabolism, etc.)
Coverage of a range of toxic effects or potencies
Chemicals that do not require metabolic activation
Appropriate negative and positive controls
Physical and chemical properties (feasibility as implied by the CAS No.)
Single chemical entities or formulations known to be of high purity
Availability
Cost

At the start of the chemical selection process, members of the CSC prepared a primary database by consolidating several authoritative lists of potential skin sensitizers. This consolidated list included chemicals that had been used in other validation studies, referenced in other test methods, recommended for use in testing by EURL ECVAM, and the reference chemicals from OECD TG 429. An extensive search of the literature was performed by the CSC to ensure that all chemicals were selected for use in accordance with the above criteria.

Emphasis was placed achieving a diverse selection of different potencies (Category 1A, Category 1B and non-sensitizer). Additionally, in order to increase the statistical power of the data analysis, the VMT determined that at least 20% of the test chemicals selected should be non-sensitizers.

5. Acquisition, coding, and distribution

5-1. Chemical Acquisition, Coding, and Distribution

The evaluation of transferability, within- and between-laboratory reproducibility, and predictive capacity was made using the results of tests conducted at the participating laboratories using coded chemicals. The coding was supervised by JaCVAM, in collaboration with the CSC. The CSC was responsible for coding and distributing all test chemicals, reference chemicals, and control chemicals used in testing for the validation study.

5-2. Handling

JaCVAM provided the chemical master (defined as “the person responsible for the handling of the chemicals”) at each participating laboratory with essential information about the test chemicals, including physical state, weight or volume of sample, specific density for liquid test chemicals, and storage

instructions. This person was responsible for storing each chemical in accordance with the storage instructions and separately received sealed safety information, including Material Safety Data Sheets (MSDS), which specified hazard identification, exposure control, and personal protection for each chemical. The test chemicals were delivered directly to these persons**.** The MSDS were to be accessed only in the event of a laboratory accident, and the information disclosed only to those who needed to know: unopened MSDSs were to be returned to the VMT at the end of the study.
No such accidents occurred during the course of the validation study, and upon completion of the study, all residual test chemicals were disposed of in compliance with the rules and regulations of the participating laboratories, and all MSDS were returned to JaCVAM still in their sealed envelopes.

6. Data management

Data collection and analysis for the validation study were performed by biostatisticians and the quality assurance group. These independent biostatisticians collected and organized data using custom data collection software, and all records were checked by the quality assurance group. Assays and quality assurance were carried out in the spirit of GLP (Balls *et al*., 1995), although not all the participating laboratories routinely worked under GLP certification. The participating laboratories were deemed to have conducted the experiments in accordance with the Study Plan and SOP provided by the VM, other than in the one instance discussed elsewhere in this report. All raw data and data analysis sheets were pre-checked for quality by each laboratory and then were reviewed by the VMT quality assurance team. The raw and collated data were found to reflect the test results accurately.

7. Statistical Analysis of Test Data

7-1. Data Analysis

Only test results that satisfied the prescribed test acceptance criteria were included in the statistical analysis, although records were provided of all test results, including those that were affected by human error or were otherwise problematic.

Reproducibility of the test method was evaluated by calculating within- and between-laboratory reproducibility. Testing for the validation study was conducted at four participating laboratories. Between-laboratory reproducibility is normally calculated using results from three participating laboratories, so, on the advice of biostatisticians, the VMT evaluated the results from four laboratories using the following method.

Calculation of between-laboratory reproducibility

Step 1. Reproducibility was calculated for each facility using test results from just three of the four participating laboratories, and between-laboratory reproducibility is calculated in this manner for all four participating laboratories.

Step 2. An average value was calculated using the between-laboratory reproducibility of each of the four participating laboratories as determined in Step 1 (immediately above).

Table1. Example of predictions made at four participating laboratories

	Lab. A	Lab. B	Lab. C	Lab. D
Chemical 1				
Chemical 2				
Chemical 3				
Chemical 4				
Chemical 5				

Please note: Table 1 is an example demonstrating how data was set out for evaluation. It does not represent data collected during the ADRA validation study.

The pink cells indicate that the test chemical was predicted to be a sensitizer, the blue cells indicate the test chemical was predicted to be a non-sensitizer.

Shown in Table 1 above are illustrative examples of predictions for five test chemicals obtained from testing at four participating laboratories.

Reproducibility (concordance) using results from just three of the four participating laboratories is calculated by the following method.

First, Lab A is left out and concordance for Labs B, C, and D is calculated. Since all three labs made concordance predictions for three (Chemicals 1, 2, and 5) of the five test chemicals, the reproducibility is 3/5 × 100% or 60%. In this same manner, when Lab B is left out, reproducibility is 1/5 × 100% or 20%. Reproducibility when Lab C is left out is 1/5 × 100% or 20%, and when Lab D is left out is 2/5 × 100% or 40%. Using these four figures, the average reproducibility across all four participating laboratories is (60 + 20 + 20 + 40)/4 x 100% or 35%. Thus, the between-laboratory reproducibility based on the results from three participating laboratories is 35%.

Calculations of reproducibility in this report are calculated based on the results from sets of three participating laboratories as described above.

7-2. Evaluation Criteria

In order to evaluate the whether the objectives of this validation study had been met, it was necessary

to define in advance the minimum performance criteria to be used to evaluate test method performance.

In doing this the VMT considered:

1. The background and objectives of the validation of the test method
2. The expected performance of a test method proposed for regulatory acceptance as a scientifically validated alternative to animal testing in this context.
3. The utility of the test method (i.e. to be used in combination with other alternative test methods.)

Based on the above, the VMT set target minimum performance standards for both within- and between-laboratory reproducibility at 80%.

Test Definition

See also ADRA SOP (Appendix 2), Articles on ADRA (Appendix 8)

1. Intended purpose of the test method

ADRA was originally conceived as a replacement for DPRA, and lacking some of the DPRA limitations, in a non-animal battery or integrated testing strategy for assessing the skin-sensitization potential of chemicals.

DPRA is currently used as an alternative to animal testing (OECD TGs 406, 429, 442A, and 442B) in place of *in vivo* tests for regulatory classification and labelling as well as to reduce the number of animals used in skin sensitization testing. ADRA will be suitable for the same purposes, but for a larger range of test chemicals. Both ADRA and DPRA operate on the same scientific principles, but rather than using cysteine peptide and lysine peptide as nucleophilic reagents as DPRA does, ADRA uses NAC and NAL, which both have high light absorption at long wavelengths and high molar extinction coefficients. Thus, the concentrations of test chemical solutions for ADRA need be only 1% of those used for DPRA. The benefits of this include elimination of virtually all precipitation of the test chemical in the reaction solution as well as eliminating potential co-elution of the test chemical and the nucleophilic reagent during HPLC analysis. These advantages mean that ADRA is significantly more versatile than DPRA, and capable of testing chemicals that are either unsuitable for or too difficult to test with DPRA.

2. Need for the test method

DPRA is currently considered a scientifically validated and accepted alternative to animal testing for the skin sensitization potential of ingredients used in cosmetics and toiletries as well as numerous chemicals that require assessment under Registration, Evaluation, and Authorisation of Chemicals (REACH).

ADRA is expected not only to be a useful means of assessing not just the same range of chemicals and yielding similar results to DPRA, but because it is conducted with much lower concentrations of test chemical, it is also designed to accommodate a much wider range of chemicals. For these reasons ADRA, if validated and deemed suitable for regulatory use, will have the potential to extend the use of this class of *in chemico* test method.

3. Development of the test method

ADRA was developed by Fujifilm Corporation.

DPRA is a scientifically validated *in chemico* test method that models the covalent bonding of chemicals to cysteine or lysine residues of *in vivo* proteins during the initial stages of the skin sensitization mechanism to predict the skin sensitization potential by quantifying the bonding of a test chemical to either of cysteine or lysine residues found in two peptides. Using DPRA the covalent bonding

to peptides is quantified by finding the mean % depletion of the two defined synthetic test peptides, which is then expressed as a percentage and the value obtained is then used to predict skin sensitization potential by classifying the test chemical as either a sensitizer or a non-sensitizer based on statistically prescribed prediction criteria.

It has been established that the great majority of test chemicals that exhibit skin sensitization potential preferentially bond with the cysteine and lysine residues of the *in vivo* proteins. Fujifilm Corporation has therefore developed an *in chemico* test method linking naphthalene rings to the N-termini of these amino acids, named NAC and NAL, which are easily detectable and quantifiable by HPLC-UV. ADRA evaluates skin sensitisation potential based on the ability of test chemicals to bond with NAC and NAL. As with DPRA, the ADRA criteria for identifying test chemicals as either sensitizers or non-sensitizers is based on mean % depletion was calculated empirically by a statistical analysis of a dataset of test results with 56 well characterized chemicals including sensitizers and non-sensitizers.

To initially evaluate ADRA, 82 chemicals previously assessed for skin sensitization potential using DPRA were retested by the test developer at the same concentrations for test chemical solutions (100 mM) and nucleophilic reagents (NAC and NAL) as those used in DPRA (Fujita *et al*. 2014). The results showed an accuracy of 88% for ADRA, which compared favorably with DPRA's accuracy of 89%.

As a next step, an ADRA derivative method was developed to take advantage of the fact that NAC and NAL are easily detected in HPLC-UV analysis. This ADRA dilutional method (ADRA-DM), uses test chemical solutions just 1% of those used in the original test method (Yamamoto *et al*. 2015). The 82 test chemicals previously assessed using DPRA and the original ADRA were retested using the ADRA-DM, with results showing an accuracy of 90%, which was in accordance with the original ADRA results. Again, as with DPRA and the original ADRA, ADRA-DM criteria for identifying test chemicals as either sensitizers or non-sensitizers based on mean % depletion of NAC/NAL was calculated empirically by a statistical analysis of a dataset of the 56 reference chemicals divided into two classifications. This validation study was based on the ADRA-DM test method referred to immediately above.

4. Scientific basis: biological and mechanistic relevance

Allergic contact dermatitis is the clinical manifestation of human skin sensitization and is caused by a wide range of chemicals after prolonged or repeated contact with the skin in susceptible subjects. Skin sensitization/allergic contact dermatitis occurs in 6 steps: (1) skin bioavailability, (2) haptenation, (3) epidermal inflammation, (4) dendritic cell (DC) activation, (5) DC migration, and (6) T-cell proliferation, and various methods for predicting or evaluating skin sensitization corresponding to each step have been developed (Adler *et al*. 2011, Basketter & Kimber. 2010).

In the initial step of skin sensitization, the sensitizers are generally electrophilic and reactive toward nucleophilic sites on proteins, and it is these characteristics have been used to develop *in chemico*

approaches for evaluating the skin sensitization potential of a number of chemicals (Ahlfors *et al*. 2003, Alvarez-Sanchez *et al*. 2003, Meschkat *et al*. 2001). DPRA is currently the most widely used scientifically validated *in chemico* method (Gerberick *et al*. 2004, Gerberick *et al.* 2007). However, the peptides used in DPRA must be detected at 220 nm because of their minimal absorption at longer wavelengths, and various test chemicals with skin sensitization potential also have UV absorption at short wavelengths such as 220 nm. Therefore, these peptides cannot be measured precisely when they co-elute with test chemicals or reaction products in HPLC, as shown by Natsch A. & Gfeller H. (2008).

Another limitation of DPRA is that test chemical solutions must be prepared the high concentration of 100 mM, and many test chemicals—even if they can be dissolved at such concentrations—have been found to precipitate when mixed with the reaction solution. In order to solve these problems, Fujifilm Corporation chemically synthesized two amino acid derivatives by introducing a naphthalene ring to the N-termini of cysteine and lysine, thereby creating amino acid derivatives with maximum absorption at 281 nm. These derivatives, named NAC and NAL respectively, are used in a novel Amino acids Derivatives Reactivity Assay (ADRA) (Fujita *et al*. 2014). In addition, Fujifilm Corporation developed an HPLC-UV method suitable for detecting and quantifying the low-concentration test chemical solutions used in a modified form of the amino acid derivative reactivity assay (ADRA) (Yamamoto *et al*. 2015). Known as the ADRA dilutional method (ADRA-DM), this derivative method features optimized reaction conditions that require test chemical solutions of just 1% of the concentrations used in the original ADRA.

Given the superiority of ADRA-DM due to its use of test chemical solutions at concentrations just 1% of those necessary for DRPA or the original ADRA, for the purposes of this validation report, the acronym ADRA should be understood to mean ADRA-DM

5. SOP (Standard Operating Procedure)

The most recent version of the ADRA SOP is version 1.1 (Appendix 2).

ADRA is an *in chemico* assay, based on the same scientific principles as DPRA. ADRA can be used to predict the skin-sensitization potential of test chemicals by introducing naphthalene rings to the N-termini of cysteine and lysine to create two types of nucleophilic reagents, known as NAC and NAL, mixing them with a test chemical solution, incubating this mixture for 24 hours at 25°C, and then calculating NAC and NAL depletion by HPLC.

In ADRA, the reaction of a test chemical with NAC and NAL as well as the subsequent HPLC analysis is performed using a 96-well microplate. Whilst it is possible to transfer the reaction solution from each well to an HPLC vial, this adds complexity and potential measurement errors, and the VMT therefore strongly recommends the use of 96-well microplates compatible with HPLC analysis.

The NAC and NAL reagents are mixed with the test chemical at a chemical ratio of 1:50. The mixture

of test chemical with either NAC or NAL is then incubated for 24 hours (plus or minus 1 hour) at 25°C (plus or minus 1ºC), after which trifluoroacetic acid (TFA) is added to stop the reaction. The levels of unreacted NAC and NAL in the reaction solution are then measured by HPLC analysis at 281 nm. The mean % depletion of NAC and NAL is then compared with the criteria specified in the prediction model to determine whether the test chemical is to be classified as a sensitizer or a non-sensitizer.

Given that the concentration of the test chemical solution used in ADRA is just 1% of that used in DPRA and the fact that the detection wavelength of 281 nm is relatively long, there is virtually no co-elution of the test chemical with NAC or NAL. Even in those rare instances where co-elution of NAL and the test chemical has been observed to date, it has been possible to accurately predict sensitization potential based on NAC value alone, just as it is possible to do so base on cysteine peptide alone in DPRA. As with DPRA, in cases where co-elution of NAC and the test chemical is observed, it is not possible to accurately predict sensitization potential based on NAL alone.

6. Modifications made to original SOP prior to the validation study

The original test method SOP for use in this validation study was prepared by the lead laboratory, Fujifilm Corporation. To address issues to do with clarity and test performance arising during the pre-training and training testing conducted in the transferability stage the SOP was revised to remove ambiguities and resolve minor technical issues. The most significant revisions were as follows.

1. In order to prevent the potential oxidation of NAC, the use of unopened, disposable labware was specified.
2. The quantities of Na_2HPO_4 aqueous solution, NaH_2PO_4 aqueous solution, and 0.1-N NaOH aqueous solution to be used in preparation of the phosphate buffer solution were clearly specified.
3. The mixer capacity as well as the length and diameter of tubing for the HPLC equipment were specified.
4. The calculation method for peak area when peaks overlap during HPLC analysis was clarified.

The following modifications were proposed, discussed and approved at a joint meeting of the VMT (on the 4th of September, 2016) and the participating laboratories prior to the start of the reliability phase of the validation study. These modifications were thus included in version 1.0 of the SOP .

1. In order to prevent oxidation of NAC, the addition of EDTA to the NAC stock solution was specified.*
2. Preparation methods for each reference control and elution control were specified in the procedure for preparing reaction solutions.
3. The acceptable range of concentration for reference control A and reference control C specified in the test acceptance criteria was revised from 3.2–4.8 μM to 3.2–4.4 μM.
4. Also, the acceptable range of concentration for reference control C when using 5% DMSO or acetonitrile in the test acceptance criteria was specified as 2.8–4.0 μM.
5. Based on background data from the lead laboratory, the acceptable range for the positive control

in the test acceptance criteria was revised from 8% ≤ NAC depletion ≤ 100% to 86% ≤ NAL depletion ≤ 100%.

*This revision required revision of the criteria for sensitizer/non-sensitizer predictions, but since the data necessary to empirically define the new criteria were not yet available at the time of the meeting, it was agreed by the VMT that the criteria would be reviewed and revised as necessary once study data was available.

The following modifications were subsequently proposed and approved at a joint meeting of the VMT (the 20th of January, 2017) and the participating laboratories held upon completion of Phase I testing. These modifications were thus included in version 1.1 of the SOP, which was used to conduct Phase II testing.

1. NAL lot number was revised.
2. A precautionary comment about pipette technique was added to section 4-2. “Preparation and Reaction of Reaction Solutions”.
3. The control criteria for the positive control reagent were revised.
4. The range of mean % depletion and NAC depletion for additional test to get an accurate predictive result was added to section 8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay.
5. Instructions on how to handle instances in which there appears to be no co-elution but depletion is less than -10% was added to section 8-4. “Handling of Co-elution”.

7. Known limitations and drawbacks

One limitation of ADRA, and all other skin sensitization potential test methods, is the need to create a test chemical solution with a suitable solvent. The ADRA SOP contains provisions for the use of four different solvents, which are to be selected by the testing laboratory by testing for suitability with each test chemical, using the prescribed solvents in the following order:

1. Water
2. Acetonitrile
3. Acetone
4. 5% DMSO/acetonitrile

ADRA differs from DPRA in that the concentration of the test chemical solution is much lower (1 mM or 20 mM), and all test chemicals evaluated thus far have been soluble in at least one of the four solvents named above. The solvent does, however, have the potential to affect the reactivity of the test chemical with NAC and NAL, and there have been test chemicals for which depletion has varied significantly depending on the solvent used.

Another limitation that was seen in DPRA is the simultaneous elution, or co-elution, of peptides and test chemicals. Since HPLC separation occurs by gradient elution (*i.e.*, the mobile phase composition

changes during the separation process), retention time of the test chemical can be influenced by the instruments with which the solvent is mixed (see below).

In DPRA, peptides are quantified through HPLC analysis of nucleophilic reagents at a wavelength of 220 nm. In ADRA, however, NAC and NAL are quantified at a wavelength of 281 nm, making it highly unlikely that co-elution of NAC and NAL with the test chemical would occur. Also, since the concentrations of nucleophilic reagents and test chemical solutions used in ADRA are a mere 1% of those used in DPRA, the peaks are commensurately smaller, and the potential for co-elution is lower. Although the VMT could not find any clear examples of test chemicals that exhibit co-elution in ADRA, additional gradient parameters have been proposed in order to avoid co-elution (Fujita *et al.* 2014).

As a case in point, salicylic acid was not tested successfully in DPRA due to co-elution with lysine peptide, and for this reason, Gerberick *et al.* (2007) lists salicylic acid as unsuitable for assessment with the cysteine peptide/lysine peptide prediction model. In ADRA, however, NAC and NAL were measured correctly with no co-elution, and for this reason Fujita *et al.* (2014) and Yamamoto *et al*. (2015) listed salicylic acid as suitable for assessment with the NAC/NAL prediction model.

8. Applicability domain

The DPRA Validation Report submitted to ECVAM by the test method developer outlined DPRA's broad applicability to a wide range of chemicals in relevant chemical classes, reaction mechanisms, skin sensitization potencies (as determined with *in vivo* studies), and physicochemical properties.

The 82 chemicals tested in house by Procter & Gamble as well as the 24 test chemicals used in the DPRA validation study have also been tested using ADRA. The results showed a predictive capacity similar to DPRA, which the VMT feels indicates and tends to confirm that ADRA is suitable for use with the same, or even a wider, range of chemicals as DPRA.

Nevertheless, just like DPRA, ADRA contains no metabolic/bioactivation system and was not designed for detecting pre-haptens (*i.e.*, chemicals requiring biochemical activation) or pro haptens (*i.e*., chemicals requiring enzymatic activation). Moreover, like DPRA, metals that are sensitive to non-covalent bonding mechanisms or that interact with amino acids other than cysteine and lysine (*e.g*., nickel) fall outside the applicability domain of the test method.

9. Test Definition: Conclusions of the Validation Management Team

- In accordance with the objective of the ADRA validation study, these pre-study results tend to confirm that the ADRA SOP has been thoroughly optimized and performs as designed.
- ADRA is based on the same scientific principles as DPRA and has been shown to be just as accurate but requires test chemical solutions to be of a concentration that is just 1% of those needed for DPRA. The VMT is not aware of chemicals that could not be tested by ADRA due to poor solubility or

co-elution; both of which are recognized limitations of the DRPA test method.

- Just like DPRA, is not considered suitable for the detection of pro haptens, pre-haptens, or metals.
- To date there has been virtually no co-elution of NAC or NAL with the test chemicals or reaction products used in ADRA, nevertheless there are provisions included in the SOP for cases where co-elution does occur.

Transferability

1. General aspects

The VMT believes that this study confirms that ADRA can be transferred to and performed at any laboratory where standard HPLC equipment and technicians with the expertise to operate such equipment are available. The equipment, labware, and reagents other than NAC and NAL needed to perform ADRA are readily available. Additionally, NAC and NAL are expected be available commercially from a chemical manufacturer by the time ADRA is adopted as an OECD test guideline.

Fujifilm, as the lead laboratory in the development of ADRA, was responsible for ensuring that the test method described in the SOP was correctly understood and properly carried out by the participating laboratories, and did so by providing guidance, training and assistance to the laboratories participating in this validation study. The laboratory technicians at each participating laboratory who received guidance from Fujifilm also undertook training in their own laboratories.

The lead laboratory was also responsible for providing detailed plans for technology transfer and training based on its experience with the test method. The lead laboratory worked with the participating laboratories to implement pre-training and training testing to ensure a common understanding of and compliance with the test acceptance criteria specified in the technology transfer plan.

The test chemicals for training phase were selected and supplied by the lead laboratory. The test chemicals were uncoded. The participating laboratories submitted their results promptly and accurately to the lead laboratory.

2. Transfer of the test method to naïve laboratories

Laboratory technicians and study directors from the four participating laboratories were provided with technology transfer training per the schedule show below, at which time information and guidance were also provided on the scientific rationale and principles of the test method, the ADRA SOP, how to quantify NAC and NAL per HPLC analysis, how to analyze results, and how to apply the prediction model.

2-1. Dates of training sessions during the Transferability Stage

- Transferability Stage, Session I
  Venue: Fujifilm Corporation Safety Evaluation Center
  Time & Date: 1:00 p.m. on Thursday, February 25, 2016
- Transferability Stage, Session II
  Venue: Fujifilm Corporation Safety Evaluation Center
  Time & Date: 1:00 p.m. on Tuesday, April 19, 2016

2-2. Training Modules

How to confirm solubility of test chemicals and select a solvent

How to prepare and handle Reference Controls A, B, and C

How to use the NAC/NAL prediction model and the NAC-only prediction model

Test acceptance criteria and retesting

How to use of the photodiode array detector and co-elution control to verify co-elution of NAC or NAL with the test chemical

3. Pre-training testing (Transferability Stage, Session I)

3-1. Goal

The lead laboratory selected and distributed to each participating laboratory five uncoded test chemicals known to be relatively easy to identify as either a sensitizer or a non-sensitizer. The participating laboratories each tested these five test chemicals in accordance with the ADRA SOP. Proficiency was reviewed by analyzing the data from each laboratory before the VMT authorized proceeding to training testing.

3-2. Test chemicals

The five test chemicals chosen by the lead laboratory are shown below in Table 1.

Table 1: Test chemicals used for pre-training

No.	Chemicals	CAS No.	LLNA category
PT-001	p-Benzoquinone	106-51-4	Extreme
PT-002	Cinnamaldehyde	14371-10-9	Moderate
PT-003	Diethyl maleate	141-05-9	Moderate
PT-004	4-Allylanisole	140-67-0	Weak
PT-005	Vanillin	121-33-5	Non-sensitizer

3-3. Pre-training results

Lion

Pre-training results obtained at Lion are shown in Table 2.

Table 2: Pre-training results at Lion

Code	Chemical	LLNA potency	Lion												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
PT-001	*p*-Benzoquinone	Strong	100.0	0.0	78.3	1.3	89.1	S	100.0	0.0	77.9	1.1	89.0	S	89.1	S
PT-002	Cinnamaldehyde	Moderate	50.1	1.3	4.5	0.9	27.3	S	92.9	2.4	5.5	0.2	49.2	S	38.2	S
PT-003	Diethyl maleate	Moderate	29.4	1.6	4.4	1.2	16.9	S	32.1	1.6	3.6	0.3	17.9	S	17.4	S
PT-004	4-Allylanisole	Weak	25.6	0.7	0.0	0.0	12.8	S	31.6	0.7	2.4	2.2	17.0	S	14.9	S
PT-005	Vanillin	Non-sensitizer	1.3	1.0	1.5	0.5	1.4	NS	6.1	3.5	0.8	0.7	3.4	NS	2.4	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

A significant discrepancy was seen in depletion of NAC between the first and the second test for cinnamaldehyde. In general, test chemicals that exhibit either strong or weak reactivity have relatively stable reproducibility, and it is moderately reactive test chemicals like cinnamaldehyde that are particularly sensitive to changes in reaction conditions.

The VMT considers this to be the most likely reason for the significant discrepancy in depletion of NAC for cinnamaldehyde. Also, because aldehyde compounds are likely to be oxidized into carboxylic acid, this may have been the factor that resulted in the change in reactivity.

The remainder of the training-set test chemicals all exhibited good reproducibility for NAC and NAL depletion between the first and second tests, and all five test chemicals were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

Mitsui

Pre-training results obtained at Mitsui are shown in Table 3.

Table 3: Pre-training results at Mitsui

Code	Chemical	LLNA potency	Mitsui												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
PT-001	*p*-Benzoquinone	Strong	100.0	0.0	68.6	0.7	84.3	S	100.0	0.0	68.4	0.4	84.2	S	84.2	S
PT-002	Cinnamaldehyde	Moderate	42.5	4.5	10.7	1.3	26.6	S	34.3	0.6	8.6	0.3	21.5	S	24.0	S
PT-003	Diethyl maleate	Moderate	22.9	2.7	0.5	0.9	11.7	S	25.6	4.6	0.0	0.0	12.8	S	12.2	S
PT-004	4-Allylanisole	Weak	13.1	0.1	0.0	0.0	6.6	S	16.2	3.2	0.0	0.0	8.1	S	7.3	S
PT-005	Vanillin	Non-sensitizer	0.0	0.0	0.0	0.0	0.0	NS	0.0	0.0	0.0	0.0	0.0	NS	0.0	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

All five test chemicals exhibited good reproducibility for NAC and NAL depletion between the first and second tests and were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

Sumitomo

Pre-training results obtained at Sumitomo are shown in Table 4.

Table 4: Pre-training results at Sumitomo

Code	Chemical	LLNA potency	Sumitomo												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
PT-001	*p*-Benzoquinone	Strong	100.0	0.0	71.8	1.0	85.9	S	100.0	0.0	72.6	0.1	86.3	S	86.1	S
PT-002	Cinnamaldehyde	Moderate	94.0	1.5	5.0	0.3	49.5	S	91.8	0.1	6.7	0.5	49.2	S	49.4	S
PT-003	Diethyl maleate	Moderate	33.6	5.1	3.3	0.3	18.4	S	24.4	0.4	4.3	0.1	14.4	S	16.4	S
PT-004	4-Allylanisole	Weak	17.8	0.5	2.2	0.3	10.0	S	15.4	0.3	3.2	0.6	9.3	S	9.6	S
PT-005	Vanillin	Non-sensitizer	8.7	0.9	1.0	0.7	4.8	NS	5.6	0.6	2.4	0.6	4.0	NS	4.4	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

All five test chemicals exhibited good reproducibility for NAC and NAL depletion between the first and second tests and were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

Nissan

Pre-training results obtained at Nissan are shown in Table 5.

Table 5: Pre-training results at Nissan

Code	Chemical	LLNA potency	Nissan												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
PT-001	*p*-Benzoquinone	Strong	100.0	0.0	73.0	1.5	86.5	S	100.0	0.0	79.3	0.3	89.7	S	88.1	S
PT-002	Cinnamaldehyde	Moderate	56.6	1.4	0.2	0.2	28.4	S	100.0	0.0	4.9	0.7	52.5	S	40.4	S
PT-003	Diethyl maleate	Moderate	32.4	1.1	0.9	0.9	16.7	S	28.6	1.1	2.8	0.4	15.7	S	16.2	S
PT-004	4-Allylanisole	Weak	14.7	0.6	0.0	0.0	7.3	S	13.9	0.5	0.2	0.2	7.0	S	7.2	S
PT-005	Vanillin	Non-sensitizer	8.8	0.6	0.0	0.0	4.4	NS	4.5	0.1	0.4	0.4	2.5	NS	3.4	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

A significant discrepancy was seen in depletion of NAC between the first and the second test run for cinnamaldehyde, the causes of which the VMT believes to be the same as those at Lion.

The other test chemicals all exhibited good reproducibility for NAC and NAL depletion between the first and second tests, and all five test chemicals were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

3-4. Issues and Solutions

The participating laboratories encountered a problem when quantifying NAC by HPLC analysis after preparation of NAC stock solution, as a result of dimer formation when a proportion of the NAC is oxidized. The lead laboratory critically evaluated the preparation method, storage, labware, and reagents used at each laboratory and after repeated testing established that NAC exhibited a tendency to be oxidized when glass labware was used. The lead laboratory then proposed, and the VMT accepted, that disposable plastic labware be used in place of glass labware. The Apparatus and Reagents section of the test method Study Plan was revised to reflect this change. The problem did not recur once disposable plastic labware was used.

Changing the capacity of the mixer used to create a uniform mixture of the two eluents used during HPLC analysis affects other conditions, such as the eluent gradient start time and slope, thus affecting peak configuration. Although the capacity of the mixers varies with the HPLC equipment manufacturer and are not standardized, in page 19 in section 5-1 of the Study Plan, to ensure uniformity the HPLC Conditions Note 1 of the SOP was revised to indicate 0.5 ml as a suitable mixer capacity, based on HLPC equipment manufactured by Shimadzu Corporation.

One of the participating laboratories experienced a very slight overlap of NAC and NAL peaks with the peak of the test chemicals. This was determined to be due to the use of very large-diameter tubing as well as relatively long tubing from the column to the detector rather than being true co-elution. Page 19 in section 5-1. HPLC Conditions Note 2 of the Study Plan was revised to indicate that 0.18-mm diameter tubing is to be used and that the length from the column to the detector is to be no more than 50 cm.

4. Training (Transferability Stage, Session II)

4-1. Goal

The lead laboratory selected and distributed to each participating laboratory ten uncoded test chemicals that are known to be relatively difficult to identify as either a sensitizer or a non-sensitizer. The participating laboratories tested these ten test chemicals in accordance with the ADRA SOP. Proficiency was evaluated, determined and confirmed by analyzing the data from each laboratory before the VMT agreed to proceed to the Reliability Stage of the validation study.

4-2. Test chemicals

The ten test chemicals chosen by the lead laboratory are shown below in Table 6.

Table 6: Test chemicals used for training

No.	Chemical	CAS No.	LLNA potency
T-001	Oxazolone	15646-46-5	Strong
T-002	Phthalic anhydride	85-44-9	Strong
T-003	Formaldehyde	50-00-0	Strong
T-004	Glyoxal	107-22-2	Moderate
T-005	Benzylideneacetone	122-57-6	Moderate
T-006	Cyclamen aldehyde	103-95-7	Weak
T-007	1-Butanol	71-36-3	Non-sensitizer
T-008	6-Methylcoumarin	92-48-8	Non-sensitizer
T-009	Lactic acid	50-21-5	Non-sensitizer
T-010	4'-Methoxyacetophenone	100-06-1	Non-sensitizer

4-3. Training results

Lion

Training results obtained at Lion for the ten test chemicals are shown in Table 7.

Table 7: Training results at Lion

Code	Chemical	LLNA potency	Lion												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
T-001	Oxazolone	Strong	78.8	0.4	69.8	2.8	74.3	S	79.5	1.3	66.8	1.2	73.2	S	73.7	S
T-002	Phthalic anhydride	Strong	0.0	0.0	88.4	0.8	44.2	S	0.4	0.7	81.3	6.2	40.8	S	42.5	S
T-003	Formaldehyde	Strong	20.3	4.5	3.1	5.3	11.7	S	16.1	2.7	2.4	3.9	9.2	S	10.5	S
T-004	Glyoxal	Moderate	13.6	1.0	0.1	0.2	6.8	S	20.2	1.2	0.3	0.4	10.3	S	8.5	S
T-005	Benzylideneacetone	Moderate	14.4	1.6	1.0	1.8	7.7	S	16.5	3.7	0.2	0.3	8.3	S	8.0	S
T-006	Cyclamen aldehyde	Weak	6.0	4.3	1.0	1.7	3.5	NS	4.6	2.6	0.4	0.7	2.5	NS	3.0	NS
T-007	1-Butanol	Non-sensitizer	2.2	3.2	4.3	6.1	3.2	NS	1.3	2.2	2.6	3.5	1.9	NS	2.6	NS
T-008	6-Methylcoumarin	Non-sensitizer	0.0	0.0	0.5	0.9	0.3	NS	0.0	0.0	0.4	0.4	0.2	NS	0.2	NS
T-009	Lactic acid	Non-sensitizer	2.1	3.6	3.1	5.1	2.6	NS	1.8	3.2	0.3	0.4	1.1	NS	1.8	NS
T-010	4'-Methoxyacetophenone	Non-sensitizer	7.1	0.1	0.1	0.1	3.6	NS	4.6	0.5	0.1	0.2	2.3	NS	3.0	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

Because the mean % depletion of NAC/NAL for cyclamen aldehyde was less than 5%, which is very

low, it was predicted to be a non-sensitizer, which does not agree with *in vivo* test results. LLNA predicts cyclamen aldehyde to be a weak sensitizer, and when selecting chemicals, the lead laboratory had expected that it would be difficult to identify correctly. In addition to Lion, results at the other three participating laboratories were between 5.7 and 12.3%, which are also low values, very near the threshold specified in the prediction criteria. Also, the depletion of aldehyde compounds is known to be generally is variable, which could be the reason that depletion was slightly lower at Lion than at the other participating laboratories.

The other nine test chemicals all exhibited good reproducibility for NAC and NAL depletion between the first and second tests and were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

Mitsui

Training results obtained at Mitsui for the ten test chemicals are shown in Table 8.

Table 8: Training results at Mitsui

Code	Chemical	LLNA potency	Mitsui												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
T-001	Oxazolone	Strong	83.5	0.3	67.4	1.0	75.4	S	87.0	0.6	63.6	0.5	75.3	S	75.4	S
T-002	Phthalic anhydride	Strong	0.1	0.2	51.3	3.3	25.7	S	4.0	0.2	54.5	4.3	29.2	S	27.5	S
T-003	Formaldehyde	Strong	31.0	0.7	0.1	0.1	15.5	S	15.7	0.2	0.3	0.5	8.0	S	11.8	S
T-004	Glyoxal	Moderate	12.0	0.3	1.1	0.3	6.5	S	15.5	0.3	0.3	0.4	7.9	S	7.2	S
T-005	Benzylideneacetone	Moderate	12.6	2.2	3.3	0.6	8.0	S	13.3	0.3	1.8	0.5	7.6	S	7.8	S
T-006	Cyclamen aldehyde	Weak	19.8	1.4	4.8	0.4	12.3	S	15.6	0.7	3.2	0.4	9.4	S	10.9	S
T-007	1-Butanol	Non-sensitizer	0.0	0.0	0.6	0.3	0.3	NS	1.9	0.1	0.4	0.2	1.2	NS	0.7	NS
T-008	6-Methylcoumarin	Non-sensitizer	1.3	2.3	3.6	0.2	2.4	NS	1.6	0.4	1.5	1.3	1.6	NS	2.0	NS
T-009	Lactic acid	Non-sensitizer	0.0	0.0	1.2	0.7	0.6	NS	2.4	1.1	1.4	2.3	1.9	NS	1.3	NS
T-010	4'-Methoxyacetophenone	Non-sensitizer	8.1	2.6	3.8	0.2	5.9	S	5.7	0.9	1.9	0.3	3.8	NS	4.9	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

The NAC peak overlapped slightly with the broadening and tailing peak of 4'-methoxyacetophenone during the first test, and so the peak area was not calculated correctly, which is why the VMT thinks it was incorrectly predicted to be a sensitizer. Prior to the second test, the length of the HPLC tubing was shortened from 50 cm to 30 cm, to improve separation performance. This lowered NAC depletion and 4'-methoxyacetophenone was correctly predicted to be a non-sensitizer.

Sumitomo

Training results obtained at Sumitomo for the ten test chemicals are shown in Table 9.

Table 9: Training results at Sumitomo

Code	Chemical	LLNA potency	Sumitomo												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
T-001	Oxazolone	Strong	78.8	0.9	81.4	0.3	80.1	S	80.7	0.9	73.4	0.8	77.0	S	78.6	S
T-002	Phthalic anhydride	Strong	0.3	0.5	86.6	7.9	43.5	S	6.1	1.1	74.6	7.5	40.4	S	41.9	S
T-003	Formaldehyde	Strong	40.1	1.1	0.0	0.1	20.1	S	16.8	0.7	0.0	0.0	8.4	S	14.2	S
T-004	Glyoxal	Moderate	13.2	1.5	0.2	0.3	6.7	S	10.8	3.8	0.0	0.0	5.4	S	6.0	S
T-005	Benzylideneacetone	Moderate	16.9	0.6	1.1	0.2	9.0	S	20.7	0.7	0.0	0.0	10.3	S	9.7	S
T-006	Cyclamen aldehyde	Weak	9.9	1.2	1.5	0.2	5.7	S	21.3	0.6	0.0	0.0	10.6	S	8.2	S
T-007	1-Butanol	Non-sensitizer	0.2	0.3	0.0	0.0	0.1	NS	0.0	0.0	3.2	5.3	1.6	NS	0.8	NS
T-008	6-Methylcoumarin	Non-sensitizer	3.9	1.1	0.9	0.2	2.4	NS	9.8	1.3	0.0	0.0	4.9	NS	3.7	NS
T-009	Lactic acid	Non-sensitizer	5.4	7.1	0.2	0.4	2.8	NS	0.0	0.0	3.9	6.7	2.0	NS	2.4	NS
T-010	4'-Methoxyacetophenone	Non-sensitizer	18.1	1.0	1.4	0.3	9.7	S	24.4	1.4	0.0	0.0	12.2	S	10.9	S

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

NAC depletion for 4'-methoxyacetophenone was quite large in both the first and second tests, and as a result it was incorrectly predicted both times to be a sensitizer. The reason for this increase in reactivity of NAC was investigated but remains unclear.

The other nine test chemicals, however, all exhibited good reproducibility for NAC and NAL depletion between the first and second tests and were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

Nissan

Training results obtained at Nissan for the ten test chemicals are shown in Table 10.

Table 10: Training results at Nissan

Code	Chemical	LLNA potency	Nissan												Mean value of mean % depletion (%)	Predicted class
			1st test						2nd test							
			NAC		NAL		Mean % depletion (%)	Predicted class	NAC		NAL		Mean % depletion (%)	Predicted class		
			Depletion (%)	SD	Depletion (%)	SD			Depletion (%)	SD	Depletion (%)	SD				
T-001	Oxazolone	Strong	84.4	0.4	69.9	0.2	77.1	S	84.2	0.6	65.6	0.2	74.9	S	76.0	S
T-002	Phthalic anhydride	Strong	0.8	1.3	89.7	1.3	45.2	S	1.7	0.3	90.1	0.2	45.9	S	45.5	S
T-003	Formaldehyde	Strong	19.6	1.5	0.5	0.4	10.0	S	18.6	1.4	0.1	0.2	9.3	S	9.7	S
T-004	Glyoxal	Moderate	18.1	0.8	0.6	0.4	9.4	S	30.4	5.1	0.0	0.0	15.2	S	12.3	S
T-005	Benzylideneacetone	Moderate	23.3	4.6	0.0	0.0	11.6	S	14.8	0.2	0.0	0.0	7.4	S	9.5	S
T-006	Cyclamen aldehyde	Weak	12.9	2.4	0.0	0.0	6.5	S	13.0	0.0	0.1	0.2	6.6	S	6.5	S
T-007	1-Butanol	Non-sensitizer	0.0	0.0	0.0	0.0	0.0	NS	1.3	0.7	0.0	0.0	0.7	NS	0.3	NS
T-008	6-Methylcoumarin	Non-sensitizer	1.9	2.2	0.2	0.2	1.1	NS	5.6	4.3	0.0	0.0	2.8	NS	1.9	NS
T-009	Lactic acid	Non-sensitizer	0.2	0.3	0.0	0.0	0.1	NS	0.0	0.0	0.1	0.2	0.1	NS	0.1	NS
T-010	4'-Methoxyacetophenone	Non-sensitizer	2.2	2.0	0.3	0.3	1.2	NS	3.6	0.4	0.2	0.3	1.9	NS	1.6	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Comments

All ten test chemicals exhibited good reproducibility for NAC and NAL depletion between the first and second tests and were accurately predicted to be either sensitizers or non-sensitizers in concordance with *in vivo* test results.

4-4. Issues and Solutions

- Although Sumitomo prepared the NAC stock solution using plastic labware, some oxidation of NAC was observed. The lead laboratory identified the cause to be the distilled water used, and problem was resolved by changing the brand of distilled water.
- Some oxidation of NAC was also observed at Nissan, but the lead laboratory was unable to identify the cause.
- Oxidation of the NAC stock solution was alleviated as described above by specifying the use of plastic labware, but the lead laboratory also identified other potential causes of oxidation and investigated and advised on potential countermeasures. In particular, the presence of minute quantities of copper and other metal ions in the aqueous solutions was identified as a likely cause of catalytic action leading to the oxidation of NAC. To alleviate this situation, the chelating agent ethylenediaminetetraacetic acid (EDTA) was added during preparation of the NAC stock solution to sequester metal ions. The addition of EDTA during preparation of the NAC stock solution at the participating laboratories where oxidation of NAC had been significant greatly reduced the oxidation. It was therefore determined that EDTA would be used as an additive for all NAC stock solution during the rest of the validation study.
- The decision to use EDTA as an additive for all NAC stock solutions meant that a new control range

for the positive control reagent phenyl acetaldehyde was needed for Reliability Stage Phase I of the validation study, and this was provisionally determined using background data from the lead laboratory for testing conducted with NAC stock solution to which EDTA had been added. Due to a lack of available background data at this time, however, the control range for Phase II was later determined using Phase I data from the participating laboratories.

5. Transferability: Conclusions of the Validation Management Team

Transfer of ADRA from the lead laboratory to the four participating laboratories was completed successfully and to the satisfaction of both the lead laboratory and the VMT.

Issues identified during technology transfer were alleviated by changing the labware, reagents and equipment as well as the settings for HPLC analysis. These changes were incorporated in the SOP and Study Plan. Of particular note were measures taken to suppress oxidation of the NAC stock solution, which included the use of plastic disposable labware and the addition of EDTA as a chelating agent to sequester metal ions in the NAC stock solution. These measures successfully resolved issues related to oxidation of NAC at the participating laboratories. Additionally, as previously described, due to a lack of background data for testing with NAC stock solution to which EDTA was added, a control range was provisionally determined for Reliability Stage Phase I of the validation study using background data from the lead laboratory, after which the control range for Phase II was determined using Phase I data from the participating laboratories.

Within-Laboratory Reproducibility

Reliability Stage Phase I (Within-Laboratory Reproducibility) was performed using SOP ver. 1.0, and as described above, acceptance criteria for the positive control reagent (phenylacetaldehyde) was provisionally set to 8–100% for NAC and 86–100% for NAL.

1. Lion

1-1. Reproducibility (concordance in prediction)

Seven sensitizers and three non-sensitizers were tested three times each, yielding results that were 100% concordant in prediction. As shown in Table 11, reproducibility was 100%.

Table 11: Reliability Stage Phase I results at Lion

No.	Chemicals	LLNA potency	Solvent	1st			1st re-test*			Solvent	2nd			2nd re-test**			Solvent	3rd		
				NAC depletion (%)	NAL depletion (%)	Ave.score (%)	NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)	NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)
1	Lauryl gallate	Strong	Acetonitrile	100.0	95.6	97.8				Acetonitrile	100.0	93.4	96.7	100.0	92.7	96.3	Acetonitrile	100.0	92.6	96.3
2	4-(Methylamino) phenol hemisulfate salt	Strong	Water	99.5	13.6	56.5	99.5	13.7	56.6	Water	99.5	12.8	56.1	99.6	12.1	55.9	Water	99.7	13.2	56.5
3	Chloramine T	Strong	Water	98.2	91.4	94.8	97.3	90.9	94.1	Water	98.7	94.4	96.6	98.7	91.4	95.0	Water	98.7	93.9	96.3
4	Cinnamaldehyde	Moderate	Acetonitrile	54.3	7.4	30.8				Acetonitrile	25.2	7.2	16.2	50.1	6.4	28.2	Acetonitrile	23.9	6.4	15.1
5	2-Mercaptobenzothiazole	Moderate	Acetonitrile	49.3	0.7	25.0				Acetonitrile	48.4	2.6	25.5	42.5	1.8	22.1	Acetonitrile	41.0	2.3	21.7
6	Ethyl acrylate	Weak	Water	95.2	4.0	49.6	87.9	3.4	45.7	Water	89.0	4.0	46.5	89.1	2.3	45.7	Water	87.7	4.5	46.1
7	Imidazolidinylurea	Weak	Water	27.5	0.0	13.8	29.7	0.1	14.9	Water	27.1	0.2	13.7	20.2	0.0	10.1	Water	22.2	0.8	11.5
8	Glycerol	NC	Water	0.0	0.0	0.0	0.1	0.5	0.3	Water	0.0	0.1	0.1	0.1	0.0	0.1	Water	0.7	0.4	0.6
9	Salicylic acid	NC	Acetonitrile	0.6	2.3	1.5				Acetonitrile	1.6	3.1	2.3	0.2	2.6	1.4	Acetonitrile	1.4	2.2	1.8
10	Isopropanol	NC	Water	0.0	1.2	0.6	0.0	0.0	0.0	Water	1.1	0.2	0.6	0.0	0.0	0.0	Water	0.0	0.0	0.0

*Cells highlighted in gray indicate chemicals that were not retested, cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

*Reason for retesting during the 1st replicate test: Concentration of NAC in aqueous solution of Reference Control C was 3.14 µM, which falls below the control range of 3.2–4.4 µM.

**Reason for retesting during the 2nd replicate test: NAL depletion of the Positive Control reagent was 83.2%, which falls below the acceptance criteria of greater than 86%.

1-2. Reproducibility in the test chemicals

The results showed good reproducibility overall, and variability in the NAC depletion across three replicate tests that met test acceptance criteria being 8% or less for all ten test chemicals except cinnamaldehyde. As previously explained, cinnamaldehyde exhibits moderate reactivity and is an aldehyde compound, both of which make it particularly sensitive to variations in the testing conditions, which could explain the variability in depletion.

Similarly, variability in NAL depletion across three replicate tests that met test acceptance criteria was 3% or less for all ten test chemicals.

1-3. Reproducibility in the control reagents

Tests that met test acceptance criteria showed good reproducibility, with NAC concentrations in Reference Controls A, B, and C for the three replicate tests and two retests being 3.40–3.86 µM as well as NAC depletion in the Positive Control reagent being 11.8–18.2%. Also, NAL concentrations in Reference Controls A, B, and C for the three replicate tests and two retests were 3.61–4.13 µM and NAC depletion in the Positive Control reagent was 83.2–100%.

1-4. Retesting

Retesting was performed in two instances. Retesting during the 2nd replicate test was performed because NAL depletion of the Positive Control reagent was 83.2%, which falls below the acceptance criteria of greater than 86%.

2. Mitsui

2-1. Reproducibility (concordance in prediction)

Seven sensitizers and three non-sensitizers were tested three times each, yielding results that were 100% concordant in prediction. As shown in Table 12, reproducibility was 100%.

Table 12: Reliability Stage Phase I results at Mitsui

No.	Chemicals	LLNA potency	Solvent	1st			1st re-test*		Solvent	2nd			2nd re-test**			2nd re-re-test**		Solvent	3rd			3rd re-test***	
				NAC depletion (%)	NAL depletion (%)	Ave.score (%)	NAC depletion (%)	Ave.score (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)	NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)	NAC depletion (%)	Mean % depletion (%)
1	Lauryl gallate	Strong	Acetone	85.8	18.5	52.2	100.0	59.3	Acetone	100.0	85.2	92.6	100.0	48.3	74.2	17.0	58.5	Acetonitrile	100.0	93.6	96.8		
2	4-(Methylamino) phenol hemisulfate salt	Strong	Water	100.0	16.0	58.0			Water	100.0	8.7	54.4	100.0	16.2	58.1			Water	98.0	13.9	56.0		
3	Chloramine T	Strong	Acetonitrile	100.0	98.0	99.0			Acetonitrile	100.0	97.8	98.9	100.0	98.6	99.3			Acetonitrile	100.0	99.0	99.5		
4	Cinnamaldehyde	Moderate	Acetonitrile	35.6	6.5	21.0			Acetonitrile	60.9	8.0	34.4	38.9	6.1	22.5			Acetonitrile	33.1	12.3	22.7		
5	2-Mercaptobenzothiazole	Moderate	Acetonitrile	52.8	1.2	27.0			Acetonitrile	55.6	2.6	29.1	55.4	0.0	27.7			Acetonitrile	68.3	6.2	37.3	48.8	27.5
6	Ethyl acrylate	Weak	Water	87.2	6.3	46.7			Water	94.9	0.9	47.9	91.4	4.8	48.1			Water	90.9	3.1	47.0		
7	Imidazolidinyhurea	Weak	Water	18.6	1.8	10.2			Water	18.0	interf.	NC	22.8	0.2	11.5			Water	20.5	0.0	10.2		
8	Glycerol	NC	Water	0.2	0.0	0.1			Water	0.0	interf.	NC	0.0	0.0	0.0			Water	0.1	0.0	0.1		
9	Salicylic acid	NC	Acetonitrile	3.7	1.9	2.8			Acetonitrile	0.6	3.7	2.1	2.9	1.1	2.0			Acetonitrile	3.0	6.7	4.9		
10	Isopropanol	NC	Water	1.0	0.0	0.5			Water	0.0	interf.	NC	0.0	0.1	0.0			Water	0.1	0.0	0.1		

*Cells highlighted in gray indicate chemicals that were not retested, cells highlighted in yellow indicate chemicals that exhibited interference, cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

*Reason for retesting during the 1st replicate test: SD of NAC depletion in Test Chemical No. 1 was 12.6%, which falls outside the acceptance criteria of less than 10%.

**Reason for retesting during the 2nd replicate test: NAC depletion of the Positive Control reagent was 7.7%, which falls below the acceptance criteria of greater than 8%.

Reason for re-retesting during the 2nd replicate test: SD of NAL depletion in Test Chemical No. 1 was 36.6%, which falls outside the acceptance criteria of less than 10%.

***Reason for retesting during the 3rd replicate test: SD of NAC depletion in Test Chemical No. 5 was 23.8%, which falls outside the acceptance criteria of less than 10%.

2-2. Reproducibility in the test chemicals

The results showed good reproducibility overall, and variability in NAC depletion across three replicate tests that met test acceptance criteria was 7% or less for all ten test chemicals. Similarly, variability in NAL depletion across three replicate tests that met test acceptance criteria was 7% or less for all ten test chemicals except lauryl gallate. NAL depletion for the three replicate tests was lauryl

gallate was 18.5%, 17.0%, and 93.6%, respectively. For each replicate test the preferred solvent was identified separately by each laboratory in line with the instructions set out in the Study Plan. The solvent used to test lauryl gallate was acetone for the 1st and 2nd replicate tests, but acetonitrile for the 3rd replicate test. Also, whereas NAL depletion for the 1st and 2nd replicate tests was 18.5% and 17.0%, respectively, for the 3rd replicate test, it was 93.6%. Lion and Nissan both used acetonitrile as the solvent for all three replicate tests, and each time the NAL depletion was consistently 92% or higher. In contrast, Sumitomo used acetone as the solvent for all three replicate tests and recorded NAL depletion levels between 20.6% and 65.9%—values that are both low and inconsistent. Lauryl gallate is a pro-hapten, it can react with NAL by oxidation. Based on the above, the VMT considers that although the use of acetonitrile as a solvent for lauryl gallate provides good reactivity with NAL by oxidation, the use of acetone as a solvent for lauryl gallate is problematic due to discrepancies in how well it is oxidized in the reaction fluid, which has the potential for inconsistent levels of NAL depletion.

2-3. Reproducibility in the control reagents

Tests that met test acceptance criteria showed good reproducibility, with NAC concentrations in Reference Controls A, B, and C for the three replicate tests and two retests being 3.23–4.19 μM as well as NAC depletion in the Positive Control reagent being 7.7–10.1%. Also, NAL concentrations in Reference Controls A, B, and C for the three replicate tests and two retests being 3.36–4.16 μM as well as NAC depletion in the Positive Control reagent being 87.3–100%.

2-4. Retesting

Retesting was performed on four occasions by this laboratory. Retesting during the 1st replicate test was performed, because the SD of NAC depletion for lauryl gallate was 12.6%, which falls outside the acceptance criteria of less than 10%. Retesting during the 2nd replicate test was performed, because NAC depletion of the Positive Control reagent was 7.7%, which falls below the acceptance criteria of greater than 8%. Re-testing during the 2nd replicate test was performed, because the SD of NAL depletion for lauryl gallate was 36.6%, which falls outside the acceptance criteria of less than 10%. Retesting during the 3rd replicate test was performed, because the SD of NAC depletion for 2-mercaptbenzothiazole was 23.8%, which falls outside the acceptance criteria of less than 10%.

3. Sumitomo

3-1. Reproducibility (concordance in prediction)

The data set was incomplete because, as explained below, tests for three test chemicals—cinnamaldehyde, 2-mercaptbenzothiazole, and salicylic acid—failed to meet test acceptance criteria during the 2nd replicate test. As shown in Table 13, the other seven test chemicals were each tested three times each, yielding results that were 100% concordant in prediction. Thus, reproducibility was 100%.

Table 13: Reliability Stage Phase I results at Sumitomo

No.	Chemicals	LLNA potency	Solvent	1st			Solvent	2nd			2nd re-test*			Solvent	3rd		
				NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)	NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)
1	Lauryl gallate	Strong	Acetone	100.0	65.9	83.0	Acetone	100.0	58.5	79.3	100.0	20.6	60.3	Acetone	100.0	43.2	71.6
2	4-(Methylamino) phenol hemisulfate salt	Strong	Water	100.0	15.8	57.9	Water	100.0	15.4	57.7	100.0	15.4	57.7	Water	100.0	12.4	56.2
3	Chloramine T	Strong	Water	98.4	96.9	97.7	Water	98.2	95.0	96.6	98.1	96.8	97.5	Water	100.0	92.9	96.5
4	Cinnamaldehyde	Moderate	Acetonitrile	98.4	9.6	54.0	Acetonitrile	98.4	7.4	52.9	–	–	–	Acetonitrile	67.0	5.4	36.2
5	2-Mercaptobenzothiazole	Moderate	Acetonitrile	48.4	0.5	24.5	Acetonitrile	53.6	0.0	26.8	–	–	–	Acetonitrile	81.2	0.0	40.6
6	Ethyl acrylate	Weak	Water	92.4	5.1	48.7	Water	89.8	4.8	47.3	49.8	0.3	25.1	Water	92.4	5.3	48.9
7	Imidazolidinylurea	Weak	Water	24.1	0.5	12.3	Water	22.5	5.3	13.9	29.8	0.3	15.1	Water	30.0	0.2	15.1
8	Glycerol	NC	Water	0.0	0.3	0.2	Water	Interf.	0.0	NC	5.7	0.0	2.8	Water	5.7	0.0	2.9
9	Salicylic acid	NC	Acetonitrile	0.0	1.3	0.6	Acetonitrile	0.0	0.0	0.0	–	–	–	Acetonitrile	12.2	0.0	6.1
10	Isopropanol	NC	Water	0.0	0.6	0.3	Water	Interf.	0.3	NC	4.7	0.2	2.5	Water	5.6	0.3	3.0

*Cells highlighted in gray indicate chemicals that were not retested, cells highlighted in yellow indicate chemicals that exhibited interference, cells containing a dash (–) indicate missing data, cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Reason for retesting during the 2nd replicate test: (1)Concentration of NAC in aqueous solution of Reference Control C was 3.14 µM, which falls below the control range of 3.2–4.4 µM. (2)The CV of the peak area for NAL of Reference Control C in acetone solution was 14%, which falls outside the acceptance criteria of less than 10%. (3) NAL depletion of the Positive Control reagent was 76.3%, which falls below the acceptance criteria of greater than 86%.
Thus, no test was conducted (a deviation from the SOP).

3-2. Reproducibility in the test chemicals

The study results showed good reproducibility overall, and variability in NAC depletion across three replicate tests that met test acceptance criteria was 6% or less for six of the ten test chemicals. The four exceptions were cinnamaldehyde, 2-mercaptbenzothiazole, ethyl acrylate, and salicylic acid. As previously explained, cinnamaldehyde exhibits moderate reactivity and is an aldehyde compound, which could explain the variability in depletion. The cause of the variability in NAC depletion for 2-mercaptbenzothiazole and ethyl acrylate is unclear, but both exhibited depletion of roughly 50% or more, which means that neither chemical exhibited enough reactivity to be predicted to be sensitizers. Since salicylic acid has no site that reacts with thiol or amino groups, the VMT concludes that the high depletion value of 12.2% in the 3rd replicate test was likely an outlier resulting from the manner in which the testing was conducted. Similarly, variability in NAL depletion across three replicate tests that met test acceptance criteria was 5% or less for all ten test chemicals except lauryl gallate. Lauryl gallate, as described above, is a pro-hapten and easily exhibits variability in oxidation, especially when in acetone solution, which is likely the cause of the variability in depletion.

3-3. Reproducibility of the control reagents

Tests that met test acceptance criteria showed good reproducibility, with NAC concentrations in Reference Controls A, B, and C for the three replicate tests and two retests being 3.39–3.91 µM as well as

NAC depletion in the Positive Control reagent being 13.2–24.2%. As is explained later, however, NAC concentration in Reference Control C in aqueous solution during the 2nd replicate test was 3.14 μM, which did not meet test acceptance criteria. Also, NAL concentrations in Reference Controls A, B, and C for the three replicate tests and two retests were 3.64–4.05 μM and NAC depletion in the Positive Control reagent was 76.3–97.4%.

3-4. Retesting

Retesting was performed on one occasion at this laboratory. Retesting was performed for two reasons, the first being that the NAC concentration of Reference Control C in aqueous solution was 3.14 μM, which was below the control range of 3.2–4.4 μM, and the second being that the CV of the peak area for NAL of Reference Control C in acetone solution was 14%, which falls outside the acceptance criteria of less than 10%.

3-5. Incomplete data

The 2nd replicate test failed to meet test acceptance criteria for the following three parameters: (1) NAC concentrations of Reference Control C in aqueous solution, (2) CV (%) for NAL of Reference Control C in acetone solution, and (3) NAL depletion of the Positive Control reagent. And although retesting was performed with regard to parameters (1) and (2), it was not performed with regard to parameter (3).

As previously described in section 5, Transferability: Conclusions of the Validation Management Team, test acceptance criteria for the Positive Control reagent during Phase I was temporarily specified as greater than 15% for NAC depletion and greater than 86% for NAL depletion, after which Phase I results were used to determine new test acceptance criteria prior to the start of Phase II. This is why Sumitomo understood that (3) NAL depletion of the Positive Control reagent did not satisfy the test acceptance criteria, but had the mistaken impression that there was no need for retesting because acceptance criteria of positive control was provisionally determined for Phase I.

Thus, since cinnamaldehyde, 2-mercaptbenzothiazole, salicylic acid all required retesting with regard to parameter (3), the data for these three test chemicals was not acceptable and the data set was left incomplete. At Sumitomo, however, it proved difficult to perform testing repeatedly due to the situation in house, the statistician determined that retesting would not be necessary for the three reasons show below, so the VMT decided to proceed with Phase II as scheduled.

1. The missing data was independent of other data, and any resulting bias would be small.
2. The validation study was performed with four participating laboratories, which is more than usual.
3. The number of missing data was small, as was the concurrent loss of precision.

4. Nissan

4-1. Reproducibility (concordance in prediction)

As shown in Table 14, seven sensitizers and three non-sensitizers were tested three times each, yielding results that were concordant in prediction except for salicylic acid. Thus, reproducibility was 90%.

Table 14: Reliability Stage Phase I results at Nissan

No.	Chemicals	LLNA potency	Solvent	1st			1st re-test*			Solvent	2nd			Solvent	3rd		
				NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)	NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)		NAC depletion (%)	NAL depletion (%)	Mean % depletion (%)
1	Lauryl gallate	Strong	Acetonitrile	100.0	92.5	96.3				Acetonitrile	99.9	92.9	96.4	Acetonitrile	100.0	93.6	96.8
2	4-(Methylamino) phenol hemisulfate salt	Strong	Water	99.4	0.0	49.7	99.5	12.8	56.2	Water	99.8	13.2	56.5	Water	100.0	13.2	56.6
3	Chloramine T	Strong	Water	100.0	2.8	51.4	100.0	0.0	50.0	Water	100.0	8.3	54.1	Water	100.0	11.2	55.6
4	Cinnamaldehyde	Moderate	Acetonitrile	40.2	9.2	24.7				Acetonitrile	27.7	4.5	16.1	Acetonitrile	48.9	4.4	26.6
5	2-Mercaptobenzothiazole	Moderate	Acetonitrile	100.0	0.1	50.0				Acetonitrile	88.5	0.0	44.2	Acetonitrile	100.0	0.0	50.0
6	Ethyl acrylate	Weak	Water	98.4	interf.	NC	98.2	4.1	51.2	Water	94.8	5.8	50.3	Water	99.0	7.1	53.1
7	Imidazolidinylurea	Weak	Water	20.2	interf.	NC	25.9	0.0	12.9	Water	29.3	0.0	14.7	Water	37.0	2.7	19.8
8	Glycerol	NC	Water	0.0	interf.	NC	1.3	0.0	0.6	Water	2.3	0.0	1.1	Water	9.6	0.0	4.8
9	Salicylic acid	NC	Acetonitrile	5.5	0.0	2.8				Acetonitrile	1.6	1.1	1.3	Acetonitrile	17.5	0.5	9.0
10	Isopropanol	NC	Water	interf.	interf.	NC	0.8	0.0	0.4	Water	3.8	0.0	1.9	Water	9.7	0.0	4.8

*Cells highlighted in gray indicate chemicals that were not retested, cells highlighted in yellow indicate chemicals that exhibited interference, cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Reason for retesting during the 1st replicate test: of the six test chemicals that were tested in aqueous solution (Nos. 3, 4, 6, 8, 9, and 10), NAC depletion for No. 4 (N = 1) and NAL depletion for Nos. 4 (N = 1), No. 6 (N = 1), No. 9 (N = 2), and No. 10 (N = 3) was less than -10%, indicating interference.

Although the NAC/NAL concentration of Reference Control C in aqueous solution was within the control range of 3.2–4.4 µM, the actual values were 3.23 µM for NAC and 3.22 µM, which are both very close to the lower limit. The VMT believes that the fact that these values were so low relative to NAL depletion of the test chemical was the likely cause.

4-2. Reproducibility of depletion values for NAC and NAL

The results showed good reproducibility overall, and variability in NAC depletion across three replicate tests that met test acceptance criteria was 9% or less for six of the ten test chemicals. The four exceptions were cinnamaldehyde 2-mercaptbenzothiazole, imidazolidinylurea, and salicylic acid. As previously explained, cinnamaldehyde exhibits moderate reactivity and is an aldehyde compound, which could explain the variability in depletion. The cause of the variability in NAC depletion for 2-mercaptbenzothiazole and imidazolidinylurea is unclear, but they exhibited depletion of roughly 80% and 20%, respectively, which means that neither chemical exhibited enough reactivity to be predicted to be sensitizers.

Since salicylic acid has no site that reacts with thiol or amino groups, the VMT thinks that the cause of the high depletion value of 17.5% for NAC depletion in the 3rd replicate test remains unclear, it was likely an outlier resulting from the manner in which testing was conducted.

4-3. Reproducibility in the control reagents

Tests that met test acceptance criteria showed good reproducibility, with NAC concentrations in Reference Controls A, B, and C for the three replicate tests and two retests being 3.20–3.73 μM as well as NAC depletion in the Positive Control reagent being 19.1–23.9%. As will be explained later, NAC concentration of Reference Control C in aqueous solution in the 1st replicate test was 3.23 μM, and despite the fact that all test acceptance criteria were met and there was no co-elution, NAC depletion for isopropanol was less than -10%. The VMT considers that this occurred because the NAC concentration of Reference Control C in aqueous solution was close to the low end of the control range of 3.2–4.4 μM. Also, NAL concentrations in Reference Controls A, B, and C for the three replicate tests and two retests were 3.64–4.05 μM and NAC depletion in the Positive Control reagent was 90.2–94.2%. As will be explained later, however, NAL concentration of Reference Control C in aqueous solution in the 1st replicate test was 3.22 μM, and despite the fact that all test acceptance criteria were met and there was no co-elution, NAC depletion for ethyl acetate, imidazolidinylurea, glycerol, and isopropanol were all less than -10%. The VMT thinks that this occurred because the NAL concentration of Reference Control C in aqueous solution was quite close to the low end of the control range of 3.2–4.4 μM.

4-4. Retesting

Retesting was performed once at this laboratory. Retesting during the 1st replicate test was performed, as mentioned above, because NAC depletion of isopropanol as well as NAL depletion of ethyl acetate, imidazolidinylurea, glycerol, and isopropanol were all less than -10%. Thus, retesting was performed, despite the fact that all test acceptance criteria were met and there was no co-elution, because either NAC or NAL depletion of these four test chemicals in aqueous solution was less than -10%.

5. Results and other considerations

Test results from each of the four participating laboratories, including NAC, NAL, and mean % depletion for ten test chemicals, are shown in Tables 15-1 to 15-2.

Table 15-1: Summary of Reliability Stage Phase I results at Lion

No.	Chemical	LLNA category	Lion																	
			Experiment 1						Experiment 2						Experiment 3					
			NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class
A	Lauryl gallate	Strong	100.0	0.0	95.6	3.8	97.8	S	100.0	0.0	92.7	0.2	96.3	S	100.0	0.0	92.6	0.1	96.3	S
B	4-(Methylamino) phenol hemisulfate salt	Strong	99.5	0.1	13.6	0.3	56.5	S	99.6	0.1	12.1	1.3	55.9	S	99.7	0.3	13.2	0.3	56.5	S
C	Chloramine T	Strong	97.3	1.1	90.9	0.8	94.1	S	98.7	0.1	91.4	0.7	95.0	S	98.7	0.1	93.9	0.7	96.3	S
D	Cinnamaldehyde	Moderate	54.3	0.7	7.4	0.2	30.8	S	50.1	0.3	6.4	0.3	28.2	S	23.9	0.6	6.4	0.0	15.1	S
E	2-Mercaptobenzothiazole	Moderate	49.3	1.2	0.7	0.6	25.0	S	42.5	1.3	1.8	0.3	22.1	S	41.0	0.4	2.3	0.1	21.7	S
F	Ethyl acrylate	Weak	87.9	0.9	3.4	1.2	45.7	S	89.1	3.3	2.3	3.8	45.7	S	87.7	7.5	4.5	1.1	46.1	S
G	Imidazolidinylurea	Weak	29.7	2.9	0.1	0.3	14.9	S	20.2	3.5	0.0	0.0	10.1	S	22.2	4.6	0.8	1.1	11.5	S
H	Glycerol	NC	0.1	0.1	0.5	0.5	0.3	NS	0.1	0.2	0.0	0.0	0.1	NS	0.7	1.2	0.4	0.7	0.6	NS
I	Salicylic acid	NC	0.6	0.5	2.3	0.1	1.5	NS	0.2	0.2	2.6	1.1	1.4	NS	1.4	1.2	2.2	0.3	1.8	NS
J	Isopropanol	NC	0.0	0.0	0.0	0.0	0.0	NS	0.0	0.0	0.0	0.0	0.0	NS	0.0	0.0	0.0	0.0	0	NS

Table 15-2: Summary of Reliability Stage Phase I results at Mitsui

No.	Chemical	LLNA category	Mitsui																	
			Experiment 1						Experiment 2						Experiment 3					
			NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class
A	Lauryl gallate	Strong	100.0	0.0	18.5	2.9	59.3	S	100.0	0.0	17.0	0.9	58.5	S	100.0	0.0	93.6	0.3	96.8	S
B	4-(Methylamino) phenol hemisulfate salt	Strong	100.0	0.0	16.0	1.4	58.0	S	100.0	0.0	16.2	3.2	58.1	S	98.0	0.3	13.9	0.8	56.0	S
C	Chloramine T	Strong	100.0	0.0	98.0	0.3	99.0	S	100.0	0.0	98.6	0.1	99.3	S	100.0	0.0	99.0	0.0	99.5	S
D	Cinnamaldehyde	Moderate	35.6	2.0	6.5	0.2	21.0	S	38.9	2.7	6.1	0.2	22.5	S	33.1	4.8	12.3	1.6	22.7	S
E	2-Mercaptobenzothiazole	Moderate	52.8	1.1	1.2	2.0	27.0	S	55.4	0.2	0.0	0.0	27.7	S	48.8	0.7	6.2	1.9	37.3	S
F	Ethyl acrylate	Weak	87.2	0.3	6.3	5.1	46.7	S	91.4	0.6	4.8	0.2	48.1	S	90.9	8.2	3.1	0.2	47.0	S
G	Imidazolidinylurea	Weak	18.6	0.9	1.8	3.1	10.2	S	22.8	0.9	0.2	0.3	11.5	S	20.5	2.0	0.0	0.0	10.2	S
H	Glycerol	NC	0.2	0.4	0.0	0.0	0.1	NS	0.0	0.0	0.0	0.0	0.0	NS	0.1	0.2	0.0	0.0	0.1	NS
I	Salicylic acid	NC	3.7	0.9	1.9	1.6	2.8	NS	2.9	0.4	1.1	0.1	2.0	NS	3.0	1.0	6.7	0.4	4.9	NS
J	Isopropanol	NC	1.0	0.9	0.0	0.0	0.5	NS	0.0	0.0	0.1	0.1	0.0	NS	0.1	0.2	0.0	0.0	0.1	NS

Table 15-3: Summary of Reliability Stage Phase I results at Sumitomo

No.	Chemical	LLNA category	Sumitomo																	
			Experiment 1						Experiment 2						Experiment 3					
			NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class
A	Lauryl gallate	Strong	100.0	0.0	65.9	3.2	83.0	S	100.0	0.0	20.6	0.3	60.3	S	100.0	0.0	43.2	2.4	71.6	S
B	4-(Methylamino) phenol hemisulfate salt	Strong	100.0	0.0	15.8	0.6	57.9	S	100.0	0.0	15.4	0.7	57.7	S	100.0	0.0	12.4	0.5	56.2	S
C	Chloramine T	Strong	98.4	0.1	96.9	0.2	97.7	S	98.1	0.0	96.8	0.1	97.5	S	100.0	0.0	92.9	0.3	96.5	S
D	Cinnamaldehyde	Moderate	98.4	0.2	9.6	0.5	54.0	S	98.5	0.1	-	-	-	-	67.0	0.4	5.4	0.7	36.2	S
E	2-Mercaptobenzothiazole	Moderate	48.4	1.5	0.5	0.6	24.5	S	56.3	2.6	-	-	-	-	81.2	1.2	0.0	0.0	40.6	S
F	Ethyl acrylate	Weak	92.4	0.1	5.1	0.1	48.7	S	49.8	0.7	0.3	0.3	25.1	S	92.4	0.4	5.3	0.1	48.9	S
G	Imidazolidinylurea	Weak	24.1	1.7	0.5	0.5	12.3	S	29.8	1.8	0.3	0.5	15.1	S	30.0	1.2	0.2	0.1	15.1	S
H	Glycerol	NC	0.0	0.0	0.3	0.6	0.2	NS	5.7	0.6	0.0	0.0	2.8	NS	5.7	0.7	0.0	0.0	2.9	NS
I	Salicylic acid	NC	0.0	0.0	1.3	0.6	0.6	NS	1.9	0.9	-	-	-	-	12.2	0.6	0.0	0.0	6.1	S
J	Isopropanol	NC	0.0	0.0	0.6	0.6	0.3	NS	4.7	0.2	0.2	0.4	2.5	NS	5.6	1.6	0.3	0.2	3.0	NS

Table 15-4: Summary of Reliability Stage Phase I results at Nissan

No.	Chemical	LLNA category	Nisssan																	
			Experiment 1						Experiment 2						Experiment 3					
			NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Mean % depletion (%)	Predicted class
A	Lauryl gallate	Strong	100.0	0.0	92.5	0.0	96.3	S	99.9	0.1	92.9	0.1	96.4	S	100.0	0.0	93.6	0.2	96.8	S
B	4-(Methylamino) phenol hemisulfate salt	Strong	99.5	0.1	12.8	0.5	56.2	S	99.8	0.2	13.2	0.2	56.5	S	100.0	0.0	13.2	0.5	56.6	S
C	Chloramine T	Strong	100.0	0.0	0.0	0.0	50.0	S	100.0	0.0	8.3	1.7	54.1	S	100.0	0.0	11.2	0.7	55.6	S
D	Cinnamaldehyde	Moderate	40.2	2.0	9.2	0.2	24.7	S	27.7	0.7	4.5	1.1	16.1	S	48.9	1.3	4.4	0.3	26.6	S
E	2-Mercaptobenzothiazole	Moderate	100.0	0.0	0.1	0.1	50.0	S	88.5	7.5	0.0	0.0	44.2	S	100.0	0.0	0.0	0.0	50.0	S
F	Ethyl acrylate	Weak	98.2	0.4	4.1	0.3	51.2	S	94.8	0.6	5.8	0.5	50.3	S	99.0	1.7	7.1	0.7	53.1	S
G	Imidazolidinylurea	Weak	25.9	4.4	0.0	0.0	12.9	S	29.3	2.0	0.0	0.0	14.7	S	37.0	0.9	2.7	4.6	19.8	S
H	Glycerol	NC	1.3	1.2	0.0	0.0	0.6	NS	2.3	2.8	0.0	0.0	1.1	NS	9.6	4.7	0.0	0.0	4.8	NS
I	Salicylic acid	NC	5.5	1.9	0.0	0.0	2.8	NS	1.6	1.5	1.1	0.6	1.3	NS	17.5	3.2	0.5	0.3	9.0	S
J	Isopropanol	NC	0.8	1.3	0.0	0.0	0.4	NS	3.8	3.8	0.0	0.0	1.9	NS	9.7	3.4	0.0	0.0	4.8	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS), and cells containing a dash (–) indicate missing data.

Within-laboratory reproducibility for Phase I was 100% (10/10) at Lion and Mitsui, 100% (7/7) at Sumitomo, and 90% (9/10) at Nissan, or an average of 97.3% (36/37).

Specific considerations for some of the ten chemicals tested during Reliability Stage Phase I are given below. Insofar as there were no significant discrepancies observed in the values for NAC, NAL, or mean % depletion of 4-(methylamino) phenol hemisulfate salt, cinnamaldehyde, 2-mercaptobenzothiazole, ethyl acrylate, imidazolidinylurea, glycerol, isopropanol at the four participating laboratories, comments on these test chemicals have been omitted.

Lauryl gallate

There were significant differences in NAL depletion across the four laboratories. As shown in Table 16, a comparison of the solvents used to prepare the test chemical solutions shows that lauryl gallate in acetonitrile solution consistently yields a NAL depletion of 92.5% or higher, but lauryl gallate in acetone solution yields NAL depletions between 17.0% and 65.9%, which is significantly lower and less consistent than in acetonitrile solution. In general, the reaction rate of organic compounds in solution will vary with the solvent, which makes sense in terms of chemistry, on that basis the VMT believes that these differences were most likely due to differences in the solvents used.

Table 16: Mean % depletion for lauryl gallate in solution at the four participating laboratories

	1st	2nd	3rd
Lion	95.6% [Solvent: Acetonitrile]	92.7% [Solvent: Acetonitrile]	92.6% [Solvent: Acetonitrile]
Mitsui	18.5% [Solvent: Acetone]	17.0% [Solvent: Acetone]	93.6% [Solvent: Acetonitrile]
Sumitomo	65.9% [Solvent: Acetone]	20.6% [Solvent: Acetone]	43.2% [Solvent: Acetone]
Nissan	92.5% [Solvent: Acetonitrile]	92.9% [Solvent: Acetonitrile]	93.6% [Solvent: Acetonitrile]

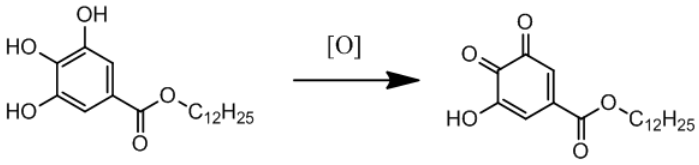
Also, significant differences in NAL depletion in acetone solution were found even at the same laboratory. Table 17 shows values for NAL depletion during the 2nd replicate test at Mitsui.

Although lauryl gallate itself has no sites that react with amino groups in NAL, but once with oxidation the formation of α,β-unsaturated ketones creates the potential that these metabolites will react with thiol and amino groups. (Figure 1) Thus, since lauryl gallate is a highly hydrophobic pre-hapten (CLOGP = 6.8), it seems highly likely that this difference in reactivity (depletion) is due to either the lauryl gallate dissolving uniformly in the reaction solution or a greater quantity of oxidation, which the VMT believes results in a low level of depletion as well as greater variability when in acetone solution.

The mean % depletion for lauryl gallate, however, were all 50% or greater, which means that differences in solvent had no effect on the prediction of its skin sensitization potential.

Table 17: Mean % depletion for lauryl gallate during 2nd replicate test at Mitsui

Mitsui	2nd test	2nd re-test	2nd re-re-test
Solvent	Acetone	Acetone	Acetone
N=1	84.6%	11.3%	18.0%
N=2	83.7%	84.4%	17.6%
N=3	87.4%	14.0%	16.1%
Mean (N=3)	85.2%	48.3%	17.0%



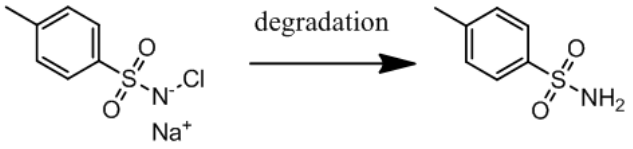
Lauryl gallate
(pre-hapten)

Lauryl gallate oxidized form
(hapten)

Figure 1: Example of oxidation of lauryl gallate

Chloramine T

NAL depletion at Lion, Mitsui, and Sumitomo during the three replicate tests was 90.9% or higher in all instances, but at Nissan was 0.0%, 8.3%, and 11.2%. (Table 15-4) Chloramine T is known to break down in aqueous solution into *p*-toluene sulfonamide, which is considered a non-sensitizer. (Figure 2) The extent of this break down varies greatly, depending on the pH of the solvent, the temperature, and the length of the treatment, which leads the VMT to conclude that the low level of NAL depletion at Nissan was most probably due to these factors, but the precise cause remains unclear.



Chloramine-T
(sensitizer)

p-Toluenesulfonamide
(non-sensitizer)

Figure 2: Break down of Chloramine-T in aqueous solution

Salicylic acid

Average value of depletion during the 3rd replicate test was 1.8% at Lion and 4.9% at Mitsui, which meant that salicylic acid was predicted to be a non-sensitizer (Tables 15-1 and 15-2), whereas 6.1% at Sumitomo and 9.0% at Nissan meant that it was predicted to be a sensitizer. Also, as shown in Table 18, the average values for the 3rd replicate test at Sumitomo and Nissan were significantly higher than those for the 1st or 2nd replicate tests, resulting in non-concordant prediction results. The VMT considers this to be an indication of a technical issue that occurred during the 3rd replicate test at these two participating laboratories, but the precise cause remains unclear.

Table 18: Mean % depletion for salicylic acid at Sumitomo and Nissan

	1st	2nd	3rd
Sumitomo	0.6% [Non-sensitizer]	–	6.1% [Sensitizer]
Nissan	2.8% [Non-sensitizer]	1.3% [Non-sensitizer]	9.0% [Sensitizer]

6. Explanation of retesting and measures implemented

Explanation of retesting

There were disparate instances of retesting at the four participating laboratories whenever one of the three replicate tests of the ten test chemicals failed to satisfy the test acceptance criteria. The reasons for retesting are summarized below in Table 19. The cells highlighted in pink indicate instances in which the test acceptance criterion shown on the left was not satisfied (9 instances in total), and the cells highlighted in blue indicate instances in which the test acceptance criteria were satisfied but the value for Reference Control C was so low that the apparent depletion was less than -10% and in spite of the fact that there was no co-elution, the data was handled as if it were interference (2 instances in total).

Table 19: Occurrences and Explanations of Retesting during Reliability Stage Phase I

Re-test in Phase I					Lion					Mitsui							Sumitomo				Nissan			
					Exp. 1	Exp. 1-1 (re-test)	Exp. 2	Exp. 2-1 (re-test)	Exp. 3	Exp. 1	Exp. 1-1 (re-test)	Exp. 2	Exp. 2-1 (re-test)	Exp. 2-2 (re-test)	Exp. 3	Exp. 3-1 (re-test)	Exp. 1	Exp. 2	Exp. 2-1 (re-test)	Exp. 3	Exp. 1	Exp. 1-1 (re-test)	Exp. 2	Exp. 3
Number of test chemicals or tests					10	6	10	10	10	10	1 (NAC)	10	10	1 (NAL)	10	1 (NAC)	10	10	10	10	10	6	10	10
Calibration curve			NAC	Calibration linearity	> 0.990																			
			NAL																					
Reference controls	A		NAC	Concentration	3.2 - 4.4μM																			
			NAL																					
	B		NAC	Peak area	CV < 10%																			
			NAL																					
	C	Acetonitrile	NAC	Concentration	3.2 - 4.4μM																			
				Peak area	CV < 10%																			
			NAL	Concentration	3.2 - 4.4μM																			
				Peak area	CV < 10%																			
		Othres	NAC	Concentration	3.2 - 4.4μM	Water 3.14μM												Water 3.14μM			Water 3.23μM			
				Peak area	CV < 10%																			
			NAL	Concentration	3.2 - 4.4μM																Water 3.22μM			
				Peak area	CV < 10%													Acetone 13.6%						
Positive control			NAC	depletion	8 - 100%							7.7%												
					SD < 10%																			
			NAL	depletion	86 - 100%		83.2%											76.3%						
					SD < 10%																			
Test chemicals			NAC	depletion	SD < 10%					12.6%					23.8%									
			NAL	depletion	SD < 10%								36.6%											
Others																								

7. Review of test acceptance criteria

The test acceptance criteria were revised as a result of the retesting that had been necessary during Reliability Stage Phase I.

Here is a summary of the changes that were agreed and implemented.

1. Revision of the control range for the positive control
2. Revision and clarification of the SOP
   2-1. Clarification of proper pipette technique
   2-2. Measures for when there is no co-elution, but the NAC or NAL depletion is less than -10%
   2-3. Retesting when the mean % depletion falls very close to the threshold value
   2-4. Revision of test record sheets

7-1. Revision of the control range for the positive control

Although there were a number of cases during Phase I in which depletion of the Positive Control reagent fell outside the control range, this was not the main reason that the control range was revised for Phase II. As previously explained, the addition of EDTA to the NAC stock solution as a means of limiting oxidation of NAC was approved during a meeting of the VMT prior to the start of Phase I. At that time, the only data for depletion of the Positive Control reagent in this type of test was from the lead laboratory, which was sufficient only for a provisional Phase I control range to be established based on the lead laboratory's historical data. When Phase I ended the additional data on for depletion of the Positive Control reagent from each of the participating laboratories were used to review the control range, after which the revised control range for Phase II was approved.

During Phase I, data on depletion of the Positive Control reagent from each of the participating laboratories was used to calculate the mean±3SD, which was used as a control criterion. In consideration of the fact that phenylacetaldehyde, which is used as a Positive Control reagent, and other chemicals that exhibit moderate levels of reactivity can easily exhibit variability in reactivity with even slight change in reaction conditions as well as the fact that the oxidation of aldehyde compounds into carboxylic acid can cause variability in depletion, ±3SD of the mean for each participating laboratory was used as a control criterion. Thus, the control range for Positive Control reagents was newly established as follows:

Control range for positive control reagents: 6% ≤ NAC depletion ≤ 30% and 75% ≤ NAL depletion ≤ 100%

For reference, the control range for Positive Control reagents in DPRA was

60.8% ≤ Cysteine peptide depletion ≤ 100% and 40.2% ≤ Lysine peptide depletion ≤ 69.4%, which is significantly wider than the control range currently used for ADRA.

7-2. Revision and clarification of the SOP

7-2-1. Clarification of proper pipette technique

Proper pipette technique helps prevent a number of potential technical problems. Listed below are some of the most common potential problems that might result from failure to use proper pipetting technique.

1. SD of NAC or NAL depletion in acetone solution is greater than 10%
2. SD of NAL depletion in acetonitrile solution is greater than 10%
3. NAC or NAL concentration of Reference Control C in aqueous solution fails to satisfy criteria
4. CV of NAL concentration of Reference Control C in acetone solution is greater than 10%
5. NAL depletion of test chemical in aqueous solution is less than -10%

In particular, acetone has a very high vapor pressure, and in order to prevent the dripping that accompanies increasing pressure inside the pipette, an instruction to "pre-rinse the pipette five times prior to use" as suggested by the manufacturer was added to page 13 in section 4-2. “Preparation and Reaction of Reaction Solutions”.

7-2-2. C/M when there is no co-elution, but the NAC or NAL depletion is -10% or less

Although NAC or NAL does not co-elute with the test chemical, there were cases where NAC or NAL depletion in Reference Control C was lower than depletion in the test chemical. Thus, the test acceptance criteria include the conditions that the CV of the peak area for both NAC and NAL in Reference Control C be 10% or less as well as the NAC and NAL concentration in Reference Control C be between 3.2 and 4.4 μM. The VMT found, however, that there were cases in which NAC or NAL concentration was within the specified range albeit very close to the lower limit and in which depletion of the test chemical was within the specified range and very close to the upper limit, for which depletion is -10% or lower irrespective of the fact there is no co-elution. Due to the nature and complexity of this phenomenon, it is very difficult to establish a countermeasure that a suitable for each individual instance, and thus an instruction was added “to implement a suitable countermeasure” on page 24 in section 8-4-1. “Co-elution: Interference” of the SOP.

7-2-3. Retesting when the mean % depletion **falls very close to the threshold value**

Just as with DPRA, retesting is required to confirm a prediction of sensitizer or non-sensitizer in ADRA whenever the mean % depletion is close to the threshold value. The conditions and requirements for retesting, and the rationale behind them, are given below.

For the NAC/NAL prediction model

Condition: When the mean % depletion is between 3.0 and 10.0%, perform retesting (page 24 in section 8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay of the SOP).

Rationale: After reviewing data on 82 test chemicals previously tested multiple times at the lead laboratory and listed in the scientific paper (Yamamoto *et al*., 2015), the VMT could only identify seven test chemicals that yielded non-concordant results across three replicate tests for predicting sensitization potential, which meant that statistical determination of a suitable criterion from such a limited dataset was highly problematic. Thus, the VMT established criteria for retesting of test chemicals that yield non-concordant results across three replicate

tests, using (1) a lower limit of the smallest mean % depletion for a test chemical predicted to be a sensitizer based on a majority decision of three replicate tests as well as (2) an upper limit of the largest mean % depletion for a test chemical predicted to be a non-sensitizer based on a majority decision. Thus, the range of values smaller than the lower limit (1) and larger than the upper limit (2) covers all test chemicals for which retesting produces a different result. As a result, the lower limit (1) was 3.5% from ethyl vanillin, and the upper limit (2) was 6.3% from α-amyl cinnamaldehyde. During Phase I, however, the highest value (2) at Nissan was 9.0%, which led to the conclusion that 3.5– 9.0% was a more suitable criterion for retesting. Also, the range used in the DPRA validation was 3–10%, which approximates to the 3.5–9.0% mentioned above and leads us to propose 3.0–10.0% as a range of mean % depletion within which retesting would be required to make a prediction using the NAC/NAL prediction model.

For the NAC-only prediction model

Condition: When NAC depletion is between 4.0 and 11.0%, perform retesting (page 24 in section 8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay of the SOP)

Rationale: There are no instances of accurate predictions made using the NAC-only prediction model in either historical data from the lead laboratory or Phase I data from the four participating laboratories. Thus, the VMT established a range of NAC depletion scores within which retesting would be required to make a prediction using the NAC-only prediction model be based on the above range for mean % depletion used to make a prediction using the NAC/NAL prediction model. The threshold value for the NAC-only prediction model is 5.6% which is 0.7% higher than the threshold value for the NAC/NAL prediction model of 4.9%. Adding 0.7% to the range for the NAC/NAL prediction model above gives a range of 3.7–10.7%. Rounding these values up led the VMT to propose 4–11% as a suitable range. Thus, the VMT established 4–11% as a range of NAC-depletion scores within which retesting would be required to make a prediction using the NAC-only prediction model.

7-2-4. Revision of test record sheets

In response to a request from the participating laboratories, the original test record sheets were replaced with a revised version that included solvent selection for the test chemical as well as for recording the details of how test chemical solutions were prepared, including the use of mixing, agitating, or sonicating, and the duration of such methods.

8. Conclusion

Within-laboratory reproducibility for Phase I was 100% (10/10) at Lion and Mitsui, 100% (7/7) at Sumitomo, and 90% (9/10) at Nissan, or an average of 97.3% (36/37) at the four participating laboratories. All of these results satisfied the 80% minimum target value established by the VMT, indicating excellent

within-laboratory reproducibility for ADRA.

Between-Laboratory Reproducibility

As previously noted, a number of improvements to issues arising in Phase I were made to the SOP, and Reliability Stage Phase II (Between-Laboratory Reproducibility) was performed using SOP ver. 1.1. Changes made between the end of Phase I and the start of Phase II included:

(1) Modification of the control range for the positive control reagent to 6–30% for NAC and 75–100% for NAL
(2) Precautions regarding pipette operation
(3) Explanations of the need for and new parameters for additional testing when the mean % depletion is close to the threshold value
(4) Countermeasures for when depletion is less that -10% in spite of a lack of co-elution.

Of these four points, we determined that Nos. 2, 3, and 4 had no effect on Phase I test results for the following reasons.

(2) affects how the test is performed but have no direct impact on test results.
(3) applies only to test chemicals that are tested only once, whereas during Phase I, all test chemicals were tested three times, which obviates the need for additional testing.
(4) was added to the SOP at this time, but similar measures were carried out during Phase I testing.

With regard to (1), in cases during Phase I for which the positive control reagent failed to satisfy control criteria and retesting was carried out, all tests satisfied the new criteria. In these cases, since we used the data for the retesting, we verified whether or not using the data for retesting changed the prediction results.

During Phase I, retesting was carried out twice at Lion and twice at Mitsui, because the positive control failed to satisfy the control criteria. We looked at test results prior to retesting for these two tests and, excluding instances where interference was detected, as shown by the yellow highlight in Table 12, there is full concordance between the results of retesting during Phase 1 and the results of applying the new control range to measurements made prior to retesting. In cases where interference was detected, since retesting would be required under the Phase II criteria, we used the results of the retesting.

Based on the above, we determined that the new control criteria for Phase II would not affect prediction results or predictive capacity even if used for Phase I data.

1. Solvent selection

Solvents for the 40 test chemicals used in Phase I and Phase II of the Reliability Stage were selected at each participating laboratory in accordance with the SOP. Prediction of sensitization potential for 32 of the 40 test chemicals was concordant at all four participating laboratories. As shown in Table 10, the eight test chemicals that were not concordant at all four participating laboratories were: lauryl gallate, chloramine T, p-benzoquinone, hydroquinone, citral, palmitoyl chloride, hydroxy citronellal, benzyl alcohol, and fumaric acid. Differences in solvent had no effect on prediction of sensitization potential for six of these eight test chemicals. The two exceptions, however, were citral and hydroxy citronellal.

LLNA predicts citral to be a sensitizer, and the three participating laboratories that used acetonitrile as the solvent—Lion, Mitsui, and Nissan—produced the same concordant result. In contrast to this, Sumitomo used water as the solvent and predicted citral to be a non-sensitizer. Similarly, LLNA predicts hydroxy citronellal to be a sensitizer, and the three participating laboratories that used acetonitrile as the solvent—Lion, Mitsui, and Sumitomo—produced concordant results. In contrast to this, Nissan used water as the solvent and predicted hydroxy citronellal to be a non-sensitizer. Based on these results, both these test chemicals yield higher mean % depletion and are predicted to be sensitizers in acetonitrile solution, but yield lower mean % depletion and are predicted to be non-sensitizers in aqueous solution. The mean % depletion for citral at all four participating laboratories were close to the threshold value and required three replicate tests to make a prediction. Also, for the same reasons, hydroxy citronellal required three replicate tests at Lion and Nissan as well as to replicate tests at Sumitomo. Based on these results, citral and hydroxy citronellal both appear to yield different results with different solvents—a sensitizer in acetonitrile solution and a non-sensitizer in aqueous solution—but in all cases the mean % depletion is very close to the threshold value, which indicates that these test chemicals are difficult to predict accurately. The VMT considers this to be a significant reason for the non-concordant predictions at the participating laboratories.

Table 20: Test chemicals and solvents used in Phase I and Phase II at the four participating laboratories

No.	Test chemicals	Lion			Mitsui			Sumitomo			Nissan		
		Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
1	Lauryl gallate	AN	AN	AN	AT	AT	AN	AT	AT	AT	AN	AN	AN
2	4-(Methylamino) phenol hemisulfate salt	W	W	W	W	W	W	W	W	W	W	W	W
3	Chloramine T	W	W	W	AN	AN	AN	W	W	W	W	W	W
4	Cinnamaldehyde	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
5	2-Mercaptobenzothiazole	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
6	Ethyl acrylate	W	W	W	W	W	W	W	W	W	W	W	W
7	Imidazolidinylurea	W	W	W	W	W	W	W	W	W	W	W	W
8	Glycerol	W	W	W	W	W	W	W	W	W	W	W	W
9	Salicylic acid	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
10	Isopropanol	W	W	W	W	W	W	W	W	W	W	W	W
11	Diphenylcyclopropenone	AN			AN			AN			AN		
12	p-Benzoquinone	W			AN			W			W		
13	Hydroquinone	W			W			AN			W		
14	2-Methyl-2H-isothiazol-3-one	W			W			W			W		
15	2-Aminophenol	AN			AN			AN			AN		
16	Iodopropynyl butylcarbamate	AN			AN			AN			AN		
17	Propyl gallate	AN			AN			AN			AN		
18	Dihydroeugenol	AN			AN			AN			AN		
19	Benzylsalicylate	AN			AN			AN			AN		
20	Squaric acid diethylester	W			W			W			W		
21	Citral	AN			AN			W			AN		
22	Palmitoyl Chloride	AT			AN			AN			AT		
23	Resorcinol	W			W			W			W		
24	Benzylcinnamate	AN			AN			AN			AN		
25	2,3-Butanedione	W			W			W			W		
26	Farnesal	AN			AN			AN			AN		
27	Eugenol	AN			AN			AN			AN		
28	Penicilln G	W			W			W			W		
29	Lilial	AN			AN			AN			AN		
30	Hydroxycitronellal	AN			AN			AN			W		
31	Benzyl alcohol	W			W			W			W		
32	Dimethylisophthalate	AN			AN			AN			AN		
33	4-Aminobenzoic acid	W			W			W			W		
34	Diethyl phthalate	AN			AN			AN			AN		
35	Methylsalicylate	AN			AN			AN			AN		
36	Dextran	W			W			W			W		
37	Coumarin	AN			AN			AN			AN		
38	Propyl paraben	AN			AN			AN			AN		
39	Sulfanilamide	AN			AN			AN			AN		
40	Fumaric acid	AT			AT			W			AT		

*W: aqueous solution, AN: acetonitrile solution, AT: acetone solution

2. Co-elution

One limitation of DPRA was co-elution of the test chemical with either cysteine peptide or lysine peptide, which affected predictions of sensitization potential. In contrast to this, an ADRA uses NAC and NAL, which are measured at a longer wavelength (λ_{max}) and a higher molar absorption coefficient (ε) than these peptides. For this reason, ADRA results have been free from measurable co-elution so far. The 40 test chemicals used in Phase I and Phase II of the Reliability Stage exhibited no co-elution with NAC or

NAL at any of the participating laboratories.

3. Between-Laboratory Reproducibility (concordance in prediction)

Between-laboratory reproducibility was evaluated based on test results from 40 coded test chemicals. Final predictions for the 10 test chemicals that were subject to three replicate tests during Phase I were based on mean % depletion. For example, the results of the three replicate tests of salicylic acid at Nissan were 2.8% (non-sensitizer), 1.3% (non-sensitizer), and 9.0% (sensitizer). Since the mean % depletion of these three results is 4.4%, which is below the threshold value of 4.9%, the test chemical was predicted to be a non-sensitizer.

3-1. Between-laboratory reproducibility of the 30 test chemicals used in Phase II

Since 10 test chemicals were subjected to three replicate tests during Phase I, and 30 test chemicals were subjected to single tests during Phase II, the results of Phase I were handled differently from the results of Phase II. Thus, a preliminary evaluation of between-laboratory reproducibility was made using just the 30 test chemicals used for Phase II. Tables 21-1 and 21-2 show NAC and NAL depletion as well as standard deviation (SD), and Table 22 shows mean % depletion and predictions at each participating laboratory for these 30 test chemicals. Between-laboratory reproducibility for these 30 test chemicals was calculated using results from three laboratories, and was 89.2%, which exceeded the minimum target value set by the VMT of 80%.

Table 21-1: NAC depletion at each participating laboratory

No.	Test chemicals	Lion						Mitsui						Sumitomo						Nissan					
		Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD	Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD	Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD	Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD
11	Diphenylcyclopropenone	32.1	0.4					26.7	0.3					32.3	0.8					25.5	1.7				
12	p-Benzoquinone	100.0	0.0					100.0	0.0					100.0	0.0					100.0	0.0				
13	Hydroquinone	100.0	0.0					100.0	0.0					100.0	0.0					100.0	0.0				
14	2-Methyl-2H-isothiazol-3-one	100.0	0.0					100.0	0.0					100.0	0.0					96.7	0.1				
15	2-Aminophenol	100.0	0.0					100.0	0.0					100.0	0.0					100.0	0.0				
16	Iodopropynyl butylcarbamate	100.0	0.0					94.3	0.5					100.0	0.0					100.0	0.0				
17	Propyl gallate	100.0	0.0					100.0	0.0					100.0	0.0					100.0	0.0				
18	Dihydroeugenol	2.3	0.9	4.3	0.3	3.4	0.1	1.7	0.1					4.4	0.1	4.0	0.2			0.3	0.1				
19	Benzylsalicylate	0.0	0.0					0.2	0.3					3.6	0.4					0.0	0.0				
20	Squaric acid diethylester	24.8	0.5					25.4	1.9					26.1	1.5					24.9	0.1				
21	Citral	7.2	0.3	9.4	0.3	10.0	0.5	9.1	0.4	4.4	4.0	12.6	0.6	11.6	0.9	0.0	0.0	5.7	0.6	6.2	0.6	13.4	0.4	19.7	0.3
22	Palmitoyl Chloride	8.2	0.2					4.2	0.2					0.0	0.0					34.3	32.0	0.0	0.0		
23	Resorcinol	5.2	0.8					1.2	1.1					12.9	0.5	6.2	0.1	7.8	0.2	6.0	0.5	6.5	0.2		
24	Benzylcinnamate	0.3	0.3					0.0	0.0					0.0	0.0					0.0	0.0				
25	2,3-Butanedione	30.5	0.9					30.8	7.6					39.0	0.8					68.5	0.4				
26	Farnesal	32.2	2.9					28.7	0.4					28.1	1.5					31.4	2.3				
27	Eugenol	19.2	0.4					29.9	1.1					28.5	3.0					18.1	1.3				
28	Penicillin G	1.0	0.4					0.0	0.0					4.0	0.3	0.0	0.0			0.5	0.2				
29	Lilial	9.8	0.4	10.3	0.5			25.6	4.6					9.4	1.1	10.9	0.3			12.8	0.5	25.2	0.5		
30	Hydroxycitronellal	5.7	0.7	16.2	0.4	10.3	0.5	20.2	1.0					6.3	3.0	8.7	0.6			7.5	0.0	13.9	6.1	7.7	0.1
31	Benzyl alcohol	0.4	0.5					0.0	0.0					4.2	0.4					0.4	0.5				
32	Dimethylisophthalate	0.4	0.6					0.9	0.3					0.0	0.0					0.5	0.9				
33	4-Aminobenzoic acid	2.2	0.2					0.0	0.0					4.1	0.9					2.7	0.5				
34	Diethyl phthalate	0.5	0.8					0.0	0.0					0.0	0.0					0.0	0.0				
35	Methylsalicylate	0.0	0.0					0.0	0.0					0.8	0.8					0.0	0.0				
36	Dextran	9.7	0.2	2.8	1.0	8.9	1.1	2.2	0.3					12.0	0.4	13.0	0.4			5.8	0.1				
37	Coumarin	7.2	4.6	1.7	0.2			1.5	0.2					3.4	1.7					0.1	0.2				
38	Propyl paraben	0.4	0.7					0.0	0.0					0.5	0.7					0.0	0.0				
39	Sulfanilamide	0.3	0.3					3.3	0.3					3.1	1.1					14.9	0.5	4.7	0.7	0.5	0.1
40	Fumaric acid	4.7	0.3	2.4	0.5	2.6	1.2	4.1	0.4					4.1	1.2					0.2	0.2				

Table 21-2: NAL depletion at each participating laboratory

No.	Test chemicals	Lion						Mitsui						Sumitomo						Nissan					
		Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD	Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD	Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD	Exp.	SD	Add. Exp.1	SD	Add. Exp.2	SD
11	Diphenylcyclopropenone	4.3	0.1					1.8	0.3					3.5	0.5					1.6	1.1				
12	p-Benzoquinone	62.3	0.2					68.4	0.8					58.5	1.7					44.4	0.9				
13	Hydroquinone	25.4	0.1					25.8	0.4					41.2	1.3					28.8	1.2				
14	2-Methyl-2H-isothiazol-3-one	0.3	0.4					0.0	0.0					0.3	0.3					0.0	0.0				
15	2-Aminophenol	36.4	0.6					32.0	0.4					32.9	3.3					26.5	0.3				
16	Iodopropynyl butylcarbamate	0.5	0.9					0.0	0.0					0.3	0.4					0.0	0.0				
17	Propyl gallate	72.9	0.1					63.9	0.5					68.6	1.2					68.4	0.7				
18	Dihydroeugenol	6.7	0.3	8.7	0.3	7.8	0.4	4.2	0.8					5.7	0.5	6.4	0.4			4.1	0.4				
19	Benzylsalicylate	1.5	0.4					0.5	0.9					0.3	0.5					0.0	0.0				
20	Squaric acid diethylester	70.6	0.2					71.5	1.3					65.9	0.5					44.2	0.4				
21	Citral	1.9	0.3	4.4	0.1	3.0	1.1	1.2	1.0	0.4	0.3	0.0	0.0	0.7	0.3	0.0	0.0	0.0	0.0	0.6	0.5	3.1	2.7	4.0	0.6
22	Palmitoyl Chloride	84.8	7.5					50.8	1.0					59.9	3.8					90.2	8.5	95.5	1.4		
23	Resorcinol	0.4	0.3					0.0	0.0					0.9	0.4	1.5	0.3	1.4	0.4	0.0	0.0	2.3	4.0		
24	Benzylcinnamate	1.3	0.2					0.0	0.0					0.9	0.4					0.2	0.3				
25	2,3-Butanedione	0.0	0.0					4.7	0.4					0.0	0.0					0.5	0.4				
26	Farnesal	8.2	0.2					8.5	0.4					9.7	0.3					6.4	0.5				
27	Eugenol	5.2	0.5					2.4	0.3					9.8	1.2					3.5	0.5				
28	Penicillin G	1.4	0.5					0.0	0.0					2.1	0.1	2.7	0.2			0.0	0.0				
29	Lilial	4.4	0.2	3.7	0.2			2.1	0.1					0.4	0.4	1.5	0.5			0.3	0.3	4.8	0.2		
30	Hydroxycitronellal	2.0	0.1	4.7	0.1	2.4	0.4	2.5	0.2					6.4	0.6	2.0	0.2			0.0	0.0	0.0	0.0	1.4	1.0
31	Benzyl alcohol	0.0	0.1					5.0	4.4					0.2	0.1					0.0	0.0				
32	Dimethylisophthalate	1.5	0.1					0.0	0.0					0.0	0.0					0.6	0.1				
33	4-Aminobenzoic acid	0.1	0.1					0.0	0.0					0.5	0.2					0.0	0.0				
34	Diethyl phthalate	1.1	0.2					0.0	0.0					0.0	0.0					0.1	0.2				
35	Methylsalicylate	1.1	0.2					0.0	0.0					0.2	0.4					0.0	0.0				
36	Dextran	0.4	0.3	0.0	0.0	0.5	0.3	0.0	0.0					2.3	0.5	2.0	0.3			0.0	0.0				
37	Coumarin	2.1	0.3	3.6	0.2			0.1	0.1					0.2	0.3					0.0	0.0				
38	Propyl paraben	1.6	0.2					0.1	0.2					0.0	0.0					0.0	0.0				
39	Sulfanilamide	1.5	0.5					0.2	0.3					0.0	0.0					0.0	0.0	1.1	1.8	4.1	1.6
40	Fumaric acid	5.9	0.5	4.6	0.3	3.6	1.2	0.7	0.4					0.7	0.1					1.0	0.6				

Table 22: Predictions at each participating laboratory for the 30 test chemicals used in Phase II

No.	Test chemicals	LION	MITSUI	SUMITOMO	NISSAN
11	Diphenylcyclopropenone	18.2	14.2	17.9	13.5
12	p-Benzoquinone	81.1	84.2	79.3	72.2
13	Hydroquinone	62.7	62.9	70.6	64.4
14	2-Methyl-2H-isothiazol-3-one	50.2	50	50.2	48.4
15	2-Aminophenol	68.2	66	66.5	63.3
16	Iodopropynyl butylcarbamate	50.3	47.2	50.2	50
17	Propyl gallate	86.5	82	84.3	84.2
18	Dihydroeugenol	4.5 / 6.5 / 5.6	2.9	5.1 / 5.2	2.2
19	Benzylsalicylate	0.8	0.4	1.9	0.0
20	Squaric acid diethylester	47.7	48.4	46	34.5
21	citral	4.6 / 6.9 / 6.5	5.1 / 2.4 / 6.3	6.1 / 0.0 / 2.8	3.4 / 8.3 / 11.9
22	Palmitoyl Chloride	46.5	27.5	29.9	47.8
23	Resorcinol	2.8	0.6	6.9 / 3.8 /4.6	3.0 / 4.4
24	Benzylcinnamate	0.8	0	0.5	0.1
25	2,3-Butanedione	15.2	17.8	19.5	34.5
26	Farnesal	20.2	18.6	18.9	18.9
27	Eugenol	12.2	16.1	19.1	10.8
28	Penicilln G	1.2	0	3.0 / 1.4	0.2
29	Lilial	7.1 / 7.0	13.8	4.9 / 6.2	6.6 / 15.0
30	Hydroxycitronellal	3.9 / 10.5 / 6.4	11.3	6.4 / 5.4	3.7 / 7.0 / 4.6
31	Benzyl alcohol	0.2	2.5	2.2	0.2
32	Dimethylisophthalate	0.9	0.5	0	0.6
33	4-Aminobenzoic acid	1.1	0	2.3	1.3
34	Diethyl phthalate	0.8	0	0	0.1
35	Methylsalicylate	0.6	0	0.5	0
36	Dextran	5.0 / 1.4 / 4.7	1.1	7.2 / 7.5	2.9
37	Coumarin	4.6 / 2.7	0.8	1.8	0.1
38	Propyl paraben	1	0.1	0.2	0
39	Sulfanilamide	0.9	1.7	1.6	7.4 / 2.9 / 2.3
40	Fumaric acid	5.3 / 3.5 / 3.1	2.4	2.4	0.6

3-2. Between-laboratory reproducibility of the 30 test chemicals used in Phase II (sorted into three categories of sensitization potency)

In section 1) above, the VMT evaluated between-laboratory reproducibility for the 30 coded test chemicals used in Phase II based on prediction of sensitization potential. The statistical analysis looked in particular at the test chemicals that were predicted as or are known to be sensitizers, and evaluated between-laboratory sensitization based on prediction of sensitization potency, using two categories: extreme/strong sensitizers and moderate/weak sensitizers, as shown in Table 23.

The results, as shown in Table 23, were 100% between-laboratory reproducibility for extreme/strong sensitizers, 80.8% between-laboratory reproducibility for moderate/weak sensitizers, and 92.5% between-laboratory reproducibility for non-sensitizers. For reference, a similar table calculated from the results of the DPRA validation study report is shown to the right.

Table 23: Between-laboratory reproducibility of the 30 test chemicals, sorted into three categories of sensitization potency

ADRA

	Number of chemicals	BLR
Extreme/Strong	7	100.0
Moderate/Weak	13	80.8
No category	10	92.5

DPRA

	Number of chemicals	BLR
Extreme/Strong	2	100.0
Moderate/Weak	4	75.0
No category	3	66.7

*BLR: Between-Laboratory Reproducibility

3-3. Between-laboratory reproducibility of the 40 test chemicals used in Phases I and II

Prediction of sensitization potential for 36 of the 40 test chemicals was concordant at all four participating laboratories. As shown in Table 24, between-laboratory reproducibility calculated similarly to DPRA using results from three laboratories was 91.9%, which exceeded the minimum target value of 80% set by the ADRA VMT.

Table 24 includes detailed results for 40 test chemicals at the four participating laboratories.

Table 24: Prediction results for 40 test chemicals at four participating laboratories.

No.	Chemicals	Lion	Mitsui	Sumitomo	Nissan	Agreement 2 classes
Average of 3 experiments						
1	Lauryl gallate	S	S	S	S	Yes
2	4-(Methylamino) phenol hemisulfate salt	S	S	S	S	Yes
3	Chloramine T	S	S	S	S	Yes
4	Cinnamaldehyde	S	S	S	S	Yes
5	2-Mercaptobenzothiazole	S	S	S	S	Yes
6	Ethyl acrylate	S	S	S	S	Yes
7	Imidazolidinylurea	S	S	S	S	Yes
8	Glycerol	NS	NS	NS	NS	Yes
9	Salicylic acid	NS	NS	NS	NS	Yes
10	Isopropanol	NS	NS	NS	NS	Yes
1 experiment						
11	Diphenylcyclopropenone	S	S	S	S	Yes
12	p-Benzoquinone	S	S	S	S	Yes
13	Hydroquinone	S	S	S	S	Yes
14	2-Methyl-2H-isothiazol-3-one	S	S	S	S	Yes
15	2-Aminophenol	S	S	S	S	Yes
16	Iodopropynyl butylcarbamate	S	S	S	S	Yes
17	Propyl gallate	S	S	S	S	Yes
18	Dihydroeugenol	S	NS	S	NS	No
19	Benzylsalicylate	NS	NS	NS	NS	Yes
20	Squaric acid diethylester	S	S	S	S	Yes
21	citral	S	S	NS	S	No
22	Palmitoyl Chloride	S	S	S	S	Yes
23	Resorcinol	NS	NS	NS	NS	Yes
24	Benzylcinnamate	NS	NS	NS	NS	Yes
25	2,3-Butanedione	S	S	S	S	Yes
26	Farnesal	S	S	S	S	Yes
27	Eugenol	S	S	S	S	Yes
28	Penicilln G	NS	NS	NS	NS	Yes
29	Lilial	S	S	S	S	Yes
30	Hydroxycitronellal	S	S	S	NS	No
31	Benzyl alcohol	NS	NS	NS	NS	Yes
32	Dimethylisophthalate	NS	NS	NS	NS	Yes
33	4-Aminobenzoic acid	NS	NS	NS	NS	Yes
34	Diethyl phthalate	NS	NS	NS	NS	Yes
35	Methylsalicylate	NS	NS	NS	NS	Yes
36	Dextran	NS	NS	S	NS	No
37	Coumarin	NS	NS	NS	NS	Yes
38	Propyl paraben	NS	NS	NS	NS	Yes
39	Sulfanilamide	NS	NS	NS	NS	Yes
40	Fumaric acid	NS	NS	NS	NS	Yes

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

3-4. Between-laboratory reproducibility of the 40 test chemicals used in Phases I and II (sorted into three categories of sensitization potency)

Section 3) above evaluated between-laboratory reproducibility for the 40 coded test chemicals used in Phases I and II based on prediction of sensitization potential, and the report now addresses test chemicals that were predicted to be sensitizers, and evaluates between-laboratory sensitization based on prediction of sensitization potency, using two categories: extreme/strong sensitizers and moderate/weak sensitizers.

The results, as shown in Table 25, confirm 100% between-laboratory reproducibility for extreme/strong sensitizers, 85.3% between-laboratory reproducibility for moderate/weak sensitizers, and 94.2% between-laboratory reproducibility for non-sensitizers. For reference, a similar table calculated from the results of the DPRA validation study report is shown to the right.

Table 25: Between-laboratory reproducibility of the 40 test chemicals, sorted into three categories of sensitization potency

ADRA

	Number of chemicals	BLR
Extreme/Strong	10	100.0
Moderate/Weak	17	85.3
No category	13	94.2

DPRA

	Number of chemicals	BLR
Extreme/Strong	8	87.5
Moderate/Weak	7	71.4
No category	9	66.7

* BLR: Between-Laboratory Reproducibility

4. Other considerations

The low level of retesting that was required tends to confirm the effectiveness of the changes made in response to the amount of retesting needed during Phase I.

There were non-concordant predictions among the four participating laboratories for four of the 30 test chemicals used during Phase II—namely, dihydroeugenol, citral, hydroxy citronellal, and dextran.

We discuss about these results below.

4-1. Test chemicals yielding non-concordant predictions among the four participating laboratories

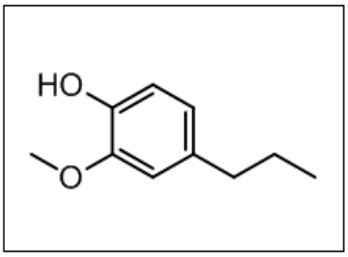
Dihydroeugenol

Dihydroeugenol is a prohapten, and for this reason is excluded from the expected applicability domain of ADRA, just as it was excluded from that of DPRA (Basketter *et al.* 2010). Dihydroeugenol is known to be oxidized into reactive sensitizer, and if oxidized sufficiently during the test procedure, will exhibit a sufficient specific reactivity with NAC or NAL and be predicted to be a sensitizer. Conversely, however, if not oxidized sufficiently, it will exhibit low reactivity with NAC or NAL and be predicted to be a non-sensitizer. Predictive results obtained at the four participating laboratories are shown in Table 26. Each of these mean % depletion is very close to the sensitization potential threshold

value of 4.9%. For this reason, as shown in Table 26, even the smallest discrepancy in oxidation can result in non-concordant predictions, making dihydroeugenol difficult to predict accurately.

Table 26 Chemical structure of dihydroeugenol and its mean % depletion at the four participating laboratories

Dihydroeugenol	Lion	Mitsui	Sumitomo	Nissan
1st	4.5	2.9	5.1	2.2
2nd	6.5	-	5.2	-
3rd	5.6	-	-	-
Prediction	Sensitizer	Non-sensitizer	Sensitizer	Non-sensitizer
Solvent	AN/AN/AN	AN	AN/AN	AN



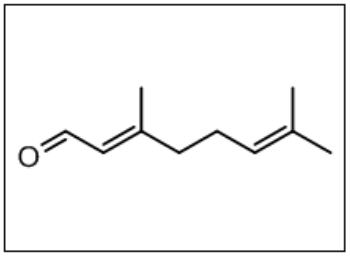
*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Citral

As shown in Table 27, citral in acetonitrile solution was predicted correctly to be a sensitizer at Lion, Mitsui and Nissan, but citral in aqueous solution was predicted incorrectly to be a non-sensitizer at Sumitomo. This raises the possibility that different solvents affect the results. The mean % depletion for citral at all four participating laboratories were close to the threshold value and required two additional tests to make a prediction. For this reason, we consider the accurate prediction of the skin-sensitization potential of citral to be highly problematic, with a strong possibility of non-concordant results across multiple participating laboratories.

Table 27 Chemical structure of citral and its mean % depletion at the four participating laboratories

Citral	Lion	Mitsui	Sumitomo	Nissan
1st	4.6	5.1	6.1	3.4
2nd	6.9	2.4	0.0	8.3
3rd	6.5	6.3	2.8	11.9
Prediction	Sensitizer	Sensitizer	Non-sensitizer	Sensitizer
Solvent	AN/AN/AN	AN/AN/AN	W/W/W	AN/AN/AN



*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

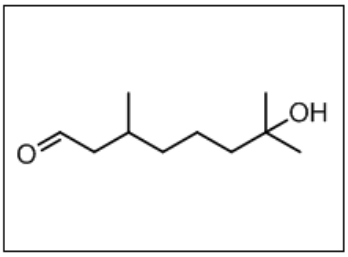
Hydroxycitronellal

As shown in Table 28 and similarly to citral, hydroxycitronellal in acetonitrile solution was predicted correctly to be a sensitizer at Lion, Mitsui and Sumitomo, but in aqueous solution was predicted incorrectly to be a non-sensitizer at Nissan. The mean % depletion for hydroxycitronellal were close to the threshold value and at three of the four participating laboratories required additional tests to make a prediction. For this reason, we consider the accurate prediction of the skin-sensitization potential of citral/hydroxycitronellal to be highly problematic, with a strong possibility of non-concordant results

across multiple participating laboratories.

Table 28 Chemical structure of hydroxycitronellal and its mean % depletion at the four participating laboratories

Hydroxycitronellal	Lion	Mitsui	Sumitomo	Nissan
1st	3.9	11.3	6.4	3.7
2nd	10.5	-	5.4	7.0
3rd	6.4	-	-	4.6
Prediction	Sensitizer	Sensitizer	Sensitizer	Non-sensitizer
Solvent	AN/AN/AN	AN	AN/AN	W/W/W



*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

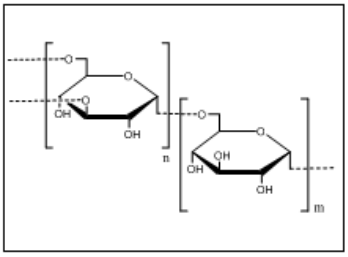
Dextran

High molecular weight test chemicals will not penetrate intact skin, and all known skin sensitizers are of relatively low molecular weights. Dextran has a mean molecular weight of 40,000 macromolecules, and for this reason could be excluded from the applicability domain of ADRA, just as it was excluded from that of DPRA. Moreover, DPRA required 100-mM test chemical solutions, which meant preparing a 4,000-mg/mL solvent and was impossible from the standpoint of solubility. In contrast, ADRA requires 1-mM test chemical solutions, which meant preparing a 40-mg/mL solvent, so that testing dextran was possible.

Dextran has no site in its chemical structure that reacts with NAC or NAL, and is considered essentially non-reactive. Nevertheless, aldehyde group at position 1 of glucose and other reducing sugar reacts with hydroxyl group at position 5 of those to form cyclic structures, which are known to generate partially aldehyde group per reverse reaction in aqueous solution (U. Satyanarayana and U. Chakrapani, 2014). This means that when the mean % depletion is more than a few percentage points, it is possible that the partially generated aldehyde groups will react with NAC or NAL. Such is likely the cause of Sumitomo recording the relatively high value of 7.2%, as shown in Table 29, which is assumed to be due to something promoting ring-open reactions of reducing termini, resulting in NAC reacting with the aldehyde groups that were generated.

Table 29 Chemical structure of dextran and its mean % depletion at the four participating laboratories

Dextran	Lion	Mitsui	Sumitomo	Nissan
1st	5.0	1.1	7.2	2.9
2nd	1.4	-	-	-
3rd	4.7	-		-
Prediction	Non-sensitizer	Non-sensitizer	Sensitizer	Non-sensitizer
Solvent	W/W/W	W	W	W



*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

4-2. The incidence of and reasons for retesting

Due to a significant incidence of retesting during Phase I, the previously described adjustments were implemented during Phase II. In order to verify the effectiveness of these measures, the reasons for retesting during Phase II are summarized below in Table 30.

Table 30: Reasons for retesting at each participating laboratory during Phase II

Re-test in Phase-2					Lion					Mitsui					Sumitomo							Nissan							
					Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
Number of test chemicals or tests					9	8	13	7	5	17	13	17	10	10	10	9	11	6	3	3	1	17	17	13	13	6 (NAC)	6	3	2
Calibration curve		NAC	Calibration linearity	> 0.990																								
		NAL																										
Reference controls	A	NAC	Concentration	3.2 - 4.4μM																								
		NAL																										
	B	NAC	Peak area	CV < 10%																								
		NAL								12.2%																		
	C Acetonitrile	NAC	Concentration	3.2 - 4.4μM																								
			Peak area	CV < 10%																								
		NAL	Concentration	3.2 - 4.4μM																								
			Peak area	CV < 10%																								
	C Others	NAC	Concentration	3.2 - 4.4μM																								
			Peak area	CV < 10%																								
		NAL	Concentration	3.2 - 4.4μM							Water 3.4μM		Water 3.6μM															
			Peak area	CV < 10%																								
Positive control		NAC	depletion	6 - 30%																					38.7%			
				SD < 10%																								
		NAL	depletion	75 - 100%																			69.6%					
				SD < 10%																								
Test chemicals		NAC	depletion	SD < 10%						14.6% 50.8% 14.7%			23.3%											32.0%				
		NAL	depletion	SD < 10%																								
Others																	problems with preparation of test chemical solutions		problems with properly setting the plate in the auto sampler			problems with the HPLC equipment						error in operation	

Also, the instances of retesting and percentage of retesting relative to the total number of tests during Phases I and II is given below for both NAC and NAL in Tables 31-1 and 31-2.

During Phase I, there was one instance out of 19 tests (5.3%) in which NAC depletion of the Positive Control reagent as well as two instances out of 18 tests (11.1%) in which NAL depletion of the Positive Control reagent failed to satisfy test acceptance criteria. During Phase II, there was one instance out of 25 tests (4.0%) in which NAC depletion of the Positive Control reagent as well as one instance out of 24 tests (4.2%) in which NAL depletion of the Positive Control reagent failed to satisfy test acceptance criteria, thus showing a slight reduction in retesting for NAL depletion. The VMT believes this improvement tends to confirm that a suitable adjustment had been made to the control range for the Positive Control reagent.

During Phase I, there were three instances out of 19 tests (15.8%) in which NAC depletion of Reference Control C as well as one instance out of 18 tests (5.6%) in which NAL depletion of Reference Control C failed to satisfy test acceptance criteria. During Phase II, there were no instances out of 25 tests (0%) in which NAC depletion of Reference Control C as well as two instances out of 24 tests (5.6%) in which NAL depletion of Reference Control C failed to satisfy test acceptance criteria, thus showing a reduction in retesting for NAC depletion. The VMT believes this improvement tends to confirm that the precautions regarding proper pipette operation had the desired effect at each of the participating laboratories.

There were a total of four instances during Phase II that were found to be human error, including problems with the HPLC equipment, preparation of test chemical solutions, or properly setting the plate in the auto sampler. In contrast to this, there were no instances during Phase I that were found to be human error. The instances of human error during Phase II appear to be random events.

Table 31-1: Incidence of and reasons for retesting during Phase I

Phase I	NAC			NAL		
	No. of Tests	No. of Errors	% Errors	No. of Tests	No. of Errors	% Errors
RC-B Errors	19	0	**0.0**	18	0	**0.0**
RC-C Errors	19	3	**15.8**	18	1	**5.6**
Positive Control Errors	19	1	**5.3**	18	2	**11.1**
Other Issues	19	0	**0.0**	-	-	-

Phase Ⅰ	NAC			NAL		
	No. of Tests	No. of Errors	% Errors	No. of Tests	No. of Errors	% Errors
Test Chemical Errors	164	2	**1.2**	163	1	**0.6**

Table 31-2: Incidence of and reasons for retesting during Phase II

Phase II	NAC			NAL		
	No. of Tests	No. of Errors	% Errors	No. of Tests	No. of Errors	% Errors
RC-B Errors	25	0	**0.0**	24	1	**4.2**
RC-C Errors	25	0	**0.0**	24	2	**8.3**
Positive Control Errors	25	1	**4.0**	24	1	**4.2**
Other Issues	25	4	**16.0**	-	-	-

Phase Ⅱ	NAC			NAL		
	No. of Tests	No. of Errors	% Errors	No. of Tests	No. of Errors	% Errors
Test Chemical Errors	229	5	**2.2**	223	0	**0.0**

4-3. Incidence of retesting and other considerations

During Phase II, in cases where the mean % depletion falls close to the threshold value, retesting was performed in accordance with section 2-3 of the SOP “Retesting when the mean % depletion falls very close to the threshold value”. This section of the report considers the incidence of retesting, the measures introduced to minimize retesting, and the results obtained.

The instances in which retesting was performed at the four participating laboratories are summarized below in Table 32. Retesting was performed for 10 test chemicals, all of which were either moderate/weak sensitizers or non-sensitizers, meaning that they exhibit little sensitization potential. 21 of 120 test chemicals were retested for an incidence of 17.5%. Of these, retesting was performed at multiple participating laboratories or six test chemicals—namely, dihydroeugenol, citral, resorcinol, lilial, hydroxy citronellal, and dextran. The testing of these six test chemicals all yielded mean % depletion very close to the threshold value even at participating laboratories that did not perform retesting, indicating that accurate prediction of sensitization potential is difficult for each of these chemicals.

Also, at one participating laboratory, retesting was performed for four test chemicals—namely, penicillin G, coumarin, sulfanilamide, and fumaric acid—each of which is either a weak sensitizer or a

non-sensitizer. Three of these—penicillin G, coumarin, and sulfanilamide—required retesting because the mean % depletion from the first replicate test was high, in spite of the fact that the mean % depletion from the second and third replicate tests were all less than 3%. The object of retesting is to enhance the accuracy of the predictions, and in these cases, that purpose was achieved by avoiding an incorrect prediction. The mean % depletion from the first replicate test for fumaric acid was also high, but retesting enabled it to be correctly predicted to be a non-sensitizer.

Table 32: Retesting at the four participating laboratories during Phase II

No.	Test chemicals	Lion	Mitsui	Sumitomo	Nissan
11	Diphenylcyclopropenone				
12	p-Benzoquinone				
13	Hydroquinone				
14	2-Methyl-2H-isothiazol-3-one				
15	2-Aminophenol				
16	Iodopropynyl butylcarbamate				
17	Propyl gallate				
18	Dihydroeugenol	*		*	
19	Benzylsalicylate				
20	Squaric acid diethylester				
21	citral	*	*	*	*
22	Palmitoyl Chloride				
23	Resorcinol			*	*
24	Benzylcinnamate				
25	2,3-Butanedione				
26	Farnesal				
27	Eugenol				
28	Penicilln G			*	
29	Lilial	*		*	*
30	Hydroxycitronellal	*	*	*	*
31	Benzyl alcohol				
32	Dimethylisophthalate				
33	4-Aminobenzoic acid				
34	Diethyl phthalate				
35	Methylsalicylate				
36	Dextran	*		*	
37	Coumarin	*			
38	Propyl paraben				
39	Sulfanilamide				*
40	Fumaric acid	*			

#Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS), and cells containing blank indicate missing data. An asterisk (*) indicates that retesting was performed.

The report now considers the results of predictions of skin sensitization potential during Phase II

between test chemicals that required retesting and those that did not. Table 33 shows the results for test chemicals that required only a single test (in other words, did not require retesting) to predict skin sensitization potential during Phase II. There were seven instances, indicated in Table 33 by bold cell borders, in which predictions that required only a single test differed from predictions that required retesting. Of these, the following five yielded incorrect predictions: dihydroeugenol at Lion, citral at Nissan, hydroxy citronellal at Lion, sulfanilamide at Nisan, and fumaric acid at Lion.

Table 33: Results for test chemicals that required only a single test (did not require retesting) to predict skin sensitization potential during Phase II

No.	Test chemicals	Lion	Mitsui	Sumitomo	Nissan
11	Diphenylcyclopropenone				
12	p-Benzoquinone				
13	Hydroquinone				
14	2-Methyl-2H-isothiazol-3-one				
15	2-Aminophenol				
16	Iodopropynyl butylcarbamate				
17	Propyl gallate				
18	Dihydroeugenol	*		*	
19	Benzylsalicylate				
20	Squaric acid diethylester				
21	citral	*	*	*	*
22	Palmitoyl Chloride				
23	Resorcinol			*	*
24	Benzylcinnamate				
25	2,3-Butanedione				
26	Farnesal				
27	Eugenol				
28	Penicilln G			*	
29	Lilial	*		*	*
30	Hydroxycitronellal	*	*	*	*
31	Benzyl alcohol				
32	Dimethylisophthalate				
33	4-Aminobenzoic acid				
34	Diethyl phthalate				
35	Methylsalicylate				
36	Dextran	*		*	
37	Coumarin	*			
38	Propyl paraben				
39	Sulfanilamide				*
40	Fumaric acid	*			

#Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS). An asterisk (*) indicates that retesting was performed.
#Red bold cell borders indicate test chemicals for which predictions that required only a single test differed from predictions that required retesting.

These results, as shown in Table 34, were 100% between-laboratory reproducibility for extreme/strong sensitizers, 75.0% between-laboratory reproducibility for moderate/weak sensitizers, and 77.5% between-laboratory reproducibility for non-sensitizers.

Between-laboratory reproducibility for test chemicals that required retesting, as shown in Table 23, was 100% for extreme/strong sensitizers, 80.8% for moderate/weak sensitizers, and 92.5% for non-sensitizers, which is an indication that retesting helped improve between-laboratory reproducibility by roughly 6% for moderate/weak sensitizers and 15% for non-sensitizers.

The above results confirm that the prescribed retesting improved between-laboratory reproducibility

Table 34: Between-laboratory reproducibility without retesting during Phase II

	Number of chemicals	BLR
Extreme/Strong	7	100.0
Moderate/Weak	13	75.0
No category	10	77.5

5. Between Laboratory Reproducibility: Conclusions of the Validation Management Team

The concordant predictions of skin sensitization potential for the 40 test chemicals by the four participating laboratories is the measure of between-laboratory reproducibility. Between-laboratory reproducibility indicating concordance of sensitizers and non-sensitizers was 91.9% when calculated for three participating laboratories, which contrasts favorably with DPRA's between-laboratory reproducibility of 75%. (Please note that, when recalculated without the three test chemicals—namely, dihydroeugenol (pro-hapten), beryllium sulfate (metal salt), and nickel chloride (metal salt)—that were later excluded from the applicability domain, DPRA's between-laboratory reproducibility is 85.7%. The VMT noted that the EURL/ECVAM DPRA validation study was conducted using a chemical set intended to evaluate the performance of DPRA and two cell-based assays.)

Additionally, for these 40 test chemicals, recalculating between-laboratory reproducibility for the three classifications of sensitization potency results in 100% for the 10 test chemicals that are extreme/strong sensitizers, 85.3% for the 17 test chemicals that are moderate/weak sensitizers, and 100% for the 13 test chemicals that are non-sensitizers (Table 25).

Similarly, recalculating DPRA's between-laboratory reproducibility for the three classifications of sensitization potency results in 87.5% for the eight test chemicals that are extreme/strong sensitizers, 71.4% for the seven test chemicals that are moderate/weak sensitizers, and 66.7% for the nine test chemicals that are non-sensitizers (Table 25). Thus, relative to DPRA, ADRA shows an improvement in between-laboratory reproducibility.

For both strong sensitizers and non-sensitizers, ADRA exhibited virtually perfect concordance between laboratories. And although differences in the number of test chemicals used to evaluate these two test methods makes it difficult to generalize, the VMT considers ADRA to have demonstrated superior between-laboratory reproducibility across all sensitization potencies relative to DPRA.

The VMT confirmed that the results more than satisfied the 80% minimum target value for between-laboratory reproducibility specified in the ADRA study plan. And having exhibited better than 90% between-laboratory reproducibility, ADRA, like DPRA, is suitable for use as an alternative method to animal testing in combination with other non-animal test methods.

Predictive Capacity

Table 35 shows the skin sensitization potential of the test chemicals predicted by each of the four participating laboratories during the ADRA validation testing.

With respect to the three replicate tests performed for the 10 test chemicals used in Phase I, the prediction of skin sensitization potential was made using the arithmetic mean value obtained from three replicate tests.

Table 35: Predicted skin sensitization potential of the 40 test chemicals at four participating laboratories (1)

No.	Chemicals	Reference result	Lion			Mitsui			Sumitomo			Nissan		
			Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
1	Lauryl gallate	Strong	S	S	S	S	S	S	S	S	S	S	S	S
2	4-(Methylamino) phenol hemisulfate salt	Strong	S	S	S	S	S	S	S	S	S	S	S	S
3	Chloramine T	strong	S	S	S	S	S	S	S	S	S	S	S	S
4	Cinnamaldehyde	Moderate	S	S	S	S	S	S	S		S	S	S	S
5	2-Mercaptobenzothiazole	moderate	S	S	S	S	S	S	S		S	S	S	S
6	Ethyl acrylate	Weak	S	S	S	S	S	S	S	S	S	S	S	S
7	Imidazolidinylurea	weak	S	S	S	S	S	S	S	S	S	S	S	S
8	Glycerol	no category	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
9	Salicylic acid	no category	NS	NS	NS	NS	NS	NS	NS		S	NS	NS	S
10	Isopropanol	no category	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
11	Diphenylcyclopropenone	Extreme	S			S			S			S		
12	p-Benzoquinone	Extreme	S			S			S			S		
13	Hydroquinone	Strong	S			S			S			S		
14	2-Methyl-2H-isothiazol-3-one	Strong	S			S			S			S		
15	2-Aminophenol	Strong	S			S			S			S		
16	Iodopropynyl butylcarbamate	Strong	S			S			S			S		
17	Propyl gallate	Strong	S			S			S			S		
18	Dihydroeugenol	Moderate	S			NS			S			NS		
19	Benzylsalicylate	Moderate	NS			NS			NS			NS		
20	Squaric acid diethylester	Moderate	S			S			S			S		
21	citral	Moderate	S			S			NS			S		
22	Palmitoyl Chloride	Moderate	S			S			S			S		
23	Resorcinol	Moderate	NS			NS			NS			NS		
24	Benzylcinnamate	Weak	NS			NS			NS			NS		
25	2,3-Butanedione	Weak	S			S			S			S		
26	Farnesal	Weak	S			S			S			S		
27	Eugenol	Weak	S			S			S			S		
28	Penicillin G	Weak	NS			NS			NS			NS		
29	Lilial	Weak	S			S			S			S		
30	Hydroxycitronellal	Weak	S			S			S			NS		
31	Benzyl alcohol	no category	NS			NS			NS			NS		
32	Dimethylisophthalate	no category	NS			NS			NS			NS		
33	4-Aminobenzoic acid	no category	NS			NS			NS			NS		
34	Diethyl phthalate	no category	NS			NS			NS			NS		
35	Methylsalicylate	no category	NS			NS			NS			NS		
36	Dextran	no category	NS			NS			S			NS		
37	Coumarin	no category	NS			NS			NS			NS		
38	Propyl paraben	no category	NS			NS			NS			NS		
39	Sulfanilamide	no category	NS			NS			NS			NS		
40	Fumaric acid	no category	NS			NS			NS			NS		

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS), and cells containing blank indicate missing data.

Between-laboratory reproducibility was evaluated based on test results from 40 coded test chemicals. Again, final predictions for the 10 test chemicals that were subject to three replicate tests during Phase I were based on mean % depletion. For example, the results of the three replicate tests of salicylic acid at Nissan were 2.8% (non-sensitizer), 1.3% (non-sensitizer), and 9.0% (sensitizer), and since the mean of these three mean % depletion is 4.4%, salicylic acid was predicted to be a non-sensitizer.

It should be noted that despite a low level of NAL depletion (76.3%) in the Positive Control reagent, which failed to satisfy the test acceptance criteria of 86–100%, no retesting was performed for the second replicate test at Sumitomo, so the data sets for the three test chemicals affected by this problem (cinnamaldehyde, 2-mercaptbenzthiazole, and salicylic acid) are incomplete (See “Module 2: Within-Laboratory Reproducibility; 3. Sumitomo; 3-5. Incomplete data”).

For this reason, only the results of the first and the third of the three replicate tests for these three test chemicals are included in the data set, and the mean of these two mean % depletion is used to predict skin sensitization potential. These results are shown in Table 36. Predictive capacity for the results shown in Table 36 is shown in Table 37. Accuracy at each participating laboratory was 90% at Lion, 87.5% at Mitsui, and 85% at both Sumitomo and Nissan; sensitivity was 85.2% at Lion, 81.5% at Mitsui, 81.5% at Sumitomo, and 77.8% at Nissan; and specificity was 100% at Lion, 100% at Mitsui, 92.3% at Sumitomo, and 100% at Nissan.

Table 36: Predicted skin sensitization potential of the 40 test chemicals at four participating laboratories (2)

No.	Chemicals	LLNA category	Lion	Mitsui	Sumitomo	Nissan	DPRA
1	Lauryl gallate	Strong	S	S	S	S	S[1,3]
2	4-(Methylamino) phenol hemisulfate salt	Strong	S	S	S	S	S[1,3]
3	Chloramine T	strong	S	S	S	S	S[2,5]
4	Cinnamaldehyde	Moderate	S	S	S	S	S[1,3]
5	2-Mercaptobenzothiazole	moderate	S	S	S	S	S[1,2,3,4]
6	Ethyl acrylate	Weak	S	S	S	S	S[1,3,5]
7	Imidazolidinylurea	weak	S	S	S	S	S[1,3,4]
8	Glycerol	no category	NS	NS	NS	NS	NS[1,2,3,4]
9	Salicylic acid	no category	NS	NS	NS	NS	NS[4] or S[3]
10	Isopropanol	no category	NS	NS	NS	NS	NS[1,2,3,4]
11	Diphenylcyclopropenone	Extreme	S	S	S	S	S[1,3]
12	p-Benzoquinone	Extreme	S	S	S	S	S[1,2,3,4]
13	Hydroquinone	Strong	S	S	S	S	S[3]
14	2-Methyl-2H-isothiazol-3-one	Strong	S	S	S	S	S[1,3]
15	2-Aminophenol	Strong	S	S	S	S	S[3]
16	Iodopropynyl butylcarbamate	Strong	S	S	S	S	S[5]
17	Propyl gallate	Strong	S	S	S	S	S[1,3,4]
18	Dihydroeugenol	Moderate	S	NS	S	NS	NS[3]
19	Benzylsalicylate	Moderate	NS	NS	NS	NS	NS[5]
20	Squaric acid diethylester	Moderate	S	S	S	S	NS[3]
21	citral	Moderate	S	S	NS	S	S[3,4]
22	Palmitoyl Chloride	Moderate	S	S	S	S	S[1]
23	Resorcinol	Moderate	NS	NS	NS	NS	NS[1,3]
24	Benzylcinnamate	Weak	NS	NS	NS	NS	NS[3]
25	2,3-Butanedione	Weak	S	S	S	S	S[1,3,4]
26	Farnesal	Weak	S	S	S	S	NS[5]
27	Eugenol	Weak	S	S	S	S	S[3,4]
28	Penicilln G	Weak	NS	NS	NS	NS	S[3]
29	Lilial	Weak	S	S	S	S	S[1,3]
30	Hydroxycitronellal	Weak	S	S	S	NS	S[1,3,4]
31	Benzyl alcohol	no category	NS	NS	NS	NS	NS[5]
32	Dimethylisophthalate	no category	NS	NS	NS	NS	NS[2]
33	4-Aminobenzoic acid	no category	NS	NS	NS	NS	NS[2,4]
34	Diethyl phthalate	no category	NS	NS	NS	NS	NS[1,3]
35	Methylsalicylate	no category	NS	NS	NS	NS	NS[1,3,4]
36	Dextran	no category	NS	NS	S	NS	-[a]
37	Coumarin	no category	NS	NS	NS	NS	NS[1,3]
38	Propyl paraben	no category	NS	NS	NS	NS	NS[1,3,4]
39	Sulfanilamide	no category	NS	NS	NS	NS	NS[1,3,4]
40	Fumaric acid	no category	NS	NS	NS	NS	S[4]

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS), and cells containing a dash (–) indicate missing data. 1) G. F. Gerberick *et al.* 2007 ; 2) DPRA validation study report, 2012 ; 3) Natsch *et al.* 2013 ; 4) Bauch *et al.* 2012 ; 5) Urbisch D *et al*. 2015 a) Dextran could not be tested with DPRA, because its high mean molecular weight precluded preparation of a 100-mM test chemical solution.

Table 37: Predictive capacity for 40 test chemicals at each of the four participating laboratories

Cumulative		
Reference result	+	-
+ (N=27)	88	20
- (N=13)	1	51
Total		

Sensitivity (%)	81.5
Specificity (%)	98.1
Accuracy (%)	86.9

LION		
	+	-
+	23	4
-	0	13
Total		

Sensitivity	85.2
Specificity	100.0
Accuracy	90.0

MITSUI		
	+	-
+	22	5
-	0	13
Total		

Sensitivity	81.5
Specificity	100.0
Accuracy	87.5

SUMITOMO		
	+	-
+	22	5
-	1	12
Total		

Sensitivity	81.5
Specificity	92.3
Accuracy	85.0

NISSAN		
	+	-
+	21	6
-	0	13
Total		

Sensitivity	77.8
Specificity	100.0
Accuracy	85.0

Thus, cumulative accuracy for the 40 test chemicals used in Phases I and II at the four participating laboratories was 86.9%, sensitivity was 81.5%, and specificity was 98.1%.

Predictive capacity for all test chemicals used at the four participating laboratories, which was calculated on the basis of 237 test results—three replicate tests of ten Phase I test chemicals at four participating laboratories, excluding the three incomplete data sets from Sumitomo, from Phase I ($3 \times 10 \times 4 - 3 = 117$) as well as one test of thirty test chemicals at four participating laboratories from Phase II ($1 \times 30 \times 4 = 120$), is shown in Table 38.

Table 38: Cumulative predictive capacity for 40 test chemicals at the four participating laboratories

Total predictive capacity		
Reference result	+	-
+ (N=162)	142	20
- (N=75)	3	72
Total		

Sensitivity (%)	87.7
Specificity (%)	96.0
Accuracy (%)	90.3

This yields an accuracy of 90.3%, a sensitivity of 87.7%, and a specificity of 96.0%.

Whichever of the above methods for calculating predictive capacity (accuracy, sensitivity, and specificity), is used, the results show a very high concordance.

1. Predictive capacity, sorted into three categories of sensitization potency

In predicting skin sensitization potential to ensure human safety it is necessary to predict with virtually 100% accuracy all chemicals with strong skin sensitization potency. For this reason, the VMT calculated

accuracy for three separate categories of sensitization potency: extreme/strong sensitizers, moderate/weak sensitizers, and non-sensitizers. For completeness, a similar calculation was made using data from the DPRA validation study report.

As can be seen below in Table 39, ADRA results exhibited the desired accuracy by correctly predicting all 10 test chemicals known to have strong sensitization potency. ADRA results also exhibited an extremely high accuracy of 98.1% for the 13 test chemicals known to be non-sensitizers. In comparison, the DPRA validation study results result in an accuracy of 79.2% for extreme/strong sensitizers and 88.9% for non-sensitizers.

Table 39: Predictive capacity for skin sensitization potential of the 40 test chemicals, sorted into three categories of sensitization potency

ADRA

	No. of chemicals	Lion	Mitsui	Sumitomo	Nissan	Predictive capacity
Extreme/Strong	10	100.0	100.0	100.0	100.0	100.0
Moderate/Weak	17	76.5	70.6	70.6	64.7	70.6
No category	13	100.0	100.0	92.3	100.0	98.1

DPRA

	No. of chemicals	Predictive capacity
Extreme/Strong	8	79.2
Moderate/Weak	7	66.7
No category	9	88.9

Since 10 test chemicals were subjected to three replicate tests during Phase I, and 30 test chemicals were subjected to single tests during Phase II, the results of Phase I were handled differently from the results of Phase II. These statistical methods were determined and agreed before study data were generated.

The VMT calculated predictive capacity for the 30 test chemicals from Phase II, sorted into three categories of sensitization potency. Again, ADRA results exhibited the desired accuracy by correctly predicting all 10 test chemicals known to have a strong sensitization potency, and exhibited an extremely high accuracy of 97.5% for the 13 test chemicals known to be non-sensitizers. For reference, DPRA results exhibited an accuracy of 83.3% for extreme/strong sensitizers and 88.9% for non-sensitizers.

Table 40: Predictive capacity for skin sensitization potential of the 30 test chemicals, sorted into three categories of sensitization potency

ADRA

	No. of chemicals	Lion	Mitsui	Sumitomo	Nissan	Predictive capacity
Extreme/Strong	7	100.0	100.0	100.0	100.0	100.0
Moderate/Weak	13	69.2	61.5	61.5	61.5	63.5
No category	10	100.0	100.0	90.0	100.0	97.5

DPRA

	No. of chemicals	Predictive capacity
Extreme/Strong	2	83.3
Moderate/Weak	4	72.6
No category	3	88.9

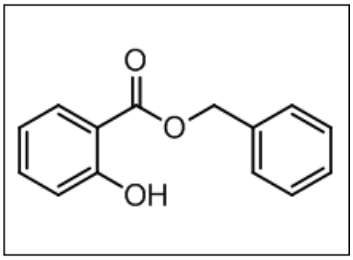
2. Four test chemicals with results that were false negatives

Benzyl salicylate

Although LLNA results classify benzyl salicylate as a sensitizer, it has no site that reacts with proteins. One potential reason for benzyl salicylate exhibiting sensitization potential *in vivo* could be that alcohol produced by hydrolysis of ester is oxidized into aldehyde, which then reacts with proteins. In this case, the test chemical itself does not react with thiol groups in cysteine or amino groups in lysine, but first becomes capable of reacting with thiol groups or amino groups after *in vivo* hydrolysis and oxidation. Thus, it is likely that the sensitization potential of benzyl salicylate cannot be correctly predicted by either ADRA or DPRA, which are both based on the same principle, and should be excluded from their applicability domains. (See Table 41 below.) In fact, benzyl salicylate was predicted to be a non-sensitizer by DPRA.

Table 41: Chemical structure of benzyl salicylate and its mean % depletion at the four participating laboratories

Benzyl salicylate	Lion	Mitsui	Sumitomo	Nissan
1st	0.8	0.4	1.9	0.0
2nd	-	-	-	-
3rd	-	-	-	-
Prediction	Non-sensitizer	Non-sensitizer	Non-sensitizer	Non-sensitizer
Solvent	AN	AN	AN	AN



*Cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers.

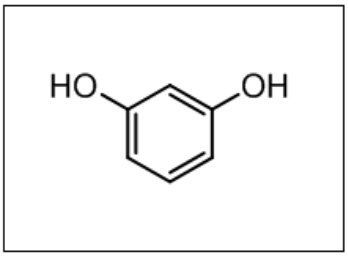
Resorcinol

Although LLNA results classify resorcinol as a sensitizer, it has no site that reacts with NAC or NAL. One potential reason for resorcinol exhibiting sensitization potential *in vivo* could be that, having oxidized into catechol derivatives, further oxidation produces ketones or α, β-unsaturated ketones, resulting in a sensitizer capable of reacting with NAC or NAL. Thus, it is likely that the sensitization potential of resorcinol cannot be correctly predicted by either ADRA or DPRA, which are both based on the same principle, and should be excluded from their applicability domains. (See Table 42 below.) In fact, resorcinol was predicted to be a non-sensitizer by DPRA. And in testing with EDTA at the four participating laboratories, resorcinol was predicted to be a non-sensitizer six of a total of seven times. In-house data from the lead laboratory indicates that even when tested without EDTA, results were divided between sensitizer and non-sensitizer (data not shown). This leads us to believe that, even though Phase II testing resulted in a false negative prediction for resorcinol, this is not due to the effects of EDTA but rather to the fact that the threshold value for mean percent depletion falls within the expected range of variability for resorcinol. As shown in Table 42 of the validation study report, resorcinol was tested using

water as the solvent once each at Lion, Mistui, and Nisson as well as three times at Sumitono. Thus we feel that the solvent used has not effect on prediction of skin sensitization potential.

Table 42: Chemical structure of resorcinol and its mean % depletion at the four participating laboratories

Resorcinol	Lion	Mitsui	Sumitomo	Nissan
1st	2.8	0.6	6.9	3.0
2nd	-	-	3.8	4.4
3rd	-	-	4.6	-
Prediction	Non-sensitizer	Non-sensitizer	Non-sensitizer	Non-sensitizer
Solvent	W	W	W/W/W	W/W/W



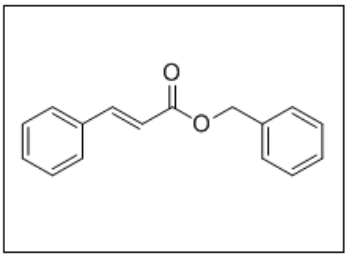
*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers, and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers.

Benzyl cinnamate

Similarly to benzyl salicylate above, LLNA results classify benzyl cinnamate as a sensitizer, and it likely transforms into aldehyde or other chemical with a sensitizing structure after *in vivo* reactions, such as hydrolysis or oxidation. Thus, it is likely that the sensitization potential of benzyl cinnamate cannot be correctly predicted by either ADRA or DPRA, which are both based on the same principle, and should be excluded from their applicability domains. (See Table 43 below.) In fact, benzyl cinnamate was predicted to be a non-sensitizer by DPRA.

Table 43: Chemical structure of benzyl cinnamate and its mean % depletion at the four participating laboratories

Benzyl cinnamate	Lion	Mitsui	Sumitomo	Nissan
1st	0.8	0.0	0.5	0.1
2nd	-	-	-	-
3rd	-	-	-	-
Prediction	Non-sensitizer	Non-sensitizer	Non-sensitizer	Non-sensitizer
Solvent	AN	AN	AN	AN



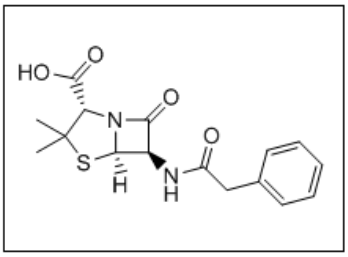
*Cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers.

Penicillin G

LLNA results classify penicillin G as a sensitizer. Penicillin G is known to react *in vivo* with β-lactam rings and hydroxy groups of serine residues (Mascaretti, 1995). It is therefore likely that penicillin G exhibits sensitization potential when it forms common bonds with serine residues of proteins that exist *in vivo*. It is possible that this test chemical was not predicted to be a sensitizer by ADRA, because it did not react with NAC or NAL. (See Table 44.) It remains unclear as to why penicillin G was correctly predicted to be a sensitizer by DPRA, even though both ADRA and DPRA are based on the same principle.

Table 44: Chemical structure of penicillin G and its mean % depletion at the four participating laboratories

Penicillin G	Lion	Mitsui	Sumitomo	Nissan
1st	1.2	0.0	3.0	0.2
2nd	-	-	1.4	-
3rd	-	-	-	-
Prediction	Non-sensitizer	Non-sensitizer	Non-sensitizer	Non-sensitizer
Solvent	W	W	W/W	W



*Cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers.

3. Predictive Capacity: Conclusions of the Validation Management Team

The results of this validation study show that, when compared to LLNA test results, ADRA exhibits a sensitivity of 80.6%, a specificity of 98.1%, and an accuracy of 86.3%.

These figures are consistent with those published by Yamamoto *et al*. (2015), who used ADRA to predict the skin sensitization potential of 82 test chemicals and obtained a sensitivity of 87%, a specificity of 97%, and an accuracy of 90%.

The VMT therefore considered ADRA to be an *in chemico* test method which, like DPRA, has shown the potential to form part of an integrated testing strategy (ITS) in the assessment of skin sensitization hazards and to play a role in enhancing the predictive capacity for skin sensitization potential as part of future ITS for comprehensive assessment.

Applicability domain

ADRA and DPRA share the same scientific rationale and therefore share similar applicability domains (EURL ECVAM Recommendation, 2013).

The ADRA applicability domain is, however, broader than that of DPRA. The nucleophilic reagents used in ADRA are more sensitive and can be measured at longer wavelengths than those used in DPRA, and as a result ARDA is conducted with lower concentrations for test chemical solutions and with virtually no co-elution between the test chemicals and the nucleophilic reagents.

ADRA's applicability domain and its characteristics are as defined below.

1. Technical limitations

1-1. Solubility of test chemicals

Chemicals that cannot be prepared in solution using the prescribed solvents are not suitable for testing.

1-2. Co-elution

The lead laboratory has heretofore never observed co-elution between any test chemical and NAC or NAL. In the event, however, that a test chemical were to exhibit co-elution with NAC, it would not be possible to predict its sensitization potential accurately based on the currently available data.

1-3. Cysteine dimerization

NAC, one of the nucleophilic reagents used in ADRA, is a cysteine derivative containing thiol groups which form dimers when oxidized and could cause depletion to be over-evaluated. Care therefore needs to taken when evaluating chemicals which may oxidize NAC.

2. Limitations on applicability

2-1. Restrictions on lysine and cysteine

The sensitization potential of chemicals which react primarily with amino acids other than cysteine or lysine (*e.g.*, nucleophilic sites in histidine) cannot be predicted accurately.

2-2. Metal compounds

Metal compounds are not suitable for testing by ADRA. Just as with DPRA, metal salts can easily be identified from their chemical structure and have their skin sensitisation potential evaluate by other test methods.

2-3. Pro-haptens and pre-haptens

ADRA encompasses neither an oxidation nor a metabolic system and is therefore not suitable for assessing pro-haptens or pre-haptens.

2-4. Oxidation

If ADRA is used to assess test chemicals that encompass an oxidation function, NAC will form dimers when oxidized. For this reason, quantification of unreacted NAC alone is likely to result in false-positive predictions.

3. ADRA's characteristics

3-1. Solubility of test chemicals

As explained above, the concentration of ADRA test chemical solutions is only 1% of those used in DPRA, which means that in practice there are very few cases in which the solubility of the test chemical is problematic, and therefore in this respect the applicability domain broader than that of DPRA.

As with DPRA, in cases where a test chemical co-elutes with NAL, a NAC-only prediction model can be used to predict sensitization. As both NAC and NAL can be measured at 281 nm, compared with DPRA, there are expected to be very few test chemicals that will exhibit co-elution.

3-2. Cysteine dimerization

As explained previously, ADRA has been optimized by adding EDTA to the NAC stock solution to prevent oxidation. Compared with DPRA, which included no means for preventing oxidation, the risk of oxidation of NAC has been reduced significantly.

3-3. Restrictions on lysine and cysteine

As mentioned previously, penicillin G, which was tested during Phase II, is known to be a human skin sensitizer that reacts preferentially *in vivo* with β-lactam rings and hydroxy groups of serine residues (Mascaretti, 1995). For this reason, it does not react with NAC or NAL, resulting in a false-negative prediction in ADRA (See "Module 5: Predictive Capacity; 2) Four test chemicals with results that were false-positives; penicillin G").

3-4. Pro-haptens and pre-haptens

There are some chemicals that are pre-haptens or pre-haptens but which are correctly predicted to be sensitizers by ADRA because they readily are sufficiently oxidized to their active form in the reaction solution. For example, lauryl gallate, hydroquinone, 2-aminophenol, propyl gallate, and eugenol are all pre-haptens, having a structure that does not react with NAC or NAL, but with during the ADRA, however they undergo a sufficient degree of oxidation while mixed with reaction solution to be correctly predicted to be sensitizers. Thus, there were some pre- and pro-haptens that were correctly predicted to be sensitizers, but in principle, these substances cannot be predicted correctly unless some form of oxidation or metabolism of the test chemical were incorporated into the test method. Accordingly, as with DPRA,

since accurate prediction of the sensitization potential of per- and pro-haptens is problematic, these substances have been excluded from the ADRA applicability domain.

3-5. Oxidation

Since oxidized NAC (NAC dimer; NAC-D) is a synthetic intermediate of NAC, it can be easily obtained. Although NAC-D can be formed under ADRA test conditions, the HPLC analysis measures both forms. Therefore, NAC and NAC-D can be quantified simultaneously under the same HPLC conditions, and it is possible to correctly evaluate unreacted NAC. This is not true for the cysteine peptide dimer encountered at times when DPRA is used cysteine peptide: and in cases where with DPRA partial precipitation seen at times when test chemical solutions are mixed with the cysteine peptide solution the DPRA HPLC analysis will not measure correctly cysteine peptides that have not reacted with the test chemical.

ADRA is an *in chemico* test method that is expected to be useful when used in combination with, for example, the ARE-Nrf2 Luciferase Test (OECD TG442D), h-CLAT (OECD TG442E), and/or other test method for which there are existing test method guidelines as part of an integrated testing strategy (ITS) in the assessment of skin sensitization hazards as well as to play a role in enhancing predictive capacity for skin sensitization potential as part of future ITS for comprehensive assessment.

4. Applicability domain: Conclusions of the Validation Management Team

Having the same scientific rationale, ADRA and DPRA essentially share the same minimum applicability domain. This means that ADRA is not suitable for the assessment of metal salts, pro haptens, pre-haptens, or similar chemicals. Nevertheless, as explained above, the use of nucleophilic reagents that are highly sensitive to UV detection represents an improvement over a number of inbuilt limitations that were problematic in DPRA, including solubility of test chemicals, co-elution of the test chemicals and nucleophilic reagents, and oxidation of cysteine component of the nucleophilic reagent. Thus, once an OECD test guideline is issued for ADRA, it will be possible to use an *in chemico* method to assess a number of test chemicals that are not suitable for assessment with DPRA, thereby broadening the applicability domain of test chemicals for which *in chemico* prediction of skin sensitization potential is possible.

VMT overall conclusions and recommendations

1. Overall conclusions

The object of this validation study was to assess the transferability, within-laboratory reproducibility, and between-laboratory reproducibility of ADRA using coded test chemicals, thereby enabling the VMT to assess the evidence for and against it being considered to have been scientifically validated and the appropriateness of developing and issuing an OECD test method guideline.

The VMT has concluded that the results of this validation study show that ADRA satisfies the requirements of Modules 1 to 4 of the ECVAM Modular Approach to Validation—namely, for test definition, within-laboratory reproducibility, between laboratory reproducibility, and transferability. Additionally, based on these results, the VMT considers that requirements of Module 5, predictive capacity, and Module 6, applicability domain, have also been satisfied when assessed against other validated test methods for assessing the skin sensitisation potential of chemicals.

The VMT considers the results of this validation study to demonstrate conclusively that ADRA is a scientifically sound a highly reliable *in chemico* alternative to testing of laboratory animals.

2. Recommendations

As with DPRA, rather than being used as a standalone test, ADRA is suitable and recommended for use as an alternative to existing *in vivo* tests within an integrated testing strategy that utilizes multiple alternative test methods to conduct a comprehensive assessment of skin sensitization potential.

ADRA removes a number of limitations that inherent to the DPRA, including the following.

1. The accuracy of measurements made during HPLC analysis using cysteine peptide and lysine peptide was enhanced in ADRA by changing the nucleophilic reagents to NAC and NAL, which are detected and measured at longer wavelengths, as well as by the use of TFA as a post-reaction fixing agent (Appendix 8 “Comparison of the accuracy of measurements of ADRA with DPRA”)
2. Difficulties in preparing solutions of hydrophobic test chemicals were mitigated by ADRA by the use of highly sensitive nucleophilic reagents which enable the concentration of test chemical solutions to be reduced to just 1% of that needed for DPRA.
3. Difficulties with precipitation and turbidity of hydrophobic test chemicals in the reaction solution were mitigated in ADRA as a consequence of the reduced concentration of test chemical solutions, as mentioned at Item 2 above.
4. Co-elution of the test chemicals with the nucleophilic reagents cysteine peptide and lysine peptide, as seen with DPRA, were virtually eliminated in ADRA by changing to nucleophilic reagents that can be measured at long wavelengths, as mentioned in Item 1.

5. Problems such as oxidation of cysteine peptide are minimised or eliminated by the addition of EDTA to the NAC stock solution.

The advantages of ADRA over DPRA can be summarized as shown below in seven categories, comprising 16 points.

The advantages of ADRA over DPRA	
1. The concentration of test chemical solutions used in DPRA is just 1% of that required for ADRA.	
	1-1. Test chemical solutions can be prepared even for test chemicals of poor solubility.
	1-2. Laboratories must choose from seven different solvents when preparing test chemical solutions for DPRA, but ADRA requires only four prescribed solvents to permit more chemicals to be tested.
	1-3. Even hydrophobic test chemicals do not precipitate easily in the reaction solution. (See Appendix 7.)
	1-4. Only very small quantities of test chemicals are required for ADRA, which means that even expensive test chemicals can be assayed.
2. The introduction of naphthalene rings to the nucleophilic reagent enables quantification at the relatively long wavelength of 281 nm.	
	2-1. There is virtually no co-elution between the test chemical and the nucleophilic reagent. (See Appendix 7.)
	2-2. Highly sensitive quantification of the nucleophilic reagent is possible, enabling the use of test chemical solutions and nucleophilic reagents at concentrations that are just 1% of those needed for DPRA.
3. Based on the above, the cost of assaying a test chemical using ADRA is between 1/5 and 1/10 that of DPRA.	
	The cost of assaying a test chemical using DPRA is roughly 10,000 JPY (Referred to "*Skin Sensitization Potential Test Method Report"* issued by JaCVAM) The cost of assaying a test chemical using ADRA is currently between 1,000 and 2,000 JPY. (TBD)
4. The potential for NAC oxidation is minimized by the use of disposable plastic labware and the addition of EDTA to the NAC stock solution.	
	4-1. Values obtained for NAC depletion are consistent.
	4-2. There is virtually no loss of NAC depletion due to oxidation, which eliminates the need for retesting due to failure to satisfy test acceptance criteria.
5. NAC stock solution can be frozen at -80°C and stored for up to one year, thereby eliminating the need for preparation prior to each use. IS THERE A REFERNCE TO CONFIRM THIS?	
6. The test procedure is performed using a single 96-well microplate for all steps from chemical reaction to measurement, which makes it possible to assay multiple test chemicals in a short period of time.	
7. The addition of a post-reaction fixing agent is expected to minimize variability of depletion values.	

It is assumed that DPRA is not suitable for assaying chemicals that are oxidizers. This is because chemicals that are oxidizers will react with the cysteine peptide, which makes accurate quantification of nonreactive peptide problematic. In contrast to this, the availability of an authentic sample for NAC-D when using ADRA means that even if there is NAC oxidation, HPLC analysis can be performed using the same parameters as those for NAC. (See Appendix 7, Figure 5.) Thus, when assaying a chemical that is an oxidizer, the quantity of NAC-D can be measured and added to the quantity of nonreactive NAC, thereby enabling an accurate calculation of NAC depletion even for a test chemical that is an oxidizer. For this reason, ADRA is expected to be able to assay test chemicals that are oxidizers.

As with DPRA, pro haptens, pre-haptens, and other chemicals that can change to sensitizers under auto oxidation or metabolic reaction as well as metal salts must be excluded from the applicability domain.

However, there is some future prospect of expanding the applicability domain to include pro-haptens and pre-haptens through the use of a derivative test system that includes *in vivo* enzymes or chemical oxidation system.

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Appendix 1

Study Plan

SOP ver.1.0

SOP ver.1.1

SOP ver.1.2

STUDY PLAN

Validation Study for Amino acid Derivative Reactivity Assay (ADRA) as Alternative Skin Sensitization Test

Version 1.0

September 13, 2016

Conducted by ADRA Validation Management Team

TABLE OF CONTENTS

LIST OF ABBREVIATION

ADRA	Amino acid Derivative Reactivity Assay
BLR	Between Laboratory Reproducibility
DPRA	Direct Peptide Reactivity Assay
ECVAM	European Centre for the Validation of Alternative Methods
GLP	Good Laboratory Practice
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM	Japanese Center for the Validation Alternative Test Method
NAC	*N*-2-(1-naphthyl)acetyl-L-cysteine
NAL	α-*N*-2-(1-naphthyl)acetyl-L-lysine
OECD	Organization for Economic Co-operation and Development
PBTG	Performance-Based Test Guideline
SD	Standard Deviation
SOP	Standard Operating Procedures
VMT	Validation Management Team
WLR	Within Laboratory Reproducibility

1. PURPOSE OF THE STUDY

An Amino acid Derivative Reactivity Assay (ADRA) for alternative sensitization test was developed by Fujifilm corp.

Fujifilm corp. and collaborators developed to discriminate between non skin sensitization and skin sensitization. A purpose of this validation study is to evaluate the skin sensitization potential for ADRA, and to submit SPSF to OECD for development of PBTG based on current OECD TG 442C including DPRA and ADRA.

ADRA is a method that improved DPRA (Direct Peptide Reaction Assay), and since the test principle of ADRA is the same as that of DPRA, validation study of ADRA was performed by reference to DPRA ECVAM Validation Report[1)].

2. INTRODUCTION

In the initial process of skin sensitization, sensitizers are generally electrophilic and reactive toward nucleophilic sites on proteins, and these characteristics have been used to develop non-animal approaches for evaluating the skin sensitization potential of chemicals[2),3),4)], among which DPRA is the most basic method[5),6)].

However, the peptides used in DPRA must be detected at 220 nm because of their minimal absorption at longer wavelengths, and various chemical substances also have UV absorption at short wavelengths such as 220 nm. These peptides cannot be measured precisely if they co-elute with test chemicals or reaction products in HPLC.[7)]

Therefore, we chemically synthesized two amino acid derivatives with an absorption maximum at 281 nm by introducing a naphthalene ring to cysteine and lysine; we named these derivatives NAC and NAL, respectively, and used them in Amino acid Derivative Reactivity Assay (ADRA). To perform the experiment effectively, we made it easy to handle multiple samples at the same time by using 96-well microplates. Moreover, we developed a method using compound concentrations 2 orders of magnitude less than those used in conventional methods[8),9)]. By making these some substantial improvements, ADRA has higher precision than the conventional methods and extends the range of application for test chemicals.

3. VALIDATION MANAGEMENT TEAM (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of Amino Derivative Reactivity Assay (ADRA) is established.

The ADRA validation management team (VMT) consisted of the members of the train coordinator, chemical selection group, chemical management group, data analysis group, record management group, and representative for assay development (lead laboratory). The lead laboratory supports the

participating laboratories. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on.

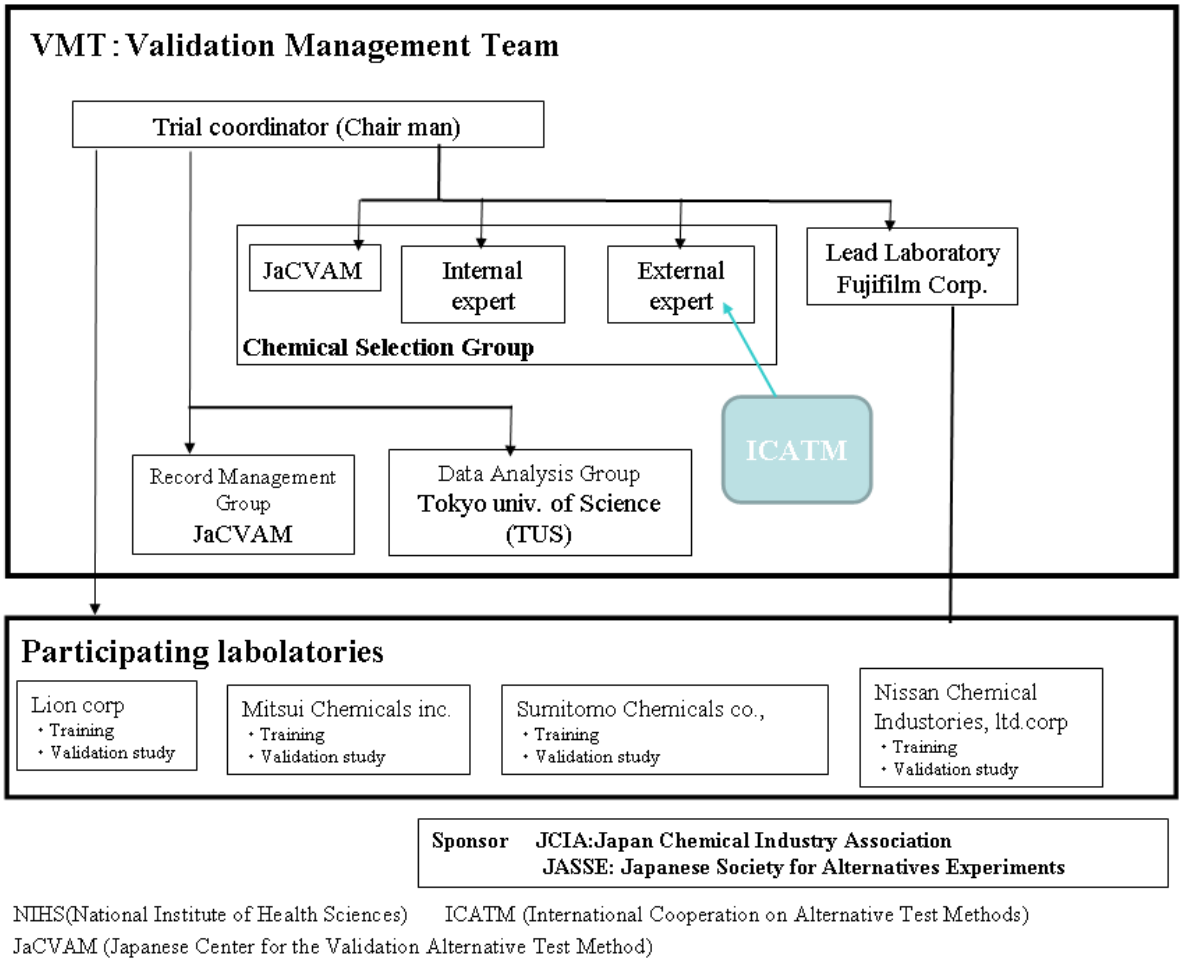

Fig.1 Management structure of the ADRA

3-1. LIST OF VALIDATION MANAGEMNT TEAM (VMT)

- Trial coordinator (Chair man) …Atsushi Ono (Okayama Univ.)
- External Expert …Kleinstreuer Nicole (NICEATM)
- External Expert …Jon Richmond (UK Home Office)
- External Expert …Kim Bae-Hwan (Keimyung Univ.)
- Internal Expert …Tsuyoshi Kawakami (National Institute of Health Sciences)
- Internal Expert …Kohichi Kojima (Hatano Research Institute Food and Drug Safety Center9
- JaCVAM …Hajime Kojima (National Institute of Health Sciences)
- JaCVAM …Shihori Tanabe (National Institute of Health Sciences)
- Analysis Expert …Takashi Sozu (Tokyo univ. of Science)
- Analysis Expert …Takuto Nakayama (Tokyo univ. of Science)
- Analysis Expert …Takeshi Kusao (Tokyo univ. of Science)

・Lead Laboratory …Toshihiko Kasahara (Fujifilm corp.)
・Lead Laboratory …Masaharu Fujita (Fujifilm corp.)
・Lead Laboratory …Yusuke Yamamoto (Fujifilm corp.)

3-2. TRIAL COORDINATOR

Trial coordinator is Dr. Atsushi Ono (Okayama Univ.). He prepares drafts of study plan, study protocol and test chemical list (draft), and convenes ad hoc VMT meetings for such reviews and finalizations of them. The trial coordinator is responsible for operational management of this validation study.

3-3. INTERNAL OR EXTERNAL EXPERT

Internal Experts are Dr. Tsuyoshi Kawakami and Dr. Kohichi Kojima and External Experts are Dr. Kleinstreuer Nicole, Jon Richmond and Kim Bae-Hwan. They must participate in ADRA VMT meeting, and must be responsible for all final documents decided by VMT meeting. In addition, they must join in preparing the OECD guidelines.

3-4. CHEMICAL SELECTION GROUP

The chemical selection group takes charges Dr. Hajime Kojima from JaCVAM (Japanese Center for the Validation Alternative Test Method), Dr. Tsuyoshi Kawakami (National Institute of Health Sciences, (NIHS)) and Dr. Kohichi Kojima (Hatano Research Institute Food and Drug Safety Center, FDSC) as Internal expert, Dr. Kleinstreuer Nicole, Dr. Jon Richmond and Dr. Kim Bae-Hwan as external experts from ICATM (International Cooperation on Alternative Test Methods). They will perform definition of selection criteria and chemical selection. The chemical selection group will proposed candidate chemicals in this validation based on chemicals used in validation study of Direct Peptide Reactivity Assay (DPRA).

3-5. CHEMICAL MANAGEMENT GROUP

The chemical management group takes charges Dr. Hajime Kojima, and JaCVAM colleagues. Dr. Tanabe prepare the list of coded test substances and distribute chemicals to the laboratories participating in this validation study.

3-6. DATA ANALYSIS GROUP

The members of data analysis group take charge Dr. Takashi Sozu (Tokyo Univ. of Science), Mr. Takuto Nakayama and Mr. Takeshi Kusao (Tokyo Univ. of Science), and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

3-7. RECORD MANAGEMENT GROUP

The members of record management group take charges Dr. Atsushi Ono, Dr. Hajime Kojima and

JaCVAM colleagues. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distribute them to the laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

3-8. LEAD LABORATORY

Fujifilm corp. supports the participating laboratories as the lead laboratory.

3-9. OBSERVERS: RESEARCHERS RESPONSIBLE FOR EXPERRIMENTAL PROCEDURES

Each delegate from each laboratory in the validation study is also an observer of the ADRA VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

4. STUDY DESIGN

The ADRA procedure is based on the measuring the remaining concentration of NAC/NAL after reaction for 24 hour. Moreover, the operation is simple and easy because it is based on 96-well microplates. This ADRA validation will evaluate within and between laboratory reproducibility, and predictive accuracy.

4-1. PARTICIPATING LABOLATORIES

Four laboratories were technical transferred from lead Laboratory through training experiment. At least three Laboratories must participate in validation study to meet a regulation requirement of this study. Laboratories that can adjust schedule of this validation study will participate in this study.

1) Lion Corp.
2) Mitsui Chemicals, Inc.
3) Nissan Chemical Industries, Ltd.
4) Sumitomo Chemical Co. Ltd.

4-2. TEST SUBSTANCES

Four laboratories will test the same 40 chemicals. This validation study will set two phases.

Table 1. Breakdown of substances used for the ADRA validation study

Phase	No. of the substances	No. of the repetitions	Examination	Remarks
I	10	3	a) WLR b) BLR	The WLR will be assessed on the basis of 10 chemicals tested 3 times. The median of 3 data obtained in the phase I will be used as data of BLR.
II	30	1	BLR	The BLR will be assessed on the basis of 40 chemicals tested (10 chemicals tested 3 times in the phase I and 30 chemicals tested once in the phase II).

4-3. ACCEPTANCE CRITERIA OF ADRA

Each Acceptance Criteria is closely prescribed in ADRA SOP. Furthermore, it is also made a table of Acceptance Criteria in the last APPENDIX

4-4. SUCCESS CRITERIA OF THE VALIDATION STUDY

The VMT considered it appropriate to define in advance indicative assessment criteria to be used to enable a transparent judgment and decision on the performance of the test methods in consideration of the study primary goal.

In consideration the above, the target performance for this study was set at 80% for the Between Laboratory Reproducibility and 80% for the Within Laboratory Reproducibility.

(1) If the Within Laboratory Reproducibility is equal to or more than 80% in the Phase I test, the next Phase II test could be performed after getting approval from VMT member. However, if it is not the case, VMT member should discuss and implement measures against the results.

(2) If the Between Laboratory Reproducibility for 40 chemicals tested, which are 10 chemicals tested 3 times in the Phase I and 30 chemicals tested once in the Phase II for each laboratories, will be equal to or more than 80%, the Validation study could be terminated after getting approval from VMT member. However, if it is not the case, VMT member should discuss and implement measures against the results.

(3) Predictive capacity of the ADRA is equal to or more than 80 % in each laboratory for 40 chemicals. However, if it is not the case, VMT member should discuss and implement measures against the results.

4-5. THE CONDITIONS FOR IMPLEMENTING RE-TEST OF THE VALIDATION STUDY

1. If Acceptance Criteria of validation study is not met:

(1) If any one of Acceptance Criteria from [1] to [6] described in APPENDIX is not met the conditions, re-test of the test conducted should be performed up to 2 times in each Validation study (Phase I and Phase II).

(2) If the SD for the depletion of NAC or NAL according to test chemicals is equal to or more than 10%, re-test of any relevant test chemicals should be performed up to 2 times in each Validation study (Phase I and Phase II), and the assay from [1] to [6] should be also performed at the same time.

2. If a human mistake is made:

(1) If an apparent human mistake is made, the reason should be recorded in the experimental report, and re-test can be performed.

4-6. STUDY DURATION

This experimental trial of validation test will be performed from October, 2016 to January, 2017. (See “6. TIMELINE”)

4-7. RECORD COLLECTION AND ANALYSIS

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the trail coordinator and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4-8. QUALITY ASSURANCE

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

5. REPORTING

1) The trial coordinator prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the ADRA validation study.

2) After obtaining scientifically pertinent validation data related to the relevance through the ADRA validation study, the trail coordinator prepares a research paper for joint publication.

6. STUDY TIMELINE

An approximate schedule for ADRA validation study is shown below.

Phase I 10 compounds three times／compound end of November, 2016

Phase II 30 compounds one time／compound end of January, 2017

7. SPONCER

To operating this validation study, research grant provided by Japan chemical Industry Association (JICA) and The Japanese Society for Alternative to Animal Experiments (JSAAE) is used as source of funds.

8. REFERENCES

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3. Alvarez-Sanchez, R., Basketter, D. A., Peace, C., & Leopoittevin, J. P. (2003). Studies of chemical selectivity of hapten, reactivity, and skin sensitization potency. 3. Synthesis and studies on the reactivity toword model nucleophiles of the 13C-labeled skin sensitizers, 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI). *Chem. Res*. **16**, 627-737.

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APPENDIX. Acceptance Criteria of ADA

	Calibration curve [1]	Reference Control A 【N=3】 (ACN)* [2]	Reference Control B 【N=6】 (ACN)* [3]	Reference Control C 【N=3】 (ACN)* [4]	Reference Control C 【N=3】 (except ACN) [5]	Positive Control 【N=3】 (ACN) [6]	Test Chemicals 【N=3】 (each solvent) [7]
r^2	>0.990	—	—	—	—	—	—
Mean concentration	—	4.0±0.8μM	—	4.0±0.8μM	4.0±0.8μM	—	—
Peak area (ACN)	—	—	CV<10% （N=9）		—	—	—
Peak area (except ACN)	—	—	—	—	CV<10% (N=3)	—	—
Depletion	—	—	—	—	—	SD<10%	SD<10%
Depletion Upper limit	—	—	—	—	—	NAC:<78.2% NAL:<25.7%	—
Depletion Lower limit	—	—	—	—	—	NAC:>47.4% NAL:>8.8%	—

* Reference Controls required in this assay independent of solvents for test chemicals

ACN : acetonitrile

STUDY PLAN

Validation Study for Amino acid Derivative Reactivity Assay (ADRA) as an Alternative Test Method for Assessing Skin Sensitization

Version 1.1

September 29, 2016

Conducted by ADRA Validation Management Team

TABLE OF CONTENTS

REVISION HISTORY

Revision	Date	Description of change:
1.0	Sep. 3, 2016	-
1.1	Sep. 29, 2016	- Addition of items in “LIST OF ABBREVIATION” - Addition and revision of items in “TABLE OF CONTENTS” - Addition of IATA and so on in “PURPOSE OF THE STUDY” - Addition of MIE and so on in “INTRODUCTION” - Addition of tasks and so on in “VALIDATION MANAGEMENT TEAM” - Revision of tasks for trial coordinator in “TRIAL COODINATOR” - Integration of “TRIAL COODINATOR” and “3-3. EXPERTS” - Addition of determination of WLR, BLR, Specificity, Sensitivity, and Accuracy in “DATA ANALYSIS GROUP” - Addition of testing without the need for the laboratories to seek advice or assistance from other laboratories in “PARTICIPATING LABORATORIES” - Addition of predictive capacity for target in “TEST SUBSTANCES” - Addition of tasks of VMT and so on in “SUCCESS CRITERIA FOR THE VALIDATION STUDY” - Addition of preliminary announcement for re-testing in “RETESTING DURING THE VALIDATION STUDY” - Addition of tasks of biostatisticians in “RECORD COLLECTION AND ANALYSIS” - Addition of requirement for Non-GLP participating laboratories in “QUALITY ASSURANCE” - Revision of tasks and so on of trial coordinator in “REPORTING” - Addition of references in “

LIST OF ABBREVIATION

ADRA	Amino acid Derivative Reactivity Assay
BLR	Between Laboratory Reproducibility
DPRA	Direct Peptide Reactivity Assay
ECVAM	European Centre for the Validation of Alternative Methods
GLP	Good Laboratory Practice
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM	Japanese Center for the Validation of Alternative Methods
NAC	*N*-2-(1-naphthyl)acetyl-L-cysteine
NAL	α-*N*-2-(1-naphthyl)acetyl-L-lysine
NICEATM	NTP (National Toxicology Program) Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organization for Economic Co-operation and Development
PBTG	Performance-Based Test Guideline
SD	Standard Deviation
SOP	Standard Operating Procedures
VMT	Validation Management Team
WLR	Within Laboratory Reproducibility

1. PURPOSE OF THE STUDY

An Amino acid Derivative Reactivity Assay (ADRA) for assessing skin sensitization potential was developed by Fujifilm Corporation as an alternative to in vivo testing.

Fujifilm Corporation and its collaborators developed this test to distinguish skin sensitizers from non-sensitizers. A purpose of this validation study is to evaluate ADRA's capacity to identify skin sensitization potential and to submit a standard project submission form (SPSF) to the OECD for the development of a performance-based test guideline (PBTG) as a derivative of the current OECD TG 442C that includes the Direct Peptide Reaction Assay (DPRA).

ADRA is an enhanced derivative of DPRA and is based on the same scientific principles, so the ADRA validation study is to be performed with reference to the DPRA ECVAM Validation Report[1] and based on the assumption that ADRA is to be used in a top-down approach to identifying chemicals having skin sensitization potential.

2. INTRODUCTION

There is a general agreement regarding the key biological events underlying skin sensitization. Existing knowledge of the chemical and biological mechanisms associated with skin sensitization has been summarized in the form of an Adverse Outcome Pathway (AOP), which starts with a molecular initiating event and continues through intermediate events that lead to the adverse effect—namely, allergic contact dermatitis in humans or contact hypersensitivity in rodents.

In the initial process of skin sensitization, sensitizers are generally electrophilic and reactive toward nucleophilic sites on proteins, and these characteristics have been used to develop non-animal approaches for evaluating the skin sensitization potential of chemicals,[2, 3, 4] among which DPRA is the most basic method.[5, 6]

The peptides used in DPRA, however, must be analyzed at 220 nm because of their minimal absorption at longer wavelengths. Moreover, many chemicals also have UV absorption at wavelengths as short as 220 nm. These peptides cannot be measured precisely if they co-elute with test chemicals or reaction products in HPLC.[7]

Therefore, we chemically synthesized two amino acid derivatives with an absorption maximum at 281 nm by introducing a naphthalene ring to cysteine and lysine; we named these derivatives NAC and NAL, respectively, and used them in ADRA. To perform the experiment effectively, we made it easy to handle multiple samples at the same time by using 96-well microplates. Moreover, we developed a method that uses chemical concentrations at two orders of magnitude less than those used in conventional methods.[8, 9] These are substantial improvements, which give ADRA greater precision than conventional methods and extend the applicability domain to even more chemicals.

3. VALIDATION MANAGEMENT TEAM

An organization is to be established to ensure both that this validation study is scientifically pertinent and that it is conducted smoothly.

The ADRA validation management team (VMT) comprises a trial coordinator, a chemical selection

group, a chemical management group, a data analysis group, a record management group, and a representative from the assay developer (lead laboratory). The lead laboratory provides support to the participating laboratories. The VMT prepares, reviews, and finalizes drafts of the study plan and study protocol. In addition, the VMT also manages the validation study by checking its progress, performing quality assurance of study records, and coordinating with participants.

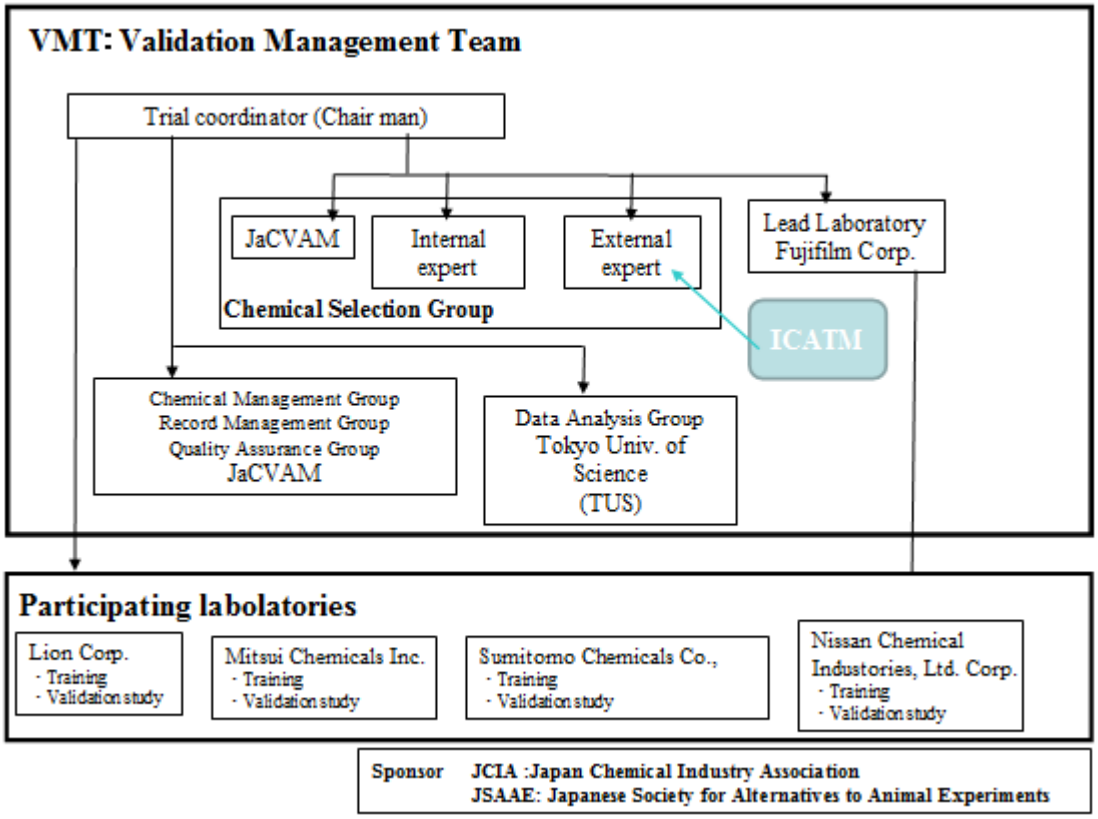

Fig.1 Management structure of the ADRA

3-1. LIST OF VALIDATION MANAGEMNT TEAM (VMT)

- Trial coordinator (Chairman): Atsushi Ono, Okayama University
- External Expert: Nicole Kleinstreuer, NICEATM
- External Expert: Jon Richmond, Ethical Biomedical Research and Testing Advice and Consultancy
- External Expert: Bae-Hwan Kim, Keimyung University
- Internal Expert: Tsuyoshi Kawakami, National Institute of Health Sciences
- Internal Expert: Kohichi Kojima, Food and Drug Safety Center
- JaCVAM: Hajime Kojima, National Institute of Health Sciences
- JaCVAM: Shihori Tanabe, National Institute of Health Sciences
- Biostatistician: Takashi Sozu, Tokyo University of Science
- Biostatistician: Takuto Nakayama, Tokyo University of Science
- Biostatistician: Takeru Kusao, Tokyo University of Science

・Lead Laboratory: Toshihiko Kasahara, Fujifilm Corporation
・Lead Laboratory: Masaharu Fujita, Fujifilm Corporation
・Lead Laboratory: Yusuke Yamamoto, Fujifilm Corporation

3-2. TRIAL COORDINATOR

Trial coordinator is Dr. Atsushi Ono of Okayama University. He prepares drafts of the study plan, study protocol and test chemical list as well as convenes VMT meetings as necessary to review and finalize these documents. The trial coordinator is responsible for operational management of this validation study.

3-3. EXPERTS

Internal Experts are Drs. Tsuyoshi Kawakami and Kohichi Kojima. Drs. Nicole Kleinstreuer, Jon Richmond and Bae-Hwan Kim were recommended by JaCVAM to serves as external experts, who support the validation study per the framework established for International Cooperation on Alternative Test Methods (ICATM). These experts participate in ADRA VMT meeting, and are responsible for all documentation finalized at VMT meetings. Additionally, they participate in the preparation of the OECD guidelines.

3-4. CHEMICAL SELECTION GROUP

The chemical selection group is led by Dr. Hajime Kojima of JaCVAM, and includes internal experts Drs. Tsuyoshi Kawakami and Kohichi Kojima as well as external experts Drs. Nicole Kleinstreuer, Jon Richmond, and Bae-Hwan Kim from ICATM. They define the selection criteria and select the test chemicals. The chemical selection group will propose candidate test chemicals for the validation based on the test chemicals used in DPRA validation study.

3-5. CHEMICAL MANAGEMENT GROUP

The chemical management group is led by Dr. Hajime Kojima and includes other JaCVAM personnel. The test chemicals required for this validation study are to be purchased by the Lead laboratory, Fujifilm Corp., which will deliver them to JaCVAM. Dr. Shihori Tanabe will prepare a list of coded test chemicals for distribution to the participating laboratories.

3-6. DATA ANALYSIS GROUP

The data analysis group is led by Dr. Takashi Sozu of Tokyo University of Science and includes Takuto Nakayama and Takeru Kusao, both of Tokyo University of Science, all of whom are responsible for analyzing the data sheets submitted by the participating laboratories as a disinterested party. They are also responsible for the statistical processing of the data obtained in this validation study.

3-7. RECORD MANAGEMENT GROUP

The record management group is led by Dr. Atsushi Ono and includes Dr. Hajime Kojima, assisted by JaCVAM personnel. They prepare and distribute to the participating laboratories the protocol, test chemical preparation record forms, blank data sheets, and other necessary documentation. They also collect and validate the completed forms and data sheets after testing is complete, including requesting correction of any omissions or errors found in the records.

3-8. QUALITY ASSURANCE (QA) GROUP

The quality assurance group is led by Dr. Atsushi Ono and includes Dr. Hajime Kojima, assisted by JaCVAM personnel. They will appoint an individual to maintain all records, documents, raw data, and reports and who will make these records available to the VMT as requested.

3-9. LEAD LABORATORY

Fujifilm Corporation is the lead laboratory and will provide support to the participating laboratories.

3-10. OBSERVERS: RESEARCHERS RESPONSIBLE FOR EXPERRIMENTAL PROCEDURES

Each participating laboratory names one or more researchers to observe ADRA VMT meetings. Personnel at the participating laboratories carry out testing according to the test protocol and under the supervision of the observers. The observers are responsible for sending copies of all record forms, data sheets, and other necessary documentation to the record management group upon completion of all testing.

4. STUDY DESIGN

The ADRA involves measuring the residual concentrations of NAC and NAL after a 24-hour incubation period. The operation is easy and simple to perform with high throughout, because it is based on the use of 96-well microplates. This validation study is designed to evaluate the within- and between-laboratory reproducibility as well as the predictive accuracy of ADRA.

4-1. PARTICIPATING LABORATORIES

Four participating laboratories underwent training to transfer technology from the lead laboratory. At least three of these laboratories must participate in the study to meet the requirements of this validation study. Laboratories that can accommodate the schedule will participate in this validation study. The four participating laboratories are:

1) Lion Corp.
2) Mitsui Chemicals, Inc.
3) Nissan Chemical Industries, Ltd.
4) Sumitomo Chemical Co. Ltd.

4-2. TEST SUBSTANCES

Four participating laboratories will test the same set of 40 chemicals in two phases.

Table 1. Breakdown of substances for the ADRA validation study

Phase	No. of substances	No. of repetitions	Target	Remarks
I	10	3	Both WLR and BLR	WLR will be assessed on the basis of 10 chemicals tested 3 times. BLR will be assessed using the mean value of the three data points obtained during Phase I.
II	30	1	BLR only	BLR will be assessed on the basis of 40 chemicals: 10 chemicals from Phase I and 30 chemicals from Phase II.

WLR: within-laboratory reproducibility, BLR: between-laboratory reproducibility

4-3. ACCEPTANCE CRITERIA FOR TEST DATA

Acceptance criteria is stipulated in the ADRA SOP. A table showing acceptance criteria is given in the appendix.

4-4. SUCCESS CRITERIA FOR THE VALIDATION STUDY

Bearing in mind the primary goal of this study, the VMT defines in advance success criteria for use in evaluating the performance of the test method. The target for both between- and within-laboratory reproducibility as well as for predictive accuracy is 80%.

(1) Within-laboratory reproducibility: The VMT will approve implementation of Phase II as long as the results of Phase I achieve a WLR of at least 80%. If the Phase I WLR is less than 80%, the VMT is to discuss and implement countermeasures as necessary to achieve the target.

(2) Between-laboratory reproducibility: The VMT will approve completion of the validation study as long as the results of testing all 40 chemicals used in Phases I and II achieve a BLR of at least 80%. These results are to be based on three tests each for the 10 chemicals in Phase I and once each for the 30 chemicals in Phase II. If the BLR is less than 80%, the VMT is to discuss and implement countermeasures as necessary to achieve the target.

(3) Predictive accuracy: The VMT will approve completion of the validation study as long as the results of testing all 40 chemicals used in Phases I and II achieve a predictive accuracy of at least 80%. If the predictive accuracy is less than 80%, the VMT is to discuss and implement countermeasures as necessary to achieve the target.

4-5. RETESTING DURING THE VALIDATION STUDY

1. In the event that the results do not satisfy the acceptance criteria:

(1) If the results of a test do not satisfy any of the Acceptance Criteria from 1 to 6 as shown in the Appendix, that test may be rerun up to two times during either Phase I or Phase II.

(2) If the SD for the depletion of NAC or NAL in a test chemical greater than 10%, as stipulated in Criterion 7, the test may be rerun up to two time during either Phase I or Phase II, and the results reevaluation for all seven criteria.

2. If an error or omission is found:

(1) If an error or omission due to a human mistake is found, the reason is to be recorded in the test records and the test rerun.

4-6. STUDY DURATION

Testing for this validation study is scheduled to be performed from October 2016 to March 2017.

Phase I	10 test chemicals	tested three times each by the end of November 2016
Phase II	30 test chemicals	tested once each by the end of March 2017

4-7. RECORD COLLECTION AND ANALYSIS

The biostatisticians are to collect and organize the collected data using a custom data collection software, after which the data will be subject to statistical analysis. The data management procedures and statistical tools are to be preapproved by the trail coordinator and data analysis group. Any deviation from these procedures or tools is to be documented along with a description of its impact on the study results.

4-8. QUALITY ASSURANCE

Participating laboratories should conduct all studies in the spirit of Good Laboratory Practices.[10] Any deviations from these practices is to be documented along with a description of their impact on the study results.

5. REPORTING

1) The trial coordinator is to prepare a report that will undergo international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada/KoCVAM) within the ICATM framework.

2) If the results of the study demonstrate the scientific validity of ADRA, the trail coordinator is to prepare a research paper for joint publication.

6. SPONSER

This validation study was supported by research grants provided by the Japan Chemical Industry Association (JCIA) and the Japanese Society for Alternative to Animal Experiments (JSAAE).

7. REFERENCES

1. Direct Peptide Reactivity Assay (DPRA) ECVAM Validation Study Report. January 2012. https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/files-dpra/DPRA%20Validation%20Study%20Report.pdf

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10. OECD (2000) OECD series on principles of good laboratory practice and compliance monitoring No 5, Compliance of Laboratory Suppliers with GLP Principles. Paris, France: Organisation for Economic Cooperation and Development.

APPENDIX. Acceptance Criteria of ADA

	Criterion 1 Calibration curve	Criterion 2 Reference Control A (ACN)* (N = 3)	Criterion 3 Reference Control B (ACN)* (N = 6)	Criterion 4 Reference Control C (ACN)* (N = 3)	Criterion 5 Reference Control C (other than ACN) (N = 3)	Criterion 6 Positive Control (ACN) (N = 3)	Criterion 7 Test Chemicals (in solution) (N = 3)
r^2	>0.990	—	—	—	—	—	—
Mean concentration	—	4.0 ±0.8 μM	—	4.0 ±0.8 μM	4.0 ±0.8 μM	—	—
Peak area (ACN)	—	—	CV < 10% (N = 9)		—	—	—
Peak area (except ACN)	—	—	—	—	CV < 10% (N = 3)	—	—
Depletion	—	—	—	—	—	SD < 10%	SD < 10%
Depletion Upper limit	—	—	—	—	—	NAC < 100% NAL < 100%	—
Depletion Lower limit	—	—	—	—	—	NAC > 15% NAL > 86%	—

* Reference Controls required in this assay independent of solvents for test chemicals

ACN : acetonitrile

STUDY PLAN

Validation Study for Amino acid Derivative Reactivity Assay (ADRA) as an Alternative Test Method for Assessing Skin Sensitization Potential

Version 1.2

October 7, 2016

Conducted by ADRA Validation Management Team

TABLE OF CONTENTS

REVISION HISTORY

Revision	Date	Description of change:
1.0	Sep. 3, 2016	-
1.1	Sep. 29, 2016	- Addition of items in “LIST OF ABBREVIATION” - Addition and revision of items in “TABLE OF CONTENTS” - Addition of IATA and so on in “PURPOSE OF THE STUDY” - Addition of MIE and so on in “INTRODUCTION” - Addition of tasks and so on in “VALIDATION MANAGEMENT TEAM” - Revision of tasks for trial coordinator in “TRIAL COODINATOR” - Integration of “TRIAL COODINATOR” and “3-3. EXPERTS” - Addition of determination of WLR, BLR, Specificity, Sensitivity, and Accuracy in “DATA ANALYSIS GROUP” - Addition of testing without the need for the laboratories to seek advice or assistance from other laboratories in “PARTICIPATING LABORATORIES” - Addition of predictive capacity for target in “TEST SUBSTANCES” - Addition of tasks of VMT and so on in “SUCCESS CRITERIA FOR THE VALIDATION STUDY” - Addition of preliminary announcement for re-testing in “RETESTING DURING THE VALIDATION STUDY” - Addition of tasks of biostatisticians in “RECORD COLLECTION AND ANALYSIS” - Addition of requirement for Non-GLP participating laboratories in “QUALITY ASSURANCE” - Revision of tasks and so on of trial coordinator in “REPORTING” - Addition of references in “
1.2	Oct. 7, 2016	- Addition of reference of ADRA Standard Operation procedure in “STUDY DESIGN”

LIST OF ABBREVIATION

ADRA	Amino acid Derivative Reactivity Assay
AOP	Adverse Outcome Pathway
BLR	Between Laboratory Reproducibility
DPRA	Direct Peptide Reactivity Assay
ECVAM	European Centre for the Validation of Alternative Methods
GLP	Good Laboratory Practice
HPLC	High Performance Liquid Chromatography
IATA	Integrated Approach to Testing and Assessment
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM	Japanese Center for the Validation of Alternative Methods
MIE	Molecular Initiating Event
NAC	*N*-2-(1-naphthyl)acetyl-L-cysteine
NAL	α-*N*-2-(1-naphthyl)acetyl-L-lysine
NICEATM	NTP (National Toxicology Program) Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organization for Economic Co-operation and Development
PBTG	Performance-Based Test Guideline
SD	Standard Deviation
SOP	Standard Operating Procedures
UV	Ultraviolet
VMT	Validation Management Team
WLR	Within Laboratory Reproducibility

1. PURPOSE OF THE STUDY

An Amino acid Derivative Reactivity Assay (ADRA) for assessing skin sensitization potential was developed by Fujifilm Corporation as an alternative to in vivo testing.

Fujifilm Corporation and its collaborators developed this test to distinguish skin sensitizers from non-sensitizers within an Integrated Approach to Testing and Assessment (IATA)[1]. The primary purpose of this validation study is to evaluate ADRA's capacity to identify skin sensitization potential and to facilitate the preparation of a standard project submission form (SPSF) to the OECD for the development of a performance-based test guideline (PBTG) as a derivative of the current OECD TG 442C based on the Direct Peptide Reactivity Assay (DPRA). [2]

ADRA is considered an enhanced derivative of DPRA and is based on the same scientific principles, so the ADRA validation study is to be performed with reference to, and taking account of, the DPRA ECVAM Validation Report[3] and has been designed based on the assumption that ADRA is to be used in a top-down approach to identifying chemicals having skin sensitization potential[1].

2. INTRODUCTION

There is a general agreement regarding the key biological events underlying skin sensitization initiated by covalent binding to skin proteins. Existing knowledge of the chemical and biological mechanisms associated with skin sensitization has been summarized in the form of an Adverse Outcome Pathway (AOP), which starts with a molecular initiating event (MIE) and continues through intermediate key events that lead to the adverse effect—namely, allergic contact dermatitis in humans or contact hypersensitivity in rodents.[4] In the initial step of the skin sensitization process, the MIE, sensitizers are generally electrophilic and reactive toward nucleophilic sites on proteins, and these characteristics have been used to develop *in-chemico* non-animal approaches for evaluating the skin sensitization potential of chemicals,[5), 6), 7)] among which DPRA is currently the best characterized test method.[8), 9)]

However, the peptides used in DPRA as a surrogate for the skin protein nucleophile sites must be detected and quantified spectroscopically at 220 nm because of their minimal absorption at longer wavelengths. Moreover, many chemicals also have Ultraviolet (UV) absorption at wavelengths as short as 220 nm. The DPRA peptides cannot be measured precisely if they co-elute with test chemicals or their reaction products in High Performance Liquid Chromatography (HPLC).[10)]

To overcome these problems with, and limitations of, the DPRA, we chemically synthesized two amino acid derivatives with a UV absorption maximum at 281 nm by introducing a naphthalene ring to cysteine and lysine; we named these derivatives NAC and NAL, respectively, and used them to develop the ADRA. To perform the test efficiently and effectively, we made it easy to handle multiple samples at the same time by using 96-well microplates. Moreover, to remedy the problem of testing poorly soluble chemicals using DPRA, we have developed a method that uses chemical concentrations at two orders of magnitude less than those used in conventional methods.[11), 12)] These are substantial improvements, which give ADRA greater precision than conventional methods and extend the applicability domain to cover more chemicals.

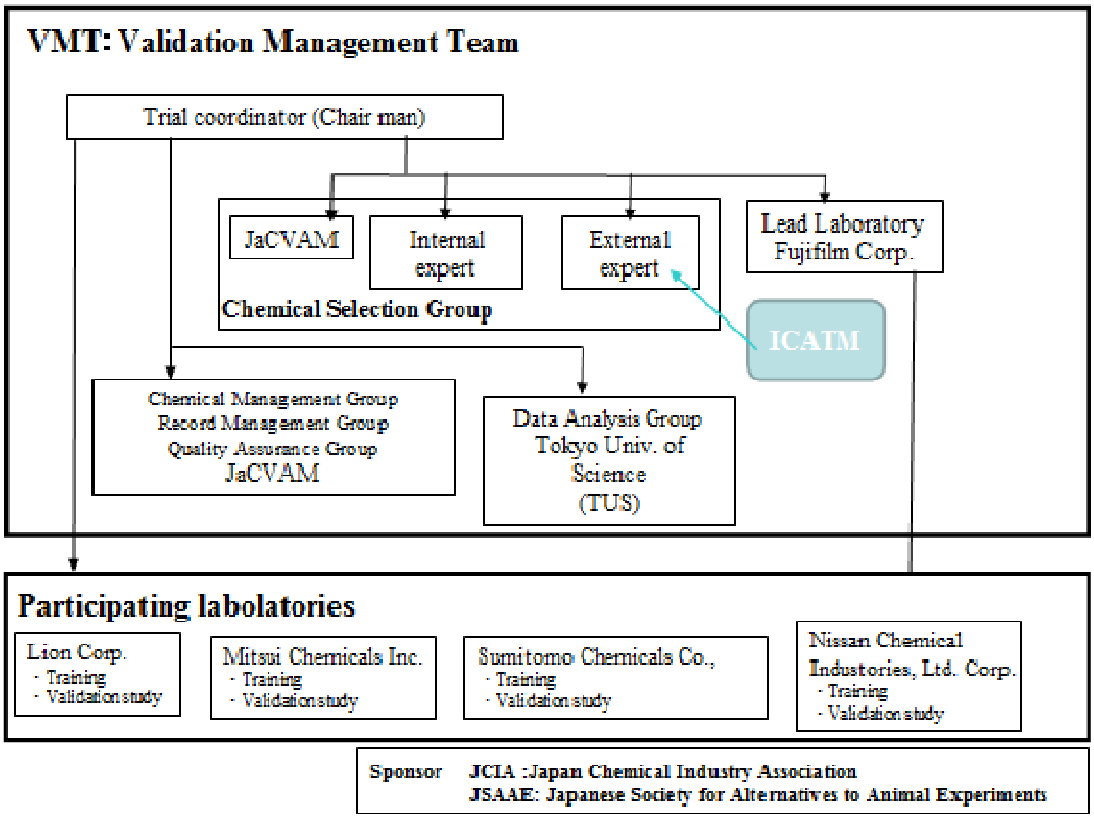
3. VALIDATION MANAGEMENT TEAM

A Validation Management Team (VMT) will be established to ensure that this validation study is scientifically sound, properly conducted, and objectively reported.

The ADRA validation management team (VMT) comprises a trial coordinator, a chemical selection group, a chemical management group, a data analysis group, a record management group, a quality assurance group and a representative from the assay developer (lead laboratory). The lead laboratory provides support to the participating laboratories. The VMT will prepare, review, and finalize drafts of the

study plan and study protocol. In addition, the VMT will manage the validation study, monitoring its progress, performing quality assurance of study records as required, and coordinating the activities, contributions and outputs of the study participants.

Fig.1 Management structure of the ADRA



3-1. LIST OF VALIDATION MANAGEMNT TEAM (VMT) MEMBERS

- Trial coordinator (Chairman): Atsushi Ono, Okayama University
- Expert: Nicole Kleinstreuer, NICEATM
- Expert: Jon Richmond, Ethical Biomedical Research and Testing Advice and Consultancy
- Expert: Bae-Hwan Kim, Keimyung University
- Expert: Tsuyoshi Kawakami, National Institute of Health Sciences
- Expert: Kohichi Kojima, Food and Drug Safety Center
- JaCVAM: Hajime Kojima, National Institute of Health Sciences
- JaCVAM: Shihori Tanabe, National Institute of Health Sciences
- Biostatistician: Takashi Sozu, Tokyo University of Science
- Biostatistician: Takuto Nakayama, Tokyo University of Science
- Biostatistician: Takeru Kusao, Tokyo University of Science
- Lead Laboratory: Toshihiko Kasahara, Fujifilm Corporation
- Lead Laboratory: Masaharu Fujita, Fujifilm Corporation
- Lead Laboratory: Yusuke Yamamoto, Fujifilm Corporation

3-2. TRIAL COORDINATOR

The Trial Coordinator, Dr. Atsushi Ono of Okayama University, is responsible for operational management of this validation study including preparing draft study plans, study protocols, test chemical lists and study reports. The Trial Coordinator chairs the VMT, and convenes VMT meetings as necessary to manage the study and review, finalize, and maintain these documents.

3-3. EXPERTS

The composition of the VMT conforms to, and complies with, the framework established for International Cooperation on Alternative Test Methods (ICATM).

Drs. Tsuyoshi Kawakami, Kohichi Kojima and Jon Richmond were nominated by JaCVAM.

Drs. Nicole Kleinstreuer and Bae-Hwan Kim were nominated by ICCVAM and KoCVAM respectively based on the ICATM framework.

All experts participate in ADRA VMT meeting, and are responsible for producing and agreeing all documentation finalized at VMT meetings. Additionally, the experts will participate in the preparation of the OECD submission.

3-4. CHEMICAL SELECTION GROUP

The chemical selection group is led by Dr. Hajime Kojima of JaCVAM, and includes internal experts Drs. Tsuyoshi Kawakami and Kohichi Kojima as well as external experts Drs. Nicole Kleinstreuer, Jon Richmond, and Bae-Hwan Kim.

The Group will both determine the selection criteria and select the test chemicals for the ADRA Validation Study.

A sufficient number of chemicals shall be used to obtain statistically significant results. The test chemicals to be used shall take account of the chemicals used to develop and optimise the ADRA test method, and the chemicals used in the DPRA validation study. All chemicals selected shall have high-quality reference data (LLNA and whenever possible human data). The chemicals selected shall cover the full range of skin sensitisation potentials, require the use of various solvents, and comprise more than one physical state.

3-5. CHEMICAL MANAGEMENT GROUP

The chemical management group is led by Dr. Hajime Kojima supported by other JaCVAM personnel. The test chemicals required for this validation study are to be purchased by the Lead laboratory, Fujifilm Corp., which will deliver them to JaCVAM. Dr. Shihori Tanabe will prepare coded test chemicals for distribution to the participating laboratories.

3-6. DATA ANALYSIS GROUP

The data analysis group is led by Dr. Takashi Sozu of Tokyo University of Science and includes Takuto Nakayama and Takeru Kusao, both of Tokyo University of Science, all of whom are responsible for independently and objectively analyzing the data sheets submitted by the participating laboratories. They are also responsible for the statistical processing of the data obtained in this validation study to determine the Within Laboratory Reproducibility (WLR), Between Laboratory Reproducibility (BLR), Specificity, Sensitivity, and Accuracy of the test method.

3-7. RECORD MANAGEMENT GROUP

The record management group is led by Dr. Atsushi Ono and includes Dr. Hajime Kojima, assisted by JaCVAM personnel. This Group will prepare and distribute to the participating laboratories the test method protocol, test chemical preparation record forms, blank data sheets, and other necessary documentation. The Group will also collect and validate the completed forms and data sheets after testing has been completed, requesting correction of any omissions or errors found in the records.

3-8. QUALITY ASSURANCE (QA) GROUP

The quality assurance group is led by Dr. Atsushi Ono and includes Dr. Hajime Kojima, assisted by JaCVAM personnel. They will appoint an individual to maintain all records, documents, raw data, and reports and make these records available to the VMT as requested.

3-9. LEAD LABORATORY

Fujifilm Corporation is the lead laboratory and will provide support to the participating laboratories. The lead laboratory representing the test method is responsible for providing the test method protocol and the necessary assay datasheets and worksheets, etc.

3-10. OBSERVERS: RESEARCHERS RESPONSIBLE FOR EXPERIMENTAL PROCEDURES

Each participating laboratory shall nominate one or more researchers to observe ADRA VMT meetings. The personnel at the participating laboratories carrying out testing according to the test protocol shall work under the supervision of the observers. The observers are responsible for sending copies of all record forms, data sheets, and other necessary documentation to the record management group upon completion of testing.

4. STUDY DESIGN[1]

The ADRA involves measuring the residual concentrations of NAC and NAL after a 24-hour incubation period. The operation is easy and simple to perform with high throughout, because it is based on the use of 96-well microplates. This validation study is designed to evaluate the Within- and Between-Laboratory Reproducibility (WLR and BLR) as well as the predictive capacity of ADRA.

4-1. PARTICIPATING LABORATORIES

The four participating laboratories previously and successfully underwent training to transfer technology from the lead laboratory. At least three of these laboratories must participate in the study to meet the requirements of this validation study. Laboratories that can accommodate the schedule will participate in this validation study. The four participating laboratories are:

1) Lion Corp.
2) Mitsui Chemicals, Inc.
3) Nissan Chemical Industries, Ltd.
4) Sumitomo Chemical Co. Ltd.

It is anticipated that the study will be conducted without the need for the participating laboratories

[1] Amino acid Derivative Reactivity Assay (ADRA) Standard Operating Procedure

to seek advice or assistance from other participating laboratories during Phase I or Phase II of the Validation Study; details on any such contact shall be notified to the VMT.

4-2. TEST SUBSTANCES

Four participating laboratories will test the same set of 40 coded chemicals in two phases. According to the assay protocol, each of these chemicals will be tested in triplicate during each repetition.

Table 1. Breakdown of substances for the ADRA validation study

Phase	No. of substances	No. of repetitions	Target	Remarks
I	10	3	Both WLR and BLR and Predictive Capacity	WLR will be assessed on the basis of 10 chemicals tested 3 times. BLR will be assessed using the mean value of the three data points obtained during Phase I.
II	30	1	BLR and Predictive Capacity	BLR will be assessed on the basis of 40 chemicals: 10 chemicals from Phase I and 30 chemicals from Phase II.

WLR: within-laboratory reproducibility, BLR: between-laboratory reproducibility

4-3. ACCEPTANCE CRITERIA FOR TEST DATA

Test data acceptance criteria are stipulated in the ADRA SOP. A table showing the acceptance criteria forms an appendix to this Study Plan. On the basis of the test data generated during the validation study, the acceptance criteria (for example acceptable positive control values) and prediction model (the value used to distinguish sensitisers from non-sensitisers) may be reviewed and/or revised by the VMT during or after the study to optimise test method performance.

4-4. SUCCESS CRITERIA FOR THE VALIDATION STUDY

Bearing in mind the primary goal of this study, the VMT defined in advance provisional success criteria for use in evaluating the performance of the test method. The target for both between- and within-laboratory reproducibility as well as for predictive accuracy is 80%.

(1) Within-laboratory reproducibility: The VMT will approve implementation of Phase II, providing that the results of Phase I achieve a WLR of at least 80%. If the Phase I WLR is less than 80%, the VMT will review the findings and consider what further action should be taken.

(2) Between-laboratory reproducibility: The VMT will approve completion of the validation study as long as the results of testing all 40 chemicals used in Phases I and II achieve a BLR of at least 80%. These results are to be based on three independent tests each for the 10 chemicals in Phase I and once each for the 30 chemicals in Phase II at each participating laboratory. If the BLR is less than 80%, the VMT will review the findings and consider what further action should be taken.

(3) Predictive accuracy: The VMT will approve completion of the validation study as long as the results of the testing of all 40 chemicals used in Phases I and II achieve a predictive accuracy of at least 80%. If the predictive accuracy is less than 80%, the VMT will review the findings and consider what further action should be taken.

4-5. RETESTING DURING THE VALIDATION STUDY

During the course of the Validation Study in the event that individual test results do not satisfy the

acceptance criteria there is limited provision for re-testing, and all test data shall be submitted to the VMT:

(1) If the results of a test do not satisfy any of the Acceptance Criteria from 1 to 6 as shown in the Appendix, that test may be repeated a further two times during either Phase I or Phase II.

(2) If the SD for the depletion of NAC or NAL in a test chemical greater than 10%, as stipulated in Criterion 7, the test may be repeated a further two times during either Phase I or Phase II, and the results reevaluated for all seven criteria.

(3) If the acceptance criteria are not met after the maximum permitted number of test runs, the results should be reported as "invalid".

If an error or omission is found:

(1) If an error or omission due to a human mistake occurs during the performance of the test, the reason is to be recorded in the test records and the test rerun.

4-6. STUDY DURATION

Testing for this validation study is scheduled to be performed from October 2016 to March 2017.

Phase I10 test chemicals tested three times each by the end of November 2016

Phase II 30 test chemicals tested once each by the end of March 2017

4-7. RECORD COLLECTION AND ANALYSIS

The biostatisticians will collect, organize, and collate the validation study data using a custom data collection software package, after which the data will be subject to statistical analysis. The data management procedures and statistical tools are to be preapproved by the trial coordinator and Data Analysis Group. The explanation and justification for any deviation from these procedures or tools is to be documented along with a description of its impact on the study results.

4-8. QUALITY ASSURANCE

Participating laboratories should conduct all studies in the spirit of Good Laboratory Practices.[13)] Any deviations from these practices is to be documented along with a description of their impact on the study results.

Non-GLP laboratories participating in the Validation Study are required, at a minimum, to comply with the relevant quality assurance provisions set out in ECVAM Workshop Report Number 5[14)]

5. REPORTING

1) The trial coordinator shall be responsible for the preparation of a final report that will be submitted for international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada/KoCVAM) within the ICATM framework.

2) If the results of the study demonstrate and confirm the scientific validity of ADRA, the trial coordinator is to prepare a research paper for joint publication in a peer reviewed journal.

6. SPONSOR

This validation study was supported by research grants provided by the Japan Chemical Industry Association (JCIA) and the Japanese Society for Alternative to Animal Experiments (JSAAE).

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APPENDIX. Acceptance Criteria of ADRA

	Criterion 1 Calibration curve	Criterion 2 Reference Control A (ACN)* (N = 3)	Criterion 3 Reference Control B (ACN)* (N = 6)	Criterion 4 Reference Control C (ACN)* (N = 3)	Criterion 5 Reference Control C (other than ACN) (N = 3)	Criterion 6 Positive Control (ACN) (N = 3)	Criterion 7 Test Chemicals (in solution) (N = 3)
r^2	>0.990	—	—	—	—	—	—
Mean concentration	—	3.2-4.4 μM	—	3.2-4.4μM	3.2-4.4 μM (2.8-4.0μM)[#]	—	—
Peak area (ACN)	—	—	CV < 10% (N = 9)		—	—	—
Peak area (except ACN)	—	—	—	—	CV < 10% (N = 3)	—	—
Depletion	—	—	—	—	—	SD < 10%	SD < 10%
Depletion Upper limit	—	—	—	—	—	NAC < 100% NAL < 100%	—
Depletion Lower limit	—	—	—	—	—	NAC > 15% NAL > 86%	—

[#] In the case of 5%DMSO/Acetonitrile

* Reference Controls required in this assay independent of solvents for test chemicals

ACN : acetonitrile

Appendix 2

Standard Operating Procedure (SOP)

SOP ver.1.0

SOP ver.1.1

SOP ver.1.2

Appendix 2

Standard Operating Procedure (SOP)

SOP ver.1.0

SOP ver.1.1

SOP ver.1.2

FUJIFILM	**Amino acid Derivative Reactivity Assay (ADRA)** **Standard Operating Procedure**	Version 1.0 Pages:27 Annexes:0

Japanese Center for Validation of Alternative Methods (JaCVAM)
Skin Sensitization Validation Study

Issued by	Fujifilm corporation	Date:
Approved by	Validation Management Team	Date:
Distributed by		N/A

Revision History

Revision	Date:	Description of change:

Table of Contents

Amino acid Derivative Reactivity Assay

PRINCIPLE AND SCOPE

The covalent binding of low molecular chemicals (haptens) to skin proteins is the molecular initiating event for skin sensitisation. The Amino acid Derivative Reactivity Assay (ADRA) is used to evaluate the reactivity of a test chemical with N- (2- (1-naphthyl)acetyl)-L-cysteine (NAC) and α-N- (2-(1-naphthyl)acetyl)-L-lysine (NAL) by combining the test chemical with a solution of NAC and NAL (abbreviated hereinafter as "NAC/NAL solution") and then measuring the residual concentration of NAC and NAL after a 24-hour incubation at 25ºC. NAC and NAL are chemically-synthesized compounds containing a naphthalene group simulating cysteine and lysine residues in skin proteins. Residual concentrations of NAC and NAL following the 24-hour incubation are determined by high performance liquid chromatography (HPLC) with gradient elution and UV detection at 281 nm. Test chemicals are prepared and analyzed in triplicate in batches of up to 17 chemicals, including controls. The method is applicable to test chemicals that are soluble in acetonitrile or other non-reactive, water-miscible solvents at a 1-mM concentration.

REFERENCES

Fujita, M., *et al*., "Development of a prediction method for skin sensitization using novel cysteine and lysine derivatives.", *J. Pharm. Toxicol. Method.* 2014; **70**; 94-105.

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1. APPARATUS AND REAGENTS

ADRA is best performed using disposable plastic labware, because NAC is susceptible to oxidization by trace metal ions which may be found in glassware. Spatulae used for weighing test chemicals and the various parts of the HPLC are not subject to this restriction.

1-1. Apparatus (1)

Apparatus	**Example of use by Lead Laboratory**
Analytical Balance	Capable of weighing up to 20 grams at an accuracy of 0.1 mg readability
・Dispensing Pipettes capable of delivering 50–150 µL ・12 Channel Dispensing Pipettes capable of delivering 50–150 µL	*Verify accuracy at time of use*
High Performance Liquid Chromatograph with light-excluding auto-sampler for 96-well microwell plates capable of delivering a 0.3 ml/min flow rate	Shimadzu Prominence series
UV detector[*1]	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector

	(281 nm)
pH meter with electrode and calibration	Capable of reading ±0.01 pH
HPLC Column	Shiseido CAPCELL CORE C_{18} column (2.7 μm, 3.0 × 150 mm) [Cat. 51112] *Alternative Column* The follow two columns have been confirmed to yield results for five test chemicals that are essentially the same as the Shiseido column. Waters, CORTECS C_{18} Column (2.7 μm, 3.0 × 150 mm) [Catalog # 186007373] Agilent, Poroshell 120 EC-C_{18} (2.7 μm, 3.0 × 150 mm) [Catalog # 693975-302]
Guard Column and Guard Column Holder[*2]	Column: Shiseido EXP GUARD CARTRIDGE CAPCELL CORE C_{18} S-2.7 2.1×5mm [Cat. 3643] Holder: Shiseido EXP DIRECT CONNECT HOLDER [Catalog # 3640]
Incubator	Capable of controlling at 25 +/ - 1ºC

*[1] If more than one wavelength is detectable, measurements should be made at both 281 nm and 291 nm to confirm peak purities of NAC and NAL.

[*2] Guard columns need not be used for test chemicals that are not precipitated in the reaction solution.

1-1. Apparatus (2)

Apparatus	**Example of use by Lead Laboratory**
Test Tube Mixer	SHIBATA Test Tube Mixer TTM-1
Plate Shaker	Heidolph Titramax 100 [Catalog # 544-11200-00]
Plate Centrifuge	KUBOTA PlateSpin
Recommended but non-essential consumables	
Dispensing Pipettes capable of delivering 1000 – 5000 μL	*Verify accuracy at time of use*

1-2. Consumables (1)

Consumables	**Recommended by Lead Laboratory**
96-well Microwell Plate for HPLC analysis	Polypropylene U bottom 96-well Microwell Plate Thermo (NUNC) U96 PP-0.5 ML NATURAL [Catalog # 267245] Greiner MICROPLATE, 96 WELL, PP, U-BOTTOM, NATURAL [Catalog # 650201]
96-well Microwell Plate seal for HPLC	Use the seal having sealing and solvent-resistant

analysis	performance[*1] Shimadzu GLC Resistant Embossed Seal [Catalog # 568-RES001]

[*1] When using a plate seal other than the recommended one, ensure and verify that no part of the reaction solution is volatilized and that the measured values are consistent before performing actual testing. Be sure to leave no gaps between the plate seal and the plate when applying the plate seal.

1-2. Consumables (2)

Consumables	**Example of use (Used by Lead Laboratory)**
Bottle with the quantity of approximately 500 ml	Sterilized bottle (AS ONE Corporation) [Catalog # 1-9475-05]
Polypropylene centrifuge tube with the quantity equal to or more than 50 ml	100 ml: Watson Clean-vessel [Catalog # 536-150S] 50 ml: FALCON 50-ml PP Conical Tube [Catalog # 352070]
Measuring pipette for 10–100ml	100-ml measuring pipette: FALCON 100-ml Serological Pipette [Catalog # 357600] 50-ml measuring pipette: FALCON 50-ml Serological Pipette [Catalog # 356550] 25-ml measuring pipette: FALCON 25-ml Serological Pipette [Catalog # 356525]
Polypropylene centrifuge tube with the quantity equal to or more than 10 ml	FALCON 15-ml Polypropylene Conical Tube [Catalog # 352196]
Tube for cryopreservation	5-ml Tube[*1]: 5-ml Outer serum tube (SUMITOMO BAKELITE CO., LTD.) [Catalog # MS-4605X] 2-ml Tube[*1]: 2.0-ml Ring rock tube (BM Equipment Co., Ltd.) [Catalog # BM-20] 1.5-ml Tube[*1]: 1.7-ml Ring rock tube (BM Equipment Co., Ltd.) [Catalog # BM-15]

[*1] A smaller or larger tube may be used per the quantity to be cryopreserved.

1-2. Consumables (3)

Recommended but non-essential consumables	**Example of use by Lead Laboratory**
Reservoir for operating 12 channel multipipette	Corning 50-ml Reagent Reservoir [Catalog # 4870]
Approximately 1.5-ml tube and cap operable by 12 channel multipipette (for dispensing test chemical solution)	Alphanumeric storage tube 1.4-ml (Thermo 4247JP) SepraSeal (Thermo 4463)
Tool for removing multiple caps simultaneously	Cap remover for SepraSeal (Thermo 4469)

1-3. Chemical and Special Materials

Chemical and Special Materials	**Example of use by Lead Laboratory**
Trifluoroacetic Acid (TFA) (MW=114.02) CASRN 76-05-1	Wako [Cat. No. 204-02743] 98.0%
Sodium Phosphate, Monobasic, Anhydrous (NaH2PO4, MW=119.98) CASRN 7558-80-7	Wako [Catalog # 197-09705]
Sodium Phosphate, Dibasic, Anhydrous (Na2HPO4, MW=141.96) CASRN 7558-79-4	Wako [Catalog # 197-02865]
0.1M Sodium Hydroxide Solution CASRN 1310-73-2	Wako [Catalog # 196-02195]
Acetonitrile, HPLC Grade CASRN 75-05-8	Wako [Catalog # 015-08633]
Distilled water	Distilled water or equivalent may be used.
Acetone, special grade CASRN 67-64-1	Wako [Catalog # 016-00346]
Dimethyl sulfoxide (DMSO), special grade CASRN 67-68-5	Wako [Catalog # 043-07216]
Ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid, disodium salt, dihydrate ($EDTA \cdot 2Na \cdot 2H_2O$) CASRN 6381-92-6	Dojindo Molecular Technologies, Inc. [Catalog #343-01861
NAC (refrigeration storage) MW=289.35, Purity> 95%	Lot No. #960074 (FUJIFILM provides synthetic NAC samples to each laboratory in the validation study)
NAL (refrigeration storage) MW=314.38 Purity>95%	Lot No. #990024 (FUJIFILM provides synthetic NAC samples to each laboratory participating in the validation study.)
Phenyl acetaldehyde (Positive control) MW=120.15 CASRN 122-78-1	SIGMA [Catalog # 107395] Note: Store phenyl acetaldehyde under the following conditions. (1) Store at a temperature of 2–8ºC. Do not store at below 0ºC. (2) Do not expose to UV light during storing or handling. (3) Handle as little as possible, keep stored under the above conditions.

2. PRE-WORK

2-1. Preparation of each solution (Typical)

2-1-1. 0.1-mM EDTA solution

1) Weigh 37.2 mg of EDTA·2Na·2H_2O into a 15-ml Conical Tube and use a 25-ml measuring pipette to add 10 ml of distilled water to dissolve it. (10-mM EDTA solution)
2) Use a 50-ml measuring pipette to add 49.5 ml of distilled water to a 100-ml tube and add 0.5 ml of the 10-mM EDTA solution described above to dilute it 100 fold. (0.1-mM EDTA solution)

2-1-2. 100-mM Phosphate buffer (pH 8.0)

To prepare an approximately 300-ml quantity

1) Weigh 0.6 g of Sodium Phosphate Dibasic Anhydrous into a 100-ml tube and use a 50-ml measuring pipette to add 50 ml of distilled water to dissolve 0.6 g of Sodium Phosphate Dibasic Anhydrous in distilled water.[*1]
2) Use a 50-ml (or 100-ml) pipette to add 300 ml of distilled water.
3) Weigh 4.26 g of Sodium Phosphate Monobasic Anhydrous and dissolve it in distilled water 2).[*1]
4) Use a 25-ml measuring pipette to add 16 ml of Sodium Phosphate Dibasic solution 1) in Sodium Phosphate Monobasic solution 3).
5) Use a 25-ml measuring pipette to remove 17 ml from solution 4) and to add 1 ml of 0.1-mM EDTA solution to solution 4). The concentrations of EDTA in this solution and final concentration of reaction solution are 0.33 μM and 0.25 μM, respectively.
6) Measure pH of solution 5) using a calibrated pH meter and verify that the pH is between 7.9 and 8.1. If the pH is out of range, prepare a new batch of phosphate buffer.
7) Use the NAC stock solution within a week or cryopreserve the unused quantity. Repackage it into 2-ml tubes to cryopreserve for Co-elution Control at -80ºC.

2-1-3. 100-mM Phosphate buffer (pH 10.2)

To prepare an approximately 300-ml quantity

1) Use a 50-ml measuring pipette to add 286 ml of distilled water to a 500-ml bottle.
2) Weigh 4.26 g of Sodium Phosphate Monobasic Anhydrous and dissolve it in the distilled water 1)[*1].
3) Use a 25-ml measuring pipette to add 14 ml of 0.1M NaOH solution to the solution 2).
4) Measure the pH of solution 3) using a calibrated pH meter and verify that it is between 10.1 and 10.3. If the pH is out of range, prepare a new batch of phosphate buffer.
5) Use the NAL stock solution within a week or cryopreserve the unused quantity. Repackage it into 2-ml tube to cryopreserve for Co-elution Control at -80ºC.

2-1-4. Reaction fixing solution (2.5% (v/v) TFA aqueous solution)

Add 2.5 ml of TFA to 100 ml of distilled water.

2-1-5. HPLC mobile phase A: 0.1% (v/v) TFA aqueous solution

Add 1.0 ml of TFA to 1000 ml of distilled water.

2-1-6. HPLC mobile phase B: 0.1% (v/v) TFA Acetonitrile solution

Add 1.0 ml of TFA to 1000 ml of HPLC grade Acetonitrile.

*[*1]: When dissolving phosphate salts, close the bottle to mix them. Do not use glass labware or other washable equipment. Verify that salts are completely dissolved after mixing.*

2-2. Preparation of NAC and NAL stock solutions

2-2-1. Preparation of NAC stock solution:

The NAC (molecular weight of 289.35) stock solution used in each assay is to all be from a single batch. We recommend storing NAC stock solution in quantities sufficient for an assay. A typical preparation as performed at the Lead Laboratory is described below.[*1]

1) Weigh 11.6 ± 0.1 mg of NAC in a 50-ml tube, add 20 ml of 100-mM phosphate buffer (pH 8.0) to the tube using a 25-ml measuring pipette, and mix it gently using a test tube mixer to dissolve the NAC. (2-mM NAC stock solution).[*2]
2) Use a 50-ml measuring pipette to add 149.5 ml of this buffer to a 500-ml bottle. Next, add 0.5 ml of 2-mM NAC stock solution to this solution and mix gently but thoroughly by inversion. (6.667-μM NAC stock solution).
3) Cryopreserve 3-ml quantities of stock solution in cryopreservable 5-ml tubes at -80ºC. Store for no more than six months before use.[*3]

2-2-2. Preparation of NAL stock solution

The NAL (molecular weight of 314.38) stock solution used in each assay is to all be from a single batch. We recommend storing NAL stock solution in quantities sufficient for an assay. A typical preparation as performed at the Lead Laboratory is described below.[*1]

1) Weigh 12.6 ± 0.1 mg of NAL in a 50-ml tube, and add 20 ml of 100-mM phosphate buffer (pH 10.2) to the tube using a 25 ml measuring pipette, and mix it gently using a test tube mixer to dissolve the NAL. (2-mM NAL stock solution).[*2]
2) Use a 50 ml measuring pipette to add 149.5 ml of this buffer to a 500 ml bottle. Next, add 0.5 ml of 2-mM NAL stock solution to this solution and mix it gently but thoroughly by inversion. (6.667-μM NAL stock solution)
3) Cryopreserve 3 ml quantities of stock solution in 5 ml tubes at -80ºC. Store for no more than six months before use.[*3]

[*1] *These quantities are typical examples only and can be adjusted as necessary.*

[*2] *The remaining 2-mM stock solution can be cryopreserved at -80*ºC *for later use. Typically, cryopreserve 1 ml of stock solution in a 1.5 ml tube at -80*ºC.

[*3] *Stability testing of NAC stock solution at temperatures of 25ºC, 4ºC, -20ºC, and -80ºC has shown that it remains stable and free of auto-oxidation only at -80* ºC. *We recommend that the NAL stock solution also be stored at -80* ºC, *although NAL is not susceptible to oxidization in the same manner as NAC.*

Note: Prior to preparing new batches of NAC/NAL stock solution, verify the stability of the NAC/NAL stock solution using the following procedure.

1) Add 150 μl of 6.667-μM NAC/NAL stock solution to three wells in each of two 96-well Microwell Plates.

2) Add 50 μl of acetonitrile to each of these six wells 1).

3) Attach a plate-seal to one of the two 96-well Microwell Plates and spin down the plate using a centrifuge, the incubate for 24 hours at 25ºC. After incubation, add 50μl of reaction fixing solution to each well, attach a new plate-seal, then mix and spin down the plate. Analyze NAC/NAL using the HPLC system.

4) Add 50 μl of reaction fixing solution to the other 96-well Microwell Plate, attach a plate-seal to it, then mix and spin down the plate. Analyze NAC/NAL using the HPLC system.

If the residual concentration of NAC/NAL falls below 90% after either 0 hour or 24 hours, the NAC and NAL are not stable, and so new batches of NAC/NAL solutions must be prepared.

2-3. Preparation of Standard Solution for Calibration Curve

The calibration curve for the acceptance criteria is obtained from a six-dose standard solution. The initial standard solution, Std1 at 5.0 μM, is cryopreserved in small quantities and used to prepare dilution series. A typical procedure for preparation of the standard solution is described below.[*1]

Procedure for preparation of 20-ml solution and 40-66 tubes

1) Use a 25-ml pipette to add 15 ml of 6.667-μM NAC/NAL stock solution to a 50 ml tube. Then use a micropipette to add 1 ml of distilled water, 100μl of TFA, and 3.9 ml of acetonitrile.

2) Cryopreserve 300–500 μl quantities of the standard solution in 1.5-ml tubes at -80ºC.

Also, solvent for preparing dilution series of the standard solution may be cryopreserved in 1000–1200-μl quantities, as described below.

1) Use a 25-ml pipette to add 15 ml of 100-mM phosphate buffer (pH 8.0 for NAC, pH 10.2 for NAL), 1 ml of distilled water, 100 μl of TFA, and 3.9 ml of acetonitrile to a 50-ml tube.

2) Typically, cryopreserve approximately 1 ml of solvent in a 1.5-ml tube at -80ºC.

[*1] These quantities are typical examples only and can be adjusted as necessary.

2-4. Solubility Assessment of Test Chemicals

The solubility of each test chemical in a suitable solvent must be assessed before performing the actual assay. A suitable solvent will dissolve the test chemical completely with no apparent turbidity or precipitate during visual inspection. The concentration of test chemical solution used in the assay is 1mM, but more than 10 mg is recommended to be weighed because of the precise weight of the test chemical. For this reason, the initial concentration of the test chemical solution is set to 20 mM, and the final 1-mM test chemical solution is prepared by 20-fold dilution of the 20-mM solution. The test chemical is dissolved using a test tube mixer and an additional 5 minutes of sonication if needed due to low solubility. The four solvents below are all suitable for used in ADRA, and the priority for the selection of the appropriate solvent is as follows.

(1) Distilled water

(2) Acetonitrile

(3) Acetone

(4) DMSO

Note 1: When the 20-mM solution is prepared using DMSO (4), use acetonitrile to prepare the 20-fold dilution down to a 1-mM solution.

Note 2: If none of these solvents are suitable for a given test chemical in a 20-mM solution, assess the solubility of the test chemical in 1-mM solution using solvents (1)–(3). Do not use DMSO to prepare a 1-mM solution. DMSO must always be diluted 20-fold with acetonitrile to prepare a 1-mM solution.

Note 3: Water is not suitable as a solvent for anhydrides due to hydrolysis.

A typical procedure is described below.

1. Use the following formula to calculate the quantity of test chemical needed to prepare approximately 5 ml of a 20-mM test chemical solution or 100 ml of a 1-mM test chemical solution. If no purity information is available, assume 100% purity.

$$\text{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{20\ \text{mM}}{1000} \times 5\ \text{ml} \quad \text{or} \quad \text{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{1\ \text{mM}}{1000} \times 100\text{ml}$$

$$= \text{MW} \times \frac{10}{\text{Purity (\%)}} = \text{Required quantity (mg)}$$

2. Weigh the required quantity of test chemical directly into a centrifuge tube or other suitable labware and record the actual weight.
3. Calculate the required quantity of solvent using the formula, and assess the solubility for the appropriate solvent.

Note 1: Even if the required quantity is calculated to be less than 10 mg, weigh more than 10 mg of test chemical for precise weight.

Note 2: If the test test chemical solution is in aqueous form, calculate necessary volume of the aqueous solution and quantity of water for dilution. If specific gravity of test chemical solution is not known, assume it as 1.0.

Preparation of 20-mM solution (5 ml)

$$\frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{1}{\text{Specific gravity}} \times \frac{1}{20\ \text{mmol} \times 5\ \text{ml}} \times 1000\ \mu\text{l}$$

$$= \frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{10}{\text{Specific gravity}}$$

= Volume of test chemical (µl)

5000 µl − Volume of test chemical (µl) = Solvent volume (µl)

Preparation of 1-mM solution (100 ml)

$$\frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{1}{\text{Specific gravity}} \times \frac{1}{1\ \text{mmol} \times 100\ \text{ml}} \times \quad 1000\ \mu\text{l}$$

$$= \frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{10}{\text{Specific gravity}}$$

= Volume of test chemical (μl)

$$\textbf{100 ml} \quad - \frac{\textbf{Volume of test chemical (μl)}}{\textbf{1000}} = \textbf{Solvent volume (ml)}$$

2-5. Preparation of HPLC System

Prepare an HPLC system with a 281-nm UV detector and mobile phase A and B, as described in section "2-1-5. HPLC mobile phase A: 0.1% (v/v) TFA aqueous solution" and in section "2-1-6. HPLC mobile phase B: 0.1% (v/v) TFA Acetonitrile solution".

1. Column Equilibration: Install the HPLC column (see section "1.1 Apparatus (1)") and equilibrate the column for at least 30 minutes at 40ºC with 50% A, 50% B before use. Then, condition the column by running the gradient at least twice before using the column.
2. System Shutdown: Following analysis, maintain a low flow (typically 0.05 ml/min) of 50% A and 50% B through the system and decrease column temperature to approximately 25ºC.
3. If the system is to be idle for more than a week, fill the column with acetonitrile (without Trifluoroacetic acid) and remove the column from the HPLC system. Cap tightly and purge acid containing mobile phases from the system using acetonitrile.

3. SOLUTION PREPARATION

3-1. Preparation of Test Chemical Solution

Solubility of the test chemical in the appropriate solvent is evaluated in section "2-4. Solubility Assessment of Test Chemicals". 20-mM or 1-mM solutions of test chemicals in the appropriate solvents are prepared immediately before use.

1. When ready to perform the assay, calculate and weigh the appropriate quantity of test chemical needed to prepare a 20-mM or 1-mM solution directly in a 15-ml or other appropriate tube. Use the following formula to calculate the required quantity of solvent needed based on the actual weight of the test chemical.

Prepare of 20-mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{20\ \text{mmol}} \times \quad 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times \quad 0.5 \quad = \text{Volume of solvent (ml)}$$

Prepare of 1-mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{1\ \text{mmol}} \times 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 10 = \text{Volume of solvent (ml)}$$

2. Add the volume of solvent calculated and mix in a centrifuge tube, using a test tube mixer to dissolve the test chemical. Slight sonication (less than 5 minutes) may be used if needed. If the test chemical is not completely dissolved, do not proceed with that specific test chemical in the selected solvent. Re-evaluate with another solvent (see section "2. PRE-WORK") to find a suitable vehicle.
3. Dilute 20-mM of test chemical solution to the final 1-mM solution with each appropriate solvent in the 1.4 ml Test chemical solution Storage Tube or other appropriate tube (For example, add 950 μl of appropriate solvent to 50 μl of 20-mM test chemical solution).[*1]
4. Record and report the final solvent choice for each chemical if the final solvent is different from the solvent selected in section "2-4. Solubility Assessment of Test Chemicals".

[*1] *96-well Deepwell Plate may be used instead of 1.4 ml Test chemical solution Storage Tube.*

3-2. Preparation of Positive Control Solution

Phenyl acetaldehyde (Molecular weight=120.15) dissolved in acetonitrile is used as Positive Control and should be contained in each run of assay. A typical preparation procedure for the Positive Control solution is described below.

1. Calculate a weight of Positive Control to be needed to prepare approximately 5 ml of 20-mM Positive Control solution from following formula. The next formula shows the case that the purity of Phenyl acetaldehyde is 90%.

$$\text{MW} \times \frac{10}{\text{Purity(\%)}} = 120 \times \frac{10}{90} = 13.35\text{(mg)} = \text{Phenyl acetaldehyde (mg)}$$

2. Weigh the phenyl acetaldehyde directly in a 10- or 15-ml centrifuge tube, and record the actual weight.
3. Calculate necessary quantity of solvent from following formula based on the actual weight

$$\text{Phenyl acetaldehyde (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 0.5 = 13.35 \times \frac{90}{120.15} \times 0.5 = 5.0\ \text{(ml)} = \text{Solvent volume (ml)}$$

4. Dissolve Phenyl acetaldehyde with acetonitrile in the quantities calculated above (20 mM).
5. Dilute the 20-mM Phenyl acetaldehyde solution 20-fold with acetonitrile in the 1.4-ml Test chemical solution Storage Tube or other appropriate tube. (final concentration or 1 mM)[*1]

[*1] 96-well Deepwell Plate may be used instead of 1.4-ml Test chemical solution Storage Tube.

3-3. Thaw of each stock solution

Thaw frozen Phosphate buffer (pH 8.0 and 10.2) and NAC/NAL stock solution at room temperature. The preparation of each stock solution is described in sections "2-1-2. 100-mM Phosphate buffer (pH 8.0)", "2-1-3. 100-mM Phosphate buffer (pH 10.2)", and "2-2. Preparation of NAC/NAL stock solutions".

4. Assay Procedure

4-1. Reference Control, Co-elution Control and Test Chemical Solution

Test chemical solutions are prepared in triplicate for NAC/NAL. Each assay (NAC/NAL) may be prepared and performed concurrently (NAC/NAL should not be incubated and analyzed on separate days). The three Reference Control A, six Reference Control C and one Co-elution Control (the solution after addition of buffer solution rather than NAC/NAL) should be prepared. Moreover, the solution (Co-elution Control, N=1) for each test chemical after addition of buffer solution rather than NAC/NAL solution should be prepared. The detail of each solution describes below.

Test chemical solution	Reaction solution of NAC/NAL with test chemical. Evaluate reactivity of NAC/NAL with the test chemical at a ratio of 1:50 for the NAC/NAL with the test chemical.
Reference Control A	Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC/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.
Co-elution Control	Control for verifying whether or not test chemicals co-elute with NAC/NAL. The Co-elution Control is used to verify absorbance at 281nm and whether retention time of test chemical is equal to that of NAC/NAL.

4-2. Preparation and Reaction of Reaction Solutions

1. Assemble the following previously prepared reagents, solvents, and solutions:
   a. 6.667-μM of NAC/NAL stock solution,
   b. Appropriate buffer solution (pH 8.0 for NAC, pH 10.2 for NAL),
   c. Test chemical solution (or solvent for Reference Controls)
2. Use a 96-well Microwell Plate and 12-channel pipette to prepare test chemical solutions by adding the reagents per the following table. Refer to the section on “Typical Test Chemical Solution Positions in 96-well Microwell Plate” for typical positions in the plate. Inspect test chemical solution after adding test chemical and record observations.

Ratio of NAC to test chemical = 1:50 5-μM NAC, 0.25-mM test chemical	**Ratio of NAL to test chemical = 1:50** 5-μM NAL, 0.25-mM test chemical
• **150-μL NAC solution** • **50-μL Test chemical solution**	• **150-μL NAL solution** • **50-μL Test chemical solution**

Co-elution Control and Reference Control A, B and C are prepared according to the following tables.

Co-elution Control

For NAC	For NAL
・150-µL Phosphate buffer (pH 8.0) **・50-µL Test chemical solution**	**・150-µL Phosphate buffer (pH 10.2)** **・50-µL Test chemical solution**

Reference Control A and B

For NAC	For NAL
・150-µL NAC solution **・50-µL Acetonitrile**	**・150-µL NAL solution** **・50-µL Acetonitrile**

Reference Control C

For NAC	For NAL
・150-µL NAC solution **・50-µL Acetonitrile and solvent used for dissolution of test chemical** [*1]	**・150-µL NAL solution** **・50-µL Acetonitrile and solvent used for dissolution of test chemical** [*1]

[*1] If the test chemicals are dissolved in acetonitrile, only acetonitrile need be prepared.

3. Seal the 96-well Microwell Plate completely with a plate seal and shake it using a Plate Shaker. Centrifuge the plate using a Plate Centrifuge and incubate the plate in the Incubator (dark) at 25ºC (plus or minus 1'C) for 24 hours (plus or minus 1 hour). Record the time and temperature at the start and end of incubation.

4-3. Procedure after the Completion of Reaction

1. After 24 hours of incubation, remove the plate seal and add 50 µL of Reaction Fixing Solution to each test chemical solution. Inspect the reacted test chemical solution and record any observations after addition of the reaction fixing solution. If precipitation is observed, centrifuge the 96-well Microwell Plate at low speed (100–400g) to force the precipitate to the bottom of the well as a precaution. If more than 100 µl of supernatant of test chemical solution can be removed using a pipette, the supernatant may be analyzed after being transferred to another plate.

2. Prepare the standard solution

Prepare NAC/NAL standard solutions by serial dilution from 5.0–0.156-µM

(1) Prepare each solution ahead of time after thawing frozen stock solution at room temperature.

[1] 5.0-µM stock solution "Std1"

[2] "solvent for preparing dilution series" (for NAC/NAL)

(2) Prepare the following concentration of the standard solution by diluting 150 µL of stock solution "Std1" with the same quantity of dilution solvent per to "Preparation procedure for dilution series" below. Add dilution solvent to a well as "Std7".

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7 (Dilution solvent)
NAC/NAL (µM)	5.0	2.5	1.25	0.625	0.313	0.156	0

"Preparation procedure of dilution series"

(1) Add 150 µl of dilution buffer to wells from well Std2 to well Std7 shown in "Typical Test Chemical Solution Positions in 96-well Microwell Plate"

(2) Add 150µl of Std1 to well Std1 and well Std2. Mix with minimal air entrapment.

(3) Transfer 150 µl from well Std2 to well Std3. Mix with minimal air entrapment.

(4) Continue in a similar manner from well Std4 to well Std6.

3. Prepare dilution series of NAC/NAL standard solution in the wells from Std1 to Std7 shown in the next section on "Typical Test Chemical Solution Positions in 96-well Microwell Plate", and reseal the plate tightly with a new plate seal. For preparation of dilution series, see section "4-3. 2. Prepare the standard solution". Mix the plate with a Plate Shaker and spin it down by centrifuge.

Note: Add the Reaction Fixing Solution within 30 minutes of the end of incubation.

Typical Test Chemical Solution Positions in 96-well Microwell Plate

Between 1 and 17 test chemicals can be tested in a single test run using two 96-well plates, based on consideration of the time needed for preparation and testing as well as the burden on laboratory technicians and the capacity of the HPLC apparatus. If more than one solvent are used a single test run, additional Reference Controls for each solvent need to be prepared and the number of test chemicals will need to be reduced. For example, if three kinds of solvent are used, the maximum number of test chemicals is 15. The number of test chemicals and Typical Test chemical solution position in 96-well Microwell Plate are shown below, but the positions may be adjusted per the number of test chemical solutions.

(1) For five test chemicals with acetonitrile as solvent

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	CC-1	CC-2	CC-3	CC-4	CC-5	—	—	—	—	—	STD 4	—
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	CC-1	CC-2	CC-3	CC-4	CC-5	—	—	—	—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control

(2) For 10 test chemicals with acetonitrile and water as solvent

First plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	—	—
	B												
	C												
	D	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	—	—
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	—	—
	F												
	G												
	H	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	—	—

Second plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	RC-A	RC-B	RC-B	RC-C (Water)	RC-C (ACN)	PC	—	—	—	—	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	—	—	—	—	—	—	—	—	—	—	STD 4	—
NAL	E	RC-A	RC-B	RC-B	RC-C (Water)	RC-C (ACN)	PC	—	—	—	—	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	—	—	—	—	—	—	—	—	—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control, ACN: Acetonitrile

(3) For 17 test chemicals and acetonitrile as solvent

First plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	Test chemical solution No.11	Test chemical solution No.12
	B												
	C												
	D	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	CC-9	CC-10
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	Test chemical solution No.11	Test chemical solution No.12
	F												
	G												
	H	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	CC-11	CC-12

Second plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.13	Test chemical solution No.14	Test chemical solution No.15	Test chemical solution No.16	Test chemical solution No.17	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	CC-3	CC-4	CC-5	CC-6	CC-7	—	—	—	—	—	STD 4	—
NAL	E	Test chemical solution No.13	Test chemical solution No.14	Test chemical solution No.15	Test chemical solution No.16	Test chemical solution No.17	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	CC-13	CC-14	CC-15	CC-16	CC-17				—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control, ACN: Acetonitrile

5. HPLC Analysis

1. Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the Mobile Phase A and Mobile Phase B at column temperature of 40°C. The HPLC analysis is performed using a flow of 0.3 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. Refer “5-1. HPLC Conditions” for details on the gradient.
2. Inject equal quantities of each standard solution, test chemical solution, and control solution. The injection quantity varies according to the system used but typically is from 10–20 μL. Systems require smaller injection quantities, because injection of 20 μL leads to unacceptably broad peaks. Absorbance is monitored at 281 nm. If using a Photodiode Array Detector, absorbance at 291 nm should also be recorded.
3. Increase the mobile phase B to 100% in order to remove the other compounds from the column after gradient, and re-equilibrate the column under initial conditions for at least 6.5 minutes. These process of acetonitrile 100% and re-equilibration for 6.5 minutes are described the following table “5-1. HPLC Conditions”.

Note: The 6.5 minute re-equilibration time was determined using a Shimadzu Prominence HPLC system. Other systems may require more or less re-equilibration time due to system mixing quantity. Shorter equilibration times will be acceptable if peak retention times are stable.

5-1. HPLC Conditions

Column	Shiseido CAPCELL CORE C_{18} column (2.7 μm, 3.0 × 150 mm) [Cat. 51112] *Alternative Column* The follow two columns have been confirmed to yield results for five test chemicals that are

<table>
<tr><td></td><td>essentially the same as the Shiseido column.
Waters, CORTECS C_{18} Column (2.7 μm, 3.0 × 150 mm) [Catalog # 186007373]
Agilent, Poroshell 120 EC-C_{18} (2.7 μm, 3.0 × 150 mm) [Catalog # 693975-302]</td></tr>
<tr><td>Column Temperature</td><td>40ºC</td></tr>
<tr><td>Test chemical solution Temperature</td><td>25ºC
If the auto-sampler has a cooling function, test chemical solutions can be kept more stable at 4ºC.</td></tr>
<tr><td>UV detector[*1]</td><td>Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector (281 nm)</td></tr>
<tr><td>Injection Quantity</td><td>10-20 μl (The injection quantity varies according to HPLC system. If peaks are too broad, the injection quantity should be decreased.)</td></tr>
<tr><td>Run Time</td><td>20 minutes</td></tr>
<tr><td>Flow Conditions</td><td>NAC flow conditions
<table><tr><th>Time</th><th>Flow</th><th>%A</th><th>%B</th></tr><tr><td>0 min</td><td>0.3 ml/min</td><td>70</td><td>30</td></tr><tr><td>9.5 min</td><td>0.3 ml/min</td><td>45</td><td>55</td></tr><tr><td>10 min</td><td>0.3 ml/min</td><td>0</td><td>100</td></tr><tr><td>13 min</td><td>0.3 ml/min</td><td>0</td><td>100</td></tr><tr><td>13.5 min</td><td>0.3 ml/min</td><td>70</td><td>30</td></tr><tr><td>20 min</td><td>End run</td><td></td><td></td></tr></table>
NAL flow conditions
<table><tr><th>Time</th><th>Flow</th><th>%A</th><th>%B</th></tr><tr><td>0 min</td><td>0.3 ml/min</td><td>80</td><td>20</td></tr><tr><td>9.5 min</td><td>0.3 ml/min</td><td>55</td><td>45</td></tr><tr><td>10 min</td><td>0.3 ml/min</td><td>10</td><td>100</td></tr><tr><td>13 min</td><td>0.3 ml/min</td><td>10</td><td>100</td></tr><tr><td>13.5 min</td><td>0.3 ml/min</td><td>80</td><td>20</td></tr><tr><td>20 min</td><td>End run</td><td></td><td></td></tr></table></td></tr>
</table>

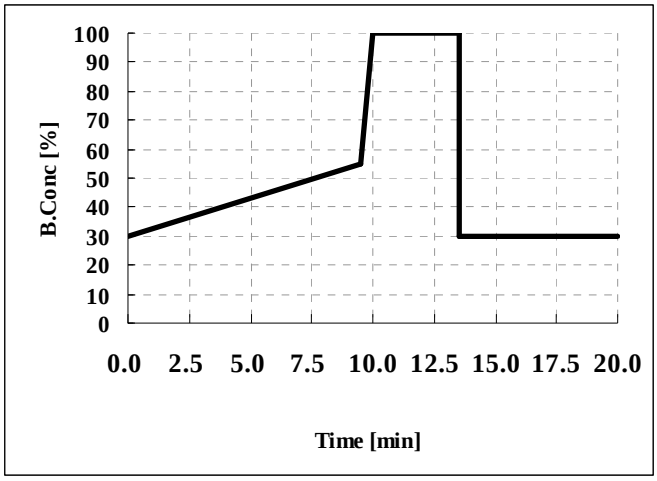
Note 1: The mixer quantity should be verified and adjusted in advance because the appropriate elution pattern of NAC/NAL peak will not be shown if the mixer quantity for mixing each mobile phase is not appropriate (For example, 0.5 ml mixing quantity is appropriate for Shimadzu prominence HPLC system).

Note 2: The inner diameter of pipe and the length of pipe from column outlet to detector inlet must be less than 0.18 mm and less than 50 cm, respectively, because the peak of NAC/NAL might be broadened depending on inner diameter and length of pipe

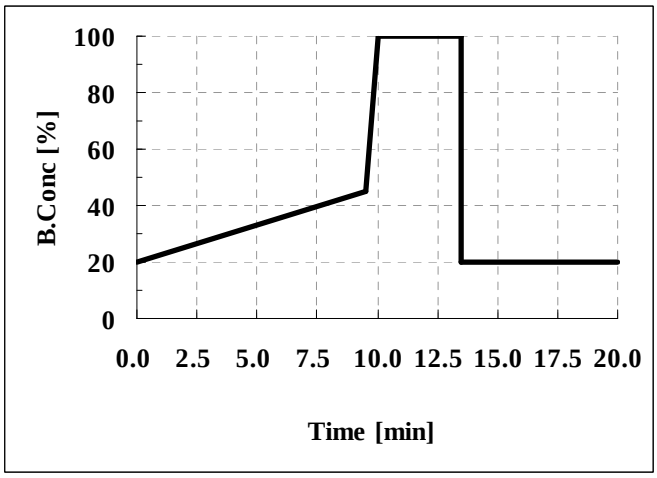
Note 3: If more than one wavelength is detected, also 291 nm besides 281 nm should be detected to check out peak purities of NAC/NAL.

Graphical Display of Gradient for NA/NAL Analysis

For NAC analysis



For NAL analysis



Note: Visual inspection of test chemical solutions must be conducted prior to HPLC analysis. Generally, precipitation is not a problem. However, if precipitation is observed, this should be noted in the data reporting template. Test chemical solutions may be centrifuged at low speed (100–400g) in vial to force precipitate to the bottom of the well as a precaution, since large quantities of precipitate may clog the HPLC tubing or columns. If more than 100 μL of supernatant can be recovered, in may be analyzed after transfer to another plate. Precipitate formation, removal, and transfer must be recorded and reported.

5-2.HPLC Sample Analysis Sequences

Each sample of HPLC analysis should be analyzed 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 analyze calibration standards and Reference Control A (N = 3).
2. The Co-elution Control does not need to be analyzed by turns if it is analyzed after analysis of standard solution and Reference Control A.
3. Reference Control B should be analyzed 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 analyzed. (After the first set of replicates of each sample is analyzed, the second set of replicates of each should be analyzed)

Example of HPLC Samples Analysis Sequences

(A more specific analysis sequence can be found at the end of the SOP)

STD7 (Buffer solution for dilution)†	Ref. 8-1. 1)
STD6	
STD5	
STD4	
STD3	
STD2	
STD1	

Reference Control A, rep 1 Reference Control A, rep 2 Reference Control A, rep 3	Ref. 8-1. 1)
Co-elution Control 1 Co-elution Control 2 Co-elution Control 3 ・・・ Co-elution Control n	Ref. 8-4.
Reference Control B, rep 1 Reference Control B, rep 2 Reference Control B, rep 3	Ref. 8-2.
Reference Control C, rep1[§†] Phenyl acetaldehyde (Positive Control), rep1 Test chemical solution 1, rep1 Test chemical solution 2, rep1 Test chemical solution 3, rep1 ・・・ Test chemical solution n, repn	***First set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control C, rep2[§†] Phenyl acetaldehyde (Positive Control), rep2 Test chemical solution 1, rep2 Test chemical solution 2, rep2 Test chemical solution 3, rep2 ・・・ Test chemical solution n, rep2	***Second set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control C, rep3[§†] Phenyl acetaldehyde (Positive Control), rep3 Test chemical solution 1, rep3 Test chemical solution 2, rep3 Test chemical solution 3, rep3 ・・・ Test chemical solution n, rep3	***Third set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control B, rep 4 Reference Control B, rep 5 Reference Control B, rep 6	Ref. 8-2.

[†] Start to analyze calibration standard immediately after addition of Reaction Fixing Solution and preparation of dilution series of standard solution.

[§] Analyze three replicates for Reference Controls C. These results are used to calculate the NAC/NAL depletion in each solvent and to verify that solvent used does not affect NAC/NAL depletion.

6. DATA ANALYSIS & CALCULATIONS

The concentration of NAC/NAL is calculated from peak area of absorbance at 281 nm for each test chemical solution based on the calibration curve derived from standard solutions Std 1 to Std7. NAC/NAL percent depletion is calculated by dividing NAC/NAL peak area of each test chemical solution by mean peak area of Reference Control C.

6-1. Calculation of Peak Area of NAC/NAL

Integrate the appropriate peaks and determine peak area for standards, test chemical solution and controls. The peak area of each integrated peak must be reported.

6-2. Calculation of Concentration of NAC/NAL

6-2-1. Generate a linear calibration curve based on the concentration of standards and the peak area. Suitable calibration curves must have an r^2>0.990.

6-2-2. Calibrate the mean NAC/NAL concentrations in Reference Controls A and C, SD and CV. The each mean should be 3.2–4.4 μM. The NAC/NAL concentration of Reference Controls A and C must be reported.

6-2-3. Calculate the mean NAC/NAL peak area, SD and CV for the Reference Controls C (N=3) for each solvent used. The each mean concentration should be 3.2–4.4 μM. However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM.

6-3. Calculation of Peak Area of NAC/NAL

6-3-1. Calculate the mean NAC/NAL peak area for the six Reference Controls B and the three Reference Control C in acetonitrile, SD and CV. The CV must be less than 10%.

6-3-2. Calculate the mean NAC/NAL peak area at 281nm for the three Reference Controls C.

6-4. Calculation of Percent Depletion of NAC/NAL

6-4-1. For the Positive Control and for each test chemical, calculate the Percent NAC/NAL Depletion in each replicate from the NAC/NAL peak area of the replicate injection and the mean NAC/NAL area in the three relevant Reference Controls C (in the appropriate solvent), using the following formula.

Percent NAC/NAL Depletion (% depletion) = [1- (NAC/NAL Peak Area in Replicate Injection/mean NAC/NAL Peak Area in Reference Controls C)]×100

6-4-2. The mean Percent NAC/NAL Depletion (Average score) of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

7. DATA REPORTING (FOR NAC AND NAL)

System Suitability

- NAC/NAL peak area at 281 nm of Standard and Reference Control B and C replicate.
- The linear calibration curve should be graphically represented and the r^2 reported.

- NAC/NAL concentration (μM) of Reference Control A replicate.
- Mean NAC/NAL concentration (μM) of Reference Controls C replicate, SD and CV.

Analysis Sequence

Reference Controls:

- NAC/NAL peak area at 281 nm of Reference Control B and C replicate.
- Mean NAC/NAL peak area at 281 nm of the nine Reference Controls B (N = 6) and C (N = 3) in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean NAC/NAL peak area at 281 nm of the three appropriate Reference Controls C replicate (for calculation of Percent NAC/NAL Depletion).
- For each solvent used in this assay, the mean NAC/NAL concentration (μM) of the appropriate Reference Control C replicate, SD and CV.

Positive Control (Phenyl acetaldehyde)

- NAC/NAL peak area at 281 nm of each replicate.
- Percent NAC/NAL Depletion of each replicate
- Mean NAC/NAL Depletion of the three replicates, SD and CV.

For Each Test Chemical:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time.
  It must be reported if precipitate was re-solubilized or centrifuged.
- NAC/NAL peak area at 281 nm of each replicate (for systems equipped with a PDA detector the peak area at 291 nm should also be reported).
- Percent NAC/NAL Depletion of each replicate.
- Mean of Percent NAC/NAL Depletion of the three replicates, SD and CV.

8. ACCEPTANCE CRITERIA

8-1. Acceptance Criteria for Amino acid Derivative Reactivity Assay Run

All criteria must be met for the whole run to be considered valid. If three criteria are not met, the run must be repeated for all test chemicals.

System Suitability:

Calibration Linearity $r^2 > 0.990$

Mean NAC/NAL concentration of Reference Controls A = 3.2–4.4 μM

Positive Control:

The mean Percent NAC/NAL Depletion value of the three replicates for phenyl acetaldehyde must fall within the range reported in the following table (Based on mean ±3SD from background data):

	Percent NAC Depletion		Percent NAL Depletion	
Positive control	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Phenyl acetaldehyde	8	100	86	100

Maximum Standard Derivatives for Positive Control replicate:

Standard Deviation for Percent NAC Depletion must be < 10%

Standard Deviation for Percent NAL Depletion must be < 10%

Stability of Reference Controls over analysis time:

For each solvent used, the mean of the NAC/NAL concentrations of the three appropriate Reference Controls C = 3.2–4.4 μM. However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM, as it is known that concentration of NAC decreases because of oxidation of SH group by DMSO.

8-2. Acceptance Criteria for Each Test Chemical

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

Maximum Standard Deviation of test chemical solution replicates:

Standard Deviation for Percent NAC Depletion must be < 10%

Standard Deviation for Percent NAL Depletion must be < 10%

Reference Controls C in the analysis sequence:

CV of NAC/NAL peak areas for the nine Reference Controls B (N = 6) and C (N = 3) in acetonitrile must be < 10%. Moreover, CV of NAC/NAL peak areas for the three Reference Controls C (N = 3) in each solvent must be < 10%.

8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay

The average score should be calculated from depletions of NAC/NAL, and the test chemicals should be predicted to be either a Sensitizer or a Non-sensitizer according to following table.

NAC/NAL **prediction model**

Average score	**Judgement**
Less than 4.9%	Non-sensitizer
4.9% or higher	Sensitizer

8-4. Handling of Co-elution

8-4-1. Co-elution : Interference

(1) Some test chemicals will co-elute with the NAC or NAL. In order to detect possible co-elution of the test chemicals with NAC or NAL, the test chemicals included in the run must be injected alone

("Co-elution Controls") at the beginning of the run sequence and their chromatograms compared to the chromatograms of Reference Controls C in the appropriate solvent.

(2) If a chemical absorbs at 281 nm and has a similar retention time as a peptide (overlap of valley-to-valley integration periods), then verify whether or not the peak of test chemical is actually separated from the peak of NAC or NAL. If the peak of test chemical is completely overlapped with the peak of NAC or NAL, and if the boundary of two peaks (valley between peaks) is located higher than baseline, co-elution of the test chemical with that NAC or NAL should be reported. The "interfering" chemical peak should have a peak area that is >10% of the mean NAC/NAL peak area in the appropriate Reference Control. If co-elution occurs and proper integration and calculation of NAC/NAL depletion is not possible, the data should be recorded as "interference" for NAC/NAL the chemical co-elutes with.

8-4-2. Peak purity of NAC/NAL : Area ratio of 281/291 nm

(1) When a Photodiode Array detector is used, co-elution of chemical and NAC/NAL may also be verified by looking at the UV spectrum at 291 nm in addition to 281 nm and calculating the area ratio of 281/291. This value should be consistent over all test chemical solutions and standards for a distilled NAC/NAL peak and thus gives a measure of peak purity. For each test chemical solution, a ratio in the following range would give a good indication that co-elution has not occurred. However, calculation of peak purity (area ratio of 281/291) might not always be possible, particularly if the test chemical is highly reactive with the NAC/NAL leading to very small peaks.

90% < Mean Area ratio of Reference Control < 110%.

8-4-3. Co-elution : Depletion < -10%

(1) If the Percent NAC/NAL Depletion is < -10%, it should be considered that this may be a situation of co-elution, inaccurate NAC/NAL addition to the reaction mixture or just baseline noise. If the NAC/NAL peak appears at the proper retention time and has the appropriate peak shape, the peak can be integrated. In this case, there may just be baseline noise causing the NAC/NAL peak to be bigger or there may be some co-elution/overlap in retention time of the NAC/NAL and test chemical.

(2) The calculated %-depletion should be reported as an estimate. In cases where a test chemical co-elutes with NAL, the NAC only prediction model can be used. In cases where a test chemical co-elutes with both NAC/NAL, the data should be reported as an inconclusive.

(3) In cases where the test chemical co-elutes with the NAC and the peak of NAC cannot be integrated, the skin sensitization of test chemical cannot be predicted from the NAL depletion alone, and the data should be reported as inconclusive.

8-4-4. Calculation of peak area for co-elution

(1) If the peak of NAC/NAL and the peak of test chemical partially overlap, the peak area of NAC/NAL should be integrated from valley of both peaks to baseline vertically.

(2) If the peak of NAC/NAL and the peak of test chemical completely overlap, the data should be reported as an Inconclusive, and the peak area should not be calculated.

8-4-5. Estimated depletion values

In some cases, a test chemical might co-elute with NAC and/or NAL though the test chemical react with NAC and /or NAL. If this is the case, co-elution will make the peak area of NAC/NAL appear to be larger than it really is, therefore the calculated percent depletion may be lower than the true value. When the overlap in retention time between the test chemical and NAC/NAL is incomplete, percent depletion can still be calculated with a notation of "co-elution – percent depletion estimates". If the average score is below the criteria, the result should be reported as Inconclusive. However, unless NAC co-elutes with test chemical, the NAC-only prediction model should be used.

Average score	No co-elution	Co-elution with NAC alone or NAC and NAL	Co-elution with NAL only
< 4.9%	Non-sensitizer	Inconclusive	Apply NAC-only prediction model
4.9% ≦	Sensitizer	Sensitizer	Apply NAC-only prediction model

NAC only prediction model

NAC Depletion	**Judgement**
less than 5.6%	Non-sensitizer
5.6% or greater	Sensitizer

Example HPLC Analysis

There are 5 test chemicals. Chemical 1,2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in distilled water.

The following 96-well Microwell Plate should be set up:

Std 7 (Dilution buffer blank)
Std 6
Std5
Std5
Std4
Std 3
Std 2
Std1
Reference Control A, rep 1 (made with acetonitrile)
Reference Control A, rep 2 (made with acetonitrile)
Reference Control A, rep 3 (made with acetonitrile)

Co-elution Control for Chemical 1 Co-elution Control for Chemical 2 Co-elution Control for Chemical 3 Co-elution Control for Chemical 4 Co-elution Control for Chemical 5
Reference Control B, rep 1 (made with acetonitrile) Reference Control B, rep 2 (made with acetonitrile) Reference Control B, rep 3 (made with acetonitrile) Reference Control C, rep 1 (made with acetonitrile) Reference Control C, rep 1 (made with distilled water) Phenyl acetaldehyde, rep 1 Chemical 1, rep 1 Chemical 2, rep 1 Chemical 3, rep 1 Chemical 4, rep 1 Chemical 5, rep 1 Reference Control C, rep 2 (made with acetonitrile) Reference Control C, rep 2 (made with distilled water) Phenyl acetaldehyde, rep 2 Chemical 1, rep 2 Chemical 2, rep 2 Chemical 3, rep 2 Chemical 4, rep 2 Chemical 5, rep 2 Reference Control C, rep 3 (made with acetonitrile) Reference Control C, rep 3 (made with distilled water) Phenyl acetaldehyde, rep 3 Chemical 1, rep 3 Chemical 2, rep 3 Chemical 3, rep 3 Chemical 4, rep 3 Chemical 5, rep 3 Reference Control B, rep 4 (made with acetonitrile) Reference Control B, rep 5 (made with acetonitrile) Reference Control B, rep 6 (made with acetonitrile)

Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean NAC/NAL peak area of the Reference Control C which are prepared with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean NAC/NAL peak area of the Reference Controls C which are prepared with distilled water.

FUJIFILM	**Amino acid Derivative Reactivity Assay (ADRA) Standard Operating Procedure**	Version 1.1 Pages:27 Annexes:0

Japanese Center for Validation of Alternative Methods (JaCVAM)
Skin Sensitization Validation Study

Issued by	Fujifilm corporation	Date:
Approved by	Validation Management Team	Date:
Distributed by		N/A

Revision History

Revision	Date:	Description of change:
Ver. 1.1	February 6, 2017	1) Revised NAL lot number on page 6. 2) Added a precautionary comment about pipette technique to section 4-2. Preparation and Reaction of Reaction Solutions on page 13. 3) Revised the control criteria for the positive control reagent on page 23. 4) Added the range of average score and NAC depletion for additional test to get an accurate predictive result in section 8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay on page 24. 5) Added instructions on how to handle instances in which there appears to be no co-elution but depletion is less than -10% in section 8-4. Handling of Co-elution on page 24.

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Amino acid Derivative Reactivity Assay

PRINCIPLE AND SCOPE

The covalent binding of low molecular chemicals (haptens) to skin proteins is the molecular initiating event for skin sensitisation. The Amino acid Derivative Reactivity Assay (ADRA) is used to evaluate the reactivity of a test chemical with N- (2- (1-naphthyl)acetyl)-L-cysteine (NAC) and α-N- (2-(1-naphthyl)acetyl)-L-lysine (NAL) by combining the test chemical with a solution of NAC and NAL (abbreviated hereinafter as "NAC/NAL solution") and then measuring the residual concentration of NAC and NAL after a 24-hour incubation at 25ºC. NAC and NAL are chemically-synthesized compounds containing a naphthalene group simulating cysteine and lysine residues in skin proteins. Residual concentrations of NAC and NAL following the 24-hour incubation are determined by high performance liquid chromatography (HPLC) with gradient elution and UV detection at 281 nm. Test chemicals are prepared and analyzed in triplicate in batches of up to 17 chemicals, including controls. The method is applicable to test chemicals that are soluble in acetonitrile or other non-reactive, water-miscible solvents at a 1-mM concentration.

REFERENCES

Fujita, M., *et al*., "Development of a prediction method for skin sensitization using novel cysteine and lysine derivatives.", *J. Pharm. Toxicol. Method.* 2014; **70**; 94-105.

Yamamoto, Y., *et al*., "A novel *in chemico* method to detect skin sensitizers in highly diluted reaction conditions. ", *J. Appl. Toxicol*. 2015; **35**: 1348-1360.

1. APPARATUS AND REAGENTS

ADRA is best performed using disposable plastic labware, because NAC is susceptible to oxidization by trace metal ions which may be found in glassware. Spatulae used for weighing test chemicals and the various parts of the HPLC are not subject to this restriction.

1-1. Apparatus (1)

Apparatus	**Example of use by Lead Laboratory**
Analytical Balance	Capable of weighing up to 20 grams at an accuracy of 0.1 mg readability
・Dispensing Pipettes capable of delivering 50–150 µL ・12 Channel Dispensing Pipettes capable of delivering 50–150 µL	*Verify accuracy at time of use*
High Performance Liquid Chromatograph with light-excluding auto-sampler for 96-well microwell plates capable of delivering a 0.3 ml/min flow rate	Shimadzu Prominence series
UV detector[*1]	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector

	(281 nm)
pH meter with electrode and calibration	Capable of reading ±0.01 pH
HPLC Column	Shiseido CAPCELL CORE C_{18} column (2.7 μm, 3.0 × 150 mm) [Cat. 51112] *Alternative Column* The follow two columns have been confirmed to yield results for five test chemicals that are essentially the same as the Shiseido column. Waters, CORTECS C_{18} Column (2.7 μm, 3.0 × 150 mm) [Catalog # 186007373] Agilent, Poroshell 120 EC-C_{18} (2.7 μm, 3.0 × 150 mm) [Catalog # 693975-302]
Guard Column and Guard Column Holder[*2]	Column: Shiseido EXP GUARD CARTRIDGE CAPCELL CORE C_{18} S-2.7 2.1×5mm [Cat. 3643] Holder: Shiseido EXP DIRECT CONNECT HOLDER [Catalog # 3640]
Incubator	Capable of controlling at 25 +/ - 1ºC

*[1] If more than one wavelength is detectable, measurements should be made at both 281 nm and 291 nm to confirm peak purities of NAC and NAL.

[*2] Guard columns need not be used for test chemicals that are not precipitated in the reaction solution.

1-1. Apparatus (2)

Apparatus	**Example of use by Lead Laboratory**
Test Tube Mixer	SHIBATA Test Tube Mixer TTM-1
Plate Shaker	Heidolph Titramax 100 [Catalog # 544-11200-00]
Plate Centrifuge	KUBOTA PlateSpin
Recommended but non-essential consumables	
Dispensing Pipettes capable of delivering 1000 – 5000 μL	*Verify accuracy at time of use*

1-2. Consumables (1)

Consumables	**Recommended by Lead Laboratory**
96-well Microwell Plate for HPLC analysis	Polypropylene U bottom 96-well Microwell Plate Thermo (NUNC) U96 PP-0.5 ML NATURAL [Catalog # 267245] Greiner MICROPLATE, 96 WELL, PP, U-BOTTOM, NATURAL [Catalog # 650201]
96-well Microwell Plate seal for HPLC	Use the seal having sealing and solvent-resistant

analysis	performance[*1] Shimadzu GLC Resistant Embossed Seal [Catalog # 568-RES001]

[*1] When using a plate seal other than the recommended one, ensure and verify that no part of the reaction solution is volatilized and that the measured values are consistent before performing actual testing. Be sure to leave no gaps between the plate seal and the plate when applying the plate seal.

1-2. Consumables (2)

Consumables	**Example of use (Used by Lead Laboratory)**
Bottle with the quantity of approximately 500 ml	Sterilized bottle (AS ONE Corporation) [Catalog # 1-9475-05]
Polypropylene centrifuge tube with the quantity equal to or more than 50 ml	100 ml: Watson Clean-vessel [Catalog # 536-150S] 50 ml: FALCON 50-ml PP Conical Tube [Catalog # 352070]
Measuring pipette for 10–100ml	100-ml measuring pipette: FALCON 100-ml Serological Pipette [Catalog # 357600] 50-ml measuring pipette: FALCON 50-ml Serological Pipette [Catalog # 356550] 25-ml measuring pipette: FALCON 25-ml Serological Pipette [Catalog # 356525]
Polypropylene centrifuge tube with the quantity equal to or more than 10 ml	FALCON 15-ml Polypropylene Conical Tube [Catalog # 352196]
Tube for cryopreservation	5-ml Tube[*1]: 5-ml Outer serum tube (SUMITOMO BAKELITE CO., LTD.) [Catalog # MS-4605X] 2-ml Tube[*1]: 2.0-ml Ring rock tube (BM Equipment Co., Ltd.) [Catalog # BM-20] 1.5-ml Tube[*1]: 1.7-ml Ring rock tube (BM Equipment Co., Ltd.) [Catalog # BM-15]

[*1] A smaller or larger tube may be used per the quantity to be cryopreserved.

1-2. Consumables (3)

Recommended but non-essential consumables	**Example of use by Lead Laboratory**
Reservoir for operating 12 channel multipipette	Corning 50-ml Reagent Reservoir [Catalog # 4870]
Approximately 1.5-ml tube and cap operable by 12 channel multipipette (for dispensing test chemical solution)	Alphanumeric storage tube 1.4-ml (Thermo 4247JP) SepraSeal (Thermo 4463)
Tool for removing multiple caps simultaneously	Cap remover for SepraSeal (Thermo 4469)

1-3. Chemical and Special Materials

Chemical and Special Materials	**Example of use by Lead Laboratory**
Trifluoroacetic Acid (TFA) (MW=114.02) CASRN 76-05-1	Wako [Cat. No. 204-02743] 98.0%
Sodium Phosphate, Monobasic, Anhydrous (NaH2PO4, MW=119.98) CASRN 7558-80-7	Wako [Catalog # 197-09705]
Sodium Phosphate, Dibasic, Anhydrous (Na2HPO4, MW=141.96) CASRN 7558-79-4	Wako [Catalog # 197-02865]
0.1M Sodium Hydroxide Solution CASRN 1310-73-2	Wako [Catalog # 196-02195]
Acetonitrile, HPLC Grade CASRN 75-05-8	Wako [Catalog # 015-08633]
Distilled water	Distilled water or equivalent may be used.
Acetone, special grade CASRN 67-64-1	Wako [Catalog # 016-00346]
Dimethyl sulfoxide (DMSO), special grade CASRN 67-68-5	Wako [Catalog # 043-07216]
Ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid, disodium salt, dihydrate (EDTA·2Na·2H_2O) CASRN 6381-92-6	Dojindo Molecular Technologies, Inc. [Catalog #343-01861
NAC (refrigeration storage) MW=289.35, Purity> 95%	Lot No. #960074 ホ゜ (FUJIFILM provides synthetic NAC samples to each laboratory in the validation study)
NAL (refrigeration storage) MW=314.38 Purity>95%	Lot No. #990029 (FUJIFILM provides synthetic NAC samples to each laboratory participating in the validation study.)
Phenyl acetaldehyde (Positive control) MW=120.15 CASRN 122-78-1	SIGMA [Catalog # 107395] Note: Store phenyl acetaldehyde under the following conditions. (1) Store at a temperature of 2–8ºC. Do not store at below 0ºC. (2) Do not expose to UV light during storing or handling. (3) Handle as little as possible, keep stored under the above conditions.

2. PRE-WORK

2-1. Preparation of each solution (Typical)

2-1-1. 0.1-mM EDTA solution

1) Weigh 37.2 mg of EDTA·2Na·2H_2O into a 15-ml Conical Tube and use a 25-ml measuring pipette to add 10 ml of distilled water to dissolve it. (10-mM EDTA solution)
2) Use a 50-ml measuring pipette to add 49.5 ml of distilled water to a 100-ml tube and add 0.5 ml of the 10-mM EDTA solution described above to dilute it 100 fold. (0.1-mM EDTA solution)

2-1-2. 100-mM Phosphate buffer (pH 8.0)

To prepare an approximately 300-ml quantity

1) Weigh 0.6 g of Sodium Phosphate Dibasic Anhydrous into a 100-ml tube and use a 50-ml measuring pipette to add 50 ml of distilled water to dissolve 0.6 g of Sodium Phosphate Dibasic Anhydrous in distilled water.[*1]
2) Use a 50-ml (or 100-ml) pipette to add 300 ml of distilled water.
3) Weigh 4.26 g of Sodium Phosphate Monobasic Anhydrous and dissolve it in distilled water 2).[*1]
4) Use a 25-ml measuring pipette to add 16 ml of Sodium Phosphate Dibasic solution 1) in Sodium Phosphate Monobasic solution 3).
5) Use a 25-ml measuring pipette to remove 17 ml from solution 4) and to add 1 ml of 0.1-mM EDTA solution to solution 4). The concentrations of EDTA in this solution and final concentration of reaction solution are 0.33 μM and 0.25 μM, respectively.
6) Measure pH of solution 5) using a calibrated pH meter and verify that the pH is between 7.9 and 8.1. If the pH is out of range, prepare a new batch of phosphate buffer.
7) Use the NAC stock solution within a week or cryopreserve the unused quantity. Repackage it into 2-ml tubes to cryopreserve for Co-elution Control at -80ºC.

2-1-3. 100-mM Phosphate buffer (pH 10.2)

To prepare an approximately 300-ml quantity

1) Use a 50-ml measuring pipette to add 286 ml of distilled water to a 500-ml bottle.
2) Weigh 4.26 g of Sodium Phosphate Monobasic Anhydrous and dissolve it in the distilled water 1)[*1].
3) Use a 25-ml measuring pipette to add 14 ml of 0.1M NaOH solution to the solution 2).
4) Measure the pH of solution 3) using a calibrated pH meter and verify that it is between 10.1 and 10.3. If the pH is out of range, prepare a new batch of phosphate buffer.
5) Use the NAL stock solution within a week or cryopreserve the unused quantity. Repackage it into 2-ml tube to cryopreserve for Co-elution Control at -80ºC.

2-1-4. Reaction fixing solution (2.5% (v/v) TFA aqueous solution)

Add 2.5 ml of TFA to 100 ml of distilled water.

2-1-5. HPLC mobile phase A: 0.1% (v/v) TFA aqueous solution

Add 1.0 ml of TFA to 1000 ml of distilled water.

2-1-6. HPLC mobile phase B: 0.1% (v/v) TFA Acetonitrile solution

Add 1.0 ml of TFA to 1000 ml of HPLC grade Acetonitrile.

*[*1]: When dissolving phosphate salts, close the bottle to mix them. Do not use glass labware or other washable equipment. Verify that salts are completely dissolved after mixing.*

2-2. Preparation of NAC and NAL stock solutions

2-2-1. Preparation of NAC stock solution:

The NAC (molecular weight of 289.35) stock solution used in each assay is to all be from a single batch. We recommend storing NAC stock solution in quantities sufficient for an assay. A typical preparation as performed at the Lead Laboratory is described below.[*1]

1) Weigh 11.6 ± 0.1 mg of NAC in a 50-ml tube, add 20 ml of 100-mM phosphate buffer (pH 8.0) to the tube using a 25-ml measuring pipette, and mix it gently using a test tube mixer to dissolve the NAC. (2-mM NAC stock solution).[*2]
2) Use a 50-ml measuring pipette to add 149.5 ml of this buffer to a 500-ml bottle. Next, add 0.5 ml of 2-mM NAC stock solution to this solution and mix gently but thoroughly by inversion. (6.667-μM NAC stock solution).
3) Cryopreserve 3-ml quantities of stock solution in cryopreservable 5-ml tubes at -80ºC. Store for no more than six months before use.[*3]

2-2-2. Preparation of NAL stock solution

The NAL (molecular weight of 314.38) stock solution used in each assay is to all be from a single batch. We recommend storing NAL stock solution in quantities sufficient for an assay. A typical preparation as performed at the Lead Laboratory is described below.[*1]

1) Weigh 12.6 ± 0.1 mg of NAL in a 50-ml tube, and add 20 ml of 100-mM phosphate buffer (pH 10.2) to the tube using a 25 ml measuring pipette, and mix it gently using a test tube mixer to dissolve the NAL. (2-mM NAL stock solution).[*2]
2) Use a 50 ml measuring pipette to add 149.5 ml of this buffer to a 500 ml bottle. Next, add 0.5 ml of 2-mM NAL stock solution to this solution and mix it gently but thoroughly by inversion. (6.667-μM NAL stock solution)
3) Cryopreserve 3 ml quantities of stock solution in 5 ml tubes at -80ºC. Store for no more than six months before use.[*3]

[*1] *These quantities are typical examples only and can be adjusted as necessary.*

[*2] *The remaining 2-mM stock solution can be cryopreserved at -80*ºC *for later use. Typically, cryopreserve 1 ml of stock solution in a 1.5 ml tube at -80*ºC.

[*3] *Stability testing of NAC stock solution at temperatures of 25ºC, 4ºC, -20ºC, and -80ºC has shown that it remains stable and free of auto-oxidation only at -80* ºC. *We recommend that the NAL stock solution also be stored at -80* ºC, *although NAL is not susceptible to oxidization in the same manner as NAC.*

Note: Prior to preparing new batches of NAC/NAL stock solution, verify the stability of the NAC/NAL stock solution using the following procedure.

1) Add 150 μl of 6.667-μM NAC/NAL stock solution to three wells in each of two 96-well Microwell Plates.

2) Add 50 μl of acetonitrile to each of these six wells 1).

3) Attach a plate-seal to one of the two 96-well Microwell Plates and spin down the plate using a centrifuge, the incubate for 24 hours at 25ºC. After incubation, add 50μl of reaction fixing solution to each well, attach a new plate-seal, then mix and spin down the plate. Analyze NAC/NAL using the HPLC system.

4) Add 50 μl of reaction fixing solution to the other 96-well Microwell Plate, attach a plate-seal to it, then mix and spin down the plate. Analyze NAC/NAL using the HPLC system.

If the residual concentration of NAC/NAL falls below 90% after either 0 hour or 24 hours, the NAC and NAL are not stable, and so new batches of NAC/NAL solutions must be prepared.

2-3. Preparation of Standard Solution for Calibration Curve

The calibration curve for the acceptance criteria is obtained from a six-dose standard solution. The initial standard solution, Std1 at 5.0 μM, is cryopreserved in small quantities and used to prepare dilution series. A typical procedure for preparation of the standard solution is described below.[*1]

Procedure for preparation of 20-ml solution and 40-66 tubes

1) Use a 25-ml pipette to add 15 ml of 6.667-μM NAC/NAL stock solution to a 50 ml tube. Then use a micropipette to add 1 ml of distilled water, 100μl of TFA, and 3.9 ml of acetonitrile.

2) Cryopreserve 300–500 μl quantities of the standard solution in 1.5-ml tubes at -80ºC.

Also, solvent for preparing dilution series of the standard solution may be cryopreserved in 1000–1200-μl quantities, as described below.

1) Use a 25-ml pipette to add 15 ml of 100-mM phosphate buffer (pH 8.0 for NAC, pH 10.2 for NAL), 1 ml of distilled water, 100 μl of TFA, and 3.9 ml of acetonitrile to a 50-ml tube.

2) Typically, cryopreserve approximately 1 ml of solvent in a 1.5-ml tube at -80ºC.

[*1] These quantities are typical examples only and can be adjusted as necessary.

2-4. Solubility Assessment of Test Chemicals

The solubility of each test chemical in a suitable solvent must be assessed before performing the actual assay. A suitable solvent will dissolve the test chemical completely with no apparent turbidity or precipitate during visual inspection. The concentration of test chemical solution used in the assay is 1mM, but more than 10 mg is recommended to be weighed because of the precise weight of the test chemical. For this reason, the initial concentration of the test chemical solution is set to 20 mM, and the final 1-mM test chemical solution is prepared by 20-fold dilution of the 20-mM solution. The test chemical is dissolved using a test tube mixer and an additional 5 minutes of sonication if needed due to low solubility. The four solvents below are all suitable for used in ADRA, and the priority for the selection of the appropriate solvent is as follows.

(1) Distilled water

(2) Acetonitrile

(3) Acetone

(4) DMSO

Note 1: When the 20-mM solution is prepared using DMSO (4), use acetonitrile to prepare the 20-fold dilution down to a 1-mM solution.

Note 2: If none of these solvents are suitable for a given test chemical in a 20-mM solution, assess the solubility of the test chemical in 1-mM solution using solvents (1)–(3). Do not use DMSO to prepare a 1-mM solution. DMSO must always be diluted 20-fold with acetonitrile to prepare a 1-mM solution.

Note 3: Water is not suitable as a solvent for anhydrides due to hydrolysis.

A typical procedure is described below.

1. Use the following formula to calculate the quantity of test chemical needed to prepare approximately 5 ml of a 20-mM test chemical solution or 100 ml of a 1-mM test chemical solution. If no purity information is available, assume 100% purity.

$$\text{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{20\ \text{mM}}{1000} \times 5\ \text{ml} \quad \text{or} \quad \text{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{1\ \text{mM}}{1000} \times 100\text{ml}$$

$$= \text{MW} \times \frac{10}{\text{Purity (\%)}} = \text{Required quantity (mg)}$$

2. Weigh the required quantity of test chemical directly into a centrifuge tube or other suitable labware and record the actual weight.
3. Calculate the required quantity of solvent using the formula, and assess the solubility for the appropriate solvent.

Note 1: Even if the required quantity is calculated to be less than 10 mg, weigh more than 10 mg of test chemical for precise weight.

Note 2: If the test test chemical solution is in aqueous form, calculate necessary volume of the aqueous solution and quantity of water for dilution. If specific gravity of test chemical solution is not known, assume it as 1.0.

Preparation of 20-mM solution (5 ml)

$$\frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{1}{\text{Specific gravity}} \times \frac{1}{20\ \text{mmol} \times 5\ \text{ml}} \times 1000\ \mu\text{l}$$

$$= \frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{10}{\text{Specific gravity}}$$

= Volume of test chemical (µl)

5000 µl − Volume of test chemical (µl) = Solvent volume (µl)

Preparation of 1-mM solution (100 ml)

$$\frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{1}{\text{Specific gravity}} \times \frac{1}{1\ \text{mmol} \times 100\ \text{ml}} \times \quad 1000\ \mu\text{l}$$

$$= \frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{10}{\text{Specific gravity}}$$

= Volume of test chemical (μl)

$$\textbf{100 ml} - \frac{\textbf{Volume of test chemical (μl)}}{\textbf{1000}} = \textbf{Solvent volume (ml)}$$

2-5. Preparation of HPLC System

Prepare an HPLC system with a 281-nm UV detector and mobile phase A and B, as described in section "2-1-5. HPLC mobile phase A: 0.1% (v/v) TFA aqueous solution" and in section "2-1-6. HPLC mobile phase B: 0.1% (v/v) TFA Acetonitrile solution".

1. Column Equilibration: Install the HPLC column (see section "1.1 Apparatus (1)") and equilibrate the column for at least 30 minutes at 40ºC with 50% A, 50% B before use. Then, condition the column by running the gradient at least twice before using the column.
2. System Shutdown: Following analysis, maintain a low flow (typically 0.05 ml/min) of 50% A and 50% B through the system and decrease column temperature to approximately 25ºC.
3. If the system is to be idle for more than a week, fill the column with acetonitrile (without Trifluoroacetic acid) and remove the column from the HPLC system. Cap tightly and purge acid containing mobile phases from the system using acetonitrile.

3. SOLUTION PREPARATION

3-1. Preparation of Test Chemical Solution

Solubility of the test chemical in the appropriate solvent is evaluated in section "2-4. Solubility Assessment of Test Chemicals". 20-mM or 1-mM solutions of test chemicals in the appropriate solvents are prepared immediately before use.

1. When ready to perform the assay, calculate and weigh the appropriate quantity of test chemical needed to prepare a 20-mM or 1-mM solution directly in a 15-ml or other appropriate tube. Use the following formula to calculate the required quantity of solvent needed based on the actual weight of the test chemical.

Prepare of 20-mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{20\ \text{mmol}} \times \quad 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times \quad 0.5 \quad = \text{Volume of solvent (ml)}$$

Prepare of 1-mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{1\ \text{mmol}} \times 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 10 = \text{Volume of solvent (ml)}$$

2. Add the volume of solvent calculated and mix in a centrifuge tube, using a test tube mixer to dissolve the test chemical. Slight sonication (less than 5 minutes) may be used if needed. If the test chemical is not completely dissolved, do not proceed with that specific test chemical in the selected solvent. Re-evaluate with another solvent (see section "2. PRE-WORK") to find a suitable vehicle.
3. Dilute 20-mM of test chemical solution to the final 1-mM solution with each appropriate solvent in the 1.4 ml Test chemical solution Storage Tube or other appropriate tube (For example, add 950 μl of appropriate solvent to 50 μl of 20-mM test chemical solution).[*1]
4. Record and report the final solvent choice for each chemical if the final solvent is different from the solvent selected in section "2-4. Solubility Assessment of Test Chemicals".

[*1] *96-well Deepwell Plate may be used instead of 1.4 ml Test chemical solution Storage Tube.*

3-2. Preparation of Positive Control Solution

Phenyl acetaldehyde (Molecular weight=120.15) dissolved in acetonitrile is used as Positive Control and should be contained in each run of assay. A typical preparation procedure for the Positive Control solution is described below.

1. Calculate a weight of Positive Control to be needed to prepare approximately 5 ml of 20-mM Positive Control solution from following formula. The next formula shows the case that the purity of Phenyl acetaldehyde is 90%.

$$\text{MW} \times \frac{10}{\text{Purity(\%)}} = 120 \times \frac{10}{90} = 13.35\text{(mg)} = \text{Phenyl acetaldehyde (mg)}$$

2. Weigh the phenyl acetaldehyde directly in a 10- or 15-ml centrifuge tube, and record the actual weight.
3. Calculate necessary quantity of solvent from following formula based on the actual weight

$$\text{Phenyl acetaldehyde (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 0.5 = 13.35 \times \frac{90}{120.15} \times 0.5 = 5.0\ \text{(ml)} = \text{Solvent volume (ml)}$$

4. Dissolve Phenyl acetaldehyde with acetonitrile in the quantities calculated above (20 mM).
5. Dilute the 20-mM Phenyl acetaldehyde solution 20-fold with acetonitrile in the 1.4-ml Test chemical solution Storage Tube or other appropriate tube. (final concentration or 1 mM)[*1]

[*1] 96-well Deepwell Plate may be used instead of 1.4-ml Test chemical solution Storage Tube.

3-3. Thaw of each stock solution

Thaw frozen Phosphate buffer (pH 8.0 and 10.2) and NAC/NAL stock solution at room temperature. The preparation of each stock solution is described in sections "2-1-2. 100-mM Phosphate buffer (pH 8.0)", "2-1-3. 100-mM Phosphate buffer (pH 10.2)", and "2-2. Preparation of NAC/NAL stock solutions".

4. Assay Procedure

4-1. Reference Control, Co-elution Control and Test Chemical Solution

Test chemical solutions are prepared in triplicate for NAC/NAL. Each assay (NAC/NAL) may be prepared and performed concurrently (NAC/NAL should not be incubated and analyzed on separate days). The three Reference Control A, six Reference Control C and one Co-elution Control (the solution after addition of buffer solution rather than NAC/NAL) should be prepared. Moreover, the solution (Co-elution Control, N=1) for each test chemical after addition of buffer solution rather than NAC/NAL solution should be prepared. The detail of each solution describes below.

Test chemical solution	Reaction solution of NAC/NAL with test chemical. Evaluate reactivity of NAC/NAL with the test chemical at a ratio of 1:50 for the NAC/NAL with the test chemical.
Reference Control A	Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC/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.
Co-elution Control	Control for verifying whether or not test chemicals co-elute with NAC/NAL. The Co-elution Control is used to verify absorbance at 281nm and whether retention time of test chemical is equal to that of NAC/NAL.

4-2. Preparation and Reaction of Reaction Solutions

Use 12-channel pipettes with 96-well plates. Proper attention is required when using pipettes: pre-rinse at least five times to ensure precise pick up and take care to avoid dripping from the tip when dispensing.

1. Assemble the following previously prepared reagents, solvents, and solutions:
   a. 6.667-μM of NAC/NAL stock solution,
   b. Appropriate buffer solution (pH 8.0 for NAC, pH 10.2 for NAL),
   c. Test chemical solution (or solvent for Reference Controls)
2. Use a 96-well Microwell Plate and 12-channel pipette to prepare test chemical solutions by adding the reagents per the following table. Refer to the section on “Typical Test Chemical Solution Positions in 96-well Microwell Plate” for typical positions in the plate. Inspect test chemical solution after adding test chemical and record observations.

Ratio of NAC to test chemical = 1:50 5-μM NAC, 0.25-mM test chemical	**Ratio of NAL to test chemical = 1:50** 5-μM NAL, 0.25-mM test chemical
・150-μL NAC solution **・50-μL Test chemical solution**	**・150-μL NAL solution** **・50-μL Test chemical solution**

Co-elution Control and Reference Control A, B and C are prepared according to the following tables.

Co-elution Control

For NAC	**For NAL**
・150-μL Phosphate buffer (pH 8.0) **・50-μL Test chemical solution**	**・150-μL Phosphate buffer (pH 10.2)** **・50-μL Test chemical solution**

Reference Control A and B

For NAC	**For NAL**
・150-μL NAC solution **・50-μL Acetonitrile**	**・150-μL NAL solution** **・50-μL Acetonitrile**

Reference Control C

For NAC	**For NAL**
・150-μL NAC solution **・50-μL Acetonitrile and solvent used for dissolution of test chemical** [*1]	**・150-μL NAL solution** **・50-μL Acetonitrile and solvent used for dissolution of test chemical** [*1]

[*1] If the test chemicals are dissolved in acetonitrile, only acetonitrile need be prepared.

3. Seal the 96-well Microwell Plate completely with a plate seal and shake it using a Plate Shaker. Centrifuge the plate using a Plate Centrifuge and incubate the plate in the Incubator (dark) at 25ºC (plus or minus 1'C) for 24 hours (plus or minus 1 hour). Record the time and temperature at the start and end of incubation.

4-3. Procedure after the Completion of Reaction

1. After 24 hours of incubation, remove the plate seal and add 50 μL of Reaction Fixing Solution to each test chemical solution. Inspect the reacted test chemical solution and record any observations after addition of the reaction fixing solution. If precipitation is observed, centrifuge the 96-well Microwell Plate at low speed (100–400g) to force the precipitate to the bottom of the well as a precaution. If more than 100 μl of supernatant of test chemical solution can be removed using a pipette, the supernatant may be analyzed after being transferred to another plate.

2. Prepare the standard solution

Prepare NAC/NAL standard solutions by serial dilution from 5.0–0.156-μM

(1) Prepare each solution ahead of time after thawing frozen stock solution at room temperature.
  [1] 5.0-μM stock solution "Std1"
  [2] "solvent for preparing dilution series" (for NAC/NAL)

(2) Prepare the following concentration of the standard solution by diluting 150 μL of stock solution "Std1" with the same quantity of dilution solvent per to "Preparation procedure for dilution series" below. Add dilution solvent to a well as "Std7".

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7 (Dilution solvent)
NAC/NAL (μM)	5.0	2.5	1.25	0.625	0.313	0.156	0

“Preparation procedure of dilution series”

(1) Add 150 μl of dilution buffer to wells from well Std2 to well Std7 shown in “Typical Test Chemical Solution Positions in 96-well Microwell Plate”

(2) Add 150μl of Std1 to well Std1 and well Std2. Mix with minimal air entrapment.

(3) Transfer 150 μl from well Std2 to well Std3. Mix with minimal air entrapment.

(4) Continue in a similar manner from well Std4 to well Std6.

3. Prepare dilution series of NAC/NAL standard solution in the wells from Std1 to Std7 shown in the next section on “Typical Test Chemical Solution Positions in 96-well Microwell Plate”, and reseal the plate tightly with a new plate seal. For preparation of dilution series, see section “4-3. 2. Prepare the standard solution”. Mix the plate with a Plate Shaker and spin it down by centrifuge.

Note: Add the Reaction Fixing Solution within 30 minutes of the end of incubation.

Typical Test Chemical Solution Positions in 96-well Microwell Plate

Between 1 and 17 test chemicals can be tested in a single test run using two 96-well plates, based on consideration of the time needed for preparation and testing as well as the burden on laboratory technicians and the capacity of the HPLC apparatus. If more than one solvent are used a single test run, additional Reference Controls for each solvent need to be prepared and the number of test chemicals will need to be reduced. For example, if three kinds of solvent are used, the maximum number of test chemicals is 15. The number of test chemicals and Typical Test chemical solution position in 96-well Microwell Plate are shown below, but the positions may be adjusted per the number of test chemical solutions.

(1) For five test chemicals with acetonitrile as solvent

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	CC-1	CC-2	CC-3	CC-4	CC-5	—	—	—	—	—	STD 4	—
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	CC-1	CC-2	CC-3	CC-4	CC-5	—	—	—	—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control

(2) For 10 test chemicals with acetonitrile and water as solvent

First plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	—	—
	B												
	C												
	D	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	—	—
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	—	—
	F												
	G												
	H	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	—	—

Second plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	RC-A	RC-B	RC-B	RC-C (Water)	RC-C (ACN)	PC	—	—	—	—	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	—	—	—	—	—	—	—	—	—	—	STD 4	—
NAL	E	RC-A	RC-B	RC-B	RC-C (Water)	RC-C (ACN)	PC	—	—	—	—	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	—	—	—	—	—	—	—	—	—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control, ACN: Acetonitrile

(3) For 17 test chemicals and acetonitrile as solvent

First plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	Test chemical solution No.11	Test chemical solution No.12
	B												
	C												
	D	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	CC-9	CC-10
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	Test chemical solution No.11	Test chemical solution No.12
	F												
	G												
	H	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	CC-11	CC-12

Second plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.13	Test chemical solution No.14	Test chemical solution No.15	Test chemical solution No.16	Test chemical solution No.17	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	CC-3	CC-4	CC-5	CC-6	CC-7	—	—	—	—	—	STD 4	—
NAL	E	Test chemical solution No.13	Test chemical solution No.14	Test chemical solution No.15	Test chemical solution No.16	Test chemical solution No.17	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	CC-13	CC-14	CC-15	CC-16	CC-17				—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control, ACN: Acetonitrile

5. HPLC Analysis

1. Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the Mobile Phase A and Mobile Phase B at column temperature of 40°C. The HPLC analysis is performed using a flow of 0.3 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. Refer “5-1. HPLC Conditions” for details on the gradient.
2. Inject equal quantities of each standard solution, test chemical solution, and control solution. The injection quantity varies according to the system used but typically is from 10–20 μL. Systems require smaller injection quantities, because injection of 20 μL leads to unacceptably broad peaks. Absorbance is monitored at 281 nm. If using a Photodiode Array Detector, absorbance at 291 nm should also be recorded.

3. Increase the mobile phase B to 100% in order to remove the other compounds from the column after gradient, and re-equilibrate the column under initial conditions for at least 6.5 minutes. These process of acetonitrile 100% and re-equilibration for 6.5 minutes are described the following table “5-1. HPLC Conditions”.

Note: The 6.5 minute re-equilibration time was determined using a Shimadzu Prominence HPLC system. Other systems may require more or less re-equilibration time due to system mixing quantity. Shorter equilibration times will be acceptable if peak retention times are stable.

5-1. HPLC Conditions

Column	Shiseido CAPCELL CORE C_{18} column (2.7 μm, 3.0 × 150 mm) [Cat. 51112] *Alternative Column* The follow two columns have been confirmed to yield results for five test chemicals that are essentially the same as the Shiseido column. Waters, CORTECS C_{18} Column (2.7 μm, 3.0 × 150 mm) [Catalog # 186007373] Agilent, Poroshell 120 EC-C_{18} (2.7 μm, 3.0 × 150 mm) [Catalog # 693975-302]
Column Temperature	40ºC
Test chemical solution Temperature	25ºC If the auto-sampler has a cooling function, test chemical solutions can be kept more stable at 4ºC.
UV detector[*1]	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector (281 nm)
Injection Quantity	10-20 μl (The injection quantity varies according to HPLC system. If peaks are too broad, the injection quantity should be decreased.)
Run Time	20 minutes
Flow Conditions	NAC flow conditions **Time Flow %A %B** 0 min 0.3 ml/min 70 30 9.5 min 0.3 ml/min 45 55 10 min 0.3 ml/min 0 100 13 min 0.3 ml/min 0 100 13.5 min 0.3 ml/min 70 30 20 min End run NAL flow conditions **Time Flow %A %B**

	0 min	0.3 ml/min	80	20
	9.5 min	0.3 ml/min	55	45
	10 min	0.3 ml/min	10	100
	13 min	0.3 ml/min	10	100
	13.5 min	0.3 ml/min	80	20
	20 min	End run		

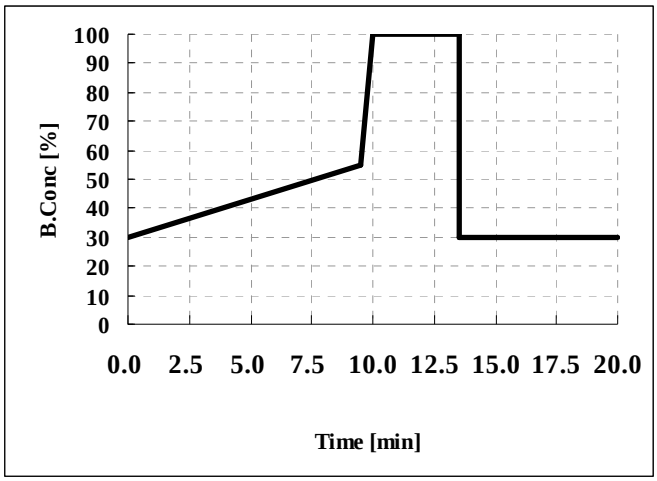
Note 1: The mixer quantity should be verified and adjusted in advance because the appropriate elution pattern of NAC/NAL peak will not be shown if the mixer quantity for mixing each mobile phase is not appropriate (For example, 0.5 ml mixing quantity is appropriate for Shimadzu prominence HPLC system).

Note 2: The inner diameter of pipe and the length of pipe from column outlet to detector inlet must be less than 0.18 mm and less than 50 cm, respectively, because the peak of NAC/NAL might be broadened depending on inner diameter and length of pipe

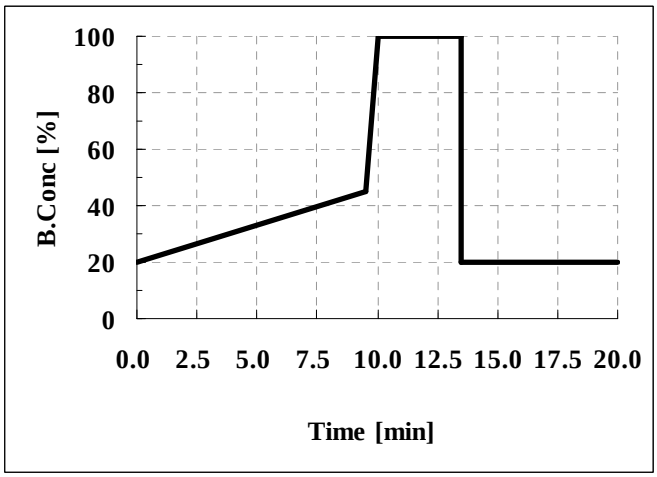
Note 3: If more than one wavelength is detected, also 291 nm besides 281 nm should be detected to check out peak purities of NAC/NAL.

Graphical Display of Gradient for NA/NAL Analysis

For NAC analysis



For NAL analysis



Note: Visual inspection of test chemical solutions must be conducted prior to HPLC analysis. Generally, precipitation is not a problem. However, if precipitation is observed, this should be noted in the data reporting template. Test chemical solutions may be centrifuged at low speed (100–400g) in vial to force precipitate to the bottom of the well as a precaution, since large quantities of precipitate may clog the HPLC tubing or columns. If more than 100 μL of supernatant can be recovered, in may be analyzed after transfer to another plate. Precipitate formation, removal, and transfer must be recorded and reported.

5-2.HPLC Sample Analysis Sequences

Each sample of HPLC analysis should be analyzed 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 analyze calibration standards and Reference Control A (N = 3).
2. The Co-elution Control does not need to be analyzed by turns if it is analyzed after analysis of standard solution and Reference Control A.
3. Reference Control B should be analyzed 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 analyzed. (After the first set of replicates of each sample is analyzed, the second set of replicates of each should be analyzed)

Example of HPLC Samples Analysis Sequences

(A more specific analysis sequence can be found at the end of the SOP)

STD7 (Buffer solution for dilution)[†] STD6 STD5 STD4 STD3 STD2 STD1	Ref. 8-1. 1)
Reference Control A, rep 1 Reference Control A, rep 2 Reference Control A, rep 3	Ref. 8-1. 1)
Co-elution Control 1 Co-elution Control 2 Co-elution Control 3 ・・・ Co-elution Control n	Ref. 8-4.
Reference Control B, rep 1 Reference Control B, rep 2 Reference Control B, rep 3	Ref. 8-2.
Reference Control C, rep1[§†] Phenyl acetaldehyde (Positive Control), rep1 Test chemical solution 1, rep1 Test chemical solution 2, rep1 Test chemical solution 3, rep1 ・・・ Test chemical solution n, repn	***First set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control C, rep2[§†] Phenyl acetaldehyde (Positive Control), rep2 Test chemical solution 1, rep2 Test chemical solution 2, rep2 Test chemical solution 3, rep2 ・・・	***Second set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.

Test chemical solution n, rep2	
Reference Control C, rep3[§†] Phenyl acetaldehyde (Positive Control), rep3 Test chemical solution 1, rep3 Test chemical solution 2, rep3 Test chemical solution 3, rep3 ・・・ Test chemical solution n, rep3	***Third set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control B, rep 4 Reference Control B, rep 5 Reference Control B, rep 6	Ref. 8-2.

[†] Start to analyze calibration standard immediately after addition of Reaction Fixing Solution and preparation of dilution series of standard solution.

[§] Analyze three replicates for Reference Controls C. These results are used to calculate the NAC/NAL depletion in each solvent and to verify that solvent used does not affect NAC/NAL depletion.

6. DATA ANALYSIS & CALCULATIONS

The concentration of NAC/NAL is calculated from peak area of absorbance at 281 nm for each test chemical solution based on the calibration curve derived from standard solutions Std 1 to Std7. NAC/NAL percent depletion is calculated by dividing NAC/NAL peak area of each test chemical solution by mean peak area of Reference Control C.

6-1. Calculation of Peak Area of NAC/NAL

Integrate the appropriate peaks and determine peak area for standards, test chemical solution and controls. The peak area of each integrated peak must be reported.

6-2. Calculation of Concentration of NAC/NAL

6-2-1. Generate a linear calibration curve based on the concentration of standards and the peak area. Suitable calibration curves must have an r^2>0.990.

6-2-2. Calibrate the mean NAC/NAL concentrations in Reference Controls A and C, SD and CV. The each mean should be 3.2–4.4 μM. The NAC/NAL concentration of Reference Controls A and C must be reported.

6-2-3. Calculate the mean NAC/NAL peak area, SD and CV for the Reference Controls C (N=3) for each solvent used. The each mean concentration should be 3.2–4.4 μM. However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM.

6-3. Calculation of Peak Area of NAC/NAL

6-3-1. Calculate the mean NAC/NAL peak area for the six Reference Controls B and the three Reference Control C in acetonitrile, SD and CV. The CV must be less than 10%.

6-3-2. Calculate the mean NAC/NAL peak area at 281nm for the three Reference Controls C.

6-4. Calculation of Percent Depletion of NAC/NAL

6-4-1. For the Positive Control and for each test chemical, calculate the Percent NAC/NAL Depletion in each replicate from the NAC/NAL peak area of the replicate injection and the mean NAC/NAL area in the three relevant Reference Controls C (in the appropriate solvent), using the following formula.

Percent NAC/NAL Depletion (% depletion) = [1- (NAC/NAL Peak Area in Replicate Injection/mean NAC/NAL Peak Area in Reference Controls C)]×100

6-4-2. The mean Percent NAC/NAL Depletion (Average score) of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

7. DATA REPORTING (FOR NAC AND NAL)

System Suitability

- NAC/NAL peak area at 281 nm of Standard and Reference Control B and C replicate.
- The linear calibration curve should be graphically represented and the r^2 reported.
- NAC/NAL concentration (μM) of Reference Control A replicate.
- Mean NAC/NAL concentration (μM) of Reference Controls C replicate, SD and CV.

Analysis Sequence

Reference Controls:

- NAC/NAL peak area at 281 nm of Reference Control B and C replicate.
- Mean NAC/NAL peak area at 281 nm of the nine Reference Controls B (N = 6) and C (N = 3) in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean NAC/NAL peak area at 281 nm of the three appropriate Reference Controls C replicate (for calculation of Percent NAC/NAL Depletion).
- For each solvent used in this assay, the mean NAC/NAL concentration (μM) of the appropriate Reference Control C replicate, SD and CV.

Positive Control (Phenyl acetaldehyde)

- NAC/NAL peak area at 281 nm of each replicate.
- Percent NAC/NAL Depletion of each replicate
- Mean NAC/NAL Depletion of the three replicates, SD and CV.

For Each Test Chemical:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time.
  It must be reported if precipitate was re-solubilized or centrifuged.
- NAC/NAL peak area at 281 nm of each replicate (for systems equipped with a PDA detector the peak

area at 291 nm should also be reported).

- Percent NAC/NAL Depletion of each replicate.
- Mean of Percent NAC/NAL Depletion of the three replicates, SD and CV.

8. ACCEPTANCE CRITERIA

8-1. Acceptance Criteria for Amino acid Derivative Reactivity Assay Run

All criteria must be met for the whole run to be considered valid. If three criteria are not met, the run must be repeated for all test chemicals.

System Suitability:

Calibration Linearity $r^2 > 0.990$

Mean NAC/NAL concentration of Reference Controls A = 3.2–4.4 μM

Positive Control:

The mean Percent NAC/NAL Depletion value of the three replicates for phenyl acetaldehyde must fall within the range reported in the following table (Based on mean ±3SD from background data):

	Percent NAC Depletion		Percent NAL Depletion	
Positive control	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Phenyl acetaldehyde	6	30	75	100

Maximum Standard Derivatives for Positive Control replicate:

Standard Deviation for Percent NAC Depletion must be < 10%

Standard Deviation for Percent NAL Depletion must be < 10%

Stability of Reference Controls over analysis time:

For each solvent used, the mean of the NAC/NAL concentrations of the three appropriate Reference Controls C = 3.2–4.4 μM. However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM, as it is known that concentration of NAC decreases because of oxidation of SH group by DMSO.

8-2. Acceptance Criteria for Each Test Chemical

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

Maximum Standard Deviation of test chemical solution replicates:

Standard Deviation for Percent NAC Depletion must be < 10%

Standard Deviation for Percent NAL Depletion must be < 10%

Reference Controls C in the analysis sequence:

CV of NAC/NAL peak areas for the nine Reference Controls B (N = 6) and C (N = 3) in acetonitrile

must be < 10%. Moreover, CV of NAC/NAL peak areas for the three Reference Controls C (N = 3) in each solvent must be < 10%.

8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay

The average score should be calculated from depletions of NAC/NAL, and the test chemicals should be predicted to be either a Sensitizer or a Non-sensitizer according to following table.

NAC/NAL **prediction model**

Average score	**Judgement**
Less than 4.9%	Non-sensitizer
4.9% or higher	Sensitizer

If an average score for NAC depletion in a test chemical falls within the borderline range described below, additional testing should be performed to confirm the validity of the prediction. If the result of the second test is not concordant with the first test, a third test should be performed to determine a prediction for the test chemical by majority of the three test results.

NAC and NAL prediction model: 3.0% ≤ average score ≤ 10.0%
NAC only prediction model: 4.0% ≤ NAC depletion ≤ 11.0%

8-4. Handling of Co-elution

8-4-1. Co-elution : Interference

(1) Some test chemicals will co-elute with the NAC or NAL. In order to detect possible co-elution of the test chemicals with NAC or NAL, the test chemicals included in the run must be injected alone (“Co-elution Controls”) at the beginning of the run sequence and their chromatograms compared to the chromatograms of Reference Controls C in the appropriate solvent.

(2) If a chemical absorbs at 281 nm and has a similar retention time as a peptide (overlap of valley-to-valley integration periods), then verify whether or not the peak of test chemical is actually separated from the peak of NAC or NAL. If the peak of test chemical is completely overlapped with the peak of NAC or NAL, and if the boundary of two peaks (valley between peaks) is located higher than baseline, co-elution of the test chemical with that NAC or NAL should be reported. The “interfering” chemical peak should have a peak area that is >10% of the mean NAC/NAL peak area in the appropriate Reference Control. If co-elution occurs and proper integration and calculation of NAC/NAL depletion is not possible, the data should be recorded as “interference” for NAC/NAL the chemical co-elutes with.

(3) Even if the test chemical does not co-elute with NAC or NAL, the Percent NAC/NAL Depletion can appear to be < -10% if the concentration of Reference Control C is comparatively low. Moreover, the Percent NAC/NAL Depletion can also appear to be < -10% due to inappropriate handling of the measurement. In such cases, retesting of the test chemicals in question or other appropriate measure should be taken.

8-4-2. Peak purity of NAC/NAL : Area ratio of 281/291 nm

(1) When a Photodiode Array detector is used, co-elution of chemical and NAC/NAL may also be verified by looking at the UV spectrum at 291 nm in addition to 281 nm and calculating the area ratio of 281/291. This value should be consistent over all test chemical solutions and standards for a distilled NAC/NAL peak and thus gives a measure of peak purity. For each test chemical solution, a ratio in the following range would give a good indication that co-elution has not occurred. However, calculation of peak purity (area ratio of 281/291) might not always be possible, particularly if the test chemical is highly reactive with the NAC/NAL leading to very small peaks.

90% < Mean Area ratio of Reference Control < 110%.

8-4-3. Co-elution : Depletion < -10%

(1) If the Percent NAC/NAL Depletion is < -10%, it should be considered that this may be a situation of co-elution, inaccurate NAC/NAL addition to the reaction mixture or just baseline noise. If the NAC/NAL peak appears at the proper retention time and has the appropriate peak shape, the peak can be integrated. In this case, there may just be baseline noise causing the NAC/NAL peak to be bigger or there may be some co-elution/overlap in retention time of the NAC/NAL and test chemical.

(2) The calculated %-depletion should be reported as an estimate. In cases where a test chemical co-elutes with NAL, the NAC only prediction model can be used. In cases where a test chemical co-elutes with both NAC/NAL, the data should be reported as an inconclusive.

(3) In cases where the test chemical co-elutes with the NAC and the peak of NAC cannot be integrated, the skin sensitization of test chemical cannot be predicted from the NAL depletion alone, and the data should be reported as inconclusive.

8-4-4. Calculation of peak area for co-elution

(1) If the peak of NAC/NAL and the peak of test chemical partially overlap, the peak area of NAC/NAL should be integrated from valley of both peaks to baseline vertically.

(2) If the peak of NAC/NAL and the peak of test chemical completely overlap, the data should be reported as an Inconclusive, and the peak area should not be calculated.

8-4-5. Estimated depletion values

In some cases, a test chemical might co-elute with NAC and/or NAL though the test chemical react with NAC and /or NAL. If this is the case, co-elution will make the peak area of NAC/NAL appear to be larger than it really is, therefore the calculated percent depletion may be lower than the true value. When the overlap in retention time between the test chemical and NAC/NAL is incomplete, percent depletion can still be calculated with a notation of “co-elution – percent depletion estimates”. If the average score is below the criteria, the result should be reported as Inconclusive. However, unless NAC co-elutes with test chemical, the NAC-only prediction model should be used.

Average score	No co-elution	Co-elution with NAC alone or NAC and NAL	Co-elution with NAL only
< 4.9%	Non-sensitizer	Inconclusive	Apply NAC-only prediction model
4.9% ≦	Sensitizer	Sensitizer	Apply NAC-only prediction model

NAC only prediction model

NAC Depletion	Judgement
less than 5.6%	Non-sensitizer
5.6% or greater	Sensitizer

Example HPLC Analysis

There are 5 test chemicals. Chemical 1,2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in distilled water.

The following 96-well Microwell Plate should be set up:

Std 7 (Dilution buffer blank) Std 6 Std5 Std5 Std4 Std 3 Std 2 Std1 Reference Control A, rep 1 (made with acetonitrile) Reference Control A, rep 2 (made with acetonitrile) Reference Control A, rep 3 (made with acetonitrile) Co-elution Control for Chemical 1 Co-elution Control for Chemical 2 Co-elution Control for Chemical 3 Co-elution Control for Chemical 4 Co-elution Control for Chemical 5
Reference Control B, rep 1 (made with acetonitrile) Reference Control B, rep 2 (made with acetonitrile) Reference Control B, rep 3 (made with acetonitrile)

Reference Control C, rep 1 (made with acetonitrile)
Reference Control C, rep 1 (made with distilled water)
Phenyl acetaldehyde, rep 1
Chemical 1, rep 1
Chemical 2, rep 1
Chemical 3, rep 1
Chemical 4, rep 1
Chemical 5, rep 1

Reference Control C, rep 2 (made with acetonitrile)
Reference Control C, rep 2 (made with distilled water)
Phenyl acetaldehyde, rep 2
Chemical 1, rep 2
Chemical 2, rep 2
Chemical 3, rep 2
Chemical 4, rep 2
Chemical 5, rep 2

Reference Control C, rep 3 (made with acetonitrile)
Reference Control C, rep 3 (made with distilled water)
Phenyl acetaldehyde, rep 3
Chemical 1, rep 3
Chemical 2, rep 3
Chemical 3, rep 3
Chemical 4, rep 3
Chemical 5, rep 3

Reference Control B, rep 4 (made with acetonitrile)
Reference Control B, rep 5 (made with acetonitrile)
Reference Control B, rep 6 (made with acetonitrile)

Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean NAC/NAL peak area of the Reference Control C which are prepared with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean NAC/NAL peak area of the Reference Controls C which are prepared with distilled water.

FUJIFILM	**Amino acid Derivative Reactivity Assay (ADRA) Standard Operating Procedure**	Version 1.2 Pages:27 Annexes:0

Japanese Center for Validation of Alternative Methods (JaCVAM)
Skin Sensitization Validation Study

Issued by	Fujifilm corporation	Date:
Approved by	Validation Management Team	Date:
Distributed by		N/A

Revision History

Revision	Date:	Description of change:
Ver. 1.1	February 6, 2017	1) Revised NAL lot number on page 6. 2) Added a precautionary comment about pipette technique to section 4-2. Preparation and Reaction of Reaction Solutions on page 13. 3) Revised the control criteria for the positive control reagent on page 23. 4) Added the range of average score and NAC depletion for additional test to get an accurate predictive result in section 8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay on page 24. 5) Added instructions on how to handle instances in which there appears to be no co-elution but depletion is less than -10% in section 8-4. Handling of Co-elution on page 24.
Ver.1.2	April 16, 2018	1) Added notes regarding solubility of test chemicals to page 9.

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Amino acid Derivative Reactivity Assay

PRINCIPLE AND SCOPE

The covalent binding of low molecular chemicals (haptens) to skin proteins is the molecular initiating event for skin sensitisation. The Amino acid Derivative Reactivity Assay (ADRA) is used to evaluate the reactivity of a test chemical with N- (2- (1-naphthyl)acetyl)-L-cysteine (NAC) and α-N- (2-(1-naphthyl)acetyl)-L-lysine (NAL) by combining the test chemical with a solution of NAC and NAL (abbreviated hereinafter as "NAC/NAL solution") and then measuring the residual concentration of NAC and NAL after a 24-hour incubation at 25ºC. NAC and NAL are chemically-synthesized compounds containing a naphthalene group simulating cysteine and lysine residues in skin proteins. Residual concentrations of NAC and NAL following the 24-hour incubation are determined by high performance liquid chromatography (HPLC) with gradient elution and UV detection at 281 nm. Test chemicals are prepared and analyzed in triplicate in batches of up to 17 chemicals, including controls. The method is applicable to test chemicals that are soluble in acetonitrile or other non-reactive, water-miscible solvents at a 1-mM concentration.

REFERENCES

Fujita, M., *et al*., "Development of a prediction method for skin sensitization using novel cysteine and lysine derivatives.", *J. Pharm. Toxicol. Method.* 2014; **70**; 94-105.

Yamamoto, Y., *et al*., "A novel *in chemico* method to detect skin sensitizers in highly diluted reaction conditions. ", *J. Appl. Toxicol*. 2015; **35**: 1348-1360.

1. APPARATUS AND REAGENTS

ADRA is best performed using disposable plastic labware, because NAC is susceptible to oxidization by trace metal ions which may be found in glassware. Spatulae used for weighing test chemicals and the various parts of the HPLC are not subject to this restriction.

1-1. Apparatus (1)

Apparatus	**Example of use by Lead Laboratory**
Analytical Balance	Capable of weighing up to 20 grams at an accuracy of 0.1 mg readability
・Dispensing Pipettes capable of delivering 50–150 µL ・12 Channel Dispensing Pipettes capable of delivering 50–150 µL	*Verify accuracy at time of use*
High Performance Liquid Chromatograph with light-excluding auto-sampler for 96-well microwell plates capable of delivering a 0.3 ml/min flow rate	Shimadzu Prominence series
UV detector[*1]	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector

	(281 nm)
pH meter with electrode and calibration	Capable of reading ±0.01 pH
HPLC Column	Shiseido CAPCELL CORE C_{18} column (2.7 μm, 3.0 × 150 mm) [Cat. 51112] *Alternative Column* The follow two columns have been confirmed to yield results for five test chemicals that are essentially the same as the Shiseido column. Waters, CORTECS C_{18} Column (2.7 μm, 3.0 × 150 mm) [Catalog # 186007373] Agilent, Poroshell 120 EC-C_{18} (2.7 μm, 3.0 × 150 mm) [Catalog # 693975-302]
Guard Column and Guard Column Holder[*2]	Column: Shiseido EXP GUARD CARTRIDGE CAPCELL CORE C_{18} S-2.7 2.1×5mm [Cat. 3643] Holder: Shiseido EXP DIRECT CONNECT HOLDER [Catalog # 3640]
Incubator	Capable of controlling at 25 +/ - 1ºC

*[1] If more than one wavelength is detectable, measurements should be made at both 281 nm and 291 nm to confirm peak purities of NAC and NAL.

[*2] Guard columns need not be used for test chemicals that are not precipitated in the reaction solution.

1-1. Apparatus (2)

Apparatus	**Example of use by Lead Laboratory**
Test Tube Mixer	SHIBATA Test Tube Mixer TTM-1
Plate Shaker	Heidolph Titramax 100 [Catalog # 544-11200-00]
Plate Centrifuge	KUBOTA PlateSpin
Recommended but non-essential consumables	
Dispensing Pipettes capable of delivering 1000 – 5000 μL	*Verify accuracy at time of use*

1-2. Consumables (1)

Consumables	**Recommended by Lead Laboratory**
96-well Microwell Plate for HPLC analysis	Polypropylene U bottom 96-well Microwell Plate Thermo (NUNC) U96 PP-0.5 ML NATURAL [Catalog # 267245] Greiner MICROPLATE, 96 WELL, PP, U-BOTTOM, NATURAL [Catalog # 650201]
96-well Microwell Plate seal for HPLC	Use the seal having sealing and solvent-resistant

analysis	performance[*1] Shimadzu GLC Resistant Embossed Seal [Catalog # 568-RES001]

[*1] When using a plate seal other than the recommended one, ensure and verify that no part of the reaction solution is volatilized and that the measured values are consistent before performing actual testing. Be sure to leave no gaps between the plate seal and the plate when applying the plate seal.

1-2. Consumables (2)

Consumables	**Example of use (Used by Lead Laboratory)**
Bottle with the quantity of approximately 500 ml	Sterilized bottle (AS ONE Corporation) [Catalog # 1-9475-05]
Polypropylene centrifuge tube with the quantity equal to or more than 50 ml	100 ml: Watson Clean-vessel [Catalog # 536-150S] 50 ml: FALCON 50-ml PP Conical Tube [Catalog # 352070]
Measuring pipette for 10–100ml	100-ml measuring pipette: FALCON 100-ml Serological Pipette [Catalog # 357600] 50-ml measuring pipette: FALCON 50-ml Serological Pipette [Catalog # 356550] 25-ml measuring pipette: FALCON 25-ml Serological Pipette [Catalog # 356525]
Polypropylene centrifuge tube with the quantity equal to or more than 10 ml	FALCON 15-ml Polypropylene Conical Tube [Catalog # 352196]
Tube for cryopreservation	5-ml Tube[*1]: 5-ml Outer serum tube (SUMITOMO BAKELITE CO., LTD.) [Catalog # MS-4605X] 2-ml Tube[*1]: 2.0-ml Ring rock tube (BM Equipment Co., Ltd.) [Catalog # BM-20] 1.5-ml Tube[*1]: 1.7-ml Ring rock tube (BM Equipment Co., Ltd.) [Catalog # BM-15]

[*1] A smaller or larger tube may be used per the quantity to be cryopreserved.

1-2. Consumables (3)

Recommended but non-essential consumables	**Example of use by Lead Laboratory**
Reservoir for operating 12 channel multipipette	Corning 50-ml Reagent Reservoir [Catalog # 4870]
Approximately 1.5-ml tube and cap operable by 12 channel multipipette (for dispensing test chemical solution)	Alphanumeric storage tube 1.4-ml (Thermo 4247JP) SepraSeal (Thermo 4463)
Tool for removing multiple caps simultaneously	Cap remover for SepraSeal (Thermo 4469)

1-3. Chemical and Special Materials

Chemical and Special Materials	**Example of use by Lead Laboratory**
Trifluoroacetic Acid (TFA) (MW=114.02) CASRN 76-05-1	Wako [Cat. No. 204-02743] 98.0%
Sodium Phosphate, Monobasic, Anhydrous (NaH2PO4, MW=119.98) CASRN 7558-80-7	Wako [Catalog # 197-09705]
Sodium Phosphate, Dibasic, Anhydrous (Na2HPO4, MW=141.96) CASRN 7558-79-4	Wako [Catalog # 197-02865]
0.1M Sodium Hydroxide Solution CASRN 1310-73-2	Wako [Catalog # 196-02195]
Acetonitrile, HPLC Grade CASRN 75-05-8	Wako [Catalog # 015-08633]
Distilled water	Distilled water or equivalent may be used.
Acetone, special grade CASRN 67-64-1	Wako [Catalog # 016-00346]
Dimethyl sulfoxide (DMSO), special grade CASRN 67-68-5	Wako [Catalog # 043-07216]
Ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid, disodium salt, dihydrate (EDTA·2Na·2H_2O) CASRN 6381-92-6	Dojindo Molecular Technologies, Inc. [Catalog #343-01861
NAC (refrigeration storage) MW=289.35, Purity> 95%	Lot No. #960074 ホ゜ (FUJIFILM provides synthetic NAC samples to each laboratory in the validation study)
NAL (refrigeration storage) MW=314.38 Purity>95%	Lot No. #990029 (FUJIFILM provides synthetic NAC samples to each laboratory participating in the validation study.)
Phenyl acetaldehyde (Positive control) MW=120.15 CASRN 122-78-1	SIGMA [Catalog # 107395] Note: Store phenyl acetaldehyde under the following conditions. (1) Store at a temperature of 2–8ºC. Do not store at below 0ºC. (2) Do not expose to UV light during storing or handling. (3) Handle as little as possible, keep stored under the above conditions.

2. PRE-WORK

2-1. Preparation of each solution (Typical)

2-1-1. 0.1-mM EDTA solution

1) Weigh 37.2 mg of EDTA·2Na·2H_2O into a 15-ml Conical Tube and use a 25-ml measuring pipette to add 10 ml of distilled water to dissolve it. (10-mM EDTA solution)
2) Use a 50-ml measuring pipette to add 49.5 ml of distilled water to a 100-ml tube and add 0.5 ml of the 10-mM EDTA solution described above to dilute it 100 fold. (0.1-mM EDTA solution)

2-1-2. 100-mM Phosphate buffer (pH 8.0)

To prepare an approximately 300-ml quantity

1) Weigh 0.6 g of Sodium Phosphate Dibasic Anhydrous into a 100-ml tube and use a 50-ml measuring pipette to add 50 ml of distilled water to dissolve 0.6 g of Sodium Phosphate Dibasic Anhydrous in distilled water.[*1]
2) Use a 50-ml (or 100-ml) pipette to add 300 ml of distilled water.
3) Weigh 4.26 g of Sodium Phosphate Monobasic Anhydrous and dissolve it in distilled water 2).[*1]
4) Use a 25-ml measuring pipette to add 16 ml of Sodium Phosphate Dibasic solution 1) in Sodium Phosphate Monobasic solution 3).
5) Use a 25-ml measuring pipette to remove 17 ml from solution 4) and to add 1 ml of 0.1-mM EDTA solution to solution 4). The concentrations of EDTA in this solution and final concentration of reaction solution are 0.33 μM and 0.25 μM, respectively.
6) Measure pH of solution 5) using a calibrated pH meter and verify that the pH is between 7.9 and 8.1. If the pH is out of range, prepare a new batch of phosphate buffer.
7) Use the NAC stock solution within a week or cryopreserve the unused quantity. Repackage it into 2-ml tubes to cryopreserve for Co-elution Control at -80ºC.

2-1-3. 100-mM Phosphate buffer (pH 10.2)

To prepare an approximately 300-ml quantity

1) Use a 50-ml measuring pipette to add 286 ml of distilled water to a 500-ml bottle.
2) Weigh 4.26 g of Sodium Phosphate Monobasic Anhydrous and dissolve it in the distilled water 1)[*1].
3) Use a 25-ml measuring pipette to add 14 ml of 0.1M NaOH solution to the solution 2).
4) Measure the pH of solution 3) using a calibrated pH meter and verify that it is between 10.1 and 10.3. If the pH is out of range, prepare a new batch of phosphate buffer.
5) Use the NAL stock solution within a week or cryopreserve the unused quantity. Repackage it into 2-ml tube to cryopreserve for Co-elution Control at -80ºC.

2-1-4. Reaction fixing solution (2.5% (v/v) TFA aqueous solution)

Add 2.5 ml of TFA to 100 ml of distilled water.

2-1-5. HPLC mobile phase A: 0.1% (v/v) TFA aqueous solution

Add 1.0 ml of TFA to 1000 ml of distilled water.

2-1-6. HPLC mobile phase B: 0.1% (v/v) TFA Acetonitrile solution

Add 1.0 ml of TFA to 1000 ml of HPLC grade Acetonitrile.

*[*1]: When dissolving phosphate salts, close the bottle to mix them. Do not use glass labware or other washable equipment. Verify that salts are completely dissolved after mixing.*

2-2. Preparation of NAC and NAL stock solutions

2-2-1. Preparation of NAC stock solution:

The NAC (molecular weight of 289.35) stock solution used in each assay is to all be from a single batch. We recommend storing NAC stock solution in quantities sufficient for an assay. A typical preparation as performed at the Lead Laboratory is described below.[*1]

1) Weigh 11.6 ± 0.1 mg of NAC in a 50-ml tube, add 20 ml of 100-mM phosphate buffer (pH 8.0) to the tube using a 25-ml measuring pipette, and mix it gently using a test tube mixer to dissolve the NAC. (2-mM NAC stock solution).[*2]
2) Use a 50-ml measuring pipette to add 149.5 ml of this buffer to a 500-ml bottle. Next, add 0.5 ml of 2-mM NAC stock solution to this solution and mix gently but thoroughly by inversion. (6.667-μM NAC stock solution).
3) Cryopreserve 3-ml quantities of stock solution in cryopreservable 5-ml tubes at -80ºC. Store for no more than six months before use.[*3]

2-2-2. Preparation of NAL stock solution

The NAL (molecular weight of 314.38) stock solution used in each assay is to all be from a single batch. We recommend storing NAL stock solution in quantities sufficient for an assay. A typical preparation as performed at the Lead Laboratory is described below.[*1]

1) Weigh 12.6 ± 0.1 mg of NAL in a 50-ml tube, and add 20 ml of 100-mM phosphate buffer (pH 10.2) to the tube using a 25 ml measuring pipette, and mix it gently using a test tube mixer to dissolve the NAL. (2-mM NAL stock solution).[*2]
2) Use a 50 ml measuring pipette to add 149.5 ml of this buffer to a 500 ml bottle. Next, add 0.5 ml of 2-mM NAL stock solution to this solution and mix it gently but thoroughly by inversion. (6.667-μM NAL stock solution)
3) Cryopreserve 3 ml quantities of stock solution in 5 ml tubes at -80ºC. Store for no more than six months before use.[*3]

[*1] *These quantities are typical examples only and can be adjusted as necessary.*

[*2] *The remaining 2-mM stock solution can be cryopreserved at -80*ºC *for later use. Typically, cryopreserve 1 ml of stock solution in a 1.5 ml tube at -80*ºC.

[*3] *Stability testing of NAC stock solution at temperatures of 25ºC, 4ºC, -20ºC, and -80ºC has shown that it remains stable and free of auto-oxidation only at -80* ºC. *We recommend that the NAL stock solution also be stored at -80* ºC, *although NAL is not susceptible to oxidization in the same manner as NAC.*

Note: Prior to preparing new batches of NAC/NAL stock solution, verify the stability of the NAC/NAL stock solution using the following procedure.

1) Add 150 μl of 6.667-μM NAC/NAL stock solution to three wells in each of two 96-well Microwell Plates.

2) Add 50 μl of acetonitrile to each of these six wells 1).

3) Attach a plate-seal to one of the two 96-well Microwell Plates and spin down the plate using a centrifuge, the incubate for 24 hours at 25ºC. After incubation, add 50μl of reaction fixing solution to each well, attach a new plate-seal, then mix and spin down the plate. Analyze NAC/NAL using the HPLC system.

4) Add 50 μl of reaction fixing solution to the other 96-well Microwell Plate, attach a plate-seal to it, then mix and spin down the plate. Analyze NAC/NAL using the HPLC system.

If the residual concentration of NAC/NAL falls below 90% after either 0 hour or 24 hours, the NAC and NAL are not stable, and so new batches of NAC/NAL solutions must be prepared.

2-3. Preparation of Standard Solution for Calibration Curve

The calibration curve for the acceptance criteria is obtained from a six-dose standard solution. The initial standard solution, Std1 at 5.0 μM, is cryopreserved in small quantities and used to prepare dilution series. A typical procedure for preparation of the standard solution is described below.[*1]

Procedure for preparation of 20-ml solution and 40-66 tubes

1) Use a 25-ml pipette to add 15 ml of 6.667-μM NAC/NAL stock solution to a 50 ml tube. Then use a micropipette to add 1 ml of distilled water, 100μl of TFA, and 3.9 ml of acetonitrile.

2) Cryopreserve 300–500 μl quantities of the standard solution in 1.5-ml tubes at -80ºC.

Also, solvent for preparing dilution series of the standard solution may be cryopreserved in 1000–1200-μl quantities, as described below.

1) Use a 25-ml pipette to add 15 ml of 100-mM phosphate buffer (pH 8.0 for NAC, pH 10.2 for NAL), 1 ml of distilled water, 100 μl of TFA, and 3.9 ml of acetonitrile to a 50-ml tube.

2) Typically, cryopreserve approximately 1 ml of solvent in a 1.5-ml tube at -80ºC.

[*1] These quantities are typical examples only and can be adjusted as necessary.

2-4. Solubility Assessment of Test Chemicals

The solubility of each test chemical in a suitable solvent must be assessed before performing the actual assay. A suitable solvent will dissolve the test chemical completely with no apparent turbidity or precipitate during visual inspection. The concentration of test chemical solution used in the assay is 1mM, but more than 10 mg is recommended to be weighed because of the precise weight of the test chemical. For this reason, the initial concentration of the test chemical solution is set to 20 mM, and the final 1-mM test chemical solution is prepared by 20-fold dilution of the 20-mM solution. The test chemical is dissolved using a test tube mixer and an additional 5–10 minutes of sonication if needed due to low solubility. Because the quantity of test chemical is small relative to the quantity of solvent, solubility is to be carefully verified macroscopically.

The four solvents below are all suitable for used in ADRA, and the priority for the selection of the appropriate solvent is as follows.

(1) Distilled water

(2) Acetonitrile
(3) Acetone
(4) DMSO

Note 1: When the 20-mM solution is prepared using DMSO (4), use acetonitrile to prepare the 20-fold dilution down to a 1-mM solution.

Note 2: If none of these solvents are suitable for a given test chemical in a 20-mM solution, assess the solubility of the test chemical in 1-mM solution using solvents (1)–(3). Do not use DMSO to prepare a 1-mM solution. DMSO must always be diluted 20-fold with acetonitrile to prepare a 1-mM solution.

Note 3: Water is not suitable as a solvent for anhydrides due to hydrolysis.

A typical procedure is described below.

1. Use the following formula to calculate the quantity of test chemical needed to prepare approximately 5 ml of a 20-mM test chemical solution or 100 ml of a 1-mM test chemical solution. If no purity information is available, assume 100% purity.

$$\text{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{20\ \text{mM}}{1000} \times 5\ \text{ml} \quad \text{or} \quad \text{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{1\ \text{mM}}{1000} \times 100\text{ml}$$

$$= \text{MW} \times \frac{10}{\text{Purity (\%)}} = \text{Required quantity (mg)}$$

2. Weigh the required quantity of test chemical directly into a centrifuge tube or other suitable labware and record the actual weight.
3. Calculate the required quantity of solvent using the formula, and assess the solubility for the appropriate solvent.

Note 1: Even if the required quantity is calculated to be less than 10 mg, weigh more than 10 mg of test chemical for precise weight.

Note 2: If the test test chemical solution is in aqueous form, calculate necessary volume of the aqueous solution and quantity of water for dilution. If specific gravity of test chemical solution is not known, assume it as 1.0.

Preparation of 20-mM solution (5 ml)

$$\frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{1}{\text{Specific gravity}} \times \frac{1}{20\ \text{mmol} \times 5\ \text{ml}} \times 1000\ \mu\text{l}$$

$$= \frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{10}{\text{Specific gravity}}$$

= Volume of test chemical (μl)

5000 μl − Volume of test chemical (μl) = Solvent volume (μl)

Preparation of 1-mM solution (100 ml)

$$\frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{1}{\text{Specific gravity}} \times \frac{1}{1\ \text{mmol} \times 100\ \text{ml}} \times \quad 1000\ \mu\text{l}$$

$$= \frac{\text{MW}}{\text{Concentration (w/v\%)}} \times \frac{10}{\text{Specific gravity}}$$

= Volume of test chemical (μl)

$$\textbf{100 ml} \quad - \frac{\textbf{Volume of test chemical (μl)}}{\textbf{1000}} = \textbf{Solvent volume (ml)}$$

2-5. Preparation of HPLC System

Prepare an HPLC system with a 281-nm UV detector and mobile phase A and B, as described in section "2-1-5. HPLC mobile phase A: 0.1% (v/v) TFA aqueous solution" and in section "2-1-6. HPLC mobile phase B: 0.1% (v/v) TFA Acetonitrile solution".

1. Column Equilibration: Install the HPLC column (see section "1.1 Apparatus (1)") and equilibrate the column for at least 30 minutes at 40ºC with 50% A, 50% B before use. Then, condition the column by running the gradient at least twice before using the column.
2. System Shutdown: Following analysis, maintain a low flow (typically 0.05 ml/min) of 50% A and 50% B through the system and decrease column temperature to approximately 25ºC.
3. If the system is to be idle for more than a week, fill the column with acetonitrile (without Trifluoroacetic acid) and remove the column from the HPLC system. Cap tightly and purge acid containing mobile phases from the system using acetonitrile.

3. SOLUTION PREPARATION

3-1. Preparation of Test Chemical Solution

Solubility of the test chemical in the appropriate solvent is evaluated in section "2-4. Solubility Assessment of Test Chemicals". 20-mM or 1-mM solutions of test chemicals in the appropriate solvents are prepared immediately before use.

1. When ready to perform the assay, calculate and weigh the appropriate quantity of test chemical needed to prepare a 20-mM or 1-mM solution directly in a 15-ml or other appropriate tube. Use the following formula to calculate the required quantity of solvent needed based on the actual weight of the test chemical.

   Prepare of 20-mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{20\ \text{mmol}} \times \quad 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times \quad 0.5 \quad = \text{Volume of solvent (ml)}$$

Prepare of 1-mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{1\ \text{mmol}} \times \quad 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times \quad 10 \quad = \text{Volume of solvent (ml)}$$

2. Add the volume of solvent calculated and mix in a centrifuge tube, using a test tube mixer to dissolve the test chemical. Slight sonication (less than 5 minutes) may be used if needed. If the test chemical is not completely dissolved, do not proceed with that specific test chemical in the selected solvent. Re-evaluate with another solvent (see section "2. PRE-WORK") to find a suitable vehicle.
3. Dilute 20-mM of test chemical solution to the final 1-mM solution with each appropriate solvent in the 1.4 ml Test chemical solution Storage Tube or other appropriate tube (For example, add 950 µl of appropriate solvent to 50 µl of 20-mM test chemical solution).[*1]
4. Record and report the final solvent choice for each chemical if the final solvent is different from the solvent selected in section "2-4. Solubility Assessment of Test Chemicals".

[*1] *96-well Deepwell Plate may be used instead of 1.4 ml Test chemical solution Storage Tube.*

3-2. Preparation of Positive Control Solution

Phenyl acetaldehyde (Molecular weight=120.15) dissolved in acetonitrile is used as Positive Control and should be contained in each run of assay. A typical preparation procedure for the Positive Control solution is described below.

1. Calculate a weight of Positive Control to be needed to prepare approximately 5 ml of 20-mM Positive Control solution from following formula. The next formula shows the case that the purity of Phenyl acetaldehyde is 90%.

$$\text{MW} \times \frac{10}{\text{Purity(\%)}} = 120 \times \frac{10}{90} = 13.35(\text{mg}) = \text{Phenyl acetaldehyde (mg)}$$

2. Weigh the phenyl acetaldehyde directly in a 10- or 15-ml centrifuge tube, and record the actual weight.
3. Calculate necessary quantity of solvent from following formula based on the actual weight

$$\text{Phenyl acetaldehyde (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 0.5 = 13.35 \times \frac{90}{120.15} \times 0.5 = 5.0\ (\text{ml}) = \text{Solvent volume (ml)}$$

4. Dissolve Phenyl acetaldehyde with acetonitrile in the quantities calculated above (20 mM).
5. Dilute the 20-mM Phenyl acetaldehyde solution 20-fold with acetonitrile in the 1.4-ml Test chemical solution Storage Tube or other appropriate tube. (final concentration or 1 mM)[*1]

[*1] 96-well Deepwell Plate may be used instead of 1.4-ml Test chemical solution Storage Tube.

3-3. Thaw of each stock solution

Thaw frozen Phosphate buffer (pH 8.0 and 10.2) and NAC/NAL stock solution at room temperature.

The preparation of each stock solution is described in sections “2-1-2. 100-mM Phosphate buffer (pH 8.0)”, “2-1-3. 100-mM Phosphate buffer (pH 10.2)”, and “2-2. Preparation of NAC/NAL stock solutions”.

4. Assay Procedure

4-1. Reference Control, Co-elution Control and Test Chemical Solution

Test chemical solutions are prepared in triplicate for NAC/NAL. Each assay (NAC/NAL) may be prepared and performed concurrently (NAC/NAL should not be incubated and analyzed on separate days). The three Reference Control A, six Reference Control C and one Co-elution Control (the solution after addition of buffer solution rather than NAC/NAL) should be prepared. Moreover, the solution (Co-elution Control, N=1) for each test chemical after addition of buffer solution rather than NAC/NAL solution should be prepared. The detail of each solution describes below.

Test chemical solution	Reaction solution of NAC/NAL with test chemical. Evaluate reactivity of NAC/NAL with the test chemical at a ratio of 1:50 for the NAC/NAL with the test chemical.
Reference Control A	Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC/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.
Co-elution Control	Control for verifying whether or not test chemicals co-elute with NAC/NAL. The Co-elution Control is used to verify absorbance at 281nm and whether retention time of test chemical is equal to that of NAC/NAL.

4-2. Preparation and Reaction of Reaction Solutions

Use 12-channel pipettes with 96-well plates. Proper attention is required when using pipettes: pre-rinse at least five times to ensure precise pick up and take care to avoid dripping from the tip when dispensing.

1. Assemble the following previously prepared reagents, solvents, and solutions:
   a. 6.667-μM of NAC/NAL stock solution,
   b. Appropriate buffer solution (pH 8.0 for NAC, pH 10.2 for NAL),
   c. Test chemical solution (or solvent for Reference Controls)
2. Use a 96-well Microwell Plate and 12-channel pipette to prepare test chemical solutions by adding the reagents per the following table. Refer to the section on “Typical Test Chemical Solution Positions in 96-well Microwell Plate” for typical positions in the plate. Inspect test chemical solution after adding test chemical and record observations.

Ratio of NAC to test chemical = 1:50 5-μM NAC, 0.25-mM test chemical	**Ratio of NAL to test chemical = 1:50** 5-μM NAL, 0.25-mM test chemical
・150-μL NAC solution **・50-μL Test chemical solution**	**・150-μL NAL solution** **・50-μL Test chemical solution**

Co-elution Control and Reference Control A, B and C are prepared according to the following tables.

Co-elution Control

For NAC	**For NAL**
・150-μL Phosphate buffer (pH 8.0) **・50-μL Test chemical solution**	**・150-μL Phosphate buffer (pH 10.2)** **・50-μL Test chemical solution**

Reference Control A and B

For NAC	**For NAL**
・150-μL NAC solution **・50-μL Acetonitrile**	**・150-μL NAL solution** **・50-μL Acetonitrile**

Reference Control C

For NAC	**For NAL**
・150-μL NAC solution **・50-μL Acetonitrile and solvent used for dissolution of test chemical** [*1]	**・150-μL NAL solution** **・50-μL Acetonitrile and solvent used for dissolution of test chemical** [*1]

[*1] If the test chemicals are dissolved in acetonitrile, only acetonitrile need be prepared.

3. Seal the 96-well Microwell Plate completely with a plate seal and shake it using a Plate Shaker. Centrifuge the plate using a Plate Centrifuge and incubate the plate in the Incubator (dark) at 25ºC (plus or minus 1'C) for 24 hours (plus or minus 1 hour). Record the time and temperature at the start and end of incubation.

4-3. Procedure after the Completion of Reaction

1. After 24 hours of incubation, remove the plate seal and add 50 μL of Reaction Fixing Solution to each test chemical solution. Inspect the reacted test chemical solution and record any observations after addition of the reaction fixing solution. If precipitation is observed, centrifuge the 96-well Microwell Plate at low speed (100–400g) to force the precipitate to the bottom of the well as a precaution. If more than 100 μl of supernatant of test chemical solution can be removed using a pipette, the supernatant may be analyzed after being transferred to another plate.

2. Prepare the standard solution

Prepare NAC/NAL standard solutions by serial dilution from 5.0–0.156-μM

(1) Prepare each solution ahead of time after thawing frozen stock solution at room temperature.

[1] 5.0-μM stock solution "Std1"

[2] "solvent for preparing dilution series" (for NAC/NAL)

(2) Prepare the following concentration of the standard solution by diluting 150 µL of stock solution "Std1" with the same quantity of dilution solvent per to "Preparation procedure for dilution series" below. Add dilution solvent to a well as "Std7".

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7 (Dilution solvent)
NAC/NAL (µM)	5.0	2.5	1.25	0.625	0.313	0.156	0

"Preparation procedure of dilution series"

(1) Add 150 µl of dilution buffer to wells from well Std2 to well Std7 shown in "Typical Test Chemical Solution Positions in 96-well Microwell Plate"

(2) Add 150µl of Std1 to well Std1 and well Std2. Mix with minimal air entrapment.

(3) Transfer 150 µl from well Std2 to well Std3. Mix with minimal air entrapment.

(4) Continue in a similar manner from well Std4 to well Std6.

3. Prepare dilution series of NAC/NAL standard solution in the wells from Std1 to Std7 shown in the next section on "Typical Test Chemical Solution Positions in 96-well Microwell Plate", and reseal the plate tightly with a new plate seal. For preparation of dilution series, see section "4-3. 2. Prepare the standard solution". Mix the plate with a Plate Shaker and spin it down by centrifuge.

Note: Add the Reaction Fixing Solution within 30 minutes of the end of incubation.

Typical Test Chemical Solution Positions in 96-well Microwell Plate

Between 1 and 17 test chemicals can be tested in a single test run using two 96-well plates, based on consideration of the time needed for preparation and testing as well as the burden on laboratory technicians and the capacity of the HPLC apparatus. If more than one solvent are used a single test run, additional Reference Controls for each solvent need to be prepared and the number of test chemicals will need to be reduced. For example, if three kinds of solvent are used, the maximum number of test chemicals is 15. The number of test chemicals and Typical Test chemical solution position in 96-well Microwell Plate are shown below, but the positions may be adjusted per the number of test chemical solutions.

(1) For five test chemicals with acetonitrile as solvent

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	CC-1	CC-2	CC-3	CC-4	CC-5	—	—	—	—	—	STD 4	—
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	CC-1	CC-2	CC-3	CC-4	CC-5	—	—	—	—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control

(2) For 10 test chemicals with acetonitrile and water as solvent

First plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	—	—
	B												
	C												
	D	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	—	—
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	—	—
	F												
	G												
	H	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	—	—

Second plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	RC-A	RC-B	RC-B	RC-C (Water)	RC-C (ACN)	PC	—	—	—	—	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	—	—	—	—	—	—	—	—	—	—	STD 4	—
NAL	E	RC-A	RC-B	RC-B	RC-C (Water)	RC-C (ACN)	PC	—	—	—	—	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	—	—	—	—	—	—	—	—	—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control, ACN: Acetonitrile

(3) For 17 test chemicals and acetonitrile as solvent

First plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	Test chemical solution No.11	Test chemical solution No.12
	B												
	C												
	D	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	CC-9	CC-10
NAL	E	Test chemical solution No.1	Test chemical solution No.2	Test chemical solution No.3	Test chemical solution No.4	Test chemical solution No.5	Test chemical solution No.6	Test chemical solution No.7	Test chemical solution No.8	Test chemical solution No.9	Test chemical solution No.10	Test chemical solution No.11	Test chemical solution No.12
	F												
	G												
	H	CC-1	CC-2	CC-3	CC-4	CC-5	CC-6	CC-7	CC-8	CC-9	CC-10	CC-11	CC-12

Second plate

		1	2	3	4	5	6	7	8	9	10	11	12
NAC	A	Test chemical solution No.13	Test chemical solution No.14	Test chemical solution No.15	Test chemical solution No.16	Test chemical solution No.17	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	B											STD 2	STD 6
	C											STD 3	STD 7
	D	CC-3	CC-4	CC-5	CC-6	CC-7	—	—	—	—	—	STD 4	—
NAL	E	Test chemical solution No.13	Test chemical solution No.14	Test chemical solution No.15	Test chemical solution No.16	Test chemical solution No.17	RC-A	RC-B	RC-B	RC-C	PC	STD 1	STD 5
	F											STD 2	STD 6
	G											STD 3	STD 7
	H	CC-13	CC-14	CC-15	CC-16	CC-17				—	—	STD 4	—

RC: Reference Control, CC: Co-elution Control, PC: Positive Control, ACN: Acetonitrile

5. HPLC Analysis

1. Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the Mobile Phase A and Mobile Phase B at column temperature of 40°C. The HPLC analysis is performed using a flow of 0.3 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. Refer “5-1. HPLC Conditions” for details on the gradient.
2. Inject equal quantities of each standard solution, test chemical solution, and control solution. The injection quantity varies according to the system used but typically is from 10–20 μL. Systems require smaller injection quantities, because injection of 20 μL leads to unacceptably broad peaks. Absorbance is monitored at 281 nm. If using a Photodiode Array Detector, absorbance at 291 nm should also be recorded.

3. Increase the mobile phase B to 100% in order to remove the other compounds from the column after gradient, and re-equilibrate the column under initial conditions for at least 6.5 minutes. These process of acetonitrile 100% and re-equilibration for 6.5 minutes are described the following table “5-1. HPLC Conditions”.

Note: The 6.5 minute re-equilibration time was determined using a Shimadzu Prominence HPLC system. Other systems may require more or less re-equilibration time due to system mixing quantity. Shorter equilibration times will be acceptable if peak retention times are stable.

5-1. HPLC Conditions

Column	Shiseido CAPCELL CORE C_{18} column (2.7 μm, 3.0 × 150 mm) [Cat. 51112] *Alternative Column* The follow two columns have been confirmed to yield results for five test chemicals that are essentially the same as the Shiseido column. Waters, CORTECS C_{18} Column (2.7 μm, 3.0 × 150 mm) [Catalog # 186007373] Agilent, Poroshell 120 EC-C_{18} (2.7 μm, 3.0 × 150 mm) [Catalog # 693975-302]
Column Temperature	40ºC
Test chemical solution Temperature	25ºC If the auto-sampler has a cooling function, test chemical solutions can be kept more stable at 4ºC.
UV detector[*1]	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector (281 nm)
Injection Quantity	10-20 μl (The injection quantity varies according to HPLC system. If peaks are too broad, the injection quantity should be decreased.)
Run Time	20 minutes
Flow Conditions	NAC flow conditions **Time Flow %A %B** 0 min 0.3 ml/min 70 30 9.5 min 0.3 ml/min 45 55 10 min 0.3 ml/min 0 100 13 min 0.3 ml/min 0 100 13.5 min 0.3 ml/min 70 30 20 min End run NAL flow conditions **Time Flow %A %B**

	0 min	0.3 ml/min	80	20
	9.5 min	0.3 ml/min	55	45
	10 min	0.3 ml/min	10	100
	13 min	0.3 ml/min	10	100
	13.5 min	0.3 ml/min	80	20
	20 min	End run		

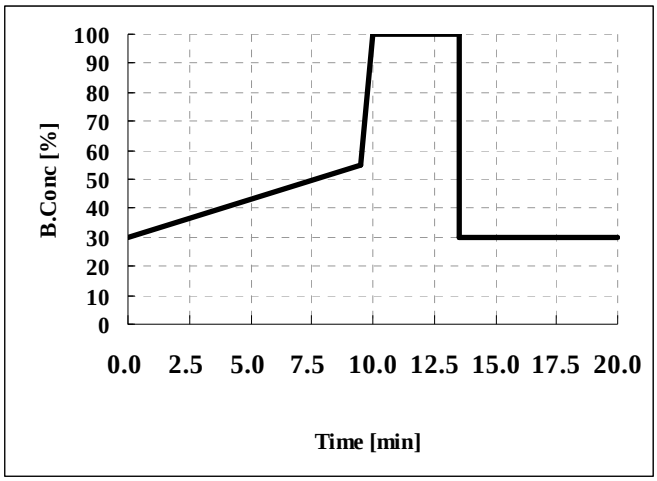
Note 1: The mixer quantity should be verified and adjusted in advance because the appropriate elution pattern of NAC/NAL peak will not be shown if the mixer quantity for mixing each mobile phase is not appropriate (For example, 0.5 ml mixing quantity is appropriate for Shimadzu prominence HPLC system).

Note 2: The inner diameter of pipe and the length of pipe from column outlet to detector inlet must be less than 0.18 mm and less than 50 cm, respectively, because the peak of NAC/NAL might be broadened depending on inner diameter and length of pipe

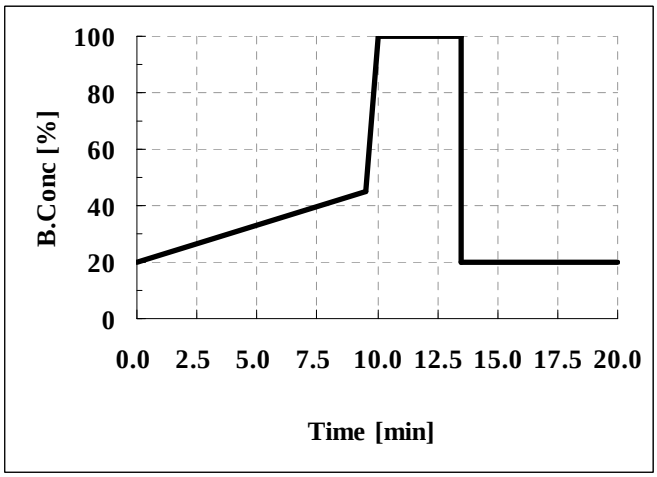
Note 3: If more than one wavelength is detected, also 291 nm besides 281 nm should be detected to check out peak purities of NAC/NAL.

Graphical Display of Gradient for NA/NAL Analysis

For NAC analysis



For NAL analysis



Note: Visual inspection of test chemical solutions must be conducted prior to HPLC analysis. Generally, precipitation is not a problem. However, if precipitation is observed, this should be noted in the data reporting template. Test chemical solutions may be centrifuged at low speed (100–400g) in vial to force precipitate to the bottom of the well as a precaution, since large quantities of precipitate may clog the HPLC tubing or columns. If more than 100 μL of supernatant can be recovered, in may be analyzed after transfer to another plate. Precipitate formation, removal, and transfer must be recorded and reported.

5-2.HPLC Sample Analysis Sequences

Each sample of HPLC analysis should be analyzed 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 analyze calibration standards and Reference Control A (N = 3).
2. The Co-elution Control does not need to be analyzed by turns if it is analyzed after analysis of standard solution and Reference Control A.
3. Reference Control B should be analyzed 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 analyzed. (After the first set of replicates of each sample is analyzed, the second set of replicates of each should be analyzed)

Example of HPLC Samples Analysis Sequences

(A more specific analysis sequence can be found at the end of the SOP)

STD7 (Buffer solution for dilution)[†] STD6 STD5 STD4 STD3 STD2 STD1	Ref. 8-1. 1)
Reference Control A, rep 1 Reference Control A, rep 2 Reference Control A, rep 3	Ref. 8-1. 1)
Co-elution Control 1 Co-elution Control 2 Co-elution Control 3 ・・・ Co-elution Control n	Ref. 8-4.
Reference Control B, rep 1 Reference Control B, rep 2 Reference Control B, rep 3	Ref. 8-2.
Reference Control C, rep1[§†] Phenyl acetaldehyde (Positive Control), rep1 Test chemical solution 1, rep1 Test chemical solution 2, rep1 Test chemical solution 3, rep1 ・・・ Test chemical solution n, repn	***First set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control C, rep2[§†] Phenyl acetaldehyde (Positive Control), rep2 Test chemical solution 1, rep2 Test chemical solution 2, rep2 Test chemical solution 3, rep2 ・・・	***Second set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.

Test chemical solution n, rep2	
Reference Control C, rep3[§†] Phenyl acetaldehyde (Positive Control), rep3 Test chemical solution 1, rep3 Test chemical solution 2, rep3 Test chemical solution 3, rep3 ・・・ Test chemical solution n, rep3	***Third set of replicates*** Ref. 8-1. 2), 8-1. 3), 8-2.
Reference Control B, rep 4 Reference Control B, rep 5 Reference Control B, rep 6	Ref. 8-2.

[†] Start to analyze calibration standard immediately after addition of Reaction Fixing Solution and preparation of dilution series of standard solution.

[§] Analyze three replicates for Reference Controls C. These results are used to calculate the NAC/NAL depletion in each solvent and to verify that solvent used does not affect NAC/NAL depletion.

6. DATA ANALYSIS & CALCULATIONS

The concentration of NAC/NAL is calculated from peak area of absorbance at 281 nm for each test chemical solution based on the calibration curve derived from standard solutions Std 1 to Std7. NAC/NAL percent depletion is calculated by dividing NAC/NAL peak area of each test chemical solution by mean peak area of Reference Control C.

6-1. Calculation of Peak Area of NAC/NAL

Integrate the appropriate peaks and determine peak area for standards, test chemical solution and controls. The peak area of each integrated peak must be reported.

6-2. Calculation of Concentration of NAC/NAL

6-2-1. Generate a linear calibration curve based on the concentration of standards and the peak area. Suitable calibration curves must have an r^2>0.990.

6-2-2. Calibrate the mean NAC/NAL concentrations in Reference Controls A and C, SD and CV. The each mean should be 3.2–4.4 μM. The NAC/NAL concentration of Reference Controls A and C must be reported.

6-2-3. Calculate the mean NAC/NAL peak area, SD and CV for the Reference Controls C (N=3) for each solvent used. The each mean concentration should be 3.2–4.4 μM. However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM.

6-3. Calculation of Peak Area of NAC/NAL

6-3-1. Calculate the mean NAC/NAL peak area for the six Reference Controls B and the three Reference Control C in acetonitrile, SD and CV. The CV must be less than 10%.

6-3-2. Calculate the mean NAC/NAL peak area at 281nm for the three Reference Controls C.

6-4. Calculation of Percent Depletion of NAC/NAL

6-4-1. For the Positive Control and for each test chemical, calculate the Percent NAC/NAL Depletion in each replicate from the NAC/NAL peak area of the replicate injection and the mean NAC/NAL area in the three relevant Reference Controls C (in the appropriate solvent), using the following formula.

Percent NAC/NAL Depletion (% depletion) = [1- (NAC/NAL Peak Area in Replicate Injection/mean NAC/NAL Peak Area in Reference Controls C)]×100

6-4-2. The mean Percent NAC/NAL Depletion (Average score) of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

7. DATA REPORTING (FOR NAC AND NAL)

System Suitability

- NAC/NAL peak area at 281 nm of Standard and Reference Control B and C replicate.
- The linear calibration curve should be graphically represented and the r^2 reported.
- NAC/NAL concentration (μM) of Reference Control A replicate.
- Mean NAC/NAL concentration (μM) of Reference Controls C replicate, SD and CV.

Analysis Sequence

Reference Controls:

- NAC/NAL peak area at 281 nm of Reference Control B and C replicate.
- Mean NAC/NAL peak area at 281 nm of the nine Reference Controls B (N = 6) and C (N = 3) in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean NAC/NAL peak area at 281 nm of the three appropriate Reference Controls C replicate (for calculation of Percent NAC/NAL Depletion).
- For each solvent used in this assay, the mean NAC/NAL concentration (μM) of the appropriate Reference Control C replicate, SD and CV.

Positive Control (Phenyl acetaldehyde)

- NAC/NAL peak area at 281 nm of each replicate.
- Percent NAC/NAL Depletion of each replicate
- Mean NAC/NAL Depletion of the three replicates, SD and CV.

For Each Test Chemical:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time.
  It must be reported if precipitate was re-solubilized or centrifuged.
- NAC/NAL peak area at 281 nm of each replicate (for systems equipped with a PDA detector the peak

area at 291 nm should also be reported).

- Percent NAC/NAL Depletion of each replicate.
- Mean of Percent NAC/NAL Depletion of the three replicates, SD and CV.

8. ACCEPTANCE CRITERIA

8-1. Acceptance Criteria for Amino acid Derivative Reactivity Assay Run

All criteria must be met for the whole run to be considered valid. If three criteria are not met, the run must be repeated for all test chemicals.

System Suitability:

Calibration Linearity $r^2 > 0.990$

Mean NAC/NAL concentration of Reference Controls A = 3.2–4.4 μM

Positive Control:

The mean Percent NAC/NAL Depletion value of the three replicates for phenyl acetaldehyde must fall within the range reported in the following table (Based on mean ±3SD from background data):

	Percent NAC Depletion		Percent NAL Depletion	
Positive control	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Phenyl acetaldehyde	6	30	75	100

Maximum Standard Derivatives for Positive Control replicate:

Standard Deviation for Percent NAC Depletion must be < 10%

Standard Deviation for Percent NAL Depletion must be < 10%

Stability of Reference Controls over analysis time:

For each solvent used, the mean of the NAC/NAL concentrations of the three appropriate Reference Controls C = 3.2–4.4 μM. However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM, as it is known that concentration of NAC decreases because of oxidation of SH group by DMSO.

8-2. Acceptance Criteria for Each Test Chemical

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

Maximum Standard Deviation of test chemical solution replicates:

Standard Deviation for Percent NAC Depletion must be < 10%

Standard Deviation for Percent NAL Depletion must be < 10%

Reference Controls C in the analysis sequence:

CV of NAC/NAL peak areas for the nine Reference Controls B (N = 6) and C (N = 3) in acetonitrile

must be < 10%. Moreover, CV of NAC/NAL peak areas for the three Reference Controls C (N = 3) in each solvent must be < 10%.

8-3. Data Acceptance for Amino Acid Derivative Reactivity Assay

The average score should be calculated from depletions of NAC/NAL, and the test chemicals should be predicted to be either a Sensitizer or a Non-sensitizer according to following table.

NAC/NAL **prediction model**

Average score	**Judgement**
Less than 4.9%	Non-sensitizer
4.9% or higher	Sensitizer

If an average score for NAC depletion in a test chemical falls within the borderline range described below, additional testing should be performed to confirm the validity of the prediction. If the result of the second test is not concordant with the first test, a third test should be performed to determine a prediction for the test chemical by majority of the three test results.

NAC and NAL prediction model: 3.0% ≤ average score ≤ 10.0%
NAC only prediction model: 4.0% ≤ NAC depletion ≤ 11.0%

8-4. Handling of Co-elution

8-4-1. Co-elution : Interference

(1) Some test chemicals will co-elute with the NAC or NAL. In order to detect possible co-elution of the test chemicals with NAC or NAL, the test chemicals included in the run must be injected alone (“Co-elution Controls”) at the beginning of the run sequence and their chromatograms compared to the chromatograms of Reference Controls C in the appropriate solvent.

(2) If a chemical absorbs at 281 nm and has a similar retention time as a peptide (overlap of valley-to-valley integration periods), then verify whether or not the peak of test chemical is actually separated from the peak of NAC or NAL. If the peak of test chemical is completely overlapped with the peak of NAC or NAL, and if the boundary of two peaks (valley between peaks) is located higher than baseline, co-elution of the test chemical with that NAC or NAL should be reported. The “interfering” chemical peak should have a peak area that is >10% of the mean NAC/NAL peak area in the appropriate Reference Control. If co-elution occurs and proper integration and calculation of NAC/NAL depletion is not possible, the data should be recorded as “interference” for NAC/NAL the chemical co-elutes with.

(3) Even if the test chemical does not co-elute with NAC or NAL, the Percent NAC/NAL Depletion can appear to be < -10% if the concentration of Reference Control C is comparatively low. Moreover, the Percent NAC/NAL Depletion can also appear to be < -10% due to inappropriate handling of the measurement. In such cases, retesting of the test chemicals in question or other appropriate measure should be taken.

8-4-2. Peak purity of NAC/NAL : Area ratio of 281/291 nm

(1) When a Photodiode Array detector is used, co-elution of chemical and NAC/NAL may also be verified by looking at the UV spectrum at 291 nm in addition to 281 nm and calculating the area ratio of 281/291. This value should be consistent over all test chemical solutions and standards for a distilled NAC/NAL peak and thus gives a measure of peak purity. For each test chemical solution, a ratio in the following range would give a good indication that co-elution has not occurred. However, calculation of peak purity (area ratio of 281/291) might not always be possible, particularly if the test chemical is highly reactive with the NAC/NAL leading to very small peaks.

90% < Mean Area ratio of Reference Control < 110%.

8-4-3. Co-elution : Depletion < -10%

(1) If the Percent NAC/NAL Depletion is < -10%, it should be considered that this may be a situation of co-elution, inaccurate NAC/NAL addition to the reaction mixture or just baseline noise. If the NAC/NAL peak appears at the proper retention time and has the appropriate peak shape, the peak can be integrated. In this case, there may just be baseline noise causing the NAC/NAL peak to be bigger or there may be some co-elution/overlap in retention time of the NAC/NAL and test chemical.

(2) The calculated %-depletion should be reported as an estimate. In cases where a test chemical co-elutes with NAL, the NAC only prediction model can be used. In cases where a test chemical co-elutes with both NAC/NAL, the data should be reported as an inconclusive.

(3) In cases where the test chemical co-elutes with the NAC and the peak of NAC cannot be integrated, the skin sensitization of test chemical cannot be predicted from the NAL depletion alone, and the data should be reported as inconclusive.

8-4-4. Calculation of peak area for co-elution

(1) If the peak of NAC/NAL and the peak of test chemical partially overlap, the peak area of NAC/NAL should be integrated from valley of both peaks to baseline vertically.

(2) If the peak of NAC/NAL and the peak of test chemical completely overlap, the data should be reported as an Inconclusive, and the peak area should not be calculated.

8-4-5. Estimated depletion values

In some cases, a test chemical might co-elute with NAC and/or NAL though the test chemical react with NAC and /or NAL. If this is the case, co-elution will make the peak area of NAC/NAL appear to be larger than it really is, therefore the calculated percent depletion may be lower than the true value. When the overlap in retention time between the test chemical and NAC/NAL is incomplete, percent depletion can still be calculated with a notation of “co-elution – percent depletion estimates”. If the average score is below the criteria, the result should be reported as Inconclusive. However, unless NAC co-elutes with test chemical, the NAC-only prediction model should be used.

Average score	No co-elution	Co-elution with NAC alone or NAC and NAL	Co-elution with NAL only
< 4.9%	Non-sensitizer	Inconclusive	Apply NAC-only prediction model
4.9% ≦	Sensitizer	Sensitizer	Apply NAC-only prediction model

NAC only prediction model

NAC Depletion	Judgement
less than 5.6%	Non-sensitizer
5.6% or greater	Sensitizer

Example HPLC Analysis

There are 5 test chemicals. Chemical 1,2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in distilled water.

The following 96-well Microwell Plate should be set up:

Std 7 (Dilution buffer blank) Std 6 Std5 Std5 Std4 Std 3 Std 2 Std1 Reference Control A, rep 1 (made with acetonitrile) Reference Control A, rep 2 (made with acetonitrile) Reference Control A, rep 3 (made with acetonitrile) Co-elution Control for Chemical 1 Co-elution Control for Chemical 2 Co-elution Control for Chemical 3 Co-elution Control for Chemical 4 Co-elution Control for Chemical 5
Reference Control B, rep 1 (made with acetonitrile) Reference Control B, rep 2 (made with acetonitrile) Reference Control B, rep 3 (made with acetonitrile)

Reference Control C, rep 1 (made with acetonitrile)
Reference Control C, rep 1 (made with distilled water)
Phenyl acetaldehyde, rep 1
Chemical 1, rep 1
Chemical 2, rep 1
Chemical 3, rep 1
Chemical 4, rep 1
Chemical 5, rep 1

Reference Control C, rep 2 (made with acetonitrile)
Reference Control C, rep 2 (made with distilled water)
Phenyl acetaldehyde, rep 2
Chemical 1, rep 2
Chemical 2, rep 2
Chemical 3, rep 2
Chemical 4, rep 2
Chemical 5, rep 2

Reference Control C, rep 3 (made with acetonitrile)
Reference Control C, rep 3 (made with distilled water)
Phenyl acetaldehyde, rep 3
Chemical 1, rep 3
Chemical 2, rep 3
Chemical 3, rep 3
Chemical 4, rep 3
Chemical 5, rep 3

Reference Control B, rep 4 (made with acetonitrile)
Reference Control B, rep 5 (made with acetonitrile)
Reference Control B, rep 6 (made with acetonitrile)

Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean NAC/NAL peak area of the Reference Control C which are prepared with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean NAC/NAL peak area of the Reference Controls C which are prepared with distilled water.

Appendix 3

Principle of DPRA and ADRA Test Methods

1. Principle of DPRA test method

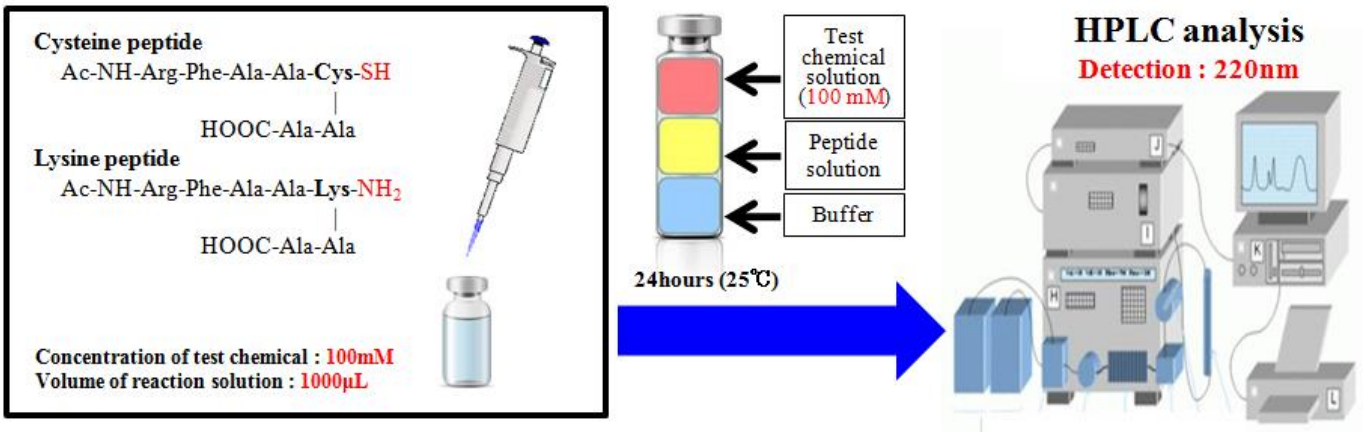


Figure 1 Principle of the DPRA test method

The DPRA test method uses two synthetic peptides: cysteine peptide (Ac-RFAACAA-COOH) and lysine peptide (Ac-RFAAKAA-COOH). A test chemical solution is mixed with each peptide in a 1-mL vial and allowed to react for 24 hours at 25°C, after which high-performance liquid chromatography (HPLC) is used to quantify the nonreactive peptide. The depletion of the cysteine peptide and the lysine peptide is calculated as a percentage, and reactivity with the test chemical is assigned to one of four categories—high, moderate, low, or minimal—based on the mean % depletion of the two peptides. In this manner, test chemicals that are assigned to the high, moderate, or low categories are predicted to be sensitizers, and those assigned to the minimal category are predicted to be non-sensitizers. (See Figure 1.)

2. Principle of ADRA test method

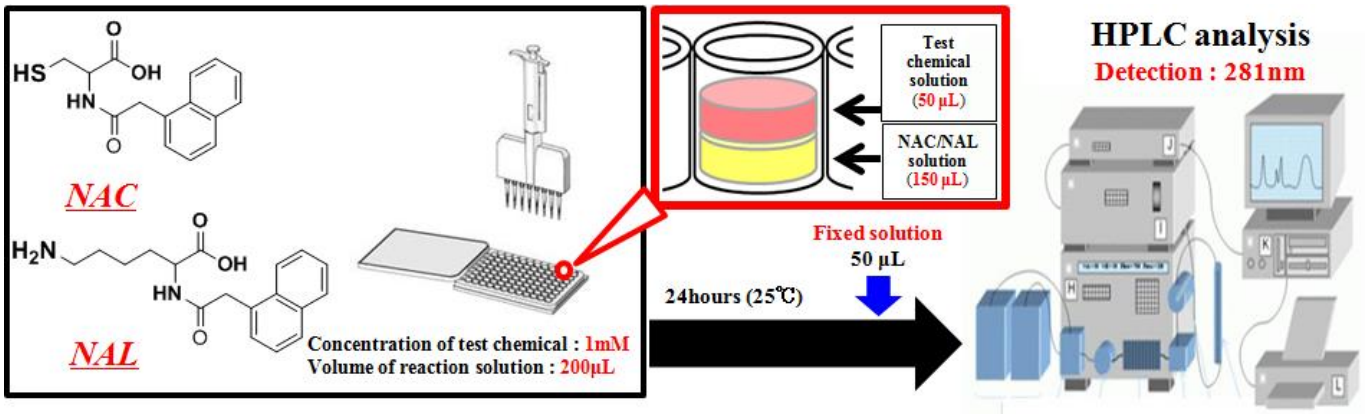


Figure 2 Principle of the ADRA test method

The ADRA test method uses two synthetic amino acid derivatives: *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine (NAL). A test chemical solution is mixed with either NAC or NAL in the 200-μL wells of a 96-well microplate and allowed to react for 24 hours at 25°C, after which a fixing solution is added and high-performance liquid chromatography (HPLC) is used to quantify the nonreactive NAC and NAL. The depletion of the NAC and NAL is calculated as a percentage, and test chemicals with a mean % depletion of 4.9% or greater are predicted to be sensitizers while those with a mean % depletion of less than 4.9% are predicted to be non-sensitizers. (See Figure 2.)

Appendix 4

Chemical Selection

Report on the selection of test chemicals for the ADRA validation study

－ ADRA Validation Management Team (VMT) －

Members for Chemical Selection Committee of the ADRA Validation Management Team

Hajime Kojima (JaCVAM, NIHS, Japan), JaCVAM representative
Nicole Kleinstreuer (NICEATM/ICCVAM, USA), ICCVMA liaison
Jon Richmond (dr.jonrichmond: Advice and Consultancy, UK)
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Toshihiko Kasahara (FUJIFILM Corporation)
Masaharu Fujita (FUJIFILM Corporation)
Yusuke Yamamoto (FUJIFILM Corporation)
Tsuyoshi Kawakami (NIHS, Japan)
Kohichi Kojima (Food and Drug Safety Center, Japan)
Atsushi Ono (Okayama University)

This report describes the process through which test chemicals for the ADRA validation study were selected.

The object of this validation study was to evaluate within- and between-laboratory reproducibility as well as predictive capacity for skin sensitization potential (*i.e.*, concordance with classification of sensitizers and non-sensitizers) of ADRA. As a complementary study, the validation management team (VMT) evaluated the predictability of the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) with three classifications (Category 1A, Category 1B and non-sensitizer).

A pre-validation study (training) and validation studies (Phase I and Phase II testing) were conducted at four participating laboratories using the test chemicals shown in Table 1.

In addition, the chemical categories or physical state and chemical properties (*e.g.*, solid, liquid, etc.) were included in the tables of these test chemicals in order to investigate the applicability domain.

Table 1. Breakdown of the ADRA validation study

Phase	The number of the test substances	The number of the repetitions	Examination	Date of experiment start
Pre-training	5	1	Transferability (non-coded)	
Training	10	1	Transferability (non-coded)	
I	10	3	Between- laboratory reproducibility and transferability (coded)	October, 2016
II	30	1	Within- and between-laboratory reproducibility (coded)	February, 2017

1. Basis for chemical selection

The selection of test chemicals by the Chemical Selection Committee (CSC) was based on published papers on *in vivo* skin sensitization tests and validation studies for *in vitro* alternative assays on skin sensitization test methods.

1-1 The applied selection criteria

Test chemicals were selected based on the ECVAM validation study and the published papers on skin sensitization.

The applied selection criteria were:

- information on mode/site of action
- coverage of range of relevant chemical classes and product classes quality and quantity of reference data (*in vivo* and *in vitro*)
- high quality data derived from animals as well as humans, if available
- knowledge of interspecies variations (*e.g.*, variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of range of toxic effects/potencies
- chemicals that do not need metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- costs

In the first phase of the selection procedure, the CSC identified and collected several existing lists of potential chemical sensitizing in order to establish a primary database. These listings had originally been compiled by international experts as reference compounds for validation studies and other purposes. An extensive literature research was performed by the CSC in order to insure that the selected chemicals fulfilled the selection criteria described above.

Emphasis was placed on selecting chemicals of varying potencies (strong, weak and no activity). In addition, it was decided that at least 20% of the total substances to be tested should be non-sensitizers in order to increase the statistical power of the data analysis.

1-2 Chemical Acquisition, Coding, and Distribution

The assessment of between-laboratory transferability as well as of within- and between-laboratory reproducibility and predictive capacity was performed at all participating laboratories with coded chemicals. The VMT made provision for the need for additional testing at all participating laboratories.

The coding was supervised by JaCVAM in collaboration with CSC. The CSC was responsible for coding and distributing the test chemicals, references, and control reagents for the validation study.

1-3 Handling

The chemical master (See “5.Acquisition, coding, and distribution; 5-2 Handling”) at each participating laboratory received from JaCVAM a complete set of essential information about the test chemicals, including physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions. Moreover, the chemical master at each participating laboratory stored each chemical in accordance with the storage instructions and received sealed safety information, including Material Safety Data Sheets (MSDS) describing hazard identification, exposure controls, and personal

protection for each chemical. The test chemicals were delivered directly to the study director, who was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. Even if the study director referred to the MSDS, he or she was not to reveal the content of the MSDS to the laboratory technicians.

Unfortunately, one JaCVAM employee who was responsible dispensing the test chemicals withdrew from the validation study. But there were no accidents at the participating laboratories during the course of the validation study, all of which returned the MSDS to JaCVAM in their sealed envelopes upon completion of the validation study. All unused test chemicals were disposed of in compliance with the rules and regulations of the participating laboratories upon completion of the validation study.

2. Pre-validation study

For evaluating transferability, the lead laboratory selected the five test substances for pre-training shown in Table 2. Later, using a newer SOP that was revised in accordance with pre-training results, the lead laboratory selected the 10 test substances for training shown in Table 3.

Table 2. Test chemicals used for pre-training

No.	Chemicals	CAS No.	LLNA category
PT-001	p-Benzoquinone	106-51-4	Extreme
PT-002	Cinnamaldehyde	14371-10-9	Moderate
PT-003	Diethyl maleate	141-05-9	Moderate
PT-004	4-Allylanisole	140-67-0	Weak
PT-005	Vanillin	121-33-5	Non-sensitizer

Table 3. Test chemicals used for training

No.	Chemical	CAS No.	LLNA category
T-001	Oxazolone	15646-46-5	Strong
T-002	Phthalic anhydride	85-44-9	Strong
T-003	Formaldehyde	50-00-0	Strong
T-004	Glyoxal	107-22-2	Moderate
T-005	Benzylideneacetone	122-57-6	Moderate
T-006	Cyclamen aldehyde	103-95-7	Weak
T-007	1-Butanol	71-36-3	Non-sensitizer
T-008	6-Methylcoumarin	92-48-8	Non-sensitizer
T-009	Lactic acid	50-21-5	Non-sensitizer
T-010	4'-Methoxyacetophenone	100-06-1	Non-sensitizer

3. Validation study -Phase I testing-

Ten test chemicals were selected by the CSC for evaluating within- and between-laboratory reproducibility. Three runs were performed, but the order of testing had no impact on the results. These chemicals were selected at the chemical selection meeting in Gotemba, Japan, on September 15, 2016, in accordance with the applied chemical selection criteria and based on similarity of their chemical properties and sensitization potential with the chemicals used in Phase I of the ECVAM validation study. The chemicals were coded by JaCVAM as shown in Appendix 1 and distributed to the test facilities. No poisonous or deleterious chemicals were used in the training stage.

4. Validation study -Phase II testing-

Thirty chemicals were selected by the CSC for evaluating between-laboratory reproducibility and predictive capacity. These chemicals were selected at the chemical selection meeting in Gotemba, Japan, on September 15, 2016, based on their chemical properties, results obtained in earlier phase studies, and sensitization potential based on human data from a list of chemicals prepared by the lead laboratory based on the data set at FUJIFILM corporation, DPRA, KeratinoSense and h-CLAT. The chemicals were coded by JaCVAM as shown in Appendix 2 and distributed to the participating laboratories. In the process of chemical selection, almost no poisonous or deleterious chemicals were used in training stage and Phase I testing.

References

1) OECD (2005). Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD Testing Series and Assessment Number 34. 281 ENV/JM/MONO(2005)14, pp 96, Paris, France: OECD. 282

2) Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, Gribaldo L, Halder M, Hoffmann S, 283 Janusch Roi A, Prieto P, Sabbioni E, Scott L, Worth A and Zuang V (2004). A Modular Approach to 284 the ECVAM Principles on Test Validity. ATLA 32, 467–472. 285

Appendix 5

Prediction of Human Skin Sensitization by ADRA and DPRA

1. Background and objectives

The predictive capacity of both DPRA and ADRA relative to LLNA has been shown by past studies as well as by the present validation results to be highly concordant with LLNA. Since no study, however, has compared the predictive capacity of DPRA and ADRA relative to human sensitization data, we identified 48 test chemicals tested using both DPRA and ADRA for which human sensitization data exists to determine the predictive capacity of DPRA and ADRA relative to human data (Basketter, *et al*.,2014).

2. Results and Discussion

Comparing the predictive capacity of each test method

Table 1 shows human sensitization data as well as the results for 48 test chemicals tested using LLNA, DPRA, and ADRA. Shown in Figure 1 are the predictive capacities of ADRA, DPRA, and LLNA relative to human data as calculated from the data given in Table 1. Although LLNA exhibits an accuracy relative to human data of 81.3%, ADRA's accuracy of 68.8% and DPRA's accuracy of 72.9% are roughly 10 percentage points lower.

As shown in Table 1 there are six human categories, with Category 1 comprising the strongest sensitizers and becoming successively weaker until Category 6, which comprises non-sensitizers. Focusing for the moment on the 10 chemicals that comprise Category 5, we see that seven of these 10 chemicals were predicted to be non-sensitizers by LLNA, while both ADRA and DPRA predicted nine of these 10 to be non-sensitizers. The definition of Category 5 states: "A rare cause of contact allergy except perhaps in special circumstances, e.g., use in topical medicaments (hexylcinnamal, isopropanol, parabens)," which is indicative of the inherent difficulty of predicting these chemicals accurately.

Setting aside for the moment the problematic Category 5 chemicals, and looking at predictive accuracy based on the remaining 38 test chemicals, an analysis gives accuracies of 84.2% for ADRA, 89.5% for DPRA, and 94.7% for LLNA. And while these results show that ADRA's accuracy is still somewhat lower than either DPRA or LLNA, 84% can be considered high. (See Figure 2.) The reason for ADRA's relatively low predictive capacity appears to be that a greater number of test chemicals were predicted to be non-sensitizers than when using DPRA. The VMT interpreted this result as described below.

Table 1 Predictive capacity of LLNA, ADRA, and DPRA relative to human sensitization data

№	Chemical	CAS No.	LLNA (EC3)	ADRA Prediction	DPRA Prediction
Human Category 1					
1	Dinitrochlorobenzene	97-00-7	0.05	S [1]	S [4,6,7]
2	Diphenylcyclopropenone	886-38-4	0.0003	S [1]	S [4,6]
3	MCI/MI (Kathon CG)	55965-84-9/ 96118-96-6	0.008	S [1]	S [4,7]
4	MI (5-Chloro-2-methyl-4-isothiazolin-3-one)	2682-20-4	0.009	S [1]	S [4,6]
5	p-Phenylenediamine	106-50-3	0.11	S [3]	S [5,6,7]
6	Tetrachlorosalicylanilide	1154-59-2	0.04	S [1]	S [4,6]
Human Category 2					
7	Benzisothiazolinone	2634-33-5	2.3	S [1]	S [4,6]
8	Diethyl maleate	141-05-9	5.8	S [1]	S [4,6,7]
9	Formaldehyde	50-00-0	0.61	S [1]	S [4,5,6,7]
10	Glutaraldehyde	111-30-8	0.1	S [1]	S [4,6]
11	Glyoxal	107-22-2	1.4	S [1]	S [4,6]
12	Isoeugenol	97-54-1	1.2	S [3]	S [6,7]
13	Lauryl gallate	1166-52-5	0.3	S [1]	S [4,6]
14	Propyl gallate	121-79-9	0.32	S [1]	S [4,6,7]
15	Thioglycerol	96-27-5	3.6	NS [3]	S [5]
Human Category 3					
16	Benzoyl Peroxide	94-36-0	0.004	S [1]	S [4,6]
17	Citral	5392-40-5	13	S [2]	S [6,7]
18	Coumarin	91-64-5	NC	NS	NS [4,6]
19	Eugenol	97-53-0	12.9	S [2]	S [6,7]
20	Farnesol	4602-84-0	28	S [3]	NS [6]
21	Hydroquinone	123-31-9	0.1	S [2]	S [6]
22	2-Hydroxyethyl acrylate	818-61-1	1.4	S [1]	S [4,6]
23	Mercaptobenzothiazole	149-30-4	1.7	S [1]	S [4,5,6,7]
24	Methyl-2,3-hexanedione	13706-86-0	26	S [1]	S [4,6]
25	Metol	55-55-0	0.8	S [1]	S [4,6]
Human Category 4					
26	Amylcinnamal	122-40-7	11	NS [1]	NS [4,6]
27	Ethyl acrylate [*a]	140-88-5	28	S [1]	S [4,6,7]
28	Ethylenglycol dimethacrylate	97-90-5	28	NS [1]	S [4,6,7]
29	Hydroxycitonellal	107-75-5	33	S [1]	S [4,6,7]
30	Methyl methacrylate	80-62-6	90	NS [3]	S [5,7]
31	Resorcinol	108-46-3	5.5	NS [1]	NS [4,6]
Human Category 5					
32	4-Aminobenzoic acid	150-13-0	NC	NS [2]	NS [5,7]
33	Benzaldehyde	100-52-7	NC	S [1]	NS [4,6]
34	Benzyl benzoate	120-51-4	17	NS [1]	NS [4,6]
35	Hexylcinnamal	101-86-0	11	NS [1]	NS [4,6,7]
36	Isopropanol	67-63-0	NC	NS [1]	NS [4,5,6,7]
37	Isopropyl miristate	110-27-0	NC	NS [1]	NS [4,6,7]
38	Limonene (not oxidized)	138-86-3/ 5989-27-5	69	NS [3]	S
39	Methyl salicylate	119-36-8	NC	NS [1]	NS [6,7]
40	Propyl paraben	94-13-3	NC	NS [1]	NS [4,5,6]
41	Propylene glycol	57-55-6	NC	NS [1]	NS [4,6,7]
Human Category 6					
42	Butanol	71-36-3	NC	NS [1]	NS [4,6,7]
43	Diethyl phthalate	84-66-2	NC	NS [1]	NS [4,6]
44	Glycerol	56-81-5	NC	NS [1]	NS [4,6,7]
45	Hexane	110-54-3	NC	NS [1]	NS [4,6,7]
46	Lactic acid	50-21-5	NC	NS [1]	NS [4,6,7]
47	Octanoic acid	124-07-2	NC	NS [1]	NS [4,6]
48	Xylene	1330-20-7	95.8 [*b]	NS [3]	NS [6]

#Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

1. Data being submitting as an article
2. Reference data of lead laboratory in validation Phase II
3. Unpublished data in lead laboratory
4. G. F. Gerberick *et al*. (2007) *Toxicol. Sci*., 97(2):417-427.
5. DPRA validation study report (2012)
6. Natsch *et al*. (2013) *J. Appl. Toxicol.* 33 (11), 1337–1352.
7. Bauch *et al*. (2012) *Regul. Toxicol. Pharmacol*. 63(3):489-504.
8. Urbisch D. *et al* (2015) *Regul. Toxicol. Pharmacol*. 71(2):337-51

Reference result (human data)	ADRA		DPRA		LLNA	
	+	-	+	-	+	-
+ (n=41)	26	15	28	13	33	8
- (n=7)	0	7	0	7	1	6
Total	26	22	28	20	34	14
Sensitivity (%)	63.4		68.3		80.5	
Specificity (%)	100.0		100.0		85.7	
Accuracy (%)	68.8		72.9		81.3	

Figure 1 Predictive capacity of 48 chemicals

Reference result (human data)	ADRA		DPRA		LLNA	
	+	-	+	-	+	-
+ (n=31)	25	6	27	4	30	1
- (n=7)	0	7	0	7	1	6
Total	25	13	27	11	31	7
Sensitivity (%)	80.6		87.1		96.8	
Specificity (%)	100.0		100.0		85.7	
Accuracy (%)	84.2		89.5		94.7	

Figure 2 Predictive capacity of 38 chemicals (chemicals of Human Category 5 were excpted)

Test chemicals that were accurately predicted to be sensitizers using DPRA but were non-sensitizers using ADRA

There were four test chemicals that have been reported to be sensitizers in human subjects and which were correctly predicted to be sensitizers using DPRA, but which were incorrectly predicted to be non-sensitizers using ADRA.

Thioglycerol

Thioglycerol has thiol groups that react and bond with the thiol groups of cysteine residues. Also, it is

known that even minute quantities of copper or other metallic ions in chemical solutions act as a catalyst to induce reactions between thiol groups and promote oxidation. In ADRA, EDTA is used as an additive to chelate metallic ions and thereby prevent NAC oxidation (dimerization) that results when thiol groups react with one another. Aside from this, it is possible that the reactivity of nucleophilic agents such as NAC (or the cysteine peptide used in DPRA) with thiolglycerol and other chemicals that have thiol groups is promoted in the presence of minute quantities of metallic ions. Because of this, we conclude that use of EDTA in so-called "reactivity assays," like ADRA and DPRA, which are designed to measure the true reactivity of a nucleophilic reagent with a test chemical, will provide accurate and essential results. In fact, thiolglycerol was correctly predicted to be a sensitizer using DPRA and a version of ADRA without EDTA, but was incorrectly predicted to be a non-sensitizer by the version of ADRA with EDTA that was used for this validation study. This discrepancy is assumed to be due to the effects of chelating metallic ions with EDTA additive, but the VMT considers it to be likely that this prediction is correct and this is a limitation for test methods based on the principles of ADRA and DPRA.

Limonene

Limonene is a prehapten, and for this reason is excluded from the applicability domain of ADRA, just as it was excluded from that of DPRA (Basketter *et al*. 2010). When oxidized, pre-haptens change to reactive sensitizers. In some cases, minute quantities of metallic ions contribute to and promote this oxidation. For this reason, although the presence of minute quantities of metallic ions promoted limonene oxidation in DPRA, the addition of EDTA in ADRA prevented limonene oxidation due to metallic ions, which is what we think might have caused the discrepancy between the DPRA and ADRA predictions results. If this is correct, as with thioglycerol above, we conclude that use of EDTA in a test method designed to measure the true reactivity of a nucleophilic reagent with a test chemical will provide accurate results.

Also, limonene is classified as Category 5 for human sensitization, and since it was predicted to be an extremely weak sensitizer with an EC3 of 69% in LLNA, the VMT thinks it was predicted to be a non-sensitizer in ADRA because of its low reactivity with proteins.

Ethylenglycol dimehtacrylate

In general, "acrylates" are known to exhibit clearly skin sensitization and other toxicity, because they react readily with thiol groups in cysteine and amino groups in lysine. In contrast to this, "methacrylates" have methyl groups in close proximity to the carbonyl groups, which play a role in reactivity with methacryloyl groups, and because they are electron-donors, are significantly less reactive than "acrylates". Thus, in general, "methacrylates" are known to exhibit significantly less skin sensitization and other toxicity than "acrylates".

Ethylenglycol dimethacrylate exhibits extremely weak sensitization, because it has a methacryloyl group. Thus the accurate prediction of skin sensitization potential using ADRA to test "methacrylates", which have extremely low reactivity and are only weak sensitizers, might be difficult.

Methyl methacrylate

As with ethylenglycol dimethacrylate above, methyl methacrylate exhibits extremely weak sensitization, because it has a methacryloyl group. In fact, it is classified as Category-4 weak sensitizer for human sensitization and was predicted to be a weak sensitizer with an EC3 of 90% in LLNA. As with ethylenglycol dimethacrylate above, the accurate prediction of skin sensitization potential using ADRA to test “methacrylates”, which have extremely low reactivity and are only weak sensitizers, might be difficult.

3. Conclusion

The VMT compared the predictive capacity of ADRA, DPRA, and LLNA relative to human sensitization. Of the 48 test chemicals common to DPRA and ADRA, ten were human Category 5 test chemicals, which in clinical use are known to cause sensitization in rare cases. When these 10 test chemicals are excluded, accuracy for the remaining 38 test chemicals is 84.2% for ADRA, 89.5% for DPRA, and 94.7% for LLNA, resulting in an overall accuracy of greater than 84% for all test methods. Although ADRA exhibits a slightly lower accuracy for these 38 test chemicals than either DPRA or LLNA, the difference with DPRA is only five percentage points, which the VMT considers to be more than sufficient for predicting human sensitization potential.

Reference

Basketter, *et al.*, (2014), Categorization of Chemicals According to Their Relative Human Skin Sensitizing Potency. *Dermatitis*, **25**:11-21.

Appendix 6

Results of Phase-1 and Phase-2 Performed by FUJIFILM

1. Background and objectives

The objective of the testing performed during this validation study is to demonstrate that the participating laboratories are able to replicate to a high degree of certainty the same accurate test results obtained and published by the lead laboratory. Given this objective and the fact that the lead laboratory is experienced in this technology, out of fairness alone, the lead laboratory itself would ordinarily not participate in validation testing. In the event, however, that the lead laboratory were to participate in validation testing under the same conditions as the participating laboratories, test results and comments from the lead laboratory would be useful in reviewing the results of the participating laboratories, and in cases where problems were encountered during the validation, could be considered helpful in finding solutions. Thus, FUJIFILM, the lead laboratory, mirrored the ADRA validation, testing the encoded chemicals and undergoing QC checks under the same conditions as the other participating laboratories. Furthermore, test results obtained by the lead laboratory were handled as reference data and used in the evaluation of the validation testing.

2. Results and Discussion

2-1. Phase I results at FUJIFILM

Table 1 below shows the % depletion, standard deviation (SD), and mean % depletion for both NAC and NAL of the ten chemicals tested at FUJIFILM during Phase I. Predictions of sensitization potential are shown in Table 3.

Table 1　Summary of Phase I results at FUJIFILM

No.	Chemical	LLNA category	FUJIFILM																	
			Experiment 1						Experiment 2						Experiment 3					
			NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Average score (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Average score (%)	Predicted class	NAC depletion (%)	SD (%)	NAL depletion (%)	SD (%)	Average score (%)	Predicted class
A	Lauryl gallate	Strong	100.0	0.0	93.3	0.0	96.7	S	100.0	0.0	93.2	0.5	96.6	S	100.0	0.0	90.4	2.3	95.2	S
B	4-(Methylamino) phenol hemisulfate salt	Strong	100.0	0.0	15.5	0.3	57.8	S	100.0	0.0	15.6	0.5	57.8	S	100.0	0.0	12.3	0.2	56.1	S
C	Chloramine T	Strong	100.0	0.0	97.8	0.1	98.9	S	98.0	0.1	97.7	0.1	97.8	S	97.9	0.0	98.1	0.1	98.0	S
D	Cinnamaldehyde	Moderate	45.4	0.3	8.6	0.4	27.0	S	43.0	1.2	9.4	0.3	26.2	S	43.7	0.6	8.5	0.4	26.1	S
E	2-Mercaptobenzothiazole	Moderate	52.1	0.6	3.2	0.1	27.7	S	52.1	0.5	3.5	0.4	27.8	S	49.0	0.6	2.5	0.3	25.8	S
F	Ethyl acrylate	Weak	97.6	0.2	7.3	0.2	52.5	S	94.2	0.4	6.1	0.3	50.1	S	96.4	0.4	3.9	0.1	50.2	S
G	Imidazolidinylurea	Weak	28.3	2.8	0.9	0.4	14.6	S	30.5	2.5	0.2	0.2	15.3	S	23.3	1.8	0.0	0.0	11.7	S
H	Glycerol	NC	1.7	0.1	0.0	0.0	0.9	NS	0.0	0.0	0.2	0.2	0.1	NS	0.0	0.0	0.0	0.0	0.0	NS
I	Salicylic acid	NC	3.6	0.3	3.4	0.3	3.5	NS	4.8	0.3	3.3	0.5	4.1	NS	0.4	0.4	3.1	0.4	1.7	NS
J	Isopropanol	NC	0.8	0.7	0.4	0.1	0.6	NS	0.0	0.0	0.5	0.4	0.2	NS	0.0	0.0	0.0	0.0	0.0	NS

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

There were no major discrepancies between the results obtained at FUJIFILM for NAC and NAL depletion of the 10 test chemicals and the results obtained at the four participating laboratories. Also, as shown in Tables 15-1 to 15-4, predictions of the test chemicals as either a sensitizer or a non-sensitizer matched perfectly with the predictions from both LION and MITSUI and did not differ significantly from the results at the other two laboratories.

Based on these results, the results from FUJIFILM were nearly identical with those from the participating laboratories, and a comparison of the results from all five laboratories indicates good within-laboratory reproducibility.

2-2. Phase II results at FUJIFILM

Table 2 shows % depletion, standard deviation (SD), and mean % depletion of both NAC and NAL obtained by FUJIFILM for the 30 chemicals tested in Phase II. Predictions of sensitization potential are shown in Table 3.

There were no major discrepancies between the results obtained at FUJIFILM for NAC and NAL depletion of the 30 test chemicals and the results obtained at the four participating laboratories. Also, predictions of the test chemicals as either a sensitizer or a non-sensitizer matched perfectly with the predictions from NISSAN and did not differ significantly from the results at the other three laboratories.

Based on these results, the results from FUJIFILM were nearly identical with those from the participating laboratories, and a comparison of the results from all five laboratories adds to the evidence that there is good between-laboratory reproducibility.

Table 2 Summary of Phase II results at FUJIFILM

■ NAC depletion

№	Chemicals	FUJIFILM					
		Exp.1	SD	Add. Exp.1	SD	Add. Exp.2	SD
11	Diphenylcyclopropenone	32.4	0.5				
12	p-Benzoquinone	100.0	0.0				
13	Hydroquinone	100.0	0.0				
14	2-Methyl-2H-isothiazol-3-one	92.4	0.1				
15	2-Aminophenol	100.0	0.0				
16	Iodopropynyl butylcarbamate	100.0	0.0				
17	Propyl gallate	100.0	0.0				
18	Dihydroeugenol	2.4	0.9	6.7	0.2	1.9	2.1
19	Benzylsalicylate	1.2	0.2				
20	Squaric acid diethylester	28.5	0.5				
21	citral	7.2	0.9	13.5	0.3		
22	Palmitoyl Chloride	2.7	0.8				
23	Resorcinol	5.7	0.4				
24	Benzylcinnamate	2.2	0.9				
25	2,3-Butanedione	29.6	0.3				
26	Farnesal	26.5	0.7				
27	Eugenol	10.4	0.6	18.4	0.7		
28	Penicilln G	2.2	0.2				
29	Lilial	12.9	0.4	16.5	0.5		
30	Hydroxycitronellal	4.9	0.1				
31	Benzyl alcohol	3.4	0.5				
32	Dimethylisophthalate	2.0	0.6				
33	4-Aminobenzoic acid	2.0	0.3				
34	Diethyl phthalate	2.1	0.2				
35	Methylsalicylate	0.1	0.1				
36	Dextran	8.9	0.1	7.7	0.3	0.2	0.4
37	Coumarin	1.3	0.3				
38	Propyl paraben	1.4	0.3				
39	Sulfanilamide	0.2	0.2				
40	Fumaric acid	0.0	0.0				

■ NAL depletion

№	Chemicals	FUJIFILM					
		Exp.1	SD	Add. Exp.1	SD	Add. Exp.2	SD
11	Diphenylcyclopropenone	4.0	0.2				
12	p-Benzoquinone	60.7	0.3				
13	Hydroquinone	25.2	0.4				
14	2-Methyl-2H-isothiazol-3-one	0.0	0.0				
15	2-Aminophenol	34.6	1.2				
16	Iodopropynyl butylcarbamate	0.4	0.3				
17	Propyl gallate	56.9	0.1				
18	Dihydroeugenol	5.5	0.4	4.8	3.2	1.1	1.3
19	Benzylsalicylate	0.8	0.6				
20	Squaric acid diethylester	70.4	0.5				
21	citral	2.6	0.6	3.0	2.3		
22	Palmitoyl Chloride	79.0	6.6				
23	Resorcinol	0.2	0.2				
24	Benzylcinnamate	1.4	0.2				
25	2,3-Butanedione	6.1	0.3				
26	Farnesal	6.9	0.4				
27	Eugenol	3.4	0.6	6.6	0.5		
28	Penicilln G	2.0	0.3				
29	Lilial	2.1	0.3	4.4	0.4		
30	Hydroxycitronellal	0.4	0.2				
31	Benzyl alcohol	0.3	0.3				
32	Dimethylisophthalate	1.9	0.2				
33	4-Aminobenzoic acid	0.7	0.3				
34	Diethyl phthalate	1.6	0.3				
35	Methylsalicylate	0.1	0.1				
36	Dextran	0.7	0.3	6.2	5.4	0.3	0.3
37	Coumarin	0.6	0.4				
38	Propyl paraben	0.9	0.4				
39	Sulfanilamide	0.0	0.0				
40	Fumaric acid	0.2	0.2				

Table 3 Summary of Phase I and Phase II results at all four participating laboratories and the lead laboratory

No.	Chemicals	LION	MITSUI	SUMITOMO	NISSAN	FUJIFILM
Average of 3 experiments						
1	Lauryl gallate	96.8	71.5	71.6	96.5	96.1
2	4-(Methylamino) phenol hemisulfate salt	56.3	57.3	57.3	56.4	57.2
3	Chloramine T	95.2	99.3	97.2	53.2	97.9
4	Cinnamaldehyde	24.7	22.1	45.1	22.5	26.4
5	2-Mercaptobenzothiazole	22.9	27.4	32.5	48.1	27.1
6	Ethyl acrylate	45.8	47.3	40.9	51.5	50.9
7	Imidazolidinylurea	12.2	10.6	14.1	15.8	13.9
8	Glycerol	0.3	0.1	1.9	2.2	0.3
9	Salicylic acid	1.5	3.2	3.3	4.4	3.1
10	Isopropanol	0.0	0.2	1.9	2.4	0.3
1 experiment						
11	Diphenylcyclopropenone	18.2	14.2	17.9	13.5	18.2
12	p-Benzoquinone	81.1	84.2	79.3	72.2	80.3
13	Hydroquinone	62.7	62.9	70.6	64.4	62.6
14	2-Methyl-2H-isothiazol-3-one	50.2	50.0	50.2	48.4	46.2
15	2-Aminophenol	68.2	66.0	66.5	63.3	67.3
16	Iodopropynyl butylcarbamate	50.3	47.2	50.2	50.0	50.2
17	Propyl gallate	86.5	82.0	84.3	84.2	78.5
18	Dihydroeugenol	4.5 / 6.5 / 5.6	2.9	5.1 / 5.2	2.2	3.9 / 5.8 / 1.5
19	Benzylsalicylate	0.8	0.4	1.9	0.0	1.0
20	Squaric acid diethylester	47.7	48.4	46.0	34.5	49.4
21	citral	4.6 / 6.9 / 6.5	5.1 / 2.4 / 6.3	6.1 / 0.0 / 2.8	3.4 / 8.3 / 11.9	4.9 / 8.3
22	Palmitoyl Chloride	46.5	27.5	29.9	47.8	40.9
23	Resorcinol	2.8	0.6	6.9 / 3.8 /4.6	3.0 / 4.4	2.9
24	Benzylcinnamate	0.8	0.0	0.5	0.1	1.8
25	2,3-Butanedione	15.2	17.8	19.5	34.5	17.8
26	Farnesal	20.2	18.6	18.9	18.9	16.7
27	Eugenol	12.2	16.1	19.1	10.8	6.9 / 12.5
28	Penicilln G	1.2	0.0	3.0 / 1.4	0.2	2.1
29	Lilial	7.1 / 7.0	13.8	4.9 / 6.2	6.6 / 15.0	7.5 / 10.5
30	Hydroxycitronellal	3.9 / 10.5 / 6.4	11.3	6.4 / 5.4	3.7 / 7.0 / 4.6	2.7
31	Benzyl alcohol	0.2	2.5	2.2	0.2	1.8
32	Dimethylisophthalate	0.9	0.5	0.0	0.6	1.9
33	4-Aminobenzoic acid	1.1	0.0	2.3	1.3	1.4
34	Diethyl phthalate	0.8	0.0	0.0	0.1	1.8
35	Methylsalicylate	0.6	0.0	0.5	0.0	0.1
36	Dextran	5.0 / 1.4 / 4.7	1.1	7.2 / 7.5	2.9	4.8 / 7.0 / 0.3
37	Coumarin	4.6 / 2.7	0.8	1.8	0.1	0.9
38	Propyl paraben	1.0	0.1	0.2	0.0	1.1
39	Sulfanilamide	0.9	1.7	1.6	7.4 / 2.9 / 2.3	0.1
40	Fumaric acid	5.3 / 3.5 / 3.1	2.4	2.4	0.6	0.1

*Cells highlighted in pink indicate test chemicals that were predicted to be sensitizers (S), and cells highlighted in blue indicate test chemicals that were predicted to be non-sensitizers (NS).

Appendix 7

Advantages of ADRA over DPRA:

No precipitation of test chemicals, No co-elution of test chemicals with NAC or NAL, and suppression of NAC oxidation by EDTA

1. Precipitation of poorly soluble test chemicals in the reaction solution

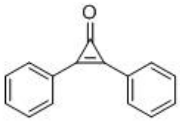
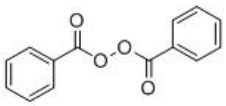
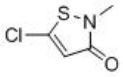
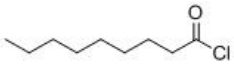
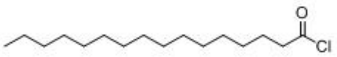
DPRA requires test chemical solutions to have a concentration of 100 mM, and even in cases where it is possible to prepare a 100-mM solution of a poorly soluble test chemical, the reaction solution, including nucleophilic reagents, is 75% water, which results in numerous cases of precipitation and turbidity of the test chemical in the reaction solution. In contrast to this, since the test chemical solution used for ADRA has a concentration of 1 mM, which is just 1% of that used in DPRA, the lead laboratory has thus far found virtually no precipitation or turbidity in the reaction solution even with poorly soluble test chemicals. Table 1 below shows some specific examples.

As can be seen in Table 1, when using DPRA, there are some cases of precipitation irrespective of the value of cLogP. Precipitation and turbidity of the test chemical in the reaction solution is an indication that the test chemical solution has fallen below the required concentration, which means that an accurate prediction cannot be obtained. Even when the test chemical solution falls below the required concentration due to precipitation or turbidity of the test chemical, there are some cases where a sufficient mean depletion is obtained to result in a prediction of sensitizer. Caution is also needed, because in some cases a low mean depletion will result in a prediction of non-sensitizer. Simply stated, it is highly desirable that there be no precipitation or turbidity of the test chemical in the reaction solution.

On the other hand, as can also be seen in Table 1, test chemical solutions prepared for ADRA almost never exhibit precipitation or turbidity. Palmitoyl chloride was the only test chemical that exhibited even the slightest turbidity in the NAC reaction solution. The cause of this turbidity is palmitoyl chloride's cLogP of 7.5, which the VMT considers to be a clear indication of how poorly soluble this test chemical is. When tested using DPRA, the test chemical solution for palmitoyl chloride exhibited a significant amount of precipitation in the reaction solutions for either peptide.

Based on the above, the VMT concludes that relative to DPRA, test chemical solutions prepared for ADRA exhibit very little precipitation in the reaction solution.

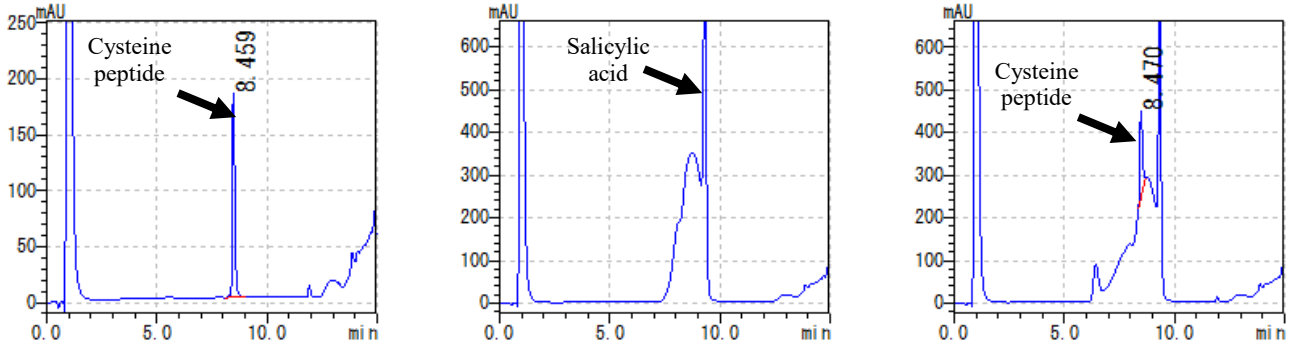
Table 1　Solubility of test chemicals in DPRA and ADRA reaction solutions

Test chemicals	Structure	cLogP	LLNA category	DPRA		ADRA	
				Cys-peptide	Lys-peptide	NAC	NAL
Diphenylcyclopropenone		1.8	Extreme				
Benzoyl peroxide		2.8	Extreme				
5-Chloro-2-methyl-4-isothiazolin-3-one		0.6	Extreme				
Nonanoyl chloride		3.8	Moderate				
Palmitoyl Chloride		7.5	Moderate				

2. Examples of co-elution of the test chemical with the nucleophilic reagent

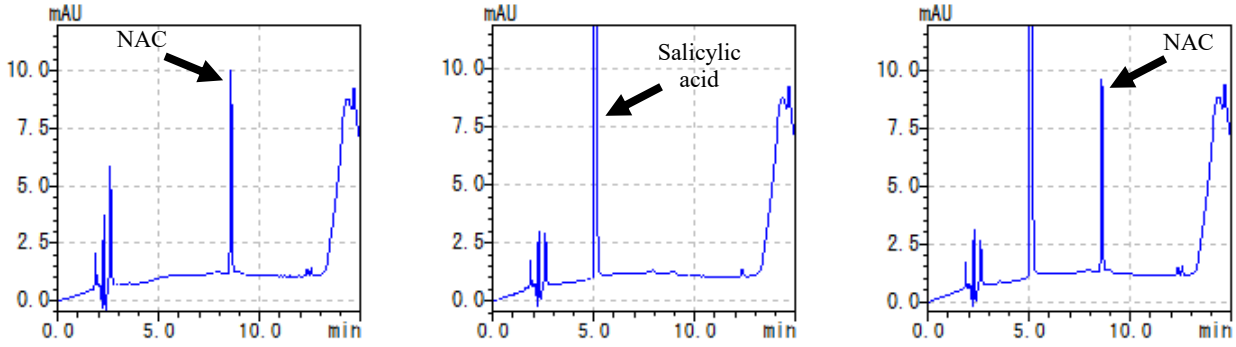
Sensitization potential is predicted in DPRA by calculating depletion from a value obtained per HPLC analysis of the unreacted cysteine peptide and lysine peptide in a mixture of test chemical solution and nucleophilic reagent. Thus, in cases where the test chemical and the nucleophilic reagent co-elute, such a value cannot be obtained and sensitization potential cannot be predicted. In cases, however, where co-elution occurs only between lysine peptide and the test chemical, predictions can be made based on the "cysteine only prediction model". Nevertheless, DPRA is designed to make predictions of sensitization based on mean depletion calculated after correctly quantifying both peptides.

The two peptides used for DPRA are known to co-elute with a number of test chemicals. In contrast to this, the lead laboratory has never experienced co-elution of NAC or NAL with a test chemical when using ADRA. For this reason, the VMT considers it very unlikely that any test chemical would co-elute with either NAC or NAL. In Figures 1 to 4 below are shown examples of co-elution with a test chemical in DPRA using the same HPLC parameters as the lead laboratory and of the same test chemical in ADRA.



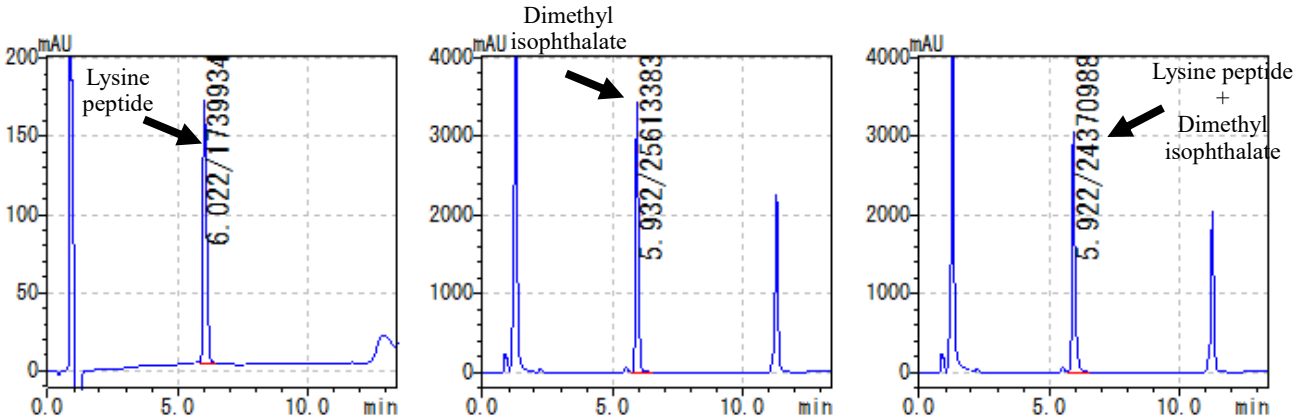
Cysteine peptide (standard) Salicylic acid (standard) Cysteine peptide reacted with salicylic acid

Figure 1 Co-elution of salicylic acid and cysteine peptide in DPRA



NAC (standard) Salicylic acid (standard) NAC reacted with salicylic acid

Figure 2 NAC and salicylic acid in ADRA



Lysine peptide (standard) Dimethyl isophthalate (standard) Lysine peptide reacted with dimethyl isophthalate

Figure 3 Co-elution of dimethyl isophthalate and lysine peptide in DPRA

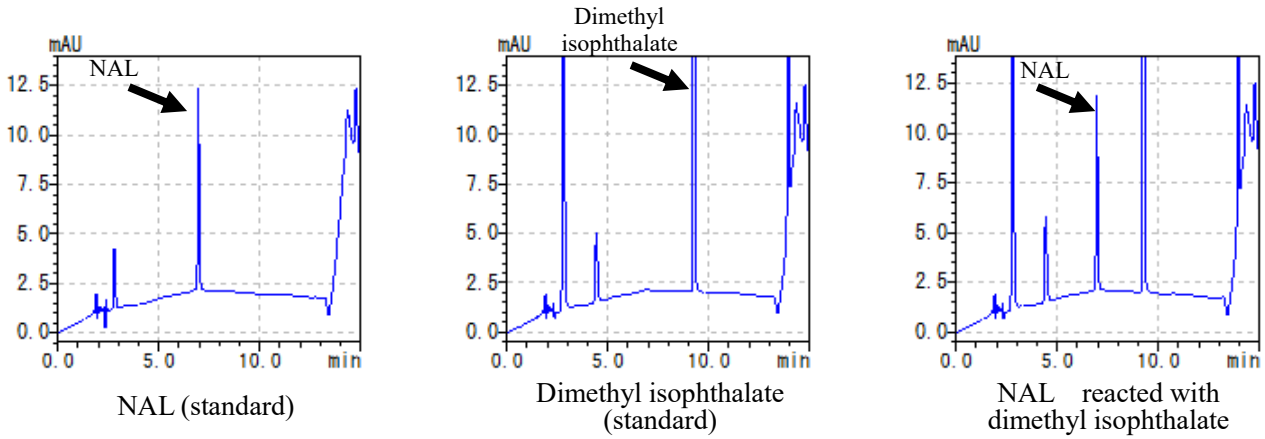


Figure 4 NAL and dimethyl isophthalate in ADRA

Based on HPLC analysis at the lead laboratory, salicylic acid and cysteine peptide co-elute when tested using DPRA, but salicylic acid and NAC do not co-elute when tested using ADRA. Similarly, dimethyl isophthalate and lysine peptide co-elute when tested using DPRA, but dimethyl isophthalate and NAL do not co-elute when tested using ADRA. In contrast to the fact that a number of test chemicals exhibit co-elution with either of the two peptides when tested using DPRA, the lead laboratory has never experienced co-elution between test chemicals and either NAC or NAL when tested using ADRA, most likely due to the following three points.

Why test chemicals exhibit co-elution when tested using DPRA but not when tested using ADRA

1) Differences in quantification wavelength

The quantification wavelength used for DPRA is 220 nm, which is relatively short and falls within the ultraviolet absorption spectrum for many test chemicals, which results in co-elution. On the other hand, the quantification wavelength used for ADRA is 281 nm, which is relatively long, and unless the test chemical structure includes a relatively long conjugated system, there will be virtually no co-elution between the test chemical and NAC or NAL, because the test chemical has no absorption of the ultraviolet spectrum in that wavelength region.

2) Differences in column

The column used in DPRA is ordinarily filled with silica gel, whereas that used in ADRA has a core shell structure with less dispersion and sharper peaks than columns that use of silica gel. Because of this, the sharper peaks co-elute less with other peaks.

3) Differences in the concentration of the sample injected into the column

The concentrations of the test chemical in solution that are injected into the column in DPRA is 5 mM for the cysteine peptide reaction solution and 25 mM for the lysine peptide reaction solution. In general, many examples for HPLC-UV method given in the technical reports from the column manufacturer use sample concentrations between approximately 1 and 100 µg/mL. For a sample with a molecular weight of 200, the concentration would normally be between 5 and 500 µM. Although DPRA calls for

quantification of two different peptides, the concentration of the test chemical in solution is between 5 and 25 mM, which is between 10 and 100 times (in case of the prediction by cysteine peptide), or 50 and 50,000 times (in case of the prediction by lysine peptide) more concentrated than the 5 to 500 μM concentrations normally used in HPLC analysis. This presents the possibility of test chemical overload, which means a strong likelihood of broadened or separated peaks overlapping with the peaks of the peptides.

In contrast to this, the concentrations of the test chemical in solution that are injected into the column in ADRA is 200 μM for both NAC or NAL. This concentration is within the range of concentration used in normal HPLC analysis, which is one of the reasons why there is no test chemical overload affecting the peaks, and therefore good separation with no overlapping.

3. Suppression of NAC oxidation by EDTA

Thiols readily undergo oxidation to form oxidants (dimers). Although oxygen itself is involved in oxidation, the presence of minute quantities of metal ions in solution is known to have a catalytic effect that promotes oxidation.

Both DPRA and ADRA test for covalent bonding between cysteine and a test chemical, and to this end use the nucleophilic reagents cysteine peptide and NAC, respectively. As described above, both these substances are oxidized readily due to the thiol groups in cysteine.

In order to suppress this oxidation, the ADRA SOP calls for the addition of EDTA to the NAC stock solution as a means of chelating metal ions. Here is an example of how EDTA suppresses NAC oxidation.

As shown in Figure 5, when EDTA is not added to the NAC stock solution, the level of residual NAC immediately after preparation of the reaction solution is roughly 98% on Day 0, but after 24 hours at 25°C falls nine percentage points to 89%. In other words, roughly 9% of the NAC has been oxidized to form dimers. Compare this with Figure 6, which shows that when EDTA is added to the NAC stock solution, the level of residual NAC immediately after preparation of the reaction solution is roughly 99% on Day 0, and even after 24 hours at 25°C remains nearly the same at 98%. Figure 7 compares the results of Figure 5 with those of Figure 6.

Thus, it can be seen that the addition of EDTA suppresses oxidation of NAC. Moreover, the NAC used in ADRA is chemically synthesized by first producing NAC dimers (NAC-D) per chemical modification of cysteine, which are then reduced to synthesize NAC. (Fujita *et al*., 2014) Thus, authentic samples of NAC-D are obtained at the same time as NAC. Furthermore, since NAC-D exhibit roughly the same solubility as NAC itself, detection and quantification is possible using the HPLC parameters specified for ADRA. This is why it is possible to assay NAC and NAC-D at the same time during ADRA testing. This permits the quantitative evaluation of the level of NAC oxidation.

Also, there are been some reports of cysteine peptide oxidation (dimerization) during DPRA testing. (K. Linke, *et al*., 2003; C.M. Cremers, *et al*., 2013; P. Nagy, 2013; J.R. Winther, *et al*., 2013) The cysteine peptides used in DPRA are synthesized by sequential linking of amino acid monomers with peptide (amide) bonds, but cysteine peptide oxidation (cysteine peptide dimerization) cannot take place during this process. Also, because it appears that cysteine peptide dimers tend to precipitate in the reaction

solution, it is difficult to detect cysteine dimers using the HPLC parameters specified for DPRA.

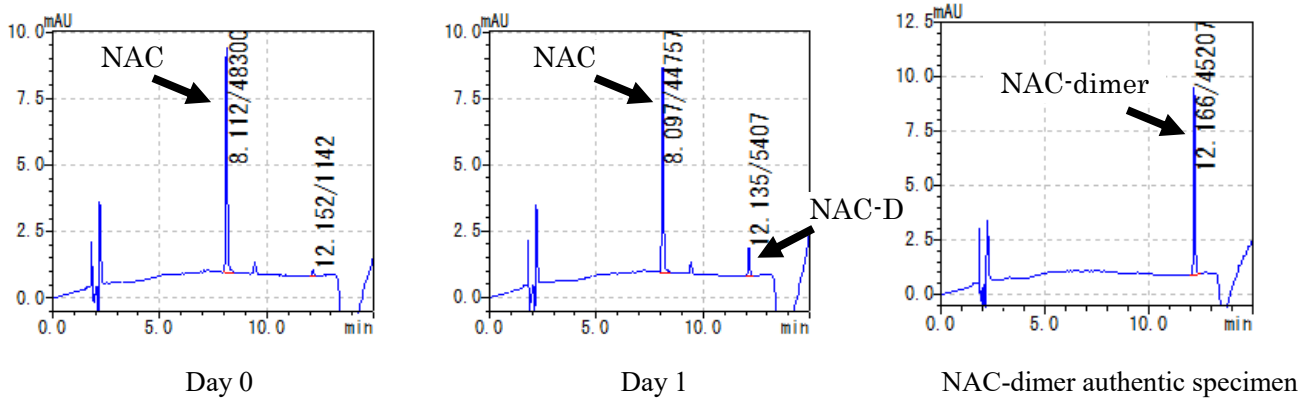


Figure 5　NAC oxidation after one day at 25°C without EDTA added

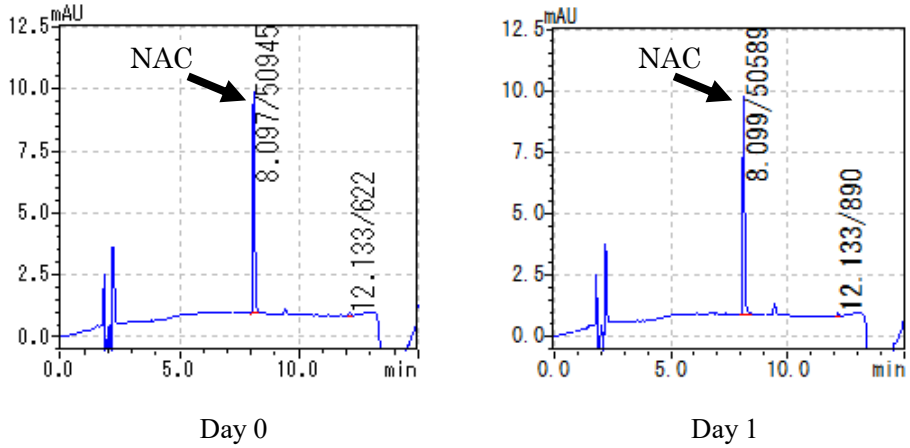


Figure 6　NAC oxidation after one day at 25°C with EDTA added

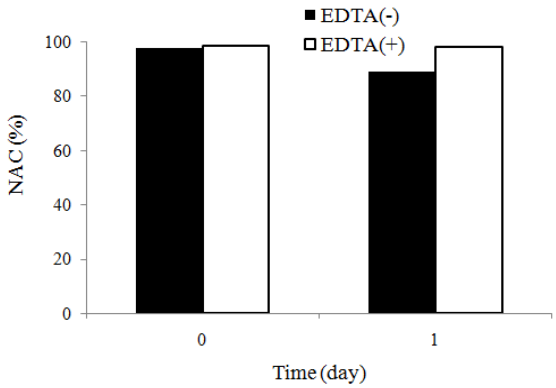


Figure 7　Changes over time at 25°C in levels of residual NAC with and without EDTA added

Appendix 8

Comparing Measurement Accuracy in ADRA with that in DPRA

1. Background and objectives

One major feature of ADRA is that the quantification wavelength of 281 nm was selected to match the maximum absorption wavelength (λ max.) of naphthalene and is roughly 60 nm longer than the 220 nm wavelength used for the nucleophilic reagents in DPRA. Also, insofar as the 220 nm wavelength used in DPRA is not λ max, there is the danger that even a small discrepancy in wavelength could result in a major discrepancy in the measured absorbency. In contrast to this, because the 281 nm wavelength used in ADRA was selected to match λ max, discrepancies in wavelength have little effect. And unless the test chemical is naphthalene or other substance having extremely long conjugated double bonds, neither will impurities have any effect. Thus, the VMT expects to achieve very stable quantification results.

To demonstrate this, we compared quantification accuracy for the 82 test chemicals tested using both ADRA and DPRA, using the measured values reported by Gerberick *et al*. (2010) for DPRA and the measured values for ADRA.

2. Results and Discussion

2-1. Extreme/strong sensitizers

Of the 82 test chemicals, 18 were classified as extreme/strong sensitizers according to LLNA categories. The VMT compared the standard deviations (SD, N = 3) for the % depletion of NAC in ADRA with the SD for the % depletion of cysteine peptide in DPRA as well as the SD for the % depletion of NAL in ADRA with the SD for the % depletion of lysine peptide in DPRA for these 18 test chemicals.

(A) SD for % depletion of NAC and cysteine peptide (B) SD for % depletion of NAL and lysine peptide

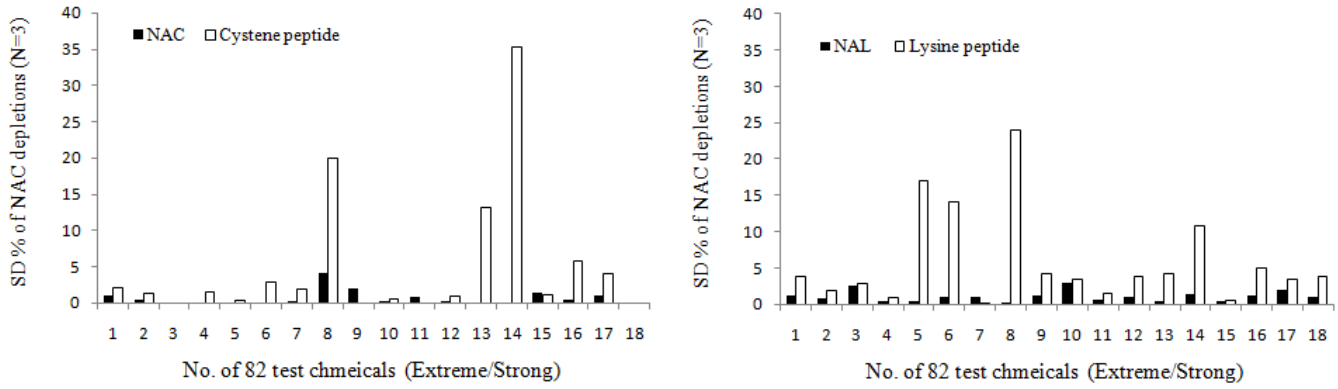


Figure 1. Comparison of the SD for % depletion in ADRA with the SD for % depletion in DPRA for 18 extreme/strong sensitizers

A great many test chemicals classified as extreme/strong sensitizers according to LLNA categories have very good reproducibility, because they have % depletion values near 100. Thus, the SD for % depletion of NAC and NAL in ADRA was less than 5% for all 18 test chemicals. On the other hand, in DPRA, there were four test chemicals with a SD of 5% or greater for % depletion of cysteine peptide

as well as four test chemicals with a SD of 5% or greater for % depletion of lysine peptide, for a total of eight.

2-2. Moderate sensitizers

Next, the VMT made the same comparison for the 20 test chemicals that were classified as moderate sensitizers according to LLNA categories. The results are shown in Figure 2.

(A) SD for % depletion of NAC and cysteine peptide (B) SD for % depletion of NAL and lysine peptide

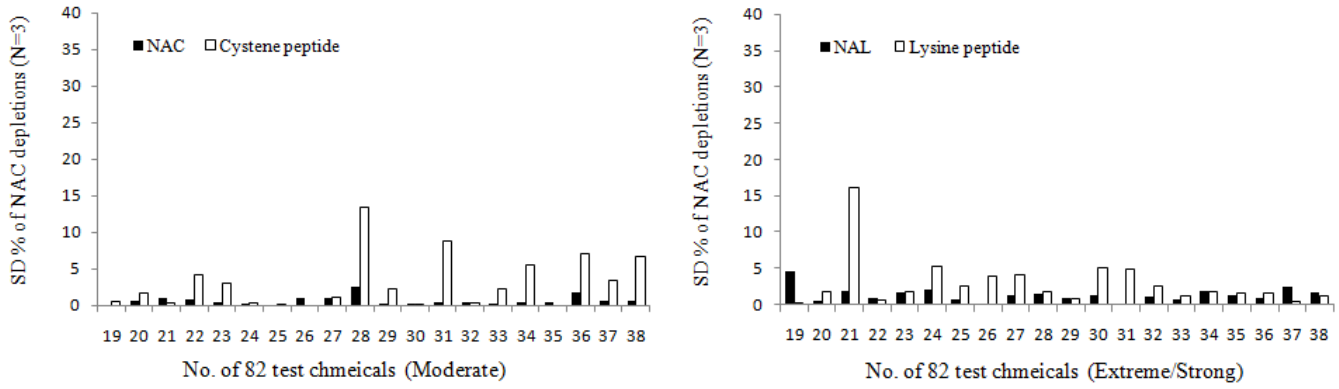


Figure 2. Comparison of the SD for % depletion in ADRA with the SD for % depletion in DPRA for 20 moderate sensitizers

A great many test chemicals classified as moderate sensitizers according to LLNA categories can be expected to have relatively poor reproducibility (relatively large SD values) compared with extreme/strong sensitizers, because they have % depletion values in the mid to high range. Nevertheless, the SD for % depletion of NAC and NAL in ADRA was less than 5% for all 20 test chemicals. On the other hand, in DPRA, there were five test chemicals with a SD of 5% or greater for % depletion of cysteine peptide as well as three test chemicals with a SD of 5% or greater for % depletion of lysine peptide, for a total of eight.

2-3. Weak sensitizers

Next, the VMT made the same comparison for the 15 test chemicals that were classified as weak sensitizers according to LLNA categories. The results are shown in Figure 3.

(A) SD for % depletion of NAC and cysteine peptide (B) SD for % depletion of NAL and lysine peptide

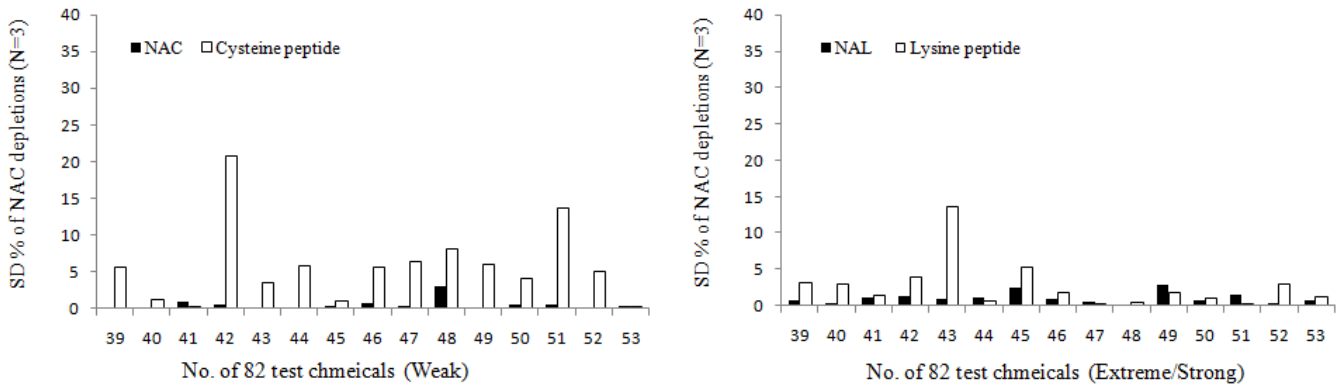


Figure 3. Comparison of the SD for % depletion in ADRA with the SD for % depletion in DPRA for 15 weak sensitizers

A great many test chemicals classified as weak sensitizers according to LLNA categories can be expected to have relatively poor reproducibility (relatively large SD values) compared with extreme/strong sensitizers, because they have % depletion values in the mid to low range.

Nevertheless, the SD for % depletion of NAC and NAL in ADRA was less than 5% for all 15 test chemicals. On the other hand, in DPRA, there were nine test chemicals with a SD of 5% or greater for % depletion of cysteine peptide as well as two test chemicals with a SD of 5% or greater for % depletion of lysine peptide, for a total of eleven.

2-4. Non-sensitizers

Next, the VMT made the same comparison for the 29 test chemicals that were classified as non-sensitizers according to LLNA categories. The results are shown in Figure 4.

(A) SD for % depletion of NAC and cysteine peptide (B) SD for % depletion of NAL and lysine peptide

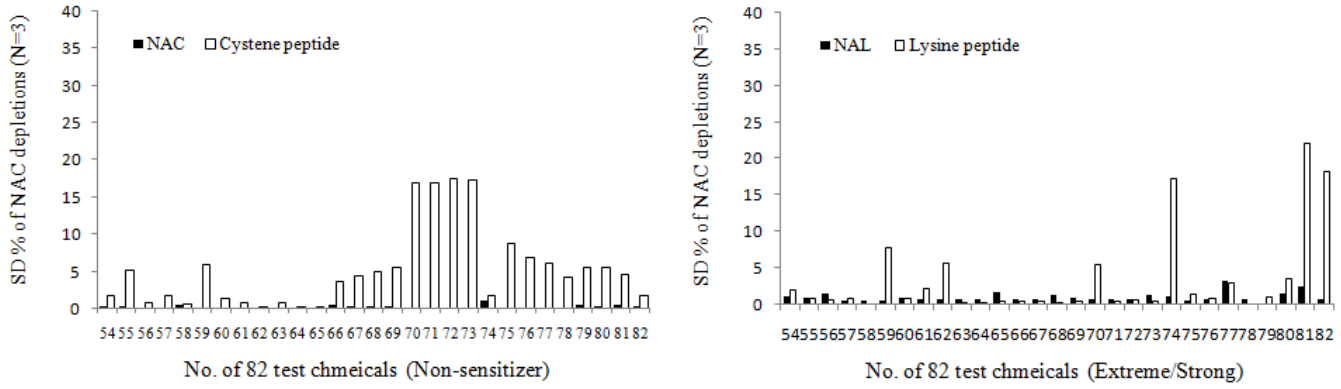


Figure 4. Comparison of the SD for % depletion in ADRA with the SD for % depletion in DPRA for 29 non-sensitizers

A great many test chemicals classified as non-sensitizers according to LLNA categories can be expected to have excellent reproducibility, because they have % depletion values near 0 (zero). Thus, the SD for % depletion of NAC and NAL in ADRA was less than 5% for all 29 test chemicals. On the other hand, in DPRA, there were 13 test chemicals with a SD of 5% or greater for % depletion of cysteine peptide as well as six test chemicals with a SD of 5% or greater for % depletion of lysine peptide, for a total of 19. Also, salicylic acid could not be quantified due to co-elution with lysine peptide.

3. Conclusion

As shown by the above results, the compared with DPRA, quantification of the depletion of the nucleophilic reagents used in ADRA is more precise than that of DPRA, based on reproducibility calculated from three measurements. The main reason for this is described in item 1 below, and items 2–4 provide supplementary reasoning.

1. The relative long quantification wavelength of 281 nm that is used for NAC and NAL was selected to match λ max, thereby achieving stability of the molar absorption coefficient. (See Recommendation No. 2 of the VMT overall conclusions and recommendations.)
2. The addition of a post-reaction fixing solution eliminates further reactivity. (See Recommendation No. 7 of the VMT overall conclusions and recommendations.)
3. The use of test chemical solutions at low concentrations eliminates precipitation in the reaction solution. (See Recommendation No. 1 of the VMT overall conclusions and recommendations.)
4. There is no co-elution of NAC or NAL with the test chemical. (See Appendix 7, 2. Examples of co-elution of the test chemical with the nucleophilic reagent.)

Appendix 9

Articles on ADRA

ADRA Article No. 1 (Fujita *et al*., 2014)
ADRA Article No. 2 (Yamamoto *et al*., 2015)

No.1

Journal of Pharmacological and Toxicological Methods 70 (2014) 94–105

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

Development of a prediction method for skin sensitization using novel cysteine and lysine derivatives

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ARTICLE INFO

Article history:
Received 28 March 2014
Accepted 2 June 2014
Available online 10 June 2014

Keywords:
Allergen
Alternatives
Skin sensitization
Reactivity assay
Prediction model
Direct Peptide Reactivity Assay (DPRA)
NAC
NAL
Amino acid Derivative Reactivity Assay (ADRA)

ABSTRACT

Introduction: The Direct Peptide Reactivity Assay (DPRA) was developed as an alternative simple and versatile method for predicting skin sensitization. Here, we describe a novel Amino acid Derivative Reactivity Assay (ADRA) involving 2 amino acid derivatives: *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine (NAL), in which each amino-terminal residue is introduced into a naphthalene ring. ADRA measurements were conducted at 281 nm, which improved baseline stability, and were less influenced by other substances in the reaction solutions than DPRA measurements that are conducted at 220 nm. **Methods:** Chemically synthesized NAC and NAL were dissolved in phosphate buffers of pH 9.5 and 12.0, respectively. Each solution, test chemical solution, and phosphate buffer, were mixed in 96-well microplates and incubated in the dark for 24 h at 25 °C. Following incubation, samples were diluted 10 times with a mixed solution of 25% acetonitrile/0.5% trifluoroacetic acid (TFA) in water, and NAC and NAL levels were quantified in each sample and control using a high-performance liquid chromatography (HPLC) system. The reactivity of NAC/NAL was calculated as the percent depletion based on the decrease in the non-reacted NAC/NAL concentration in the samples relative to the average concentration in the control; the average NAC/NAL reduction score was calculated. A 2-class classification model was developed using ADMEWORKS/ModelBuilder, and an optimal average score that could discriminate between sensitizers and non-sensitizers was determined. **Results:** A total of 82 test chemicals were applied to ADRA for comparison against DPRA. The prediction accuracy of ADRA was 88%, which was similar to that of DPRA. **Discussion:** ADRA was used to quantify NAC/NAL at 281 nm, which showed high accuracy for the prediction of skin sensitization, similar to that of DPRA. Therefore, ADRA could be used to expand the range of chemicals tested in skin sensitization analyses.

1. Introduction

Allergic contact dermatitis is the clinical manifestation of skin sensitization and is caused by a wide range of chemicals after prolonged or repeated contact with the skin. Skin sensitization/allergic contact dermatitis occurs in 6 steps: (1) skin bioavailability, (2) haptenation, (3) epidermal inflammation, (4) dendritic cell (DC) activation, (5) DC migration, and (6) T-cell proliferation, and various methods for predicting or evaluating skin sensitization corresponding to each step have been developed (Adler et al., 2010; Basketter & Kimber, 2010). For example, for steps 2–6, DPRA, the KeratinoSens test method (Aeby, Python, & Goebel, 2007; Basketter & Kimber, 2009; Emter, Ellis, & Natsch, 2010; Natsch, Emter, & Ellis, 2009; Natsch & Gfeller, 2008; Uchino, Taketzava, & Ikarashi, 2009; Vandebriel & van Loveren, 2010), the myeloid U937 skin sensitization test (MUSST) (Python, Goebel, & Aeby, 2007), the human cell line activation test (h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2009), the in vitro DC-based migration assay (Ouwehand et al., 2010), and the CAATC assay (Aliahmadi et al., 2009), have been proposed, respectively.

In the initial process of skin sensitization, the sensitizers are generally electrophilic and reactive toward nucleophilic sites on proteins, and these characteristics have been used to develop non-animal approaches for evaluating the skin sensitization potential of a number of chemicals (Ahlfors, Sterner, & Hansson, 2003; Alvarez-Sanchez, Basketter, Peace, & Leopoittevin, 2003; Meschkat, Barratt, & Leopoittevin, 2001), among which DPRA is currently the most widely used method (Gerberick et al., 2004; Gerberick et al., 2007). Subsequently, a method using the heptapeptide Cor1-C420

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http://dx.doi.org/10.1016/j.vascn.2014.06.001
1056-8719/

(Natsch & Gfeller, 2008) and similar approaches (Aleksic et al., 2009; Böhme et al., 2009; Roberts, Schultz, Wolf, & Aptula, 2010; Schultz, Yarbrough, & Johnson, 2005) were developed. Among these methods, DPRA has been the most extensively validated, and a prediction accuracy of 89% was demonstrated for a data set that included 82 chemicals (Gerberick et al., 2007). To detect pro-hapten skin sensitizers, a peroxidase peptide reactivity assay using horseradish peroxidase/peroxide was recently proposed (Gerberick et al., 2009; Troutman et al., 2011).

However, the peptides used in DPRA must be detected at 220 nm because of their minimal absorption at longer wavelengths, and various chemical substances also have UV absorption at short wavelengths such as 220 nm. Therefore, these peptides cannot be measured precisely when they co-elute with test chemicals or reaction products in HPLC, as shown by Natsch and Gfeller (2008). Therefore, we chemically synthesized 2 amino acid derivatives with an absorption maximum at 281 nm by introducing a naphthalene ring to the cysteine and lysine residues. We named these derivatives NAC and NAL, respectively, and used them in a novel Amino acid Derivative Reactivity Assay (ADRA). First, to identify the most appropriate conditions for ADRA, we determined the appropriate buffer and buffer pH for the reaction. Furthermore, to allow the experiments to be performed effectively, we facilitated the handling of multiple samples at the same time by using 96-well microplates. Next, to determine the efficacy of ADRA for predicting skin sensitization, we performed a validation test for 82 test chemicals, as reported by Gerberick et al. (2007).

2. Materials and methods

2.1. Test chemicals

The test chemicals for evaluation were those used in the Direct Peptide Reactivity Assay (Gerberick et al., 2007). The chemicals used as test chemicals for ADRA are summarized in Table 1. CD3 (25646-71-3) was supplied by the Synthetic Organic Chemistry Laboratories of FUJIFILM Corporation.

L-cysteine and ε-*N*-BOC-L-lysine for synthesis of NAC and NAL were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 2-(1-naphthyl)acetic acid for synthesis was purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Synthesis of NAC and NAL

2-(1-Naphthyl)acetyl chloride (11.3 g, 55 mmol) was added dropwise into an ice-cooled solution of L-cystine (6.0 g, 25 mmol) and sodium hydroxide (4.2 g, 105 mmol) in water (105 ml), and the mixture was stirred at room temperature for 2 h. Concentrated hydrochloric acid (5 ml) was added to the mixture and the resulting precipitate was collected by filtration. The crude product was recrystallized from ethyl acetate (200 ml) to obtain *N,N'*-di[2-(1-naphthyl)acetyl]-L-cystine (8.7 g, 60%). Zinc powder (9.8 g, 150 mmol) was added to a mixture of *N,N'*-di[2-(1-naphthyl)acetyl]-L-cystine (14.5 g, 25 mmol), and acetic acid (150 ml) was then added and stirred for 6 h at 60 °C. The reaction mixture was poured into 0.5 M hydrochloric acid (300 ml), extracted with ethyl acetate (450 ml), washed with water (150 ml), washed with brine (150 ml), dried over magnesium sulfate, filtered, and evaporated. The residue was crystallized from isopropanol (40 ml) to give NAC (*N*-2-(1-naphthyl)acetyl-L-cysteine) (7.4 g, 54%) (Fig. 1).

2-(1-Naphthyl)acetyl chloride (4.9 g, 24 mmol) was added dropwise to an ice-cooled solution of ε-*N*-Boc-L-lysine (0.5 g, 20 mmol) and sodium hydroxide (0.8 g, 20 mmol) in water (20 ml), and the pH was maintained at 11 by adding 10% sodium hydroxide aqueous solution. The reaction mixture was stirred at room temperature for 4 h and then acidified with concentrated hydrochloric acid (1.6 ml). The mixture was extracted with hot ethyl acetate (30 ml) and the extract was cooled in an ice bath. The precipitated white solid was filtered to obtain ε-*N*-Boc-α-*N*-2-(1-naphthyl)acetyl-L-lysine (6.1 g, 74%). Trifluoroacetic acid (22 ml, 280 mmol) was added to the solution of ε-*N*-Boc-α-*N*-2-(1-naphthyl)acetyl-L-lysine (5.9 g, 14 mmol) in dichloromethane (35 ml). The mixture was stirred for 1.5 h and then the solvent was removed in vacuo. The residue was dissolved in water (20 ml) and the pH was adjusted to 6 by adding 20% sodium hydroxide aqueous solution. The mixture was extracted with ethyl acetate (50 ml) and evaporated. The residue was purified by column chromatography on a silica gel. The obtained product was crystallized from ethyl acetate/methanol to give pure NAL (α-*N*-2-(1-naphthyl)acetyl-L-lysine) (0.5 g, 11%) (Fig. 1). NAC and NAL were identified using the 3200 QTRAP LC/MS/MS system (AB SCIEX, Ontario, Canada). MS of NAC m/z (%): 290(9) ($[M + H]^+$), 141(100), 122(26), 115(48), and 76(9) (Fig. 2). MS of NAL m/z (%): 315(54) ($[M + H]^+$), 141(100), 129(21), 115(63), and 84(75) (Fig. 2).

2.3. NAC and NAL reactivity assay

Disodium hydrogen phosphate for buffer preparation, acetonitrile, trifluoroacetic acid (TFA) for HPLC, DMSO, and acetone were purchased from Wako Pure Chemical Industries.

Sodium phosphate buffers for NAC and NAL (100 mM; pH 9.5 and pH 12.0) were prepared with disodium hydrogen phosphate and sodium hydride solution, respectively.

Stock solutions of NAC and NAL were prepared to a final concentration of 1.25 mM in 100 mM phosphate buffer at pH 9.5 and pH 12.0, respectively. Test chemical solutions at a concentration of 100 mM were prepared in acetonitrile, water, acetone, or acetonitrile containing 10% or 20% DMSO. Triplicate reactivity samples were prepared in a 96-well microplate. For NAL reactivity, 80 μl of the NAL stock solution and 50 μl of the test chemical stock solution were added to 70 μl of sodium phosphate buffer (pH 12.0). For NAC reactivity, 80 μl of the NAC stock solution, 10 μl of the test chemical stock solution, and 40 μl of acetonitrile were added to 70 μl of sodium phosphate buffer (pH 9.5). As controls, samples containing solvent rather than test chemicals were also prepared in triplicate. The 96-well microplates were sealed by Plate Seal (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and gently shaken and incubated in the dark for 24 h at 25 °C in an incubator (MIR-253, SANYO Electric Co., Ltd. Osaka, Japan). Following incubation, samples were diluted 10 times with a mixed solution of 25% acetonitrile/0.5% TFA in water, and 96-well microplates were sealed by Sealig Mat (AxyGen Scientific, Inc., Union City, CA). In addition, standards used for defining the calibration curve were prepared without the test chemicals for each amino acid derivative at concentrations ranging from 0.0625 mM to 0.5 mM.

2.4. HPLC analysis of NAC and NAL

NAC and NAL in all samples and standards were quantified using a LC-20A HPLC system (Prominence, Shimadzu Scientific Instruments, Kyoto, Japan) on a CAPCELL CORE C18 column (2.7 μm, 3.0 × 150 mm, Shiseido Co., Ltd., Tokyo, Japan) with UV detection at 281 nm using a photodiode array detector (SPD-M20A, Shimadzu), which scanned wavelengths from 200 nm to 400 nm. The UV spectrum was collected from 200 nm to 400 nm to verify the identity of the NAC and NAL. The temperature of the column oven (CTO-20A, Shimadzu) and autosampler (SIL-20 AC, Shimadzu) was controlled at 40 °C and 4 °C, respectively.

The mobile phase had a flow rate of 0.3 ml/min and consisted of 98/2 water/acetonitrile with 0.1% TFA (A) and 90/10 acetonitrile/water with 0.1% TFA (B). Separation was achieved by holding at the initial conditions (20% B) for 7 min, followed by a linear gradient to 100% B for 3.5 min and then back to the initial conditions for a total analysis

Table 1
Chemicals used as test chemicals in ADRA and their sources.

	Test substance	CAS no.	Source	Solvent
Extreme/strong				
1	Diphenylcyclopropenone	886-38-4	Wako	Acetonitrile
2	Oxazolone	15646-46-5	Wako	Acetonitrile
3	Benzoyl peroxide	94-36-0	TCI	Acetonitrile
4	Kathon CG	56965-84-9	Sigma-Aldrich	H_2O
5	Bandrowski's base	20048-27-5	Alfa Aesar	20% DMSO/acetonitrile
6	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	Santa Cruz	H_2O
7	p-Benzoquinone	106-51-4	Wako	Acetonitrile
8	Tetrachlorosalicylanilide	1154-59-2	AccuStandard	Isopropanol
9	2,4-Dinitrochlorobenzene	97-00-7	Wako	Acetonitrile
10	Glutaraldehyde	111-30-8	Wako	Acetonitrile
11	Fluorescein isothiocyanate	3326-32-7	Dojindo	Acetonitrile
12	Phthalic anhydride	85-44-9	Wako	Acetonitrile
13	Lauryl gallate	1166-52-5	Wako	20% DMSO/acetonitrile
14	Propyl gallate	121-79-9	Wako	Acetonitrile
15	CD3	25646-71-3	FF	20% DMSO/acetonitrile
16	Trimellitic anhydride	552-30-7	Wako	Acetonitrile
17	Formaldehyde	50-00-0	Wako	Acetonitrile
18	Metol	55-55-0	Wako	H_2O
Moderate				
19	2-Hydroxyethyl acrylate	818-61-1	Wako	Acetonitrile
20	Glyoxal	107-22-2	Wako	H_2O
21	Vinyl pyridine	1337-81-1	Wako	Acetonitrile
22	2-Mercaptobenzothiazole	149-30-4	Wako	10% DMSO/acetonitrile
23	Nonanoyl chloride	764-85-2	TCI	Acetonitrile
24	2-Methyl-2H-isothiazol-3-one	2682-20-4	Sigma-Aldrich	Acetonitrile
25	1,2-Benzisothiazoline-3-one	2634-33-5	TCI	20% DMSO/acetonitrile
26	Methyl-2-nonynoate	111-80-8	TCI	Acetonitrile
27	Cinnamaldehyde	14371-10-9	Wako	Acetonitrile
28	Phenylacetaldehyde	122-78-1	Alfa Aesar	Acetonitrile
29	Benzylideneacetone	122-57-6	Wako	Acetonitrile
30	2,4-Heptadienal	881395	Wako	Acetonitrile
31	Squaric acid	2892-51-5	Wako	Acetonitrile
32	Trans-2-hexenal	6728-26-3	Wako	Acetonitrile
33	Resorcinol	108-46-3	Wako	Acetonitrile
34	Diethyl maleate	141-05-9	Wako	Acetonitrile
35	2-Phenylpropionaldehyde	93-53-8	Sigma-Aldrich	Acetonitrile
36	Perillaldehyde	2111-75-3	Wako	Acetonitrile
37	Palmitoyl Chloride	112-67-4	Wako	Acetone
38	1-(4-Methoxyphenyl)-1-penten-3-one	104-27-8	AccuStandard	Acetonitrile
Weak				
39	α-Hexylcinnnamaldehyde	101-86-0	Wako	Acetonitrile
40	α-Amylcinnamaldehyde	122-40-7	Wako	Acetonitrile
41	2,3-Butanedione	431-03-8	Wako	Acetonitrile
42	Farnesal	19317-11-4	Frinton	Acetonitrile
43	Oxalic acid	144-62-7	Wako	Acetonitrile
44	Benzyl benzoate	120-51-4	Wako	Acetonitrile
45	4-Allylanisole	140-67-0	TCI	Acetonitrile
46	Lilial	80-54-6	Wako	Acetonitrile
47	Cyclamen aldehyde	103-95-7	Sigma-Aldrich	Acetonitrile
48	Imidazolidinyl urea	39236-46-9	Sigma-Aldrich	H_2O
49	5-Methyl-2,3-hexanedione	13706-86-0	TCI	Acetonitrile
50	2,2,6,6-Tetramethyl-3,5-heptanedione	1118-71-4	TCI	Acetonitrile
51	Ethyleneglycol dimethacrylate	97-90-5	Wako	Acetonitrile
52	Ethyl acrylate	140-88-5	Wako	Acetonitrile
53	Hydroxycitronellal	107-75-5	Wako	Acetonitrile
Non-sensitizer				
54	Glycerol	56-81-5	Wako	Acetonitrile
55	Hexane	110-54-3	Wako	Acetonitrile
56	Diethyl phthalate	84-66-2	Wako	Acetonitrile
57	Octanoic acid	124-07-2	Wako	Acetonitrile
58	2-Hydroxypropyl methacrylate	923-26-2	Wako	Acetonitrile
59	1-Butanol	71-36-3	Wako	Acetonitrile
60	4-Hydroxybenzoic acid	99-96-7	Wako	Acetonitrile
61	6-Methyl coumatrin	92-48-8	Sigma-Aldrich	Acetonitrile
62	Methyl salicylate	119-36-8	Wako	Acetonitrile
63	Chlorobenzene	108-90-7	Wako	Acetonitrile
64	Lactic acid	50-21-5	Sigma-Aldrich	Acetonitrile
65	1-Bromobutane	109-65-9	Wako	Acetonitrile
66	2-Acethylcyclohexanone	874-23-7	Wako	Acetonitrile
67	4'-Methoxyacetophenone	100-06-1	Wako	Acetonitrile
68	Ethyl benzoylacetate	94-02-0	Wako	Acetonitrile
69	Ethyl vanillin	121-32-4	TCI	Acetonitrile

Table 1 (continued)

	Test substance	CAS no.	Source	Solvent
70	Isopropanol	67-63-0	Wako	Acetonitrile
71	Propylene glycol	57-55-6	Wako	Acetonitrile
72	Sulfanilamide	63-74-1	Wako	Acetonitrile
73	Isopropyl myristate	110-27-0	Wako	Acetonitrile
74	Benzaldehyde	100-52-7	Sigma-Aldrich	Acetonitrile
75	Methylparaben	99-76-3	Wako	Acetonitrile
76	Nonanoic acid	112-05-0	TCI	Acetonitrile
77	Propyl paraben	94-13-3	Wako	Acetonitrile
78	Salicylic acid	69-72-7	Wako	Acetonitrile
79	Sulphanilic acid	121-57-3	Wako	H_2O
80	Vanillin	121-33-5	Wako	Acetonitrile
81	Coumarin	91-64-5	Wako	Acetonitrile
82	Vinylidene dichloride	75-35-4	AccuStandard	Acetonitrile

ADRA, Amino acid Derivative Reactivity Assay.
AccuStandard: AccuStandard, Inc., New Haven, CT, USA; Alfa Aesar: Alfa Aesar, Ward Hill, MA, USA; Dojindo: Dojindo Molecular Technologies, Inc., Kumamoto, Japan; FF, Synthetic Organic Chemistry Laboratories of FUJIFILM Corporation; Frinton: Frinton Laboratories, Inc., Hainesport, NJ, USA; Santa Cruz: Santa Cruz Biotechnolo; Sigma-Aldrich: Sigma-Aldrich Inc., St. Louis, MO, USA; TCI: Tokyo Chemical Industry Co. Ltd., Tokyo, Japan; Wako: Wako Pure Chemical Industries, Ltd., Osaka, Japan.

time of 15 min per sample. The details for the extra conditions are shown in Fig. 4.

Each sample (10 μl) was separated into fractions containing NAC/NAL and the test chemicals on a CAPCELL CORE column. The reactivity of NAC/NAL was reported as the percent depletion based on the decrease in the non-reacted NAC/NAL concentration in the samples relative to the average concentration measured in the control, and the average NAC/NAL reduction was presented as the average score.

2.5. Optimization of the steric conformation of NAC and NAL

The structures of NAC and NAL were optimized using the SCIGRESS 1.2.0 software package containing MM3, PM5, and CONFLEX (Fujitsu, Tokyo, Japan). The geometry of the NAC and NAL structures was optimized by using MM3 for molecular mechanics. Iterative energy minimizing routines were conducted using a conjugate gradient minimizer algorithm. The CONFLEX conformational search procedure was used to find low-energy conformations of the NAC and NAL molecules. The semi-empirical method PM5 was also used for geometry optimization.

2.6. 2-Class classification model

The classification model was developed using ADMEWORKS/ModelBuilder V4.5 (Fujitsu Kyushu Systems Limited, Fukuoka, Japan). In particular, the "Iterative Least Square Linear Decision Surface" in the ADMEWORKS/ModelBuilder was used to build the classification model, which classified the samples into 2 classes (sensitizer and non-sensitizer) using a linear equation with depletion as the single parameter. An optimal average score that categorized sensitizers as non-sensitizers was calculated by the following formula.

$$\text{Optimal average score} = \text{Average} - (\text{Standard Deviation}/\text{Weight}) \times \text{Constant}$$

where Average, Standard Deviation, Weight, and Constant were obtained from the Iterative Least Square Linear Decision Surface calculation.

3. Results

3.1. Synthesis, identification and steric conformation of NAC and NAL

We chemosynthesized NAC and NAL as shown in Fig. 1 and obtained white powders with >98% purity. Measurement using the 3200QTRAP LC/MS/MS system assigned m/z = 290 as $[M + H]^+$, and this compound was identified as NAC from fragments such as m/z = 141 and m/z = 122 (Fig. 2A). Similarly, m/z = 315 was assigned as $[M + H]^+$, and this compound was identified as NAL from fragments such as m/z = 141 and m/z = 129 (Fig. 2B). Moreover, the semi-empirical molecular orbital method was performed to identify the steric conformations. NAC was folded around the carbonyl group in the *N*-acetyl group, and an SH group existed near the naphthalene ring (Fig. 3A). In contrast, the structure of NAL linearly extended from the naphthalene ring to the ε-NH_2 group, which was the reactive site; thus, these groups were sterically distant (Fig. 3B).

3.2. Reactivity and its reproducibility of NAC/NAL

To establish our method, we evaluated the reactivity of NAC/NAL with the 82 test chemicals previously evaluated by Gerberick et al. (2007), and the results are shown in Table 2. The chemicals are listed in the order of lowest EC3 value (i.e., highest allergic potency), including 52 sensitizers and 30 non-sensitizers based on an existing LLNA categorization scheme (Kimber et al., 2003). The LLNA EC3 data reported in this manuscript are derived from previous studies (Ashby, Basketter, Patton, & Kimber, 1995; Basketter, Sanders, & Jowsey, 2007; Basketter & Scholes, 1992; Basketter et al., 2001; Dearman, Spence, & Kimber, 1992; Estrada, Patlewicz, Chamberlain, Basketter, & Larbey, 2003; Gerberick et al., 2005; Kimber et al., 1998; Loveless et al., 1996; Ryan et al., 2000). The mean values and SD (N = 3) for the depletion of NAC and NAL in the present study, and those of cysteine and lysine reported by Gerberick et al. (2007), are shown in Table 2 (right row), in which the DPRA values are subtracted from the ADRA values for each test chemical to compare the reactivity of ADRA with that of DPRA (Fig. 5). The depletion values of NAC and NAL in ADRA were generally higher than those of the corresponding peptides in DPRA, and therefore, ΔDepletion was generally positive. ΔAverage score was also positive for most test chemicals. These findings indicate that the reactivity of NAC/NAL with the test chemicals in ADRA is higher than that of the corresponding peptides in DPRA. As reported in Table 2, the concentrations of NAC and NAL in the reaction solution are higher than those in the control solution when the depletion has a negative value, and in such cases, it is likely that NAC and NAL in ADRA, or the corresponding peptides in DPRA, were co-eluted with the test chemicals or compounds derived from the test chemicals in the reaction solution. Depletions with a negative value in ADRA had values of almost −5.0% at the minimum, whereas depletions with a negative value in DPRA had values lower than −10%. Moreover, the SD ranges for NAC and NAL were 0.0 to 7.8 and 0.0 to 9.5, respectively, whereas those for the cysteine and lysine peptides in DPRA were 0.0 to 35.2 and 0.1 to 24.0, respectively.

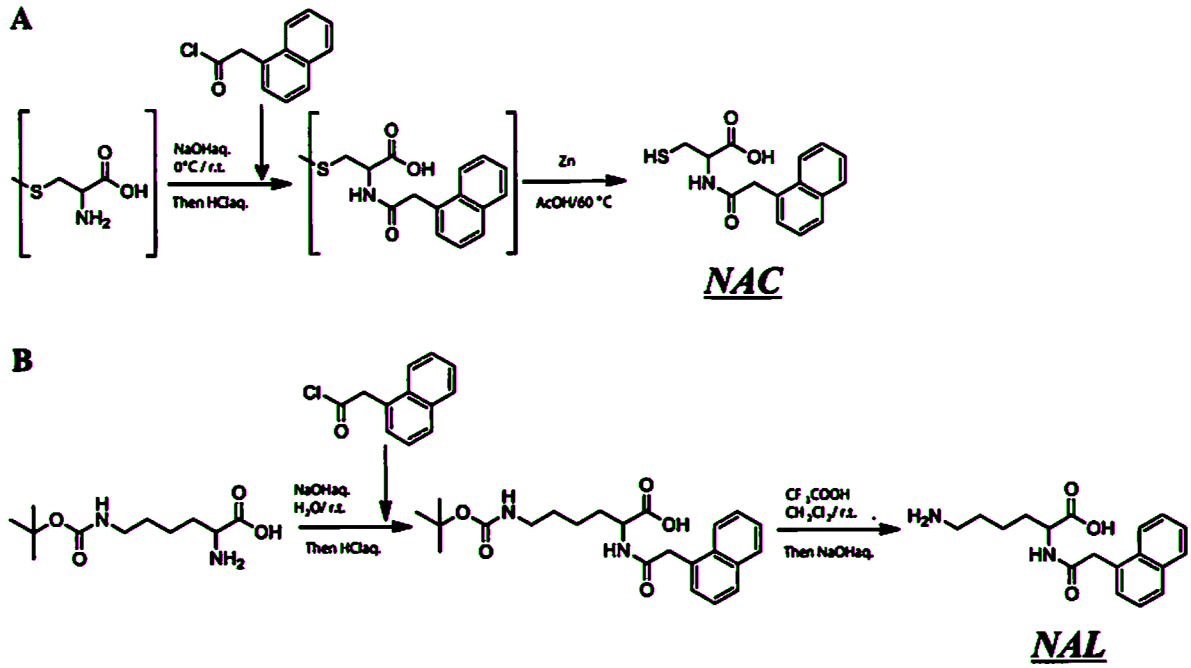


Fig. 1. Synthesis scheme for NAC and NAL. NAC was synthesized be reacting cystine with 2-(1-naphthyl)acetyl chloride (A). NAL was synthesized by reacting ε-*N*-Boc-L-lysine with 2-(1-naphthyl)acetyl chloride (B).

3.3. Prediction model based on 2-class classification

By using ModelBuilder in ADMEWORKS, we developed a classification model to classify test chemicals into sensitizers and non-sensitizers based on the average score, and the optimal average score to categorize chemicals as sensitizers or non-sensitizers was calculated to be 7.750. Based on this threshold, the sensitivity, specificity, positive predictivity, negative predictivity, and accuracy were calculated by Cooper statistics for comparison with the DPRA values. The accuracy of ADRA was found to be 88%, which was identical to the accuracy of DPRA. Similarly, the sensitivity and specificity of ADRA were also identical to those of DPRA. Seven known sensitizers were incorrectly classified as non-sensitizers in DPRA, whereas the corresponding number was only 5 in ADRA (Fig. 6, Table 3).

4. Discussion

The semi-empirical molecular orbital method demonstrated that NAC was folded around the carbonyl group, and a SH group existed near the naphthalene ring. Therefore, it was expected that the SH group, which was the reactive site, existed in a highly hydrophobic environment generated by the nearby naphthalene group (Jao et al., 2006; Kortemme & Creighton, 1995), and other chemicals were thus prevented from approaching by steric hindrance. Thus, NAC was considered to present slightly lower reactivity than cysteine peptide.

However, the ε-NH_2 group of NAL, which was the reactive site, was found to linearly extend from the naphthalene ring. Therefore, NAL was considered to show equivalent reactivity to native lysine because the naphthalene ring had only a slight influence on the ε-NH_2 group.

In DPRA, the model based on the average score, where the ratios of peptide to chemical were 1:10 for cysteine and 1:50 for lysine, showed the best prediction accuracy (Gerberick et al., 2007). It is therefore likely that each peptide interacts with the chemical in excess, and the reactions are expected to progress quantitatively as pseudo-first order reactions. We therefore also examined the reactivity of NAC/NAL to chemicals under the same conditions used for DPRA.

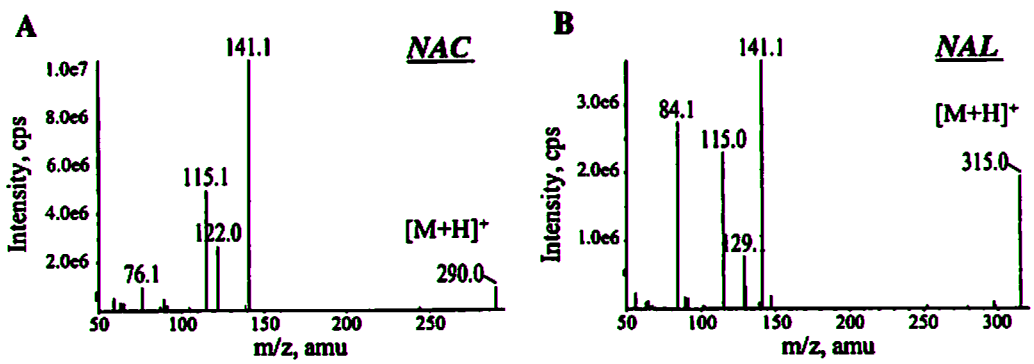


Fig. 2. Mass spectra of NAC and NAL. NAC and NAL were identified by MS. The m/z 290.0 (A) and m/z 315.0 (B) indicate the predominant protonated ion $[M + H]^+$ of NAC and NAL, respectively. The m/z 141.1 and 115.1 common to A and B show fragment ions containing the naphthalene ring and derived from the naphthalene ring, respectively.

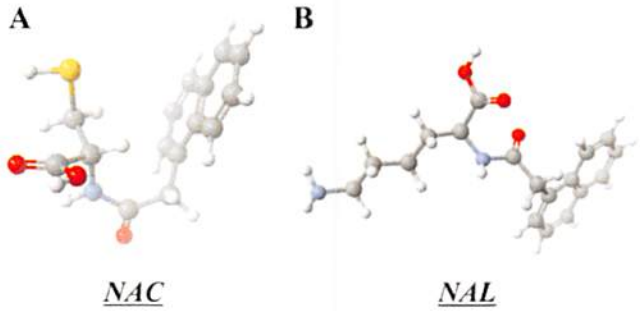


Fig. 3. Steric conformations of NAC and NAL. The steric conformations of NAC (A) and NAL (B) were optimized by the semi-empirical molecular orbital method. The gray, white, red, purple, and yellow balls show carbon, hydrogen, oxygen, nitrogen, and sulfur, respectively.

To determine appropriate reaction conditions for NAC/NAL, we evaluated the reactivity of NAC/NAL with 82 test chemicals under the same buffer and pH conditions used for DPRA (data not shown). The reactivity of NAL was almost identical to that of lysine peptide, but the reactivity of NAC was lower than that of cysteine peptide. It was therefore considered that the reactivity of NAC with chemicals may decreased by the 3D structural characteristics of NAC. However, it was considered necessary to react NAC/NAL with chemicals under pH conditions at which NAC/NAL would sufficiently dissociated because the goal of the protein binding assay to predict skin sensitization is to determine whether chemicals react with proteins in vivo. Therefore, we selected pH 9.5 phosphate buffer for NAC as the highest pH at which NAC did not dimerise even after 24 h and selected pH 12.0 phosphate buffer for NAL.

To perform the experiment effectively, 96-well microplates were used for mDPRA rather than the vials used for DPRA, and the amount of each reaction solution was scaled down to 80% by this change. Moreover, it became possible to analyze more than 20 test chemicals in triplicate per plate, simultaneously. Even with these changes in experimental conditions, extensive differences did not appear when we compared differences in reactivity between 96-well microplates and vials (data not shown). We therefore judged that the use of 96-well microplates would not affect the reactivity of NAC/NAL with chemicals. Furthermore, the use of TFA, which was used to stop the reactions in ADRA, allowed numerous samples to be analyzed simultaneously, whereas HPLC was performed in DPRA, without any steps to stop the reactions. Furthermore, we established analytical conditions under which NAC/NAL could be measured in 15 min by using a core–shell column, whereas a gradient of two mobile phases over 25 min was used in DPRA (Gerberick et al., 2004). However, we also included extra conditions because we assumed that NAC/NAL may co-elute with chemicals or reaction products if only one condition was used, as in DPRA (Fig. 4).

We evaluated the reactivity of NAC/NAL with 82 test chemicals according to these conditions. The depletions for NAC/NAL were higher than those observed for the peptide in DPRA were (Table 2). In particular, focusing on the Schiff base formation of aldehydes with NAL, depletions of 16 of the 18 aldehydes (except glyoxal and 2-phenylpropionaldehyde) in the 82 test chemicals were higher than those of lysine peptide in DPRA. We speculate that this may be due to the higher pH and conversion of the buffer from ammonium acetate to Na-phosphate (Table 2). Furthermore, ADRA provided high analytical precision, as few negative depletion values were observed, and high reproducibility was achieved, as demonstrated by the lower standard deviations observed relative to DPRA.

We calculated the average depletion score for the 82 test chemicals and conducted prediction modeling for 2-class classification of sensitizers and non-sensitizers. Iterative Least Square Linear Decision Surface analysis in ModelBuilder using 56 training set chemicals arbitrarily selected from the 82 test chemicals revealed the highest precision

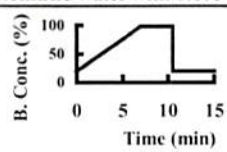
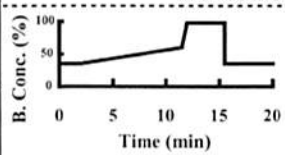
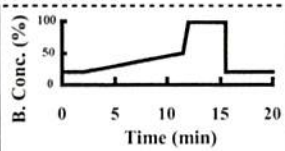
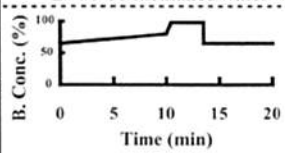
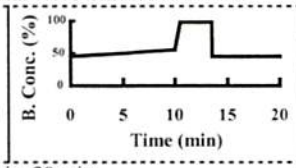
		NAC	NAL
Normal conditions	Mobile phase	A: 98/2 water/acetonitorile with 0.1% trifluoroacetic acid B: 90/10 acetonitrile/water with 0.1% trifluoroacetic acid	
	Flow conditions		
	Flow rate, Run time	0.3 ml/min, 15 min	
Extra conditions -1	Mobile phase	same as normal conditions	
	Gradient conditions		
	Flow rate, Run time	0.3 ml/min, 20 min	
Extra conditions -2	Mobile phase	A: 98/2 water/methanol with 0.1% trifluoroacetic acid B: 90/10 methanol/water with 0.1% trifluoroacetic acid	
	Gradient conditions		
	Flow rate, Run time	0.2 ml/min, 20 min	

Fig. 4. Mobile phase conditions of HPLC analysis for NAC and NAL. The two types of mobile phase (A and B), gradient conditions, flow rate, and run time are shown. Normal conditions are shown at the top of the table. Extra condition-1 and extra condition-2 are shown at the middle and bottom of the table, respectively.

Table 2
Reactivity of test chemicals with NAC/NAL and synthetic peptides with results expressed as percent depletion of non-reacted NAC/NAL and peptides.

No.	Test substance	ADRA					DPRA[a]				
							Results according to Gerberick et al.				
		NAC		NAL		Ave. score	Cys peptide		Lys peptide		Ave. score
		Depletion	SD	Depletion	SD		Depletion	SD	Depletion	SD	
Extreme/strong											
1	Diphenylcyclopropenone	99.8	2.1	99.6	1.4	99.7	98.8	2.0	−0.7	3.8	49.1
2	Oxazolone	76.5	0.4	30.1[b]	1.8	53.3	75.5	1.4	49.6	1.8	62.6
3	Benzoyl peroxide	100.0	0.0	100.0	1.4	100.0	100.0	0.0	81.3	2.9	90.7
4	Kathon CG	98.9	0.5	3.6	0.5	51.3	99.1	1.6	3.9	1.0	51.5
5	Bandrowski's base	100.0	0.0	38.8	1.1	69.4	87.5	0.3	4.2	17.0	45.9
6	5-Chloro-2-methyl-4-isothiazolin-3-one	99.3	0.6	63.3	9.2	81.3	96.3	2.8	35.1	14.0	65.7
7	p-Benzoquinone	98.9	2.1	97.5	1.4	98.2	99.0	1.8	91.0	0.2	95.0
8	Tetrachlorosalicylanilide	23.3	0.4	14.9	0.7	19.1	36.8	20.0	9.0	24.0	22.9
9	2,4-Dinitrochlorobenzene	100.0	0.0	83.2	0.9	91.6	100.0	0.0	14.7	4.2	57.4
10	Glutaraldehyde	49.2[c]	0.6	96.2	1.4	72.7	30.2	0.5	85.4	3.5	57.8
11	Fluorescein isothiocyanate	100.0	0.0	100.0[b]	0.0	100.0	100.0	0.0	61.1	1.5	80.6
12	Phthalic anhydride	1.0	1.7	84.4	1.3	42.7	−1.9	1.0	75.0	3.9	36.6
13	Lauryl gallate	99.6	0.3	33.8	0.8	66.7	90.9	13.1	8.7	4.2	49.8
14	Propyl gallate	93.7	2.0	75.2	1.4	84.5	59.9	35.2	26.6	10.7	43.3
15	CD3	100.0	0.0	40.8	1.1	70.4	90.1	1.1	13.6	0.5	51.9
16	Trimellitic anhydride	1.0	1.6	99.4	1.4	50.2	−1.1	5.7	43.7	4.9	21.3
17	Formaldehyde	81.7	1.9	65.6[d]	2.4	73.7	60.4	4.1	11.2	3.5	35.8
18	Metol	97.5	0.6	60.8[b]	0.4	79.2	100.0	0.0	44.7	3.8	72.4
Moderate											
19	2-Hydroxyethyl acrylate	100.0	0.0	81.5	1.1	90.7	92.6	0.5	88.9	0.3	90.8
20	Glyoxal	33.3[c]	2.5	21.3[b]	0.8	27.3	56.5	1.7	67.8	1.9	62.2
21	Vinyl pyridine	74.8	2.0	6.4	2.1	40.6	92.1	0.4	−16.9	16.2	37.6
22	2-Mercaptobenzothiazole	40.0[c]	1.9	−0.9	0.8	19.5	97.5	4.2	−3.0	0.6	47.3
23	Nonanoyl chloride	8.1	1.6	100.0	0.0	54.1	18.2	3.0	−6.3	1.8	6.0
24	2-Methyl-2H-isothiazol-3-one	100.0	0.0	−0.2	8.2	49.9	97.9	0.3	−5.6	5.2	46.2
25	1,2-Benzisothiazoline-3-one	100.0[c]	0.0	0.1	1.5	50.1	97.7	0.1	9.7	2.5	53.7
26	Methyl-2-nonynoate	99.6	2.1	10.2	1.6	54.9	100.0	0.0	3.2	4.0	51.6
27	Cinnamaldehyde	95.5	1.6	81.3	1.8	88.4	70.6	1.0	43.2	4.1	56.9
28	Phenylacetaldehyde	99.8	0.4	99.2	1.0	99.5	60.7	13.3	22.6	1.9	41.7
29	Benzylideneacetone	95.3	2.1	15.0	1.1	55.1	94.7	2.3	1.5	0.9	48.1
30	2,4-Heptadienal	100.0	0.0	98.4	1.4	99.2	97.3	0.1	23.9	5.0	60.6
31	Squaric acid	−2.0	0.4	1.5	0.6	−0.3	46.9	8.7	4.8	4.9	25.9
32	Trans-2-hexenal	96.7	2.1	97.6	1.4	97.1	97.9	0.3	3.6	2.6	50.8
33	Resorcinol	10.0	1.4	64.1	0.6	37.1	1.6	2.3	−0.8	1.3	0.4
34	Diethyl maleate	98.9	0.4	18.7	0.8	58.8	100.0	5.6	85.5	1.9	92.8
35	2-phenylpropionaldehyde	70.6	7.8	8.5	9.5	39.6	48.2	0.0	21.2	1.6	34.7
36	Perillaldehyde	94.0	2.1	66.0	1.2	80.0	31.9	7.1	13.8	1.6	22.9
37	Palmitoyl Chloride	17.1	1.0	100.0	0.0	58.5	25.5	3.3	26.6	0.5	26.1
38	1-(4-Methoxyphenyl)-1-penten-3-one	88.7	2.1	6.4	1.7	47.6	29.9	6.6	14.3	1.3	22.1
Weak											
39	α-Hexylcinnnamaldehyde	0.1	1.9	1.8	1.5	1.0	−0.3	5.6	−1.6	3.2	−1.0
40	α-Amylcinnamaldehyde	2.2	1.6	6.0	2.0	4.1	0.6	1.2	3.9	2.9	2.3
41	2,3-Butanedione	100.0	0.0	73.1	2.8	86.5	79.0	0.2	27.0	1.5	53.0
42	Farnesal	41.3	0.8	38.6	1.9	39.9	16.4	20.8	8.5	3.9	12.5
43	Oxalic acid	−4.3	1.6	4.5	1.0	0.1	0.9	3.5	−0.9	13.6	0.0
44	Benzyl benzoate	3.3	2.0	3.0	1.2	3.1	0.2	5.8	3.0	0.7	1.6
45	4-Allylanisole	66.0	0.8	10.9	1.0	38.4	20.6	1.1	−0.8	5.3	9.9
46	Lilial	4.5	2.7	54.7	1.8	29.6	14.0	5.6	0.7	1.8	7.4
47	Cyclamen aldehyde	10.5	2.3	11.1	1.0	10.8	18.9	6.4	1.0	0.2	10.0
48	Imidazolidinyl urea	80.0	0.5	83.7	0.5	81.8	52.3	8.1	1.3	0.4	26.8
49	5-Methyl-2,3-hexanedione	23.2	1.9	98.9	1.3	61.0	25.8	6.0	7.5	1.9	16.7
50	2,2,6,6-Tetramethyl-3,5-heptanedione	8.1	1.8	7.6	1.4	7.8	1.4	4.0	0.0	1.1	0.7
51	Ethyleneglycol dimethacrylate	100.0	0.0	24.3	1.3	62.1	87.3	13.6	12.4	0.2	49.9
52	Ethyl acrylate	100.0	0.0	96.7	1.2	98.4	96.4	5.0	93.7	3.0	95.1
53	Hydroxycitronellal	14.8	4.6	21.9	1.2	18.3	17.5	0.3	6.5	1.3	12.0
Non-sensitizer											
54	Glycerol	4.3	2.3	4.3	1.9	4.3	−3.8	1.7	2.1	2.0	−0.9
55	Hexane	3.0	2.0	5.8	1.5	4.4	−0.4	5.2	−5.1	0.9	−2.8
56	Diethyl phthalate	4.8	1.8	6.8	1.8	5.8	0.8	0.8	−0.7	0.6	0.1
57	Octanoic acid	1.8[c]	1.9	5.0	1.0	3.4	−1.0	1.7	0.9	0.9	−0.1
58	2-Hydroxypropyl methacrylate	98.1	1.7	10.9	1.3	54.5	58.4	0.7	−13.6	0.1	22.4
59	1-Butanol	3.9	2.4	2.5	1.6	3.2	−0.4	5.9	1.2	7.8	0.4
60	4-Hydroxybenzoic acid	0.5	1.8	2.2	3.4	1.3	−0.3	1.4	2.2	0.8	1.0
61	6-Methyl coumatrin	6.1	1.8	4.5	1.0	5.3	1.4	0.8	4.0	2.1	2.7
62	Methyl salicylate	−0.7	2.0	9.1	1.1	4.2	0.3	0.3	1.6	5.6	1.0
63	Chlorobenzene	2.1	2.4	4.6	1.0	3.3	0.4	0.8	1.3	0.3	0.9
64	Lactic acid	−2.4	1.8	2.8	1.9	0.2	−0.9	0.2	0.8	0.2	−0.1
65	1-Bromobutane	6.0	3.6	2.9	0.8	4.4	13.8	0.3	1.2	0.5	7.5

Table 2 (continued)

No.	Test substance	ADRA					DPRA[a]				
							Results according to Gerberick et al.				
		NAC		NAL		Ave. score	Cys peptide		Lys peptide		Ave. score
		Depletion	SD	Depletion	SD		Depletion	SD	Depletion	SD	
66	2-Acethylcyclohexanone	7.2[e]	1.9	14.6	2.6	10.9	18.2	3.6	12.5	0.4	15.4
67	4′-Methoxyacetophenone	4.1	1.3	−1.4	2.3	1.3	4.7	4.4	0.1	0.5	2.4
68	Ethyl benzoylacetate	3.6	2.9	5.4	1.6	4.5	2.3	5.0	1.9	0.3	2.1
69	Ethyl vanillin	−3.2	2.1	63.9	1.2	30.4	1.1	5.5	9.7	0.4	5.4
70	Isopropanol	−0.2	1.8	−5.2	6.7	−2.7	−10.0	17.0	0.5	5.5	−4.8
71	Propylene glycol	−5.0	2.1	−1.2	1.0	−3.1	−0.9	17.0	0.6	0.5	−0.2
72	Sulfanilamide	−4.4	2.3	−2.3	1.0	−3.3	−1.3	17.5	0.8	0.7	−0.3
73	Isopropyl myristate	−0.9	1.7	−0.9	0.8	−0.9	0.8	17.3	−4.0	0.5	−1.6
74	Benzaldehyde	12.5	1.5	5.3	0.5	8.9	7.2	1.7	−1.7	17.3	2.8
75	Methylparaben	2.6	1.4	3.7	0.6	3.2	3.6	8.8	−0.4	1.4	1.6
76	Nonanoic acid	0.9	1.5	3.8	0.6	2.4	−3.7	6.8	−9.6	0.8	−6.7
77	Propyl paraben	−3.3	1.2	1.0	0.9	−1.2	8.2	6.1	−0.2	2.9	4.0
78	Salicylic acid	−2.3	1.4	1.5	0.7	−0.4	3.5	4.2		-	-
79	Sulphanilic acid	−1.5	0.6	0.9	0.4	−0.3	5.3	5.5	0.5	1.0	2.9
80	Vanillin	−0.2	2.3	62.7	0.6	31.3	3.2	5.5	−6.6	3.6	−1.7
81	Coumarin	2.5	1.7	1.5	1.5	2.0	1.0	4.6	−14.9	22.0	−7.0
82	Vinylidene dichloride	4.9	1.6	0.7	0.9	2.8	2.4	1.7	−4.3	18.2	−1.0

DPRA, Direct Peptide Reactivity Assay; ADRA, Amino acid Derivative Reactivity Assay; NAC, *N*-(2-(1-naphthyl)acetyl)-L-cysteine; NAL, α-*N*-(2-(1-naphthyl)acetyl)-L-lysine; Ave. score, Average score; SD, standard deviation.

[a] Depletion and SD of Cys peptide (cysteine peptide) and Lys peptide (lysine peptide) (Gerberick et al., 2007).
[b] Depletion and SD of NAL analyzed under extra condition-1.
[c] Depletion and SD of NAC analyzed under extra condition-2.
[d] Depletion and SD of NAL analyzed under extra condition-2.
[e] Depletion and SD of NAC analyzed under extra condition-1.

when the optimum average score was set to 7.750; therefore, we decided to classify chemicals with an average score of ≥7.750 as sensitizers and those with average score of <7.750 as non-sensitizers. Furthermore, Cooper statistics indicated that ADRA resulted in a prediction accuracy of 88%, with 10 misclassifications. While DPRA gave a prediction accuracy of 89%, if we consider that Basketter et al. reclassified resorcinol, which was originally classified as a non-sensitizer by LLNA (Gerberick et al., 2005), to a sensitizer (Basketter et al., 2007), the prediction accuracy of DPRA would be changed from 89% to 88%, and the accuracy would be identical to that of ADRA. However, whereas 7 sensitizers were incorrectly classified as non-sensitizers by DPRA, only 5 sensitizers were incorrectly classified as non-sensitizers by ADRA; therefore, ADRA would be expected to have a slightly lower false negative rate than DPRA. The reason for the lower false negative rate may be that the reactions of NAC/NAL with chemicals were performed under higher pH to ensure the completeness of the reactions.

Squaric acid was one of the 7 sensitizers incorrectly predicted to be a non-sensitizer by ADRA (Table 3). The cysteine peptide depletion in DPRA was 46.9%, and squaric acid was thus classified as a sensitizer in DPRA. Squaric acid was also categorized as sensitizer by LLNA. However,

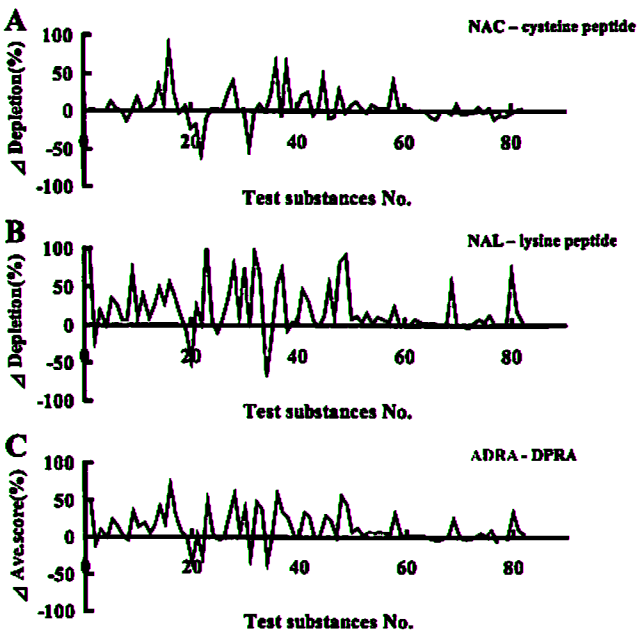


Fig. 5. Differences in reactivity between ADRA and DPRA. Each horizontal axis shows the 82 test chemicals indicated in Table 2. ΔDepletions indicates subtraction of the depletion of cysteine from the depletion of NAC for 82 test chemicals (A) and subtraction of the depletion of lysine from the depletion of NAL for 82 test chemicals (B), respectively. ΔAve. score indicates the subtraction of the average score in DPRA from the average score in ADRA for 82 test chemicals (C). The reactivity of ADRA is higher than that of DPRA if the values on the vertical axis are positive.

		Predicted Classification		
		Sensitizer	Non-sensitizer	total
Chemical Classification[a]	Sensitizer	48	5	53
	Non-sensitizer	5	24	29
	total	53	29	82

sensitivity:	91%
specificity:	83%
positive predictivity:	91%
negative predictivity:	83%
accuracy:	88%

[a] Based primarily on LLNA data

Fig. 6. Cooper statistics (sensitizers vs. non-sensitizers) for the NAC and NAL prediction model.

Table 3
Comparison of prediction results for skin sensitization for ADRA and DPRA and potency data.

No.	Test substance	EC3 value[a]	LLNA category[a]	ADRA prediction	DPRA prediction[b]
1	Diphenylcyclo propenone	0.0003	Extreme	S	S
2	Oxazolone	0.003	Extreme	S	S
3	Benzoyl peroxide	0.004	Extreme	S	S
4	Kathon CG	0.008	Extreme	S	S
5	Bandrowski's base	0.008	Extreme	S	S
6	5-Chloro-2-methyl-4-isothiazolin-3-one	0.009	Extreme	S	S
7	p-Benzoquinone	0.0099	Extreme	S	S
8	Tetrachlorosalicylanilide	0.04	Extreme	S	S
9	2,4-Dinitrochlorobenzene	0.05	Extreme	S	S
10	Glutaraldehyde	0.1	Strong	S	S
11	Fluorescein isothiocyanate	0.14	Strong	S	S
12	Phthalic anhydride	0.16	Strong	S	S
13	Lauryl gallate	0.3	Strong	S	S
14	Propyl gallate	0.32	Strong	S	S
15	CD3	0.6	Strong	S	S
16	Trimellitic anhydride	0.6	Strong	S	S
17	Formaldehyde	0.61	Strong	S	S
18	Metol	0.8	Strong	S	S
19	2-Hydroxyethyl acrylate	1.4	Moderate	S	S
20	Glyoxal	1.4	Moderate	S	S
21	Vinyl pyridine	1.6	Moderate	S	S
22	2-Mercaptobenzothiazole	1.7	Moderate	S	S
23	Nonanoyl chloride	1.8	Moderate	S	NS
24	2-Methyl-2H-isothiazol-3-one	1.9	Moderate	S	S
25	1,2-Benzisothiazoline-3-one	2.3	Moderate	S	S
26	Methyl-2-nonynoate	2.5	Moderate	S	S
27	Cinnamaldehyde	3	Moderate	S	S
28	Phenylacetaldehyde	3	Moderate	S	S
29	Benzylideneacetone	3.7	Moderate	S	S
30	2,4-Heptadienal	4	Moderate	S	S
31	Squaric acid	4.3	Moderate	NS	S
32	Trans-2-hexenal	5.5	Moderate	S	S
33	Resorcinol	5.5	Moderate	S	NS
34	Diethyl maleate	5.8	Moderate	S	S
35	2-phenylpropionaldehyde	6.3	Moderate	S	S
36	Perillaldehyde	8.1	Moderate	S	S
37	Palmitoyl Chloride	8.8	Moderate	S	S
38	1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	S	S
39	α-Hexylcinnnamaldehyde	11	Weak	NS	NS
40	α-Amylcinnamaldehyde	11	Weak	NS	NS
41	2,3-Butanedione	11	Weak	S	S
42	Farnesal	12	Weak	S	S
43	Oxalic acid	15	Weak	NS	NS
44	Benzyl benzoate	17	Weak	NS	NS
45	4-Allylanisole	18	Weak	S	S
46	Lilial	19	Weak	S	S
47	Cyclamen aldehyde	22	Weak	S	S
48	Imidazolidinyl urea	24	Weak	S	S
49	5-Methyl-2,3-hexanedione	26	Weak	S	S
50	2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	S	NS
51	Ethyleneglycol dimethacrylate	28	Weak	S	S
52	Ethyl acrylate	28	Weak	S	S
53	Hydroxycitronellal	33	Weak	S	S
54	Glycerol	NC	NS	NS	NS
55	Hexane	NC	NS	NS	NS
56	Diethyl phthalate	NC	NS	NS	NS
57	Octanoic acid	NC	NS	NS	NS
58	2-Hydroxypropyl methacrylate	NC	NS	S	S
59	1-Butanol	NC	NS	NS	NS
60	4-Hydroxybenzoic acid	NC	NS	NS	NS
61	6-Methyl coumatrin	NC	NS	NS	NS
62	Methyl salicylate	NC	NS	NS	NS
63	Chlorobenzene	NC	NS	NS	NS
64	Lactic acid	NC	NS	NS	NS
65	1-Bromobutane	NC	NS	NS	S
66	2-Acethylcyclohexanone	NC	NS	S	S
67	4'-Methoxyacetophenone	NC	NS	NS	NS
68	Ethyl benzoylacetate	NC	NS	NS	NS
69	Ethyl vanillin	NC	NS	S	NS
70	Isopropanol	NC	NS	NS	NS
71	Propylene glycol	NC	NS	NS	NS
72	Sulfanilamide	NC	NS	NS	NS
73	Isopropyl myristate	NC	NS	NS	NS
74	Benzaldehyde	NC	NS	S	NS
75	Methylparaben	NC	NS	NS	NS

Table 3 (continued)

No.	Test substance	EC3 value[a]	LLNA category[a]	ADRA prediction	DPRA prediction[b]
76	Nonanoic acid	21 (False +)	NS	NS	NS
77	Propyl paraben	NC	NS	NS	NS
78	Salicylic acid	NC	NS	NS	-
79	Sulphanilic acid	NC	NS	NS	NS
80	Vanillin	NC	NS	S	NS
81	Coumarin	NC	NS	NS	NS
82	Vinylidene dichloride	NC	NS	NS	NS

DPRA, Direct Peptide Reactivity Assay; mDPRA, ADRA, Amino acid Derivative Reactivity Assay; EC3, estimated concentration required to produce a 3-fold increase for control; NC, not calculated; S, sensitizer; NS, non-sensitizer; -, not evaluated.

[a] EC3 values and LLNA category (Gerberick et al., 2007).

[b] Results judged from reactivity based on Cys and Lys peptide data (Gerberick et al., 2007).

because squaric acid is a strong acid once dissolved even though it cannot be dissolved easily, it is considered to be not susceptible to reaction with nucleophilic agents. In contrast, the NAC depletion in ADRA was −2.0%, thus presenting almost no reaction. This difference in the results between DPRA and ADRA was subtle, but they may have resulted from the lack of sufficient dissolution of squaric acid. In both DPRA and ADRA, oxalic acid and benzyl benzoate were also predicted to be non-sensitizers, although these chemicals were categorized as sensitizers by LLNA. However, these chemicals have no reaction sites, and therefore, the reason why the results in LLNA were different from those in DPRA and ADRA is unclear.

Moreover, vanillin is a non-sensitizer that was predicted to be a sensitizer by ADRA (Table 3). Vanillin has a terminal aldehyde group and it is therefore likely that this group reacted with the ε-NH_2 of NAL and formed a Schiff base. Depletion of NAL by vanillin was 62.7%, indicating high reactivity. As vanillin has been reported to be an extremely weak allergen (Basketter et al., 2001), the observed depletion may only reflect reactivity of NAL with vanillin, although the discrepancy may also have been caused by a species difference.

Among the 82 test chemicals, only salicylic acid co-eluted with lysine peptide, indicating that it could not be evaluated using DPRA (Gerberick et al., 2007). However, salicylic acid could be easily separated from NAL using ADRA, and its depletion could hence be determined (data not shown).

On the other hand, NAC and NAL were co-eluted with some of the test chemicals applied under normal HPLC conditions in ADRA. For example, NAC co-eluted with 2-mercaptobenzothiazole, and the reaction product of NAC with glutaraldehyde (Fig. 7). However, it is extremely difficult to distinguish the peptides, NAC, and NAL from all chemicals using only one HPLC condition; it is therefore useful to analyze NAC and NAL under additional HPLC conditions when they are co-eluted with test chemicals. We therefore performed additional

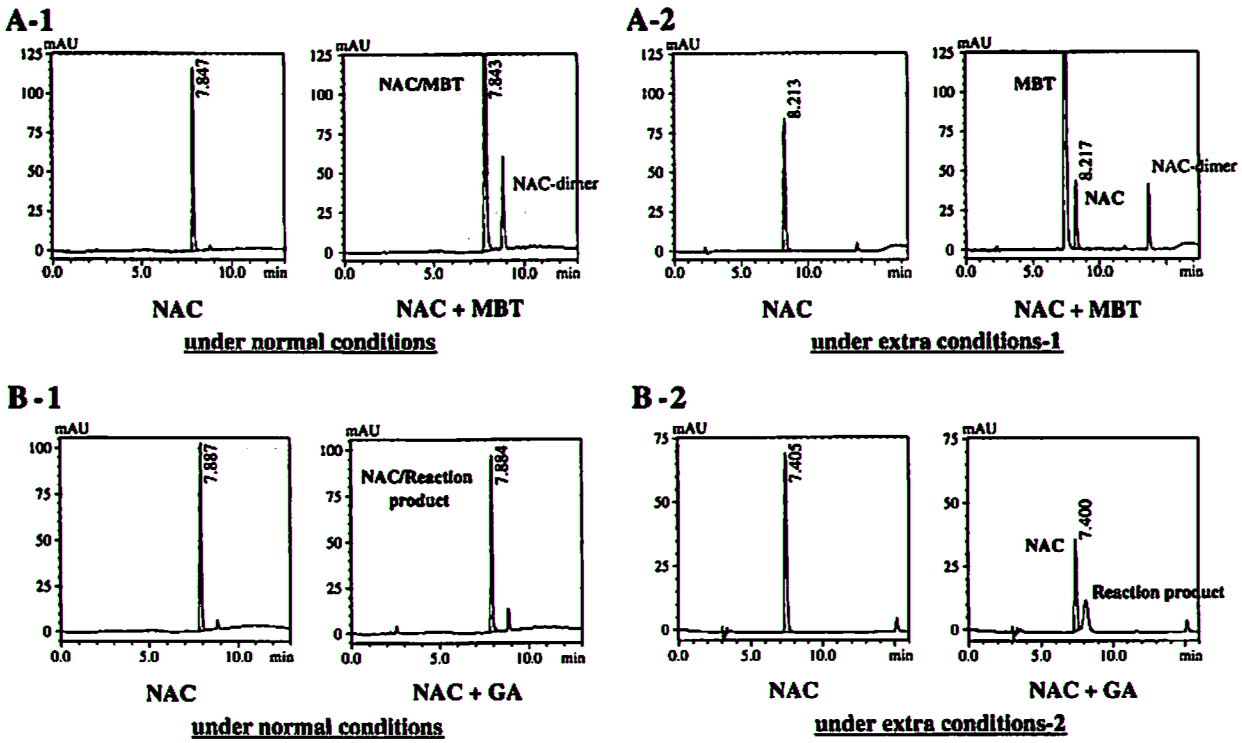


Fig. 7. Chromatograms of reaction solutions after reaction of NAC with MBT or GA under normal and extra conditions. Chromatograms of NAC and 2-mercaptobenzothiazole (MBT) analyzed under normal conditions are shown (A-1). NAC completely co-eluted with MBT. The peak obtained at approximately 9 min shows NAC dimerization (NAC disulfide form). Chromatograms of NAC and MBT analyzed under extra condition-1 are shown (A-2). NAC was completely separated from MBT. Chromatograms of NAC and glutaraldehyde (GA) analyzed under normal conditions (B-1) are shown. As GA has no absorbance in the UV spectrum, the peak overlapped with that of NAC shows the reaction product of NAC and GA. Chromatograms after the reaction of NAC with GA analyzed under extra condition-2 are shown (B-2). NAC was completely separated from the reaction product of NAC and G.

HPLC analyses in 3 steps. Initially, NAC and NAL were analyzed under normal conditions, and showed no co-elution with 93% of the test chemicals. NAC and NAL were then analyzed under extra condition-1 for the remaining 5 chemicals, except for glutaraldehyde and formaldehyde. For these low-molecular weight chemicals whose reaction products co-elute with NAC and NAL, it was more useful to analyze NAC and NAL under extra condition-2 (Fig. 7B). Therefore, using these 3 steps, it was expected that nearly all chemicals tested should be evaluable by ADRA.

Furthermore, the use of NAC/NAL in ADRA is expected to have several advantages over DPRA. Natsch and Gfeller (2008) identified several limitations of DPRA, including the relatively high concentration of test chemicals required, especially in the lysine peptide-based assay (25 mM), which is impractical for many hydrophobic test chemicals.

Moreover, as NAC/NAL could be analyzed at 281 nm, which improved the baseline stability compared with DPRA, in which analyses are conducted at 220 nm, NAC/NAL in ADRA could be quantified precisely even if the concentrations of each reaction solution, including relatively high concentrations of test chemicals in DPRA, were reduced to 1/100 of that used in DPRA (data not shown).

This dilution may enable the evaluation of chemicals whose solubility in water is very low and thus cannot be evaluated using DPRA. In addition, because peptides must be dissolved in water for DPRA, relatively insoluble chemicals have to be dissolved in a reaction solution containing a high amount of water; thus, the range of chemicals that could be evaluated is limited by this requirement. However, because NAC and NAL can be dissolved in both water and organic solvents, ADRA provides the possibility to change the reaction solution from an aqueous system to an organic solvent system.

In this study, ADRA was used to evaluate skin sensitizers using low-molecular weight compounds such as NAC/NAL instead of oligopeptides, which are conventionally used in DPRA, and achieved the same prediction accuracy as DPRA. Therefore, we conclude that ADRA is a useful and versatile prediction method. Further analyses conducted at a 281 nm detection wavelength using ADRA might facilitate the precise quantification of chemicals that could not previously be evaluated using DPRA.

Conflict of interest statement

There is no conflict of interest for any of the authors.

Acknowledgment

The authors wish to acknowledge Ms. Nanako Aihara for technical contributions to this work.

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Research article

Journal of Applied Toxicology

Received: 6 October 2014, Revised: 1 February 2015, Accepted: 2 February 2015 Published online in Wiley Online Library: 25 March 2015

(wileyonlinelibrary.com) DOI 10.1002/jat.3139

A novel *in chemico* method to detect skin sensitizers in highly diluted reaction conditions

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ABSTRACT: The direct peptide reactivity assay (DPRA) is a simple and versatile alternative method for the evaluation of skin sensitization that involves the reaction of test chemicals with two peptides. However, this method requires concentrated solutions of test chemicals, and hydrophobic substances may not dissolve at the concentrations required. Furthermore, hydrophobic test chemicals may precipitate when added to the reaction solution. We previously established a high-sensitivity method, the amino acid derivative reactivity assay (ADRA). This method uses novel cysteine (NAC) and novel lysine derivatives (NAL), which were synthesized by introducing a naphthalene ring to the amine group of cysteine and lysine residues. In this study, we modified the ADRA method by reducing the concentration of the test chemicals 100-fold. We investigated the accuracy of skin sensitization predictions made using the modified method, which was designated the ADRA-dilutional method (ADRA-DM). The predictive accuracy of the ADRA-DM for skin sensitization was 90% for 82 test chemicals which were also evaluated via the ADRA, and the predictive accuracy in the ADRA-DM was higher than that in the ADRA and DPRA. Furthermore, no precipitation of test compounds was observed at the initiation of the ADRA-DM reaction. These results show that the ADRA-DM allowed the use of test chemicals at concentrations two orders of magnitude lower than that possible with the ADRA. In addition, ADRA-DM does not have the restrictions on test compound solubility that were a major problem with the DPRA. Therefore, the ADRA-DM is a versatile and useful method. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: skin sensitization; prediction; direct peptide reactivity assay (DPRA); amino acid derivative reactivity assay (ADRA); NAC; NAL; ADRA-DM (ADRA-dilutional method)

Introduction

The formation of covalent adducts between a skin sensitizer and endogenous proteins or peptides in the skin is a very important step in the skin sensitization process (Smith and Hotchkiss, 2001). The interaction of skin sensitizers with proteins, peptides and model nucleophiles representing proteins or peptides was found to be predominantly covalent bonding between electrophiles and nucleophiles (Aleksic *et al.*, 2008). Based on these findings, it may be possible to predict skin sensitization based on *in vitro* data of the reactivity of test chemicals with peptides and proteins (Divkovic *et al.*, 2005; Gerberick *et al.*, 2008). To evaluate the reactivity of peptide and proteins with test compounds, a method for directly detecting and characterizing adducts between these compounds has been sought. The analysis of adducts between peptides and test chemicals using nuclear magnetic resonance (NMR) was reported by Ahlfors *et al.* (2003) and Alvarez-Sánchez *et al.* (2004). In addition, Ahlfors *et al.* (2003) and Nilsson *et al.* (2005) developed an analysis using liquid chromatography-mass spectrometry (LC-MS).

In contrast, Gerberick *et al.* (2004, 2007) developed the quantitative peptide depletion assay. This assay characterizes the skin sensitizing potential of chemicals based on their ability to deplete two nucleophilic heptapeptides using high-performance liquid chromatography (HPLC). In addition, Natsch and Gfeller (2008) explored a quantitative method using LC-MS. The oxidation of the heptapeptide, Cor1-C420, was also characterized for the formation of adducts. Moreover, a kinetic spectrophotometric assay was developed by Chipinda *et al.* (2010) to evaluate the reactivity of chemicals with 4-nitrobenzenethiol using a stopped-flow technique and UV spectrophotometry. The direct peptide reactivity assay (DPRA), first reported by Gerberick *et al.* (2007), is a simple and versatile assay that will soon be adopted by the Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) as a test method for assessing the skin-sensitizing potential of chemicals.

However, Natsch and Gfeller (2008) have reported some limitations of the DPRA such as the need for highly concentrated test chemicals. This is particularly true in the lysine-peptide-based assay (25 mM) and limits the use of highly hydrophobic test chemicals. High concentrations of test chemicals are required because the DPRA uses peptides with low UV absorption in its HPLC-UV analysis. In contrast, the concentration of test chemical used in the method of Natsch and Gfeller (2008) was 1/25 of that required for the DPRA. However, this assay was costly and had poor versatility, because the peptides were analysed using LC-MS/MS. The stopped-flow analysis developed by Chipinda *et al.* (2010) made it possible to reduce the concentration of test substances to 1/10–1/10 000 of the concentrations required for the DPRA. However, with this method, the reaction solution was quan-

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tified by stopped-flow analysis without separating test chemicals from peptides and reactive products, which may reduce the precision of the method, as the reactive products could interfere with peptides at the measurement wavelength.

In order to solve these problems, we developed an HPLC-UV method that uses low-concentration solutions of test chemicals by modifying the amino acid derivative reactivity assay (ADRA), which was previously reported by our group (Fujita *et al.*, 2014). In this study, we optimized the ADRA reaction conditions to develop a method designated as the ADRA-dilutional method (ADRA-DM), in which the concentration of test chemical was 1/100 of that used in the ADRA.

In this study, we measured the predictive accuracy of the ADRA-DM for skin sensitization by evaluating 82 test substances that have been previously tested using the ADRA (Fujita *et al.*, 2014) and the DPRA (Gerberick *et al.*, 2007).

Materials and methods

Test chemicals

The cysteine and lysine derivatives, NAC and NAL were synthesised in our laboratory (Fig. 1) (Fujita *et al.*, 2014). The 82 test chemicals evaluated in this study are listed in Table 1. These test chemicals were previously evaluated via the ADRA (Fujita *et al.*, 2014) and the DPRA (Gerberick *et al.*, 2007).

NAC and NAL reactivity assay

Sodium dihydrogen phosphate, disodium hydrogen phosphate (for buffer preparation), acetonitrile, trifluoroacetic acid (TFA for HPLC), isopropanol, DMSO and acetone were purchased from Wako Pure Chemical Industries (Osaka, Japan). The sodium phosphate buffer for NAC (100 mM; pH 8.0) was prepared with sodium dihydrogen phosphate and disodium hydrogen phosphate. The buffer for NAL (100 mM; pH 10.2) was prepared with disodium hydrogen phosphate and sodium hydride solution. Stock solutions of NAC and NAL were prepared to a final concentration of 12.5 μM in 100 mM phosphate buffer at pH 8.0 and pH 10.2, respectively.

Test chemical solutions (1 mM) were prepared in acetonitrile, water, isopropanol, acetone, or acetonitrile containing 5% dimethyl sulphoxide (DMSO). Triplicate reactivity samples were prepared in 96-well microplates. For the reaction, 80 μL of the NAC or NAL stock solution and 50 μL of the test chemical stock solution were added to 70 μL of sodium phosphate buffer (pH 8.0 or pH 10.2). As a control, wells without test chemicals were prepared in triplicate. The 96-well microplates were sealed using Plate Seal (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), gently shaken, and incubated in the dark for 24 h at 25 °C. After incubation, 50 μL of 25% acetonitrile/2.25% TFA in water was added to the samples, and the microplates were sealed by using sealing mats (AxyGen Scientific, Inc., Union City, CA, USA). In addition, standards used for defining the calibration curve were prepared without the test chemicals for NAC and NAL at concentrations ranging from 0.05 to 0.4 μM.

HPLC analysis of NAC and NAL

The HPLC analysis conditions for the ADRA-DM were the same as those used for the normal ADRA (Fujita *et al.*, 2014). Briefly, NAC and NAL in all samples and standards were quantified by using a LC-20A HPLC system (Prominence, Shimadzu Scientific Instruments, Kyoto, Japan) on a CAPCELL CORE C18 column (2.7 μm, 3.0 × 150 mm; Shiseido Co., Ltd., Tokyo, Japan) with UV detection at 281 nm using a photodiode array detector (SPD-M20A, Shimadzu Scientific Instruments, Kyoto, Japan). The temperatures of the column oven (CTO-20A; Shimadzu Scientific Instruments, Kyoto, Japan) and auto-sampler (SIL-20 AC; Shimadzu Scientific Instruments) were maintained at 40 °C and 4 °C, respectively. The mobile phase had a flow rate of 0.3 ml min^{-1} and consisted of 98/2 water/acetonitrile with 0.1% TFA (A) and 90/10 acetonitrile/water with 0.1% TFA (B). Separation was achieved by holding under the initial conditions (20% B) for 7 min, followed by a linear gradient to 100% B for 3.5 min and then back to the initial conditions for a total analysis time of 15 min per sample. Each sample (10 μL) was separated into fractions containing NAC/NAL and test chemicals on a CAPCELL CORE column. The reactivity of NAC/NAL was calculated as the percent depletion based on the decrease in the NAC/NAL concentration in the samples relative to the average concentration measured in the control. Finally, the NAC/NAL depletion was presented as the average of triplicate samples.

Class classification model

The quantitative structure–activity relationship (QSAR) modelling program was built using Statistics Gradient Perceptron Model Settings in ADMEWORKS/ModelBuilder V4.5 (Fujitsu Kyushu Systems Limited, Fukuoka, Japan). In this software, samples were divided into two classes (sensitizer and non-sensitizer) using a linear equation with depletion as the single parameter. An optimal average score that categorized sensitizers or non-sensitizers was calculated using the following formulas.

$$\text{Optimal average score} = \text{average} - (\text{standard deviation}/\text{weight}) \times \text{constant}$$

The average, standard deviation, weight and constant were obtained from the Statistics Gradient Perceptron Model Settings calculation.

Results

Initial experiments were conducted to determine the optimal reaction conditions for NAC and NAL. This allowed determination of whether it was possible to quantify NAC and NAL at concentrations of test chemicals 1/100 of those used in the ADRA. Experiments were conducted to determine the optimal pH for both the NAC and NAL reactions and to evaluate the optimal NAC/NAL:test chemical ratios of 1:10 and 1:50. For NAL, the NAL to test chemical ratio was 1:50 in the ADRA. The ratio was fixed at 1:50 in this study because of limitation on additional NAL under these reaction conditions.

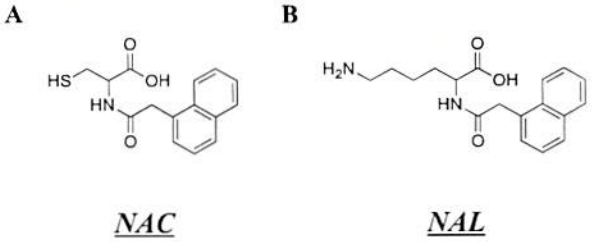

Figure 1. The chemical structures of NAC and NAL. NAC, *N*-(2-(1-naphthyl)acetyl)-L-cysteine (A) and NAL is α-*N*-(2-(1-naphthyl)acetyl)-L-lysine (B).

Table 1. Test chemicals used in the amino acid derivative reactivity assay-dilutional method (ADRA-DM), their sources and solvents

№	Test substance	CAS No.	Scource[a]	Solvent[b]
Extreme/strong				
1	Diphenylcyclopropenone	886-38-4	Wako	Acetonitrile
2	Oxazolone	15646-46-5	Wako	Acetonitrile
3	Benzoyl peroxide	94-36-0	TCI	Acetonitrile
4	Kathon CG	56965-84-9	Sigma-Aldrich	H_2O
5	Bandrowski's base	20048-27-5	Alfa Aesar	5%DMSO/Acetonitrile
6	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	Santa Cruz	H_2O
7	p-Benzoquinone	106-51-4	Wako	Acetonitrile
8	Tetrachlorosalicylanilide	1154-59-2	AccuStandard	Isopropanol
9	2,4-Dinitrochlorobenzene	97-00-7	Wako	Acetonitrile
10	Glutaraldehyde	111-30-8	Wako	Acetonitrile
11	Fluorescein isothiocyanate	3326-32-7	Dojindo	5%DMSO/Acetonitrile
12	Phthalic anhydride	85-44-9	Wako	Acetonitrile
13	Lauryl gallate	1166-52-5	Wako	Isopropanol
14	Propyl gallate	121-79-9	Wako	Acetonitrile
15	CD3	25646-71-3	FF	H_2O
16	Trimellitic anhydride	552-30-7	Wako	Acetonitrile
17	Formaldehyde	50-00-0	Wako	Acetonitrile
18	Metol	55-55-0	Wako	H_2O
Moderate				
19	2-Hydroxyethyl acrylate	818-61-1	Wako	Acetonitrile
20	Glyoxal	107-22-2	Wako	H_2O
21	Vinyl pyridine	1337-81-1	Wako	Acetonitrile
22	2-Mercaptobenzothiazole	149-30-4	Wako	Isopropanol
23	Nonanoyl chloride	764-85-2	TCI	Acetonitrile
24	2-Methyl-2H-isothiazol-3-one	2682-20-4	Sigma-Aldrich	Acetonitrile
25	1,2-Benzisothiazoline-3-one	2634-33-5	TCI	Isopropanol
26	Methyl-2-nonynoate	111-80-8	TCI	Acetonitrile
27	Cinnamaldehyde	14371-10-9	Wako	Acetonitrile
28	Phenylacetaldehyde	122-78-1	Alfa Aesar	Acetonitrile
29	Benzylideneacetone	122-57-6	Wako	Acetonitrile
30	2,4-Heptadienal	881395	Wako	Acetonitrile
31	Squaric acid	2892-51-5	Wako	Acetonitrile
32	Trans-2-hexenal	6728-26-3	Wako	Acetonitrile
33	Resorcinol	108-46-3	Wako	Acetonitrile
34	Diethyl maleate	141-05-9	Wako	Acetonitrile
35	2-phenylpropionaldehyde	93-53-8	Sigma-Aldrich	Acetonitrile
36	Perillaldehyde	2111-75-3	Wako	Acetonitrile
37	Palmitoyl Chloride	112-67-4	Wako	Acetone
38	1-(4-Methoxyphenyl)-1-penten-3-one	104-27-8	AccuStandard	Acetonitrile
Weak				
39	α-Hexylcinnnamaldehyde	101-86-0	Wako	Acetonitrile
40	α-Amylcinnamaldehyde	122-40-7	Wako	Acetonitrile
41	2,3-Butanedione	431-03-8	Wako	Acetonitrile
42	Farnesal	19317-11-4	Frinton	Acetonitrile
43	Oxalic acid	144-62-7	Wako	Acetonitrile
44	Benzyl benzoate	120-51-4	Wako	Acetonitrile
45	4-Allylanisole	140-67-0	TCI	Acetonitrile
46	Lilial	80-54-6	Wako	Acetonitrile
47	Cyclamen aldehyde	103-95-7	Sigma-Aldrich	Acetonitrile
48	Imidazolidinyl urea	39236-46-9	Sigma-Aldrich	H_2O
49	5-Methyl-2,3-hexanedione	13706-86-0	TCI	Acetonitrile
50	2,2,6,6-Tetramethyl-3,5-heptanedione	1118-71-4	TCI	Acetonitrile
51	Ethyleneglycol dimethacrylate	97-90-5	Wako	Acetonitrile
52	Ethyl acrylate	140-88-5	Wako	Acetonitrile
53	Hydroxycitronellal	107-75-5	Wako	Acetonitrile

(Continues)

Table 1. (Continued)

№	Test substance	CAS No.	Scource[a]	Solvent[b]
Non-sensitizer				
54	Glycerol	56-81-5	Wako	Acetonitrile
55	Hexane	110-54-3	Wako	Acetonitrile
56	Diethyl phthalate	84-66-2	Wako	Acetonitrile
57	Octanoic acid	124-07-2	Wako	Acetonitrile
58	2-Hydroxypropyl methacrylate	923-26-2	Wako	Acetonitrile
59	1-Butanol	71-36-3	Wako	Acetonitrile
60	4-Hydroxybenzoic acid	99-96-7	Wako	Acetonitrile
61	6-Methyl coumatrin	92-48-8	Sigma-Aldrich	Acetonitrile
62	Methyl salicylate	119-36-8	Wako	Acetonitrile
63	Chlorobenzene	108-90-7	Wako	Acetonitrile
64	Lactic acid	50-21-5	Sigma-Aldrich	Acetonitrile
65	1-Bromobutane	109-65-9	Wako	Acetonitrile
66	2-Acethylcyclohexanone	874-23-7	Wako	Acetonitrile
67	4'-Methoxyacetophenone	100-06-1	Wako	Acetonitrile
68	Ethyl benzoylacetate	94-02-0	Wako	Acetonitrile
69	Ethyl vanillin	121-32-4	TCI	Acetonitrile
70	Isopropanol	67-63-0	Wako	Acetonitrile
71	Propylene glycol	57-55-6	Wako	Acetonitrile
72	Sulfanilamide	63-74-1	Wako	Acetonitrile
73	Isopropyl myristate	110-27-0	Wako	Acetonitrile
74	Benzaldehyde	100-52-7	Sigma-Aldrich	Acetonitrile
75	Methylparaben	99-76-3	Wako	Acetonitrile
76	Nonanoic acid	112-05-0	TCI	Acetonitrile
77	Propyl paraben	94-13-3	Wako	Acetonitrile
78	Salicylic acid	69-72-7	Wako	Acetonitrile
79	Sulphanilic acid	121-57-3	Wako	H_2O
80	Vanillin	121-33-5	Wako	Acetonitrile
81	Coumarin	91-64-5	Wako	Acetonitrile
82	Vinylidene dichloride	75-35-4	AccuStandard	Acetonitrile

[a]AccuStandard, AccuStandard, Inc., New Haven, CT, USA; Alfa Aesar, Alfa Aesar, Ward Hill, MA, USA; Dojindo, Dojindo Molecular Technologies, Inc., Kumamoto, Japan;FF, Synthetic Organic Chemistry Laboratories of FUJIFILM Cororation; Frinton, Frinton Laboratories, Inc., Hainesport, NJ, USA; Santa Cruz, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Sigma-Aldrich, Sigma-Aldrich Corporation, St Louis, MO, USA; TCI, Tokyo Chemical Industry Co Ltd., Tokyo, Japan; Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan
[b]Solvent of 1 mM test chemical solutions

Optimal pH for the NAC reaction solution (NAC:test chemical = 1:10)

Experiments were conducted to determine optimal NAC reaction conditions in the ADRA-DM at pH 7.5 and 8.0 at a NAC:test chemical ratio of 1:10, which is equal to that used in the ADRA. For this evaluation, we selected 19 test chemicals with various skin sensitization potencies from a group of 82 chemicals tested using the ADRA and DPRA. Depletion of NAC by 2,4-dinitrochlorobenzene, phenylacetaldehyde, diethylmaleate and farnesal at pH 8.0 was more than 5% higher than the depletion by these substances at pH 7.5, but the depletion by cinnamaldehyde was more than 5% less than that observed at pH 7.5. Therefore, depletion by all test chemicals at pH 8.0, except cinnamaldehyde, was greater than or similar to the level observed at pH 7.5 (Table 2).

Optimal pH of the NAL reaction solution (NAL:test chemical = 1:50)

Experiments were conducted to determine optimal NAL reaction conditions in the ADRA-DM at pH 10.2 and 12.0 at a NAL:test chemical ratio of 1:50, which is equal to that used in the ADRA. For this evaluation, the same 19 test chemicals were used as in the NAC reaction optimization experiment. The reaction of NAL with benzoyl peroxide, fluorescein isothiocyanate and ethyl benzoylacetate could not be quantified precisely because of co-elution of NAL with degradation products of the test chemicals at pH 12.0. With the exception of tetrachlorosalicylanilide, 2,4-dinitrochlorobenzene, α-hexylcinnnamaldehyde and farnesal, depletion of NAL by all the test chemicals at pH 10.2 was higher than that at pH 12.0 (Table 3).

Optimal ratio of NAC to the test chemical in the reaction solution

Experiments were conducted to determine the optimal ratio of NAC to test chemical in the ADRA-DM reaction solution. NAC to test chemical ratios of 1:10 and 1:50 were tested. Depletion of NAC by 11 test chemicals at a ratio of 1:50 was higher than the depletion at a ratio of 1:10. NAC depletion at a ratio of 1:50 was 100% for five test chemicals that showed 100% depletion at 1:10. (Table 4).

Table 2. Reactivity of novel cysteine (NAC) with test chemicals at pH 7.5 and pH 8.0 with results expressed as percent depletion of non-reacted NAC

Nº	Test substance	ADRA-DM				ADRA[a]	
		pH 7.5		pH 8.0			
		Depletion	SD	Depletion	SD	Depletion	SD
Extreme/strong							
3	Benzoyl peroxide	100.0	0.4	100.0	0.0	100.0	0.0
6	5-Chloro-2-methyl-4-isothiazolin-3-one	100.0	1.1	100.0	0.0	99.3	0.6
8	Tetrachlorosalicylanilide	5.4	0.9	10.1	0.3	23.3	0.4
9	2,4-Dinitrochlorobenzene	17.1	0.4	44.5	0.6	100.0	0.0
11	Fluorescein isothiocyanate	45.3	0.6	48.8	0.9	100.0	0.0
13	Lauryl gallate	100.0	2.5	100.0	0.0	99.6	0.3
18	Metol	100.0	1.1	100.0	0.0	97.5	0.6
Moderate							
24	2-Methyl-2H-isothiazol-3-one	100.0	0.4	100.0	0.0	100.0	0.0
27	Cinnamaldehyde	45.6	0.3	15.0	1.0	95.5	1.6
28	Phenylacetaldehyde	3.2	0.8	11.6	1.8	99.8	0.4
34	Diethyl maleate	2.6	0.6	10.9	0.9	98.9	0.4
37	Palmitoyl Chloride	0.1	0.9	-0.2	2.5	17.1	1.0
Weak							
39	α-Hexylcinnnamaldehyde	0.9	1.0	4.3	1.5	0.1	1.9
42	Farnesal	3.0	0.4	11.2	1.3	41.3	0.8
45	4-Allylanisole	3.4	0.4	7.6	2.2	66.0	0.8
Non-sensitizer							
65	1-Bromobutane	-0.4	0.5	3.6	1.6	6.0	3.6
68	Ethyl benzoylacetate	3.3	0.5	7.4	1.4	3.6	2.9
73	Isopropyl myristate	0.5	0.4	-1.0	1.4	-0.9	1.7
77	Propyl paraben	0.0	0.8	0.9	1.5	-3.3	1.2

[a]Depletion and SD of NAC (Fujita *et al.*, 2014).

Validation of 82 test chemicals by ADRA-DM

We validated the ADRA-DM using 82 test chemicals that were previously evaluated in the ADRA and the DPRA. The pH of the reaction solutions was fixed at 8.0 for NAC and 10.2 for NAL. The ratio of NAC and NAL to the test chemicals was fixed at 1:50. The results of these experiments are shown in Table 5. Depletion of NAC and NAL in the ADRA-DM tended to be equivalent to or less than that seen in the ADRA. However, depletion of NAC with propyl gallate, 2-mercaptobenzothiazole, lilial, ethyl vanillin, and benzaldehyde and depletion of NAL with oxazolone, phthalic anhydride, and 2-methyl-2H-isothiazol-3-one was more than 5% higher than their respective levels in the ADRA.

The standard deviation (SD) of NAC and NAL depletion measurements in the ADRA ranged from 0% to 7.8% and 0% to 9.5%, respectively. The SD of NAC and NAL depletion measurements in the ADRA-DM ranged from 0% to 7.1% and 0% to 4.7%, respectively. Therefore, the variability of the depletion measurements in the ADRA-DM tended to be less than that in the ADRA (Table 5).

Prediction model based on 2-class classification

Using ModelBuilder in ADMEWORKS, we developed a classification model to classify test chemicals as sensitizers and non-sensitizers based on the average score, and the optimal average score was calculated to be 5.050%. Based on this threshold, the sensitivity, specificity, positive predictivity, negative predictivity and accuracy of the ADRA-DM measurements were calculated by Cooper statistics and compared with those calculated for the ADRA. The accuracy of the ADRA-DM was found to be 90%, which was almost exactly the same as the accuracy of the ADRA. Similarly, the sensitivity and specificity of the ADRA-DM were approximately equal to or greater than those of ADRA.

Using the ADRA, five known sensitizers were incorrectly classified as non-sensitizers, and five known non-sensitizers were incorrectly classified as sensitizers. Using the ADRA-DM, seven known sensitizers were incorrectly classified as non-sensitizers, and one known non-sensitizers were incorrectly classified as sensitizers (Fig. 2, Table 6). 2-Hydroxypropyl methacrylate, 2-acethylcyclohexanone, ethyl vanillin and vanillin were incorrectly categorized as sensitizers by the ADRA but were correctly categorized as non-sensitizers by the ADRA-DM. In contrast, 1-(4-methoxyphenyl)-1-penten-3-one and 2,2,6,6-tetramethyl-3,5-heptanedione were correctly categorized as sensitizers by the ADRA but were incorrectly categorized as non-sensitizers by the ADRA-DM (Table 6).

Discussion

The ADRA, which we recently reported (Fujita *et al.*, 2014), is a novel method in which residual levels of two amino acid derivatives, NAC and NAL, are measured after reaction with test compounds. For the modified amino acids, the amine groups of

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Table 3. Reactivity of novel lysine derivatives (NAL) with test chemicals at pH 10.2 and pH 12.0 with results expressed as percent depletion of NAL

№	Test substance	ADRA-DM					
		pH 10.2		pH 12.0		ADRA[a]	
		Depletion	SD	Depletion	SD	Depletion	SD
Extreme/strong							
3	Benzoyl peroxide	50.6	1.3	-	-	100.0	1.4
6	5-Chloro-2-methyl-4-isothiazolin-3-one	17.7	1.1	2.1	0.8	61.6	9.2
8	Tetrachlorosalicylanilide	2.1	1.3	2.4	0.9	14.9	0.7
9	2,4-Dinitrochlorobenzene	6.1	1.3	8.1	1.4	83.2	0.9
11	Fluorescein isothiocyanate	98.1	0.0	-	-	100.0	0.0
13	Lauryl gallate	19.0	1.1	11.2	0.7	33.8	0.8
18	Metol	22.8	1.1	11.8	0.4	60.8	0.4
Moderate							
24	2-Methyl-2H-isothiazol-3-one	7.0	2.2	1.5	1.6	-0.2	8.2
27	Cinnamaldehyde	13.1	1.3	4.2	1.7	81.3	1.8
28	Phenylacetaldehyde	98.3	1.5	95.4	1.6	99.2	1.0
34	Diethyl maleate	7.7	1.9	3.4	1.2	18.7	0.8
37	Palmitoyl Chloride	95.9	2.5	87.3	1.3	100.0	0.0
Weak							
39	α-Hexylcinnnamaldehyde	1.1	2.9	2.4	1.7	1.8	1.5
42	Farnesal	8.8	1.4	13.4	1.5	38.6	1.9
45	4-Allylanisole	3.9	2.6	1.4	1.5	10.9	1.0
Non-sensitizer							
65	1-Bromobutane	2.4	1.6	-0.5	1.5	2.9	0.8
68	Ethyl benzoylacetate	5.3	1.3	-	-	5.4	1.6
73	Isopropyl myristate	1.8	1.4	0.5	1.7	-0.9	0.8
77	Propyl paraben	1.9	3.1	-0.4	1.5	1.0	0.9

[a]Depletion and SD of NAL (Fujita *et al.*, 2014).

cysteine and lysine residues are bound to a naphthalene ring giving an ultraviolet absorption maximum at 281 nm, which was quantified by HPLC analysis. The concentration of the NAC and NAL stock solutions was 1.25 mM, because reaction conditions for the ADRA were established based on those for the DPRA. However, because peptides in the DPRA must be quantified by HPLC analysis at 220 nm, an absorption wavelength for many test chemicals, peptides in the DPRA might be difficult to determine by HPLC because test compounds easily mask them.

A distinctive feature of ADRA is that NAC/NAL can be quantified precisely via HPLC analysis at 281 nm; therefore, other substances in the reaction solution would not affect NAC/NAL detection. Because of the high sensitivity of the ADRA, it was suggested that NAC and NAL might be quantified at 1/100 of the concentrations normally used. Therefore, the concentrations of the stock solutions of test chemicals used in the ADRA could reduce from 100 to 1 mM. This should allow stock solutions to be prepared easily for test chemicals that are poorly soluble in water or other solvents. Moreover, it was expected that NAC/NAL could be quantified more precisely by using lower concentrations of test chemicals, because the test chemicals would be less likely to precipitate and produce a cloudy reaction solution.

Before evaluating lower concentrations of test chemical solutions via the ADRA-DM, we conducted experiments to determine optimal reaction conditions. It was hypothesized that decreasing the concentrations of the test chemicals in the reaction solutions to 1/100 of their normal levels would decrease the probability of collisions between NAC/NAL and the test chemicals and therefore, decrease their reactivity. In order to avoid reduced interaction between NAC/NAL and the test chemicals, it was considered necessary for the reaction pH to be high and for the ratio of NAC to the test chemical to be increased from 1:10 to 1:50; therefore, appropriate experiments were conducted to test these hypotheses. For NAL, the ratio of NAL to the test chemical is 1:50 in the ADRA, and so its ratio was fixed at 1:50 in the ADRA-DM, because the reaction conditions limited the amount of NAL that could be added.

For NAC in the ADRA, a pH of 9.5 was set as the higher limit to prevent auto-oxidation, but it was hypothesized that a lower pH would be needed in the ADRA-DM, because of the susceptibility of the lower concentration of NAC to oxidation by dissolved oxygen in the reaction solution. In fact, because oxidized NAC was detected in the reaction solution at pH 9.0 (data not shown), we explored conditions at pH 7.5 and 8.0 using 19 test chemicals with various skin sensitization potencies. It was found that depletion of test chemicals in the ADRA-DM was generally less than that observed in the ADRA, and this tendency grew stronger as the pH was decreased (Table 2). Because we did not observe NAC oxidation at pH 8.0, it was decided that the optimal reaction pH of NAC in the ADRA-DM was 8.0.

In a similar manner, we explored the optimal conditions for NAL in the ADRA-DM at pH 10.2 and 12.0 using the same 19 test chemicals. At pH 12.0, NAL could not be quantified owing to co-elution with benzoyl peroxide, fluorescein isothiocyanate and ethyl benzoylacetate. It was hypothesized that the increase in

Table 4. Reactivity of test chemicals with novel cysteine (NAC) at different ratios of NAC to test chemicals with results expressed as percent depletion of NAC

Nº	Test substance	ADRA-DM					
		1:10		1:50		ADRA[a]	
		Depletion	SD	Depletion	SD	Depletion	SD
Extreme/strong							
3	Benzoyl peroxide	100.0	0.0	100.0	0.0	100.0	0.0
6	5-Chloro-2-methyl-4-isothiazolin-3-one	100.0	0.0	100.0	0.0	99.3	0.6
8	Tetrachlorosalicylanilide	10.1	0.3	22.9	1.0	23.3	0.4
9	2,4-Dinitrochlorobenzene	44.5	0.6	87.6	2.9	100.0	0.0
11	Fluorescein isothiocyanate	48.8	0.9	94.1	3.9	100.0	0.0
13	Lauryl gallate	100.0	0.0	100.0	0.0	99.6	0.3
18	Metol	100.0	0.0	100.0	0.0	97.5	0.6
Moderate							
24	2-Methyl-2H-isothiazol-3-one	100.0	0.0	100.0	0.0	100.0	0.0
27	Cinnamaldehyde	15.0	1.0	100.0	0.0	95.5	1.6
28	Phenylacetaldehyde	11.6	1.8	21.2	2.4	99.8	0.4
34	Diethyl maleate	10.9	0.9	22.6	3.3	98.9	0.4
37	Palmitoyl Chloride	-0.2	2.5	6.9	1.4	17.1	1.0
Weak							
39	α-Hexylcinnnamaldehyde	4.3	1.5	2.5	2.3	0.1	1.9
42	Farnesal	11.2	1.3	17.1	2.3	41.3	0.8
45	4-Allylanisole	7.6	2.2	23.9	2.3	66.0	0.8
Non-sensitizer							
65	1-Bromobutane	3.6	1.6	6.7	2.2	6.0	3.6
68	Ethyl benzoylacetate	7.4	1.4	-6.6	2.7	3.6	2.9
73	Isopropyl myristate	-1.0	1.4	-1.6	2.4	-0.9	1.7
77	Propyl paraben	0.9	1.5	2.7	2.2	-3.3	1.2

[a]Depletion and SD of NAC (Fujita *et al.*, 2014).

co-elution was caused by increased degradation of test chemicals at the higher pH, where they are more susceptible to hydroxyl ion attack because they are present at a lower concentration (1 mM) than in the ADRA. Moreover, because NAL depletion at pH 10.2 was equal to or greater than depletion at pH 12.0 (with the exceptions of tetrachlorosalicylanilide, 2,4-dinitrochlorobenzene, α-hexylcinnnamaldehyde and farnesal), the optimal reaction pH of NAL was determined to be 10.2 (Table 3).

In addition, we explored the optimal ratio of NAC to test chemicals by testing ratios of 1:10 and 1:50 for 19 test chemicals. As expected, NAC depletion at a ratio of 1:50 was generally greater than depletion at the 1:10 ratio (Table 4). Therefore, it was decided that the optimal NAC to test chemical ratio was 1:50.

Under the optimal conditions described above, the depletion of NAC and NAL with the 82 test chemicals in the ADRA-DM was compared with the depletion of the same chemical observed with the ADRA (Table 5), which we previously reported (Fujita *et al.*, 2014). It was found that the depletion of NAC and NAL in the ADRA-DM was generally less than that observed in the ADRA for each test chemical. This trend was thought to be caused by the lower collision probability between NAC/NAL and the test chemical, which was due to the greater dilution of the reaction solution. However, the reduced reactivity observed in the ADRA-DM may not influence the accuracy of skin sensitization predictions made with this method.

Initially, we considered the results of test chemicals with comparatively high reactivity (extreme/strong sensitizers) (Table 5). Twelve test chemicals produced NAC depletion of 90% or more in the ADRA. Ten test chemicals produced NAC depletion of 90% or more in the ADRA-DM, including many of the same chemicals that produced this effect in the ADRA. In contrast, six test chemicals produced NAL depletion of 90% or more in the ADRA, but only three of these test chemicals produced NAL depletion of 90% or more in the ADRA-DM. However, the three test chemicals that produced more than 90% depletion in the ADRA but less than 90% in the ADRA-DM, showed depletion of 50% or more, suggesting that these chemicals maintained some reactivity.

Next, we considered results using test chemicals with very low reactivity in all of the sensitizing categories (Table 5). Eight test chemicals depleted NAC and NAL by 5% or less in the ADRA. However, 10 test chemicals depleted NAC by 5% or less, and 20 test chemicals depleted NAL by 5% or less in the ADRA-DM. In the ADRA-DM, the depletion of NAL was smaller than that observed in the ADRA. These results showed that the reactivity of the thiol group of NAC might be able to compensate for the lack of reactivity, even if the reactivity of the amino group of NAL was decreased. The number of test chemicals for which the average score of NAC and NAL was 5% or less was approximately equal between the ADRA and the ADRA-DM, whereas depletion of NAL in the ADRA-DM was less than that observed in the ADRA. Additionally, it was hypothesized that the ADRA-DM could provide predictive accuracy for skin sensitization equal to that of the ADRA, when the criterion used to distinguish sensitizers from non-sensitizers was defined as a 5% average score.

Table 5. Reactivity of test chemicals with novel cysteine (NAC)/novel lysine derivatives (NAL) in the amino acid derivative reactivity assay (ADRA) and the ADRA-dilutional method (DM) with results expressed as percent depletion of NAC/NAL

Nº	Test substance	ADRA-DM				Ave. score	ADRA[a]				Ave. score
		NAC		NAL			NAC		NAL		
		Depletion	SD	Depletion	SD		Depletion	SD	Depletion	SD	
Extreme/strong											
1	Diphenylcyclopropenone	23.9	1.5	6.3	1.2	15.1	99.8	2.1	99.6 [p]	1.4	99.7
2	Oxazolone	80.2	1.6	80.1	0.8	80.1	76.5	0.4	30.1	1.8	53.3
3	Benzoyl peroxide	100.0	0.0	50.6	2.7	75.3	100.0 [p]	0.0	100.0 [p]	1.4	100.0
4	Kathon CG	100.0	0.0	-0.4	0.5	49.8	98.9 [p]	0.5	3.6 [p]	0.5	51.3
5	Bandrowski's base	100.0	0.0	5.7	0.4	52.9	100.0 [p]	0.0	38.8	1.1	69.4
6	5-Chloro-2-methyl-4-isothiazolin-3-one	100.0	0.0	17.7	1.1	58.8	99.3 [p]	0.6	63.3 [p]	9.2	81.3
7	p-Benzoquinone	97.2	2.0	83.5	1.1	90.3	98.9	2.1	97.5	1.4	98.2
8	Tetrachlorosalicylanilide	22.9	4.2	2.1	0.3	12.5	23.3 [p]	0.4	14.9	0.7	19.1
9	2,4-Dinitrochlorobenzene	87.6	2.9	6.1	1.3	46.9	100.0	0.0	83.2	0.9	91.6
10	Glutaraldehyde	4.6	1.7	53.1	2.9	28.8	49.2	0.6	96.2	1.4	72.7
11	Fluorescein isothiocyanate	94.1	0.9	98.1	0.6	96.1	100.0	0.0	100.0	0.0	100.0
12	Phthalic anhydride	-1.8	1.5	96.9	1.1	47.5	1.0	1.7	84.4	1.3	42.7
13	Lauryl gallate	100.0	0.0	19.0	0.4	59.5	99.6 [p]	0.3	33.8	0.8	66.7
14	Propyl gallate	100.0	0.0	56.4	1.4	78.2	93.7	2.0	75.2	1.4	84.5
15	CD3	100.0	0.0	16.5	0.5	58.3	100.0	0.0	40.8	1.1	70.4
16	Trimellitic anhydride	1.8	1.6	97.0	1.2	49.4	1.0	1.6	99.4	1.4	50.2
17	Formaldehyde	24.4	2.7	1.6	1.9	13.0	81.7	1.9	65.6	2.4	73.7
18	Metol	100.0	0.0	22.8	1.1	61.4	97.5	0.6	60.8	0.4	79.2
Moderate											
19	2-Hydroxyethyl acrylate	100.0	0.0	16.3	4.7	58.1	100.0	0.0	81.5	1.1	90.7
20	Glyoxal	12.5	0.9	0.8	0.5	6.7	33.3	2.5	21.3	0.8	27.3
21	Vinyl pyridine	27.8	2.1	7.7	1.8	17.8	74.8	2.0	6.4	2.1	40.6
22	2-Mercaptobenzothiazole	100.0	0.0	0.3	0.8	50.2	40.0	1.9	-0.9	0.8	19.5
23	Nonanoyl chloride	7.5	2.1	39.4	1.8	23.5	8.1 [t]	1.6	100.0 [t]	0.0	54.1
24	2-Methyl-2H-isothiazol-3-one	100.0	0.0	7.0	2.2	53.5	100.0	0.0	-0.2	8.2	49.9
25	1,2-Benzisothiazoline-3-one	100.0	0.0	0.4	0.7	50.2	100.0	0.0	0.1	1.5	50.1
26	Methyl-2-nonynoate	11.1	0.8	1.4	0.2	6.2	99.6	2.1	10.2	1.6	54.9
27	Cinnamaldehyde	100.0	0.0	13.1	1.3	56.5	95.5	1.6	81.3	1.8	88.4
28	Phenylacetaldehyde	21.2	2.4	98.3	1.5	59.8	99.8	0.4	99.2	1.0	99.5
29	Benzylideneacetone	19.4	1.3	7.2	1.0	13.3	95.3	2.1	15.0 [p,t]	1.1	55.1
30	2,4-Heptadienal	35.3	2.6	50.1	1.3	42.7	100.0	0.0	98.4	1.4	99.2
31	Squaric acid	-2.9	1.8	0.5	0.2	-1.2	-2.0	0.4	1.5	0.6	-0.3
32	Trans-2-hexenal	83.5	2.4	12.3	1.1	47.9	96.7	2.1	97.6	1.4	97.1
33	Resorcinol	9.8	3.5	2.2	0.7	6.0	10.0	1.4	64.1	0.6	37.1
34	Diethyl maleate	22.6	3.3	7.7	1.9	15.1	98.9	0.4	18.7	0.8	58.8
35	2-phenylpropionaldehyde	8.1	0.8	4.6	1.3	6.3	70.6	7.8	8.5	9.5	39.6
36	Perillaldehyde	36.9	2.5	18.5	1.0	27.7	94.0	2.1	66.0	1.2	80.0
37	Palmitoyl Chloride	6.9	1.4	95.9	2.5	51.4	17.1 [p]	1.0	100.0 [p]	0.0	58.5
38	1-(4-Methoxyphenyl)-1-penten-3-one	2.9	2.4	4.2	1.6	3.5	88.7	2.1	6.4 [t]	1.7	47.6
Weak											
39	α-Hexylcinnnamaldehyde	2.5	2.2	1.1	0.9	1.8	0.1	1.9	1.8 [p]	1.5	1.0
40	α-Amylcinnamaldehyde	1.3	1.5	1.6	0.4	1.4	2.2	1.6	6.0 [t]	2.0	4.1
41	2,3-Butanedione	71.3	1.4	25.5	1.2	48.4	100.0	0.0	73.1	2.8	86.5
42	Farnesal	17.1	2.3	8.8	1.4	12.9	41.3	0.8	38.6	1.9	39.9
43	Oxalic acid	0.4	2.9	3.6	1.0	2.0	-4.3	1.6	4.5	1.0	0.1
44	Benzyl benzoate	1.5	3.4	2.8	1.1	2.2	3.3	2.0	3.0	1.2	3.1
45	4-Allylanisole	23.9	2.3	3.9	2.6	13.9	66.0	0.8	10.9	1.0	38.4
46	Lilial	20.7	1.3	3.9	0.9	12.3	4.5	2.7	54.7	1.8	29.6
47	Cyclamen aldehyde	14.5	2.6	1.7	0.6	8.1	10.5	2.3	11.1	1.0	10.8
48	Imidazolidinyl urea	35.4	2.2	2.0	0.2	18.7	80.0	0.5	83.7	0.5	81.8

(Continues)

Table 5. (Continued)

№	Test substance	ADRA-DM				Ave. score	ADRA[a]				Ave. score
		NAC		NAL			NAC		NAL		
		Depletion	SD	Depletion	SD		Depletion	SD	Depletion	SD	
49	5-Methyl-2,3-hexanedione	15.9	1.1	34.8	2.8	25.3	23.2	1.9	98.9	1.3	61.0
50	2,2,6,6-Tetramethyl-3,5-heptanedione	0.9	2.4	1.6	0.8	1.2	8.1	1.8	7.6	1.4	7.8
51	Ethyleneglycol dimethacrylate	8.9	1.1	2.6	1.6	5.8	100.0	0.0	24.3	1.3	62.1
52	Ethyl acrylate	89.8	1.9	13.7	0.4	51.7	100.0	0.0	96.7	1.2	98.4
53	Hydroxycitronellal	11.2	2.2	1.5	0.7	6.3	14.8	4.6	21.9	1.2	18.3
Non-sensitizer											
54	Glycerol	1.8	2.4	0.7	1.2	1.2	4.3	2.3	4.3	1.9	4.3
55	Hexane	0.4	1.8	0.9	0.9	0.7	3.0	2.0	5.8	1.5	4.4
56	Diethyl phthalate	0.1	2.0	1.7	1.4	0.9	4.8	1.8	6.8	1.8	5.8
57	Octanoic acid	-2.1	1.1	1.2	0.5	-0.4	1.8	1.9	5.0	1.0	3.4
58	2-Hydroxypropyl methacrylate	1.3	1.0	2.2	0.6	1.8	98.1	1.7	10.9	1.3	54.5
59	1-Butanol	-1.7	1.5	0.8	0.5	-0.4	3.9	2.4	2.5	1.6	3.2
60	4-Hydroxybenzoic acid	0.6	2.3	-1.4	1.0	-0.4	0.5	1.8	2.2	3.4	1.3
61	6-Methyl coumatrin	2.2	2.2	1.0	0.7	1.6	6.1	1.8	4.5	1.0	5.3
62	Methyl salicylate	-2.9	0.9	0.4	0.6	-1.3	-0.7	2.0	9.1	1.1	4.2
63	Chlorobenzene	1.6	2.1	0.7	0.7	1.2	2.1	2.4	4.6	1.0	3.3
64	Lactic acid	3.8	2.0	2.8	0.6	3.3	-2.4	1.8	2.8	1.9	0.2
65	1-Bromobutane	6.7	3.5	2.4	1.6	4.6	6.0	3.6	2.9	0.8	4.4
66	2-Acethylcyclohexanone	0.3	3.1	0.3	0.6	0.3	7.2	1.9	14.6	2.6	10.9
67	4'-Methoxyacetophenone	3.9	7.1	3.3	0.6	3.6	4.1	1.3	-1.4	2.3	1.3
68	Ethyl benzoylacetate	-6.6	2.7	5.3	1.3	-0.6	3.6	2.9	5.4	1.6	4.5
69	Ethyl vanillin	7.3	1.5	2.1	0.8	4.7	-3.2	2.1	63.9	1.2	30.4
70	Isopropanol	3.6	1.4	2.8	0.6	3.2	-0.2	1.8	-5.2	6.7	-2.7
71	Propylene glycol	3.5	1.3	2.2	0.7	2.9	-5.0	2.1	-1.2	1.0	-3.1
72	Sulfanilamide	-2.7	2.5	1.4	0.7	-0.7	-4.4	2.3	-2.3	1.0	-3.3
73	Isopropyl myristate	-1.6	2.4	1.8	1.4	0.1	-0.9	1.7	-0.9	0.8	-0.9
74	Benzaldehyde	24.4	0.8	2.9	1.0	13.7	12.5	1.5	5.3	0.5	8.9
75	Methylparaben	2.8	1.1	1.3	0.5	2.0	2.6	1.4	3.7	0.6	3.2
76	Nonanoic acid	-5.3	2.2	1.3	0.7	-2.0	0.9	1.5	3.8	0.6	2.4
77	Propyl paraben	2.7	2.7	1.9	3.1	2.3	-3.3	1.2	1.0	0.9	-1.2
78	Salicylic acid	-5.0	2.2	0.4	0.6	-2.3	-2.3	1.4	1.5	0.7	-0.4
79	Sulphanilic acid	-0.2	0.6	0.4	0.2	0.1	-1.5	0.6	0.9	0.4	-0.3
80	Vanillin	0.5	2.7	1.9	1.5	1.2	-0.2	2.3	62.7	0.6	31.3
81	Coumarin	-3.1	2.7	3.9	2.4	0.4	2.5	1.7	1.5	1.5	2.0
82	Vinylidene dichloride	1.4	2.4	-0.3	0.7	0.5	4.9	1.6	0.7	0.9	2.8

p: precipitaion at 0 h
t: turbidity at 0 h
[a]Depletion and SD of NAC and NAL (Fujita *et al.*, 2014).

With regard to variation in depletion levels between samples, the SD of NAC and NAL depletion in the ADRA-DM ranged from 0% to 7.1% and from 0% to 4.7%, respectively. In contrast, the SD of NAC and NAL depletion in the ADRA ranged from 0% to 7.8% and from 0% to 9.5%, respectively (Table 5). Moreover, the maximum SDs for depletion of NAC and NAL by the 29 non-sensitizers in the ADRA-DM were 7.1% and 3.1%, respectively (Table 5). These values are a slightly higher and lower than the 5.050% cut-off criterion for sensitizers in the ADRA-DM.

In contrast, the maximum SDs for depletion of Cys and Lys by the same 29 non-sensitizers in the DPRA were 17.5% and 22.0%, respectively (Gerberick *et al.*, 2007). These values surpassed by far the criterion for classification of sensitizers in the DPRA (6.38%), suggesting that the 5.050% criterion for the ADRA is a reasonable value. Furthermore, after the test chemicals reacted with NAC and NAL for 24 h at 25 °C, these reactions were completely stopped by adding trifluoroacetic acid to the reaction solution in the ADRA/ADRA-DM. Therefore, the factors contributing to experimental variation are limited to variations in the preparation of reaction solutions and HPLC analysis. The results of this study showed that the variations in depletion were only slightly influenced by dilution, even if the concentration of each substance in the reaction solution was diluted to 1/100 of the concentration used in the ADRA.

The prediction accuracy for skin sensitization in the ADRA-DM is shown in Fig. 2. The criterion to distinguish sensitizers from non-sensitizers based on statistical analysis was set at 5.050% depletion. This value was nearly equivalent to the 5% threshold that was set as a criterion to distinguish sensitizers from non-sensitizers

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		Predicted Classification		
		Sensitizer	Non-sensitizer	total
Chemical Classification[a]	Sensitizer	46	7	53
	Non-sensitizer	1	28	29
	total	47	35	82

sensitivity:	87%
specificity:	97%
positive predictivity:	98%
negative predictivity:	80%
accuracy:	**90%**

[a]Based primarily on LLNA data

Figure 2. Cooper statistics (sensitizers versus non-sensitizers) for the NAC and NAL prediction models. NAC, *N*-(2-(1-naphthyl)acetyl)-L-cysteine; NAL, α-N-(2-(1-naphthyl)acetyl)-L-lysine.

described above. Furthermore, the accuracy of the ADRA-DM calculated based on this criterion was 90%, whereas the ADRA and DPRA were only 88% accurate.

The skin sensitization predictions (sensitizers/ non-sensitizers) for 82 test chemicals are shown in Table 6. There were 5 seven false-negative results, in which test chemicals were incorrectly categorized as non-sensitizers, which was a slightly more than the number of false-negatives predicted by the ADRA. However, the ADRA-DM was less likely than the ADRA to produce false-positive results; the ADRA-DM incorrectly categorized 2 one non-sensitizer as sensitizer, whereas the ADRA produced five false-positive results. The false positives likely decreased because the reactivity of NAC/NAL with the test chemicals was very low in the ADRA-DM as compared with the ADRA, except for chemicals that strongly reacted with NAC/NAL.

Precipitation and/or turbidity appeared after the addition of 13 test chemicals to the reaction solution in the ADRA (Table 5). These test chemicals were highly hydrophobic and do not easily dissolve in highly aqueous solutions. Indeed, five of the 13 test chemicals that precipitated in the ADRA possessed calculated partition coefficients (ClogP) ≥ 4. For chemicals with ClogP < 4, reaction products may have precipitated because of the high reactivity with NAC/NAL.

In contrast, no test chemicals precipitated or produced cloudy solutions when added to reaction solutions for use in the ADRA-DM because the concentration of each chemical in the reaction solution was much lower than in the ADRA. Therefore, it seems likely that depletion was more accurately evaluated using the ADRA-DM than the ADRA or the DPRA, because test chemicals in the ADRA-DM reaction solution remained in solution.

We tested chemicals with a range of predicted results in the ADRA-DM, ADRA and DPRA (Tables 5 and 6). Although nonanoyl chloride and resorcinol were incorrectly categorized as non-sensitizers by the DPRA, they were correctly categorized as sensitizers by the ADRA-DM and ADRA. Acyl chlorides have been shown to react readily with amino groups. When nonanoyl chloride was tested, NAL depletion was about 40% or more in the ADRA-DM and ADRA, which was similar to results for palmitoyl chloride which is a kind of acyl chloride. However, depletion of NAL by acyl chlorides was lower in the DPRA - about 0% for nonanoyl chloride and slightly more than 20% for palmitoyl chloride. Therefore, for acyl chlorides, ADRA and ADRA-DM predicted skin sensitization more accurately than DPRA.

Squaric acid was correctly categorized as a sensitizer by the DPRA, but was incorrectly categorized as a non-sensitizer by the ADRA-DM and ADRA. For this chemical, the average score in the DPRA was different from that in the ADRA-DM and ADRA. As discussed in our previous work (Fujita *et al.*, 2014), squaric acid might not be sufficiently soluble in the reaction solution for accurate measurements to be performed.

Only 1-(4-methoxyphenyl)-1-penten-3-one was correctly categorized as a sensitizer in the ADRA and DPRA, but incorrectly categorized as a non-sensitizer in the ADRA-DM. The reactivity of NAC with this chemical in the ADRA-DM was markedly lesser than that in the ADRA, and so this chemical was incorrectly categorized as a non-sensitizer in the ADRA-DM. This reason why reactivity of NAC decreased might be that this reactivity was extremely dependent of pH because pH of the reaction solution in the ADRA-DM was 8.0 in spite of pH 9.5 of that in the ADRA.

α-Hexylcinnamaldehyde, α-amylcinnamaldehyde, oxalic acid and benzyl benzoate were incorrectly categorized as non-sensitizers by the ADRA-DM, ADRA and DPRA. These types of test chemicals might be hard to evaluate correctly in these assays.

We also evaluated compounds classified as non-sensitizers that gave different results in the ADRA-DM, ADRA and DPRA (Tables 4 and 5). 1-Bromobutane was incorrectly categorized as a sensitizer by the DPRA, but was correctly categorized as a non-sensitizer by the ADRA-DM and ADRA. Moreover, 2-hydroxypropyl methacrylate was incorrectly categorized as a sensitizer by the ADRA and DPRA but was correctly categorized as a non-sensitizer by the ADRA-DM. In general, the reactivity of methacrylates was lower than that of acrylates. ADRA-DM might be able to detect slight differences between methacrylates categorized as sensitizers and non-sensitizers, because ethylene glycol dimethacrylate and 2-hydroxypropyl methacrylate were correctly categorized. The results of the ADRA-DM corresponded to the results of the local lymph node assay.

Benzaldehyde was correctly categorized as non-sensitizers by the DPRA but was incorrectly categorized as sensitizers by the ADRA-DM and ADRA. Benzaldehyde was categorized as a non-sensitizer in the local lymph node assay, but it has the aldehyde group in its chemical structure, indicating that this chemical may react with Cys and Lys. In fact, it was reported that benzaldehyde was found as a positive human sensitizer (Natsch *et al.*, 2012). Therefore, benzaldehyde may be able to react with proteins (either the thiol-group of Cys or the amino-group of Lys) so that it is categorized as sensitizer in both the ADRA and the ADRA-DM. A non-sensitizer, which was incorrectly categorized as a sensitizer in the ADRA-DM, is only benzaldehyde, indicating that ADRA-DM is a prediction method that causes almost no false-negative on the selected set of chemicals.

Resorcinol was correctly categorized as a sensitizer in the ADRA-DM and the ADRA but incorrectly categorized as a non-sensitizer in the DPRA. The amino-group of NAL may have reacted with benzoquinone that was formed by oxidation of resorcinol owing to the high pH in the reaction solution in the ADRA-DM and the ADRA.

The prediction accuracy for skin sensitization in the ADRA-DM was 90% (Fig. 2), which was slightly higher than the 88% accuracy of the ADRA and DPRA (Gerberick *et al.*, 2007; Fujita *et al.*, 2014). Moreover, this high accuracy was probably due to the selective reactivity of NAC and NAL with the test chemicals at the low concentrations used in the ADRA-DM, which were 1/100 of the concentrations used in the ADRA. At higher test chemical concentrations used in the ADRA, accuracy may be negatively affected by non-specific reactions that are not predictive of skin sensitization.

Table 6. Comparison of prediction results for skin sensitization for amino acid derivative reactivity assay-dilutional method (ADRA-DM), ADRA, direct peptide reactivity assay (DPRA) and potency data

№	Test substance	EC_3 value	LLNA category	ADRA-DM Prediction	ADRA Prediction	DPRA Prediction
1	Diphenylcyclo propenone	0.0003	Extreme	S[c]	S	S
2	Oxazolone	0.003	Extreme	S	S	S
3	Benzoyl peroxide	0.004	Extreme	S	S	S
4	Kathon CG	0.008	Extreme	S	S	S
5	Bandrowski's base	0.008	Extreme	S	S	S
6	5-Chloro-2-methyl-4-isothiazolin-3-one	0.009	Extreme	S	S	S
7	p-Benzoquinone	0.0099	Extreme	S	S	S
8	Tetrachlorosalicylanilide	0.04	Extreme	S	S	S
9	2,4-Dinitrochlorobenzene	0.05	Extreme	S	S	S
10	Glutaraldehyde	0.1	Strong	S	S	S
11	Fluorescein isothiocyanate	0.14	Strong	S	S	S
12	Phthalic anhydride	0.16	Strong	S	S	S
13	Lauryl gallate	0.3	Strong	S	S	S
14	Propyl gallate	0.32	Strong	S	S	S
15	CD3	0.6	Strong	S	S	S
16	Trimellitic anhydride	0.6	Strong	S	S	S
17	Formaldehyde	0.61	Strong	S	S	S
18	Metol	0.8	Strong	S	S	S
19	2-Hydroxyethyl acrylate	1.4	Moderate	S	S	S
20	Glyoxal	1.4	Moderate	S	S	S
21	Vinyl pyridine	1.6	Moderate	S	S	S
22	2-Mercaptobenzothiazole	1.7	Moderate	S	S	S
23	Nonanoyl chloride	1.8	Moderate	S	S	NS
24	2-Methyl-2H-isothiazol-3-one	1.9	Moderate	S	S	S
25	1,2-Benzisothiazoline-3-one	2.3	Moderate	S	S	S
26	Methyl-2-nonynoate	2.5	Moderate	S	S	S
27	Cinnamaldehyde	3	Moderate	S	S	S
28	Phenylacetaldehyde	3	Moderate	S	S	S
29	Benzylideneacetone	3.7	Moderate	S	S	S
30	2,4-Heptadienal	4	Moderate	S	S	S
31	Squaric acid	4.3	Moderate	NS	NS	S
32	Trans-2-hexenal	5.5	Moderate	S	S	S
33	Resorcinol	5.5	Moderate	S	S	NS
34	Diethyl maleate	5.8	Moderate	S	S	S
35	2-phenylpropionaldehyde	6.3	Moderate	S	S	S
36	Perillaldehyde	8.1	Moderate	S	S	S
37	Palmitoyl Chloride	8.8	Moderate	S	S	S
38	1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	NS	S	S
39	α-Hexylcinnnamaldehyde	11	Weak	NS	NS	NS
40	α-Amylcinnamaldehyde	11	Weak	NS	NS	NS
41	2,3-Butanedione	11	Weak	S	S	S
42	Farnesal	12	Weak	S	S	S
43	Oxalic acid	15	Weak	NS	NS	NS
44	Benzyl benzoate	17	Weak	NS	NS	NS
45	4-Allylanisole	18	Weak	S	S	S
46	Lilial	19	Weak	S	S	S
47	Cyclamen aldehyde	22	Weak	S	S	S
48	Imidazolidinyl urea	24	Weak	S	S	S
49	5-Methyl-2,3-hexanedione	26	Weak	S	S	S
50	2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	NS	S	NS
51	Ethyleneglycol dimethacrylate	28	Weak	S	S	S
52	Ethyl acrylate	28	Weak	S	S	S
53	Hydroxycitronellal	33	Weak	S	S	S
54	Glycerol	NC[a]	NS[b]	NS	NS	NS
55	Hexane	NC	NS	NS	NS	NS
56	Diethyl phthalate	NC	NS	NS	NS	NS

(Continues)

Table 6. (Continued)

№	Test substance	EC_3 value	LLNA category	ADRA-DM Prediction	ADRA Prediction	DPRA Prediction
57	Octanoic acid	NC	NS	NS	NS	NS
58	2-Hydroxypropyl methacrylate	NC	NS	NS	S	S
59	1-Butanol	NC	NS	NS	NS	NS
60	4-Hydroxybenzoic acid	NC	NS	NS	NS	NS
61	6-Methyl coumatrin	NC	NS	NS	NS	NS
62	Methyl salicylate	NC	NS	NS	NS	NS
63	Chlorobenzene	NC	NS	NS	NS	NS
64	Lactic acid	NC	NS	NS	NS	NS
65	1-Bromobutane	NC	NS	NS	NS	S
66	2-Acethylcyclohexanone	NC	NS	NS	S	S
67	4'-Methoxyacetophenone	NC	NS	NS	NS	NS
68	Ethyl benzoylacetate	NC	NS	NS	NS	NS
69	Ethyl vanillin	NC	NS	NS	S	NS
70	Isopropanol	NC	NS	NS	NS	NS
71	Propylene glycol	NC	NS	NS	NS	NS
72	Sulfanilamide	NC	NS	NS	NS	NS
73	Isopropyl myristate	NC	NS	NS	NS	NS
74	Benzaldehyde	NC	NS	S	S	NS
75	Methylparaben	NC	NS	NS	NS	NS
76	Nonanoic acid	21 (False +)	NS	NS	NS	NS
77	Propyl paraben	NC	NS	NS	NS	NS
78	Salicylic acid	NC	NS	NS	NS	-
79	Sulphanilic acid	NC	NS	NS	NS	NS
80	Vanillin	NC	NS	NS	S	NS
81	Coumarin	NC	NS	NS	NS	NS
82	Vinylidene dichloride	NC	NS	NS	NS	NS

[a]Not calculated.
[b]Non-sensitizer.
[c]Sensitizer

Therefore, ADRA-DM may be a better assay than ADRA for the classification of sensitizers and non-sensitizers.

Moreover, the ability to evaluate NAC and NAL in a homogeneous reaction solution is another advantage of the ADRA-DM. As seen by the depletion values for NAC and NAL in Table 5, precipitation or turbidity of the reaction solutions was found with 13 test chemicals in the ADRA. It was thought that a similar phenomenon was seen in the DPRA owing to similarity of the reaction conditions. In contrast, no precipitation or turbidity was seen in the reaction solutions in the ADRA-DM because of the 100-fold reduction in test compound concentrations. Due to the absence of compound precipitation or test solution turbidity, more accurate depletion values can be obtained and skin sensitization of all test chemicals can be accurately predicted.

However, a highly hydrophobic compound, for example, palmitoyl chloride (ClogP = 7.5) precipitated as soon as it was added to the reaction solution, but the depletion of NAL was 100% in the DPRA (Gerberick *et al.*, 2007) and the ADRA (Table 5). It was correctly categorized as sensitizer in both assays. Precipitation of palmitoyl chloride and its reaction with NAL may have been competitive processes. Palmitoyl chloride showed 100% depletion of NAL because the reaction rate with NAL may have been faster than the precipitation rate. Therefore, in the case of similar chemicals that precipitate easily or have a slow rate of reaction, these chemicals may be incorrectly categorized as non-sensitizers in spite of categorization as sensitizers *in vivo*. However, our data showed that skin sensitization of test chemicals will be accurately predicted by the ADRA-DM.

We developed the ADRA to resolve the significant problem of the poor sensitivity of HPLC analysis in the DPRA (Fujita *et al.*, 2014). However, the ADRA required test chemicals at concentrations as high as those required for the DPRA. Therefore, we developed a new evaluation method, in which the concentration of test chemicals was reduced by 100-fold from the concentration used in the ADRA. This new method, ADRA-DM, utilized the highly sensitive detection of NAC and NAL by HPLC at 281 nm.

The concentration of test chemical in the reaction solution of the peroxidase peptide reactivity assay (PPRA), developed by Gerberick *et al.* (2009), Troutman *et al.* (2011) and Lalko *et al.* (2013), was extremely low at 200 μM. This concentration is similar to that used in the ADRA-DM (250 μM) described in this study. However, the PPRA uses an expensive and complicated LC-MS/MS apparatus, as the sensitivity of UV detection for Cys and Lys peptides is too low to allow the use of an HPLC-UV apparatus. In contrast, the sensitivities of an HPLC-UV apparatus for NAC and NAL are high enough that a 100-fold reduction in concentration is feasible for the ADRA-DM.

The DPRA makes it difficult to test insoluble chemicals owing to high concentration, whereas the lower concentration in the ADRA-DM facilitates solubility of test chemicals in solvent. It is difficult for the DPRA to predict accurately skin sensitization of test chemicals if test chemicals are not soluble at a final concentration of 100 mM

in solvent as described in *'In chemico* skin sensitization: Direct Peptide Reactivity Assay (DPRA) (OECD, 2015), but there are little or no problems of solubility of test chemicals for the ADRA-DM due to a low concentration. Moreover, the lower concentrations of each substance used in ADRA-DM could drastically reduce the amounts of test chemicals and NAC/NAL used in skin sensitization assays. Furthermore, concentrations could also avoid precipitation and/or turbidity in reaction solutions. In addition, the skin sensitization predictions made using the ADRA-DM were slightly more accurate than those made using the ADRA and DPRA.

The results of this study show that the ADRA-DM was more accurate than the ADRA and DPRA, and ADRA-DM can be used with a wider range of test chemicals. Thus, the ADRA-DM is a promising method that may be useful in future skin sensitization studies.

Acknowledgments

The authors wish to acknowledge Nanako Aihara for technical contributions to this work.

Conflict of interest

The Authors did not report any conflict of interest.

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Appendix 10

Reactivity and Concentration-Dependence of NAC and EDTA

Appendix 10: Reactivity and Concentration-Dependence of NAC and EDTA

Background and objectives

As is mentioned in the validation study report, we know that Cu^{2+} or other metal ions that contaminate NAC solution act as catalysts in promoting NAC oxidation. For this reason, the ADRA SOP specifies that EDTA be added to NAC stock solution as a countermeasure to suppress this effect. Although the 0.25-μM concentration of EDTA in the reaction solution is a mere 0.1% of the 250-μM concentration of the test chemical in the reaction solution, we felt it was necessary to perform testing to answer the following three questions.

1. Does EDTA react directly with NAC?
2. If it does react, does it exhibit concentration dependence?
3. Does EDTA oxidize NAC?

Testing

We prepared a standard NAC stock solution without EDTA as well as several aqueous solutions of EDTA to be used in place of a test chemical solution at the concentrations shown below. We then followed the ADRA SOP in preparing 50 μL of both solutions as well as 150 μL of both solutions in three cells each of a 96-well plate. Of these, we added 50 μL of fixing solution to three wells and measured residual NAC per HPLC analysis. The remaining plates were allowed to stand for 24 h at 25°C in an incubator, after which we added fixing solution to three wells and measured per HPLC analysis.

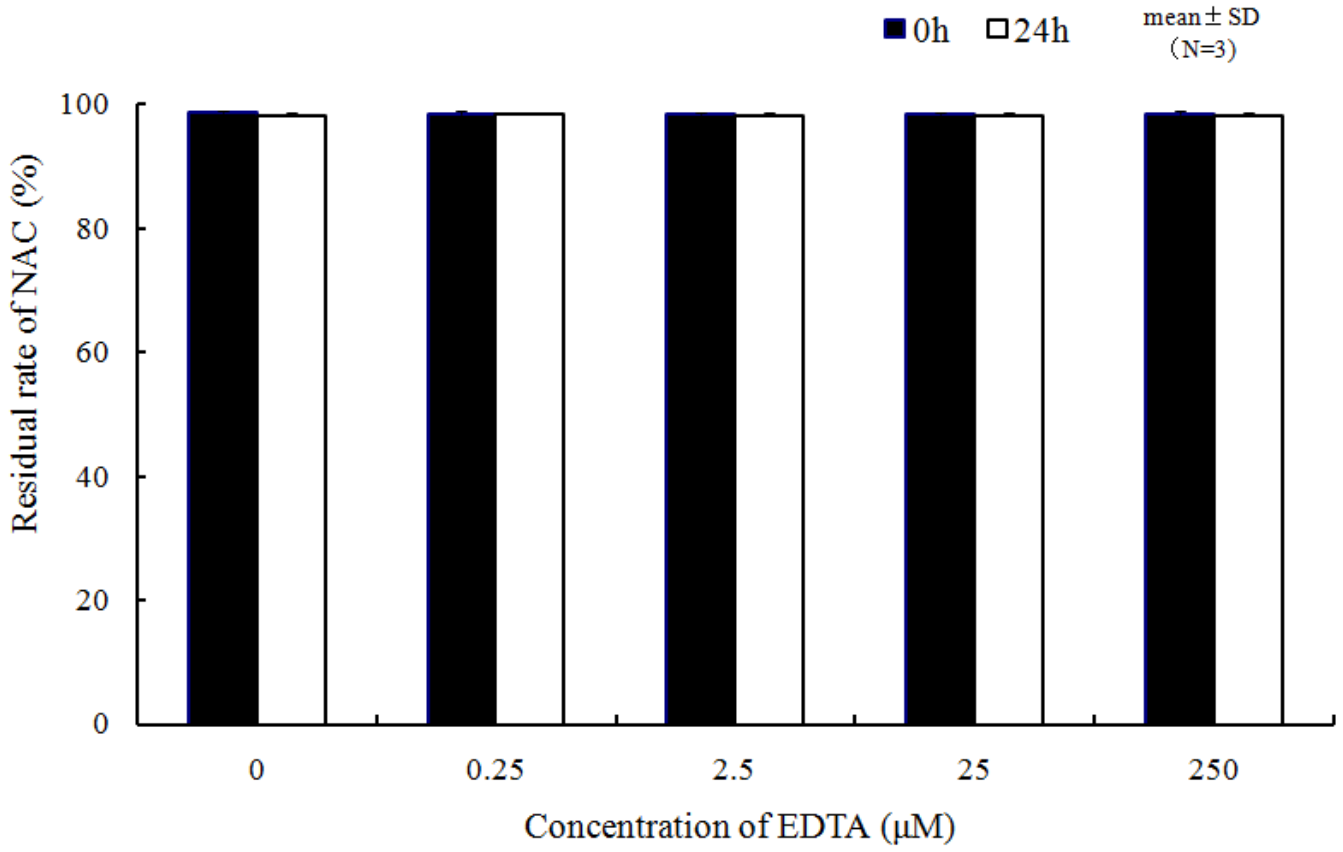
Final concentrations in reaction fluid for aqueous solutions of EDTA: 0, 0.25, 2.5, 25, 250 μM

Ordinarily, ADRA testing is performed with a 0.25-μM concentration of EDTA and a 250-μM concentration of a test chemical in the reaction fluid.

Results

The results shown in Fig. 1 below. Tests were conducted with four different final concentrations of EDTA in the reaction solution in ten-fold increments from 0.25 to 250 μM, with residual NAC immediately after preparation (0 h) between 98.4 and 98.7% (N = 3), which was within measurement tolerances and concordant with the control sample at 98.7%. Also, residual NAC after incubating for 24 h at 25°C was between 98.2 and 98.5% (N = 3), which was also within measurement tolerances and concordant with the control sample at 98.3%.

Table 1 Effect of EDTA on Residual NAC



Other considerations

Our answers to the three questions mentioned above are as follows:

First, with regard to the question of whether or not EDTA reacts directly with NAC, we measured residual levels of NAC in reaction solution containing both NAC and EDTA, both immediately after preparation and after incubating for 24 h. The results were all 98% or greater and concordant with the control, irrespective of the concentration of EDTA. These results indicate that EDTA does not react directly with NAC at all.

The molecular structure of EDTA has neither nucleophilic nor electrophilic sites. We find difficult to conceive of EDTA either attacking or reacting with other substances, and the above results are in electronic theory of organic chemistry. The possibility, however, that EDTA is either a pre- or pro-hapten that acquires sensitization potential through oxidation or metabolism cannot be denied. Henck *et al.* (1980) reported that a 10% EDTA·3Na solution, which was subjected to a repeated insult patch test on

Hartley albino guinea pigs, did not cause sensitization in any of 10 guinea pigs. In contrast to this, Raymond and Gross (1969) reported that three out of 50 (6%) subjects exhibited sensitization in a study of the human skin sensitization potential of EDTA·Ca. Henck *et al*, however, contend that since two of these three subjects had previously exhibited sensitization when exposed to EDTA, this rate of incidence was not applicable to the public at large. It is therefore extremely unlikely that EDTA sodium salt causes sensitization in humans (Henck *et al*., 1980), nor is it likely to be a pre- or pro-hapten.

Each of these *in vivo* tests involved the application of extremely high concentrations of EDTA, which precludes direct comparison with ADRA. Nevertheless, ADRA test parameters call for a 1:50 ratio of NAC to test chemical, and the 1:0.05 ratio of EDTA to NAC used to suppress NAC oxidation is extremely low, so that even in the unlikely event that *in vivo* test results do exhibit sensitization, we still consider it highly unlikely that NAC and EDTA react under ADRA test parameters. These results lead us to conclude that the EDTA added to suppress NAC oxidation reacts with NAC only rarely if at all.

Next, with regard to the question of whether or not EDTA exhibits concentration dependence, since the above results indicate that EDTA does not react with NAC irrespective of concentration, there is not dose dependence.

Finally, with regard to whether or not EDTA oxidizes NAC, as shown above, the levels of residual NAC were 98% or greater irrespective of concentration, which indicates that EDTA does not affect NAC oxidation. This is congruent with the fact that the chemical structure of EDTA makes it hard to conceive of it oxidizing thiol groups.

Appendix 11

The effects of EDTA on the reactivity of NAC with test chemicals

Appendix 11: The effects of EDTA on the reactivity of NAC with test chemicals

NAC is a nucleophilic reagent used in ADRA, that contains thiol groups and oxidizes into NAC dimers. It is known that Cu^{2+} and other metal ions present in solution act as a catalyst to promote NAC oxidation. To prevent this, the ADRA SOP stipulates that EDTA, an agent for chelating metal ions, be added during the preparation of NAC stock solution. We consider the 0.25-μM concentration of EDTA in the reaction solution to be sufficiently low relative to the 250-μM concentration of the test chemical in the reaction solution so as to have no effect on the reactivity of NAC with the test chemical.

We verified this by testing, both with and without EDTA, the reactivity of NAC with 82 test chemicals used during the development of DPRA and ADRA.

Results

1. NAC depletion as well as *in vivo* predictions for the 82 test chemicals

NAC depletion both with and without EDTA as well as *in vivo* predictions for the 82 test chemicals are shown in Table 1. Also included in this table is mean % depletion as calculated from NAC and NAL depletion. Although there were some test chemicals that exhibited discrepancies of 10 percentage points or greater in NAC depletion between testing with and without EDTA, only three test chemicals were found to have prediction results that were non-concordant with *in vivo* predictions when tested with EDTA.

2. Concordance with *in vivo* predictions

Concordance with and predictive capacity relative to *in vivo* predictions for ADRA both with and without EDTA are shown in Table 2. Predictions for the 82 test chemicals using ADRA with EDTA exhibited a sensitivity of 81%, a specificity of 97%, and an accuracy of 87%. Although specificity was unchanged relative to predictions using ADRA without EDTA, sensitivity was 6 percentage points lower, and accuracy was 3 percentage points lower.

Table 1 NAC and NAL depletion with and without EDTA as well as *in vivo* predictions

№	Test substance	EC_3 value	LLNA category	NAC with EDTA		NAC without EDTA		NAL		mean % depletion	Prediction by ADRA with EDTA	Prediction by ADRA without EDTA
				Depletion (%)	SD	Depletion (%)	SD	Depletion (%)	SD			
Extreme/strong												
1	Diphenylcyclopropenone	0.0003	Extreme	28.7	1.0	23.9	1.5	6.3	1.2	17.5	S[c]	S
2	Oxazolone	0.003	Extreme	85.3	0.3	80.2	1.6	80.1	0.8	82.7	S	S
3	Benzoyl peroxide	0.004	Extreme	100.0	0.0	100.0	0.0	50.6	2.7	75.3	S	S
4	Kathon CG	0.008	Extreme	100.0	0.0	100.0	0.0	-0.4	0.5	49.8	S	S
5	Bandrowski's base	0.008	Extreme	100.0	0.0	100.0	0.0	5.7	0.4	52.9	S	S
6	5-Chloro-2-methyl-4-isothiazolin-3-one	0.009	Extreme	100.0	0.0	100.0	0.0	17.7	1.1	58.8	S	S
7	p-Benzoquinone	0.0099	Extreme	96.5	0.2	97.2	2.0	83.5	1.1	90.0	S	S
8	Tetrachlorosalicylanilide	0.04	Extreme	43.4	4.2	22.9	4.2	2.1	0.3	22.7	S	S
9	2,4-Dinitrochlorobenzene	0.05	Extreme	89.3	2.0	87.6	2.9	6.1	1.3	47.7	S	S
10	Glutaraldehyde	0.1	Strong	1.6	0.2	4.6	1.7	53.1	2.9	27.3	S	S
11	Fluorescein isothiocyanate	0.14	Strong	73.6	0.8	94.1	0.9	98.1	0.6	85.8	S	S
12	Phthalic anhydride	0.16	Strong	0.3	0.3	-1.8	1.5	96.9	1.1	48.6	S	S
13	Lauryl gallate	0.3	Strong	100.0	0.0	100.0	0.0	19.0	0.4	59.5	S	S
14	Propyl gallate	0.32	Strong	97.1	0.1	100.0	0.0	56.4	1.4	76.7	S	S
15	CD3	0.6	Strong	76.6	1.3	100.0	0.0	16.5	0.5	46.6	S	S
16	Trimellitic anhydride	0.6	Strong	1.9	0.4	1.8	1.6	97.0	1.2	49.4	S	S
17	Formaldehyde	0.61	Strong	25.7	1.1	24.4	2.7	1.6	1.9	13.6	S	S
18	Metol	0.8	Strong	100.0	0.0	100.0	0.0	22.8	1.1	61.4	S	S
Moderate												
19	2-Hydroxyethyl acrylate	1.4	Moderate	100.0	0.0	100.0	0.0	16.3	4.7	58.1	S	S
20	Glyoxal	1.4	Moderate	12.8	0.5	12.5	0.9	0.8	0.5	6.8	S	S
21	Vinyl pyridine	1.6	Moderate	18.1	1.0	27.8	2.1	7.7	1.8	12.9	S	S
22	2-Mercaptobenzothiazole	1.7	Moderate	56.6	0.8	100.0	0.0	0.3	0.8	28.4	S	S
23	Nonanoyl chloride	1.8	Moderate	7.4	0.4	7.5	2.1	39.4	1.8	23.4	S	S
24	2-Methyl-2H-isothiazol-3-one	1.9	Moderate	94.0	0.3	100.0	0.0	7.0	2.2	50.5	S	S
25	1,2-Benzisothiazoline-3-one	2.3	Moderate	100.0	0.0	100.0	0.0	0.4	0.7	50.2	S	S
26	Methyl-2-nonynoate	2.5	Moderate	15.0	0.9	11.1	0.8	1.4	0.2	8.2	S	S
27	Cinnamaldehyde	3	Moderate	37.0	0.9	100.0	0.0	13.1	1.3	25.0	S	S
28	Phenylacetaldehyde	3	Moderate	22.4	2.5	21.2	2.4	98.3	1.5	60.3	S	S
29	Benzylideneacetone	3.7	Moderate	44.9	0.2	19.4	1.3	7.2	1.0	26.1	S	S
30	2,4-Heptadienal	4	Moderate	35.8	0.2	35.3	2.6	50.1	1.3	42.9	S	S
31	Squaric acid	4.3	Moderate	3.7	0.3	-2.9	1.8	0.5	0.2	2.1	NS	NS
32	Trans-2-hexenal	5.5	Moderate	80.4	0.4	83.5	2.4	12.3	1.1	46.3	S	S
33	Resorcinol	5.5	Moderate	4.0	0.3	9.8	3.5	2.2	0.7	3.1	NS	S
34	Diethyl maleate	5.8	Moderate	31.8	0.5	22.6	3.3	7.7	1.9	19.8	S	S
35	2-phenylpropionaldehyde	6.3	Moderate	8.0	0.3	8.1	0.8	4.6	1.3	6.3	S	S
36	Perillaldehyde	8.1	Moderate	29.1	1.7	36.9	2.5	18.5	1.0	23.8	S	S
37	Palmitoyl Chloride	8.8	Moderate	5.8	0.6	6.9	1.4	95.9	2.5	50.8	S	S
38	1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	1.6	0.7	2.9	2.4	4.2	1.6	2.9	NS	NS
Weak												
39	α-Hexylcinnnamaldehyde	11	Weak	0.0	0.0	2.5	2.2	1.1	0.9	0.6	NS	NS
40	α-Amylcinnamaldehyde	11	Weak	0.0	0.0	1.3	1.5	1.6	0.4	0.8	NS	NS
41	2,3-Butanedione	11	Weak	41.2	0.9	71.3	1.4	25.5	1.2	33.4	S	S
42	Farnesal	12	Weak	17.2	0.6	17.1	2.3	8.8	1.4	13.0	S	S
43	Oxalic acid	15	Weak	0.0	0.0	0.4	2.9	3.6	1.0	1.8	NS	NS
44	Benzyl benzoate	17	Weak	0.0	0.0	1.5	3.4	2.8	1.1	1.4	NS	NS
45	4-Allylanisole	18	Weak	27.0	0.4	23.9	2.3	3.9	2.6	15.4	S	S
46	Lilial	19	Weak	7.0	0.8	20.7	1.3	3.9	0.9	5.4	S	S
47	Cyclamen aldehyde	22	Weak	3.1	0.4	14.5	2.6	1.7	0.6	2.4	NS	S
48	Imidazolidinyl urea	24	Weak	35.4	3.1	35.4	2.2	2.0	0.2	18.7	S	S
49	5-Methyl-2,3-hexanedione	26	Weak	6.4	0.2	15.9	1.1	34.8	2.8	20.6	S	S
50	2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	0.5	0.5	0.9	2.4	1.6	0.8	1.1	NS	NS
51	Ethyleneglycol dimethacrylate	28	Weak	5.9	0.5	8.9	1.1	2.6	1.6	4.3	NS	S
52	Ethyl acrylate	28	Weak	87.8	0.1	89.8	1.9	13.7	0.4	50.8	S	S
53	Hydroxycitronellal	33	Weak	17.5	0.3	11.2	2.2	1.5	0.7	9.5	S	S
Non-sensitizer												
54	Glycerol	NC[a]	NS[b]	0.3	0.3	1.8	2.4	0.7	1.2	0.5	NS	NS
55	Hexane	NC	NS	0.2	0.3	0.4	1.8	0.9	0.9	0.5	NS	NS
56	Diethyl phthalate	NC	NS	0.0	0.0	0.1	2.0	1.7	1.4	0.9	NS	NS
57	Octanoic acid	NC	NS	0.0	0.0	-2.1	1.1	1.2	0.5	0.6	NS	NS
58	2-Hydroxypropyl methacrylate	NC	NS	2.4	0.5	1.3	1.0	2.2	0.6	2.3	NS	NS
59	1-Butanol	NC	NS	0.1	0.2	-1.7	1.5	0.8	0.5	0.5	NS	NS
60	4-Hydroxybenzoic acid	NC	NS	0.0	0.0	0.6	2.3	-1.4	1.0	-0.7	NS	NS
61	6-Methyl coumatrin	NC	NS	0.0	0.0	2.2	2.2	1.0	0.7	0.5	NS	NS
62	Methyl salicylate	NC	NS	0.0	0.0	-2.9	0.9	0.4	0.6	0.2	NS	NS
63	Chlorobenzene	NC	NS	0.0	0.0	1.6	2.1	0.7	0.7	0.4	NS	NS
64	Lactic acid	NC	NS	0.0	0.0	3.8	2.0	2.8	0.6	1.4	NS	NS
65	1-Bromobutane	NC	NS	0.1	0.1	6.7	3.5	2.4	1.6	1.2	NS	NS
66	2-Acethylcyclohexanone	NC	NS	1.3	0.6	0.3	3.1	0.3	0.6	0.8	NS	NS
67	4'-Methoxyacetophenone	NC	NS	3.3	0.4	3.9	7.1	3.3	0.6	3.3	NS	NS
68	Ethyl benzoylacetate	NC	NS	4.4	0.3	-6.6	2.7	5.3	1.3	4.9	NS	NS
69	Ethyl vanillin	NC	NS	1.4	0.3	7.3	1.5	2.1	0.8	1.7	NS	NS
70	Isopropanol	NC	NS	0.1	0.1	3.6	1.4	2.8	0.6	1.5	NS	NS
71	Propylene glycol	NC	NS	0.1	0.2	3.5	1.3	2.2	0.7	1.1	NS	NS
72	Sulfanilamide	NC	NS	0.0	0.0	-2.7	2.5	1.4	0.7	0.7	NS	NS
73	Isopropyl myristate	NC	NS	0.0	0.0	-1.6	2.4	1.8	1.4	0.9	NS	NS
74	Benzaldehyde	NC	NS	25.3	1.0	24.4	0.8	2.9	1.0	14.1	S	S
75	Methylparaben	NC	NS	0.1	0.1	2.8	1.1	1.3	0.5	0.7	NS	NS
76	Nonanoic acid	21 (False +)	NS	0.0	0.0	-5.3	2.2	1.3	0.7	0.6	NS	NS
77	Propyl paraben	NC	NS	0.1	0.2	2.7	2.7	1.9	3.1	1.0	NS	NS
78	Salicylic acid	NC	NS	0.0	0.0	-5.0	2.2	0.4	0.6	0.2	NS	NS
79	Sulphanilic acid	NC	NS	0.5	0.4	-0.2	0.6	0.4	0.2	0.5	NS	NS
80	Vanillin	NC	NS	1.5	0.3	0.5	2.7	1.9	1.5	1.7	NS	NS
81	Coumarin	NC	NS	0.5	0.5	-3.1	2.7	3.9	2.4	2.2	NS	NS
82	Vinylidene dichloride	NC	NS	0.9	0.4	1.4	2.4	-0.3	0.7	0.3	NS	NS

Table 2 Predictive capacity relative to *in vivo* predictions for ADRA with and without EDTA

	Sensitizer	Non-sensitizer	Total
Sensitizer	43 (46)	10 (7)	53 (53)
Non-sensitizer	1 (1)	28 (28)	29 (29)
Total	44 (47)	38 (35)	82 (82)

Sensitivity	81% (87%)
Specificity	97% (97%)
Positive predictivity	98% (98%)
Negative predictivity	74% (80%)
Accuracy	87% (90%)

Note: Figures not in parentheses indicate results for ADRA with EDTA. Figures in parentheses indicate results for ADRA without EDTA.

Other considerations

1. Variability of depletion in ADRA

Variation of the positive control for both NAC and NAL in ADRA is between 10 and 20%. In order to determine whether this level of variability is a characteristic of ADRA itself or is common with DPRA, we compared variation in both ADRA and DPRA for 82 test chemicals from the literature. The SD of depletion for NAC in these 82 chemicals is from 0.0 to 7.1% without EDTA and 0.0 to 4.2% with EDTA. Assuming that variation of the depletion value is ±2SD, variation of NAC depletion would be ±8.4% or a maximum of 16.8%, and variation of NAL depletion would be ± 9.4% or a maximum of 18.8%. Assuming that variation of the depletion value is ±2SD, variation of NAC depletion would be ±8.4% or a maximum of 16.8%, and variation of NAL depletion would be ± 9.4% or a maximum of 18.8%. Since the ADRA prediction model is based on mean % depletion of NAC and NAL, variation of the mean % depletion could be as high as 17.8%.

In DPRA, however, variation of cysteine peptide depletion is ± 70.4% or a maximum of 140.8%, and variation of lysine peptide depletion is ±24.0% or a maximum of 48.0%, both of which are extremely high. This results in variation of the mean % depletion as high as 94.4%, which is roughly five times larger than that of ADRA.

Even when there are discrepancies in mean % depletion based on whether EDTA is or is not added, as long as they are within 18%, they can be considered within the expected range of variability and unlikely to be a result of the effects of EDTA. Because of these factors, and particularly when the mean % depletion is close to the threshold value, ADRA could potentially yield false-positive or false-negative

predictions. In order to reduce the potential for false-positive and false-negative predictions, the ADRA test protocol conforms with DPRA's OECD TG 442C in calling for additional testing to be performed on test chemicals with a mean % depletion close to the threshold value (specifically, from 3 to 10%) to confirm reproducibility and increase concordance with *in vivo* test results.

2. Three chemicals for which predictions were non-concordant with *in vivo* results when tested using ADRA with EDTA

Table 1 shows that there were three test chemicals for which prediction results differed between ADRA with EDTA and ADRA without EDTA.

Resorcinol

Resorcinol is a pro-hapten that becomes a sensitizer when metabolically oxidized (Report and Recommendations of an EURL ECVAM Expert Meeting, 2016). Thus, it is difficult to predict accurately with either ADRA or DPRA. In fact, resorcinol was incorrectly predicted to be a non-sensitizer with DPRA.

Also, although the lead laboratory used acetonitrile as the solvent when testing without EDTA, the four participating laboratories each used water as the solvent every time resorcinol was tested. And in testing with EDTA at the four participating laboratories, resorcinol was predicted to be a non-sensitizer six of a total of seven times.

In-house data from the lead laboratory indicates that even when tested without EDTA, results were divided between sensitizer and non-sensitizer. (Data not shown.) This leads us to believe that, even though Phase II testing resulted in a false negative prediction for resorcinol, this is not due to the effects of EDTA but rather to the fact that the threshold value for mean % depletion falls within the expected range of variability for resorcinol.

As shown in Table 42 of the validation study report, resorcinol was tested using water as the solvent once each at Lion, Mitsui, and Nissan as well as three times at Sumitomo. Thus we feel that the solvent has no effect on prediction of skin sensitization potential.

Cyclamen aldehyde

NAC depletion for cyclamen aldehyde was 14.5% when tested by ADRA without EDTA and 3.1% when tested by ADRA with EDTA, so the addition of EDTA did have an effect. Aldehydes have strong reactivity and are used in both DPRA and ADRA as positive control reagents. There is, however, significant variability in reactivity. For example, ADRA test acceptance criteria stipulates that a variation of 25% is acceptable for the positive control (phenylacetaldehyde). Similarly, DPRA test acceptance criteria stipulates that a variation of between 30 and 40% is acceptable for the positive control (cyclamen aldehyde). Thus, considerable variation can be expected.

During multiple tests at both the lead laboratory and the participating laboratories, cyclamen aldehyde was predicted to be both a sensitizer and a non-sensitizer. (Data not shown.) Thus, a reduction of 10 percentage points in mean % depletion falls within the expected range of variability for ADRA. Also, since acetonitrile was used at the solvent for testing both with and without EDTA, there is no reason to think that false negative predictions were due to choice of solvent. Moreover, Aleksic *et al*. (2009) has reported that aldehydes induce the formation of cysteine dimers, which could result in over-predicting the

reactivity of cysteine. And since aldehydes are known to oxidize to carboxylic acids, accurate prediction of aldehydes can be considered problematic.

When tested using ADRA with EDTA, cyclamen aldehyde exhibits significant NAC depletion and is predicted to be a non-sensitizer, which is an indication of the need to account for the formation cysteine dimers and other factors when making a careful assessment of the skin sensitization potential of any aldehyde.

Ethylene glycol dimethacrylate

NAC depletion for ethylene glycol dimethacrylate was 5.9% when tested using ADRA without EDTA and 8.9% when tested using ADRA with EDTA, which is a mere 3% difference. Since the ADRA prediction model is based on mean % depletion of NAC and NAL, even though this variation is less than one-fifth of DPRA, it could still be as high as 17.8%. Therefore, NAC depletion in glycol dimethacrylate varies by roughly 3% depending on whether or not EDTA is added, but we consider this to be within the expected range of variability. Also, since acetonitrile was used at the solvent for testing both with and without EDTA, there is no reason to think that false negative predictions were due to choice of solvent.

3. Other considerations related to predictive capacity

We retested the 82 test chemicals (Gerberick *et al*., 2004; Fujita *et al*., 2014; Yamamoto *et al*., 2015) used in developing DPRA and ADRA to determine that 4.9% is a suitable threshold value for predicting a test chemical to be either a sensitizer or a non-sensitizer when EDTA is added to the NAC stock solution. Using this threshold value resulted in an accuracy of 87% for predicting skin sensitization potential, which is roughly similar to the 90% accuracy achieved by ADRA without EDTA. We therefore conclude that the addition of EDTA to the NAC stock solution has virtually no effect on either NAC reactivity or predictive capacity.

Appendix 12

Between-Laboratory Reproducibility Calculated as the Mean of Four Laboratories

Appendix 12: Between-Laboratory Reproducibility Calculated as the Mean of Four Laboratories

Between-laboratory reproducibility was evaluated based on test results from 40 test chemicals in three sets of ten chemicals and one set of thirty chemicals, and in the validation study report was calculated as the mean of three laboratories, so that the figures could be compared directly to those from other validation studies. Here we example between-laboratory reproducibility calculated as the mean of four participating laboratories.

1. Between-laboratory reproducibility of the 30 test substances used in Phase II

Since 10 test chemicals were subjected to three replicate tests during Phase I, and 30 test chemicals were subjected to single tests during Phase II, the results of Phase I were handled differently from the results of Phase II. Thus, as indicated in the validation study report, a preliminary evaluation of between-laboratory reproducibility was made using just the 30 test chemicals for Phase II. Between-laboratory reproducibility for these 30 test chemicals when calculated as the mean of four laboratories was 86.7% (compared with 89.2% when calculated as the mean of three laboratories), which exceeded the target value of 80%.

Table 1 Between-laboratory reproducibility for Phase II as the mean of four laboratories

	No. of chemicals	No. of chemicals with concordant results at all four laboratories.	BLR
Phase Ⅱ	30	26	**86.7**

BLR: Between-laboratory reproducibility

2. Between-laboratory reproducibility per sensitization potency of 30 test substances in Phase II

In section 1 above, we evaluated between-laboratory reproducibility for the 30 test chemicals used in Phase II based on prediction of sensitization potential. We further looked at the test chemicals that were predicted to be sensitizers, and evaluated between-laboratory sensitization based on prediction of sensitization potency, using two categories: extreme/strong sensitizers and moderate/weak sensitizers.

The results, as shown in Table 2, were 100% between-laboratory reproducibility for extreme/strong sensitizers, 76.9% between-laboratory reproducibility for moderate/weak sensitizers, and 90.0% between-laboratory reproducibility for non-sensitizers. When calculated as the mean of three laboratories rather than four, the results were 100% for extreme/strong sensitizers, 80.8% for moderate/weak sensitizers, and 92.5% for non-sensitizers.

Table 2　Between-laboratory reproducibility per sensitization potency for Phase II as the mean of four laboratories

Phase Ⅱ	No. of chemicals	No. of chemicals with concordant results at all four laboratories.	BLR
Extreme/Strong	7	7	**100.0**
Moderate/Weak	13	10	**76.9**
No category	10	9	**90.0**

3. Between-laboratory reproducibility of the 40 test substances used in Phases I and II

We calculated between-laboratory reproducibility for the 40 test substances used in Phases I and II as the mean of four laboratories. The results show that prediction of sensitization potential for 36 of the 40 test chemicals was concordant. As shown in Table 3, between-laboratory reproducibility for these 40 test chemicals as calculated as the mean of four laboratories was 90.0% (compared with 91.9% when calculated as the mean of three laboratories), which exceeded the target value of 80%.

Table 3　Between-laboratory reproducibility for Phases I and II as the mean of four laboratories

	No. of chemicals	No. of chemicals with concordant results at all four laboratories.	BLR
Phase Ⅰ & Phase Ⅱ	40	36	**90.0**

4. Between-laboratory reproducibility per sensitization potency of 40 test substances in Phases I and II

In section 3 above, we evaluated between-laboratory reproducibility for the 40 test chemicals used in Phases I and II based on prediction of sensitization potential. We further looked at the test chemicals that were predicted to be sensitizers, and evaluated between-laboratory sensitization based on prediction of sensitization potency, using two categories: extreme/strong sensitizers and moderate/weak sensitizers.

The results, as shown in Table 4, were 100% between-laboratory reproducibility for extreme/strong sensitizers, 82.4% between-laboratory reproducibility for moderate/weak sensitizers, and 92.3% between-laboratory reproducibility for non-sensitizers. When calculated as the mean of three laboratories rather than four, the results were 100% for extreme/strong sensitizers, 85.3% for moderate/weak sensitizers, and 94.2% for non-sensitizers.

Table 4　Between-laboratory reproducibility per sensitization potency for Phases I and II as the mean of four laboratories

Phase Ⅰ & Phase Ⅱ	No. of chemicals	No. of chemicals with concordant results at all four laboratories.	BLR
Extreme/Strong	10	10	**100.0**
Moderate/Weak	17	14	**82.4**
No category	13	12	**92.3**

5. Other considerations

We compared between-laboratory reproducibility when calculated as the mean of three laboratories with between-laboratory reproducibility when calculated as the mean of four laboratories for just the 30 test chemicals used in Phase II as well as for all 40 test chemicals used in Phases I and II. Between-laboratory reproducibility for the 30 chemicals tested during Phase II when calculated as the mean of four laboratories was 2.5 percentage points lower than when calculated as the mean of three laboratories. Also, between-laboratory reproducibility for the 40 chemicals tested during Phases I and II when calculated as the mean of four laboratories was 1.9 percentage points lower than when calculated as the mean of three laboratories. In either case, between-laboratory reproducibility when calculated as the mean of four laboratories satisfied the target values by a significant margin.

Additionally, when evaluating per three categories of sensitization potency, between-laboratory reproducibility for just the 30 chemicals tested during Phase II and calculated as the mean of four laboratories was as much as 3.9 percentage points lower than when calculated as the mean of three laboratories, while that for all 40 chemicals tested during Phases I and II was as much as 2.9 percentage points lower, but in either case this discrepancy was less than 5 percentage points, indicating that ADRA exhibited excellent reproducibility even when calculated as the mean of four laboratories.

Appendix 13

Definitions and Literature

Appendix 13: Definitions and Literature

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). (Formula shown below.)

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).

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

Coefficient of variation: a measure of variability that is calculated for a group of replicate data by dividing the standard deviation by the mean. It can be multiplied by 100 for expression as a percentage.
Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

DPRA: Direct Peptide Reactivity Assay issued as OECD TG 442C in 2015

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

EDTA: Ethylenediaminetetraacetic acid

GPMT: Guinea Pig Maximization Test issued as OECD TG 406 in 1992

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.

h-CLAT: human Cell Line Activation Test issued as OECD TG 442E in 2016

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.

JaCVAM: Japanese Center for the Validation of Alternative Methods

KeratinoSens: ARE-Nrf2 Luciferase Test Method issued as OECD TG 442D in 2015

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

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

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

NAC: A nucleophilic reagent which is a cysteine derivative (*N*-(2-(1-naphthyl)acetyl)-*L*-cysteine)

NAL: A nucleophilic reagent which is a lysine derivative (α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine)

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 sensitizers through abiotic transformation

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitization 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 (21).

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

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

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

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

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

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

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

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to 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 (1).

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

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

Calculating depletion of either NAC or NAL

Depletion is calculated as follows:

Percent depletion of either NAC or NAL = {1- (NAC or NAL peak area in replicate injection ÷ mean NAC or NAL peak area in reference control C)} × 100

Calculating predictive capacity

There are several terms that are commonly used along with the description of sensitivity, specificity and accuracy. They are true positive (TP), true negative (TN), false negative (FN), and false positive (FP). Sensitivity, specificity and accuracy are described in terms of TP, TN, FN and FP.

Sensitivity: Number of true positives ÷ Number of all positive chemicals, TP ÷ (TP + FN)

Specificity: Number of true negatives ÷ Number of all negative chemicals, TN ÷ (TN + FP)

Accuracy: Number of correct predictions ÷ Number of all predictions, (TN + TP) ÷ (TN+TP+FN+FP)

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Appendix 14

ADRA as a standalone test for predicting skin sensitization potency

Appendix 14: ADRA as a standalone test for predicting skin sensitization potency

In addition to predicting the skin sensitization potential of test substances, the DPRA prediction model was design also to classify test chemicals into one of four levels of reactivity—high, moderate, low, and "minimal or no" reactivity—which could perhaps prove useful to inform potency assessment within the framework of an IATA. These four levels of reactivity, however, did not always correspond accurately with the four skin sensitization categories of extreme/strong, moderate, weak, and non-sensitizer. (Gerberick et al., 2007) Insofar as ADRA is based on the same scientific principles as DPRA, and the four reactivity levels used in DPRA were not sufficiently accurate, we had no expectation that classification of reactivity in ADRA would be any better than that of DPRA, therefore the lead laboratory did not investigate this matter.

On the other hand, the ability to use ADRA not just to predict skin sensitization potential but also skin sensitization potency would be highly advantageous for accurately classifying test chemicals according to GHS category. To this end, we investigated the possibility of using ADRA as a standalone test for predicting skin sensitization potency. For the purposes of this study, we attempted to use ADRA test results to classify test chemicals into one of three categories: extreme/strong, moderate/weak, and non-sensitizer.

Methodology

As with DPRA, the criteria used to predict the skin sensitization potential of test chemicals was established using a training data set comprising 56 of 82 test chemicals to establish a binary classification based on statistical analysis. Simply put, we used ADMEWORKS/ModelBuilder ver. 4.5 from Fujitsu Kyushu Systems Limited, Japan, to build a qualitative QSAR model based on a stochastic gradient perceptron algorithm to perform binary classification of test chemicals into either sensitizer or non-sensitizer using a linear equation for a single parameter, namely "mean % depletion." As a result, we established a threshold value of 4.9% as the mean % depletion above which test chemicals are predicted to be sensitizers and below which they are predicted to be non-sensitizers.

Similarly, sensitizers were further predicted to be either extreme/strong or moderate/weak.

Results

Determining a threshold value for binary classification of sensitization potency

Using the method described above, we established a threshold value of 46.4% as the mean % depletion above which sensitization potency was predicted to be extreme/strong and below which it was predicted to be moderate/weak.

ADRA as a standalone test for predicting skin-sensitization potency

Using these two threshold values of 4.9% and 46.4%, we classified 82 test chemicals into one of the above described three categories, the results of which are shown in Table 1. The majority of chemicals predicted to be extreme/strong sensitizers using LLNA were also predicted to be extreme/strong sensitizers using ADRA, as indicated by the color red in the Prediction column of Table 1. Furthermore, many of the chemicals predicted to be moderate/weak sensitizers using LLNA were also predicted to be moderate/weak sensitizers using ADRA, as indicated by the color pink in the Prediction column of Table 1, but some of the chemicals predicted to be either extreme/strong sensitizers or non-sensitizers using LLNA were predicted to be moderate/weak sensitizers using ADRA.

Moreover, with only one exception, chemicals predicted to be non-sensitizers using LLNA were also predicted to be non-sensitizers using ADRA.

Table 1 ADRA as a standalone test for predicting skin-sensitization potency

№	Test substance	EC_3 value	LLNA category	NAC EDTA (+)		NAL		Mean % depletion	Prediction
				Depletion (%)	SD	Depletion (%)	SD		
Extreme/strong									
1	Diphenylcyclopropenone	0.0003	Extreme	28.7	1.0	6.3	1.2	17.5	
2	Oxazolone	0.003	Extreme	85.3	0.3	80.1	0.8	82.7	
3	Benzoyl peroxide	0.004	Extreme	100.0	0.0	50.6	2.7	75.3	
4	Kathon CG	0.008	Extreme	100.0	0.0	-0.4	0.5	49.8	
5	Bandrowski's base	0.008	Extreme	100.0	0.0	5.7	0.4	52.9	
6	5-Chloro-2-methyl-4-isothiazolin-3-one	0.009	Extreme	100.0	0.0	17.7	1.1	58.8	
7	p-Benzoquinone	0.0099	Extreme	96.5	0.2	83.5	1.1	90.0	
8	Tetrachlorosalicylanilide	0.04	Extreme	43.4	4.2	2.1	0.3	22.7	
9	2,4-Dinitrochlorobenzene	0.05	Extreme	89.3	2.0	6.1	1.3	47.7	
10	Glutaraldehyde	0.1	Strong	1.6	0.2	53.1	2.9	27.3	
11	Fluorescein isothiocyanate	0.14	Strong	73.6	0.8	98.1	0.6	85.8	
12	Phthalic anhydride	0.16	Strong	0.3	0.3	96.9	1.1	48.6	
13	Lauryl gallate	0.3	Strong	100.0	0.0	19.0	0.4	59.5	
14	Propyl gallate	0.32	Strong	97.1	0.1	56.4	1.4	76.7	
15	CD3	0.6	Strong	76.6	1.3	16.5	0.5	46.6	
16	Trimellitic anhydride	0.6	Strong	1.9	0.4	97.0	1.2	49.4	
17	Formaldehyde	0.61	Strong	25.7	1.1	1.6	1.9	13.6	
18	Metol	0.8	Strong	100.0	0.0	22.8	1.1	61.4	
Moderate									
19	2-Hydroxyethyl acrylate	1.4	Moderate	100.0	0.0	16.3	4.7	58.1	
20	Glyoxal	1.4	Moderate	12.8	0.5	0.8	0.5	6.8	
21	Vinyl pyridine	1.6	Moderate	18.1	1.0	7.7	1.8	12.9	
22	2-Mercaptobenzothiazole	1.7	Moderate	56.6	0.8	0.3	0.8	28.4	
23	Nonanoyl chloride	1.8	Moderate	7.4	0.4	39.4	1.8	23.4	
24	2-Methyl-2H-isothiazol-3-one	1.9	Moderate	94.0	0.3	7.0	2.2	50.5	
25	1,2-Benzisothiazoline-3-one	2.3	Moderate	100.0	0.0	0.4	0.7	50.2	
26	Methyl-2-nonynoate	2.5	Moderate	15.0	0.9	1.4	0.2	8.2	
27	Cinnamaldehyde	3	Moderate	37.0	0.9	13.1	1.3	25.0	
28	Phenylacetaldehyde	3	Moderate	22.4	2.5	98.3	1.5	60.3	
29	Benzylideneacetone	3.7	Moderate	44.9	0.2	7.2	1.0	26.1	
30	2,4-Heptadienal	4	Moderate	35.8	0.2	50.1	1.3	42.9	
31	Squaric acid	4.3	Moderate	3.7	0.3	0.5	0.2	2.1	
32	Trans-2-hexenal	5.5	Moderate	80.4	0.4	12.3	1.1	46.3	
33	Resorcinol	5.5	Moderate	4.0	0.3	2.2	0.7	3.1	
34	Diethyl maleate	5.8	Moderate	31.8	0.5	7.7	1.9	19.8	
35	2-phenylpropionaldehyde	6.3	Moderate	8.0	0.3	4.6	1.3	6.3	
36	Perillaldehyde	8.1	Moderate	29.1	1.7	18.5	1.0	23.8	
37	Palmitoyl Chloride	8.8	Moderate	5.8	0.6	95.9	2.5	50.8	
38	1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	1.6	0.7	4.2	1.6	2.9	
Weak									
39	α-Hexylcinnnamaldehyde	11	Weak	0.0	0.0	1.1	0.9	0.6	
40	α-Amylcinnamaldehyde	11	Weak	0.0	0.0	1.6	0.4	0.8	
41	2,3-Butanedione	11	Weak	41.2	0.9	25.5	1.2	33.4	
42	Farnesal	12	Weak	17.2	0.6	8.8	1.4	13.0	
43	Oxalic acid	15	Weak	0.0	0.0	3.6	1.0	1.8	
44	Benzyl benzoate	17	Weak	0.0	0.0	2.8	1.1	1.4	
45	4-Allylanisole	18	Weak	27.0	0.4	3.9	2.6	15.4	
46	Lilial	19	Weak	7.0	0.8	3.9	0.9	5.4	
47	Cyclamen aldehyde	22	Weak	3.1	0.4	1.7	0.6	2.4	
48	Imidazolidinyl urea	24	Weak	35.4	3.1	2.0	0.2	18.7	
49	5-Methyl-2,3-hexanedione	26	Weak	6.4	0.2	34.8	2.8	20.6	
50	2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	0.5	0.5	1.6	0.8	1.1	
51	Ethyleneglycol dimethacrylate	28	Weak	5.9	0.5	2.6	1.6	4.3	
52	Ethyl acrylate	28	Weak	87.8	0.1	13.7	0.4	50.8	
53	Hydroxycitronellal	33	Weak	17.5	0.3	1.5	0.7	9.5	
Non-sensitizer									
54	Glycerol	NC[a]	NS	0.3	0.3	0.7	1.2	0.5	
55	Hexane	NC	NS	0.2	0.3	0.9	0.9	0.5	
56	Diethyl phthalate	NC	NS	0.0	0.0	1.7	1.4	0.9	
57	Octanoic acid	NC	NS	0.0	0.0	1.2	0.5	0.6	
58	2-Hydroxypropyl methacrylate	NC	NS	2.4	0.5	2.2	0.6	2.3	
59	1-Butanol	NC	NS	0.1	0.2	0.8	0.5	0.5	
60	4-Hydroxybenzoic acid	NC	NS	0.0	0.0	-1.4	1.0	-0.7	
61	6-Methyl coumatrin	NC	NS	0.0	0.0	1.0	0.7	0.5	
62	Methyl salicylate	NC	NS	0.0	0.0	0.4	0.6	0.2	
63	Chlorobenzene	NC	NS	0.0	0.0	0.7	0.7	0.4	
64	Lactic acid	NC	NS	0.0	0.0	2.8	0.6	1.4	
65	1-Bromobutane	NC	NS	0.1	0.1	2.4	1.6	1.2	
66	2-Acethylcyclohexanone	NC	NS	1.3	0.6	0.3	0.6	0.8	
67	4'-Methoxyacetophenone	NC	NS	3.3	0.4	3.3	0.6	3.3	
68	Ethyl benzoylacetate	NC	NS	4.4	0.3	5.3	1.3	4.9	
69	Ethyl vanillin	NC	NS	1.4	0.3	2.1	0.8	1.7	
70	Isopropanol	NC	NS	0.1	0.1	2.8	0.6	1.5	
71	Propylene glycol	NC	NS	0.1	0.2	2.2	0.7	1.1	
72	Sulfanilamide	NC	NS	0.0	0.0	1.4	0.7	0.7	
73	Isopropyl myristate	NC	NS	0.0	0.0	1.8	1.4	0.9	
74	Benzaldehyde	NC	NS	25.3	1.0	2.9	1.0	14.1	
75	Methylparaben	NC	NS	0.1	0.1	1.3	0.5	0.7	
76	Nonanoic acid	21 (False +)	NS	0.0	0.0	1.3	0.7	0.6	
77	Propyl paraben	NC	NS	0.1	0.2	1.9	3.1	1.0	
78	Salicylic acid	NC	NS	0.0	0.0	0.4	0.6	0.2	
79	Sulphanilic acid	NC	NS	0.5	0.4	0.4	0.2	0.5	
80	Vanillin	NC	NS	1.5	0.3	1.9	1.5	1.7	
81	Coumarin	NC	NS	0.5	0.5	3.9	2.4	2.2	
82	Vinylidene dichloride	NC	NS	0.9	0.4	-0.3	0.7	0.3	

■: Extreme/Strong sensitizer, ■: Modereta/Weak sensitizer, ■: Non-sensitizaer

ADRA as a standalone test for predicting skin-sensitization potency

Predictive capacity for skin sensitization potency based on Table 1 is shown in Table 2.

Table 1　ADRA as a standalone test for predicting skin-sensitization potency

		Prediction classification		
		Extreme/ Strong	Moderate/ Weak	Non-sensitizer
Chemical classification	Extreme/Strong	14[a]	4[b]	0[c]
	Moderate/Weak	6[d]	19[e]	10[f]
	Non-sensitizer	0[g]	1[h]	28[i]
	Overprediction rate (%)	9		
	Underprediction rate (%)	17		
	Accuracy (%)	**74**		

		Prediction of Skin Sensitization Potency per ADRA		
		Extreme/strong	Moderate/weak	Non-sensitizer
Prediction of Skin Sensitization Potency per LLNA	Extreme/strong (18)	14[a]	4[b]	0[c]
	Moderate/weak (35)	6[d]	19[e]	10[f]
	Non-sensitizer (29)	0[g]	1[h]	28[i]

Overprediction rate: 9%

Underprediction rate: 17%

Accuracy: 74%

Overprediction rate is the number of chemicals overpredicted in ADRA divided by the total number of chemicals:

(d + g + h) ÷ 82 chemicals × 100 = 9%

Underprediction rate is the number of chemicals underpredicted in ADRA divided by the total number of chemicals:

(b + c + f) ÷ 82 chemicals × 100 = 17%

Table 2　DPRA as a standalone test for predicting skin-sensitization potency

		Prediction classification		
		Extreme/ Strong	Moderate/ Weak	Non-sensitizer
Chemical classification	Extreme/Strong	14[a]	4[b]	0[c]
	Moderate/Weak	14[d]	14[e]	7[f]
	Non-sensitizer	0[g]	3[h]	25[i]
	Overprediction rate (%)	21		
	Underprediction rate (%)	14		
	Accuracy (%)	**65**		

Although DPRA classified reactivity into four categories, the results here are arranged so that chemicals predicted to have high reactivity are labelled extreme/strong, those predicted to have moderate or low reactivity are labelled moderate/weak, and those to be predicted to have minimal or no reactivity are labelled non-sensitizer.

As shown in Table 1, when using ADRA as a standalone test to predict skin sensitization potency, ADRA predictions for the majority (42 of 47) of both the 18 extreme/strong sensitizers and the 29 non-sensitizers were concordant with LLNA results. Of the 35 chemicals, however, that were predicted to be either moderate or weak sensitizers in LLNA, even though many (19) of these had concordant results in ADRA, a significant number were non-concordant.

On the other hand, when DPRA results—which originally were sorted into four reactivity categories according to mean % depletion of cysteine peptide and lysine peptide—are sorted into three sensitization potency categories, they are largely concordant with ADRA results. Relative to DPRA, overprediction in ADRA was 12 percentage points lower, and even though underprediction was 3 percentage points higher, overall accuracy was 9 percentage points higher.

Other considerations

We established a threshold value of 46.4% as the mean % depletion above which sensitization potency was predicted to be extreme/strong and below which it was predicted to be moderate/weak. In the four reactivity categories used in DPRA, the threshold value between high and moderate reactivity was 42.47%. Compared with DPRA, overpredictions of skin sensitization potency are rare but underpredictions are slightly more common when predicting skin sensitization potency with ADRA. Also, ADRA's accuracy is 9 percentage points better than DPRA, which is an indication that ADRA is superior to DPRA as a standalone test for predicting skin sensitization potency. Compared with DPRA, there appears to be greater gap between NAC and NAL depletion of strong sensitizers compares with that of weak sensitizers. Thus, we consider the predictive capacity of ADRA as a standalone test for predicting sensitization potency to be excellent.

Appendix 15

Comparison of the ADRA and DPRA test methods

Appendix 15: Comparison of the ADRA and DPRA test methods

ADRA is a reactivity assay that was developed as a derivative of DPRA. There are, however, significant differences between the nucleophilic reagents used in these two test methods, which means that the test protocols themselves differ significantly.

The following comparison of the DPRA and ADRA test methods is intended to elucidate these differences.

Results

Table 1 shows how ADRA differs from DPRA and highlights the most significant changes in red.

The five most significant changes are as follows:

1. ADRA uses NAC and NAL as nucleophilic reagents.
2. Concentrations of test chemicals and nucleophilic reagents are just 1% of those used in DPRA.
3. EDTA is added to suppress NAC oxidation.
4. A fixing solution is added to suppress reactivity of the nucleophilic reagent and the test chemical after 24 h.
5. ADRA uses a quantification wavelength of 281 nm.

Other significant changes include the following:

1. ADRA uses a 96-well plate as a reaction vessel.
2. ADRA specifies that NAC have a pH of 8.0 and that NAL have a pH of 10.2 (phosphate buffer).
3. ADRA specifies a reaction volume of 200 μL.
4. ADRA specifies phenylacetaldehyde as a positive control reagent.
5. ADRA recommends the use of a CAPCELL CORE C_{18} core-shell column for high-performance separation.
6. ADRA specifies column temperature, flow speed, and other HPLC parameters.
7. ADRA specified a mean % depletion of 4.9% as the prediction criteria threshold value.

Other considerations

The five most significant changes described above have resulted in the following improvements in performance over DPRA.

1. The use of NAC and NAL as nucleophilic reagents enable highly sensitive detection, thereby allowing the use of test chemical concentrations just 1% of those used in DPRA.
2. The use of a 281-nm quantification wavelength virtually eliminates co-elution of test chemicals and nucleophilic reagents.
3. The use of a 281-nm quantification wavelength reduces the SD of depletion, thereby improving accuracy.
4. The use of lower test chemical concentrations virtually eliminates precipitation of the test chemical in the reaction solution.
5. The addition of EDTA to suppress NAC oxidation reduces the incidence of retesting.

Table 1　ADRA と DPRA の比較

Preparation of reaction solution

	DPRA		ADRA	
	cysteine peptide	lysine peptide	NAC	NAL
container	1 mL autosampler vial		96-well plate	
nucleophlic reagent solution	cysteine peptide solution concentration: 667μM pH: 7.5 (phosphate buffer) volume: 750 μL	lysine peptide solution concentration: 667μM pH : 10.2 (ammonium acetate buffer) volume: 750 μL	NAC solution concentration: 6.67μM pH: 8.0 (phosphate buffer) EDTA: 0.33 μM volume: 150 μL	NAL solution concentration: 6.67μM pH : 10.2 (phosphate buffer) volume: 150 μL
test chemical solution	test chemical solution concentration: 100 mM volume: 50 μL	test chemical solution concentration: 100 mM volume: 250 μL	test chemical solution concentration: 1 mM volume: 50 μL	
positive control	cinnamaldehyde		phenylacetaldehyde	
acetonitrile	volume : 200 μL	none	none	

Reaction

reaction temperature	25°C	25°C
reaction time	24 hour	24 hour

Reaction fixation

Reaction fixed solution	none	solution: 2.5%(v/v) trifluoroacetic acid volume: 50 μL

HPLC measurement

HPLC conditions	HPLC system : Waters 2695 Alliance column : Zorbax SB-C18 (2.1 mmx100 mmx3.5 μm) column temperature: 30°C eluent : mobile phase A: 0.1%(v/v) trifluoroacetic acid in water mobile phase B: 0.085%(v/v) trifluoroacetic acid in acetonitrile flow rate: 0.35 mL/min	HPLC system : Shimadzu LC-20A column : CAPCELL CORE C18 (3.0 mm×150 mmx2.7 μm) column temperature: 40°C eluent : mobile phase A: 0.1%(v/v) trifluoroacetic acid in water mobile phase B: 0.1%(v/v) trifluoroacetic acid in acetonitrile flow rate: 0.3 mL/min	
HPLC conditions	gradient condition: Time %A %B 0 min 90 10 10 min 75 25 11 min 10 90 13 min 10 90 13.5 min 90 10 20 min end run wave lenghth : 220 nm run time: 20 minutes	gradient condition: Time %A %B 0 min 70 30 9.5 min 45 55 10 min 0 100 13 min 0 100 13.5 min 70 30 20 min end run wave lenghth : 281 nm run time: 20 minutes	gradient condition: Time %A %B 0 min 80 20 9.5 min 55 45 01 min 10 90 13 min 10 90 13.5 min 80 20 20 min end run wave lenghth : 281 nm run time: 20 minutes

Prediction of skin sensitization

Criteria	mean % depletion<6.28%: Negative mean % depletion>=6.28%: Positive	mean % depletion<4.9%: Negative mean % depletion>=4.9%: Positive

Appendix 16

List of test chemicals tested using ADRA

Appendix 16: List of test chemicals tested using ADRA

There were 82 test chemicals tested during the development of ADRA, and during the validation study, there were five test chemicals tested in pretraining as well as 10 test chemicals tested in training for technology transfer, 10 test chemicals tested in Phase I, and 30 test chemicals tested in Phase II. Of these, there are 46 test chemicals for which human data is available. The following table shows which test chemicals were used in which tests as well as the results of ADRA, LLNA, and human data, where available. All ADRA results shown in this table—S for sensitizer and NS for non-sensitizer—are from the lead laboratory.

Results

The following table lists all test chemicals and their results, as described above. A total of 107 different test chemicals were tested.

Table 1 List of test chemicals tested using ADRA

№	Test substance	EC_3 value	LLNA category	ADRA						
				Evaluation of 82 chemicals (NAC without EDTA) [1] (See Appendix 9)	Evaluation of 82 chemicals (NAC with EDTA) [2] (See Appendix 10)	Transferability [3]		Validation testing [3]		Camparision of ADRA with human data [1),2),3),4)] (See Appendix 5)
						Pre-training	Training	Phase I	Phase II	
1	Diphenylcyclo propenone	0.0003	Extreme	S [c]	S				S	S
2	Oxazolone	0.003	Extreme	S	S		S			
3	Benzoyl peroxide	0.004	Extreme	S	S					S
4	Kathon CG	0.008	Extreme	S	S					S
5	Bandrowski's base	0.008	Extreme	S	S					
6	5-Chloro-2-methyl-4-isothiazolin-3-one	0.009	Extreme	S	S					S
7	p-Benzoquinone	0.0099	Extreme	S	S	S			S	
8	Tetrachlorosalicylanilide	0.04	Extreme	S	S					S
9	2,4-Dinitrochlorobenzene	0.05	Extreme	S	S					S
10	Glutaraldehyde	0.1	Strong	S	S					S
11	Hydroquinone	0.1	Strong						S	S
12	4-Phenylenediamine	0.11	strong							S
13	Fluorescein isothiocyanate	0.14	Strong	S	S					
14	Phthalic anhydride	0.16	Strong	S	S		S			
15	Lauryl gallate	0.3	Strong	S	S			S		S
16	Propyl gallate	0.32	Strong	S	S				S	S
17	Chloramine T	0.4	strong					S		
18	2-Aminophenol	0.4	Strong						S	
19	CD3	0.6	Strong	S	S					
20	Trimellitic anhydride	0.6	Strong	S	S					
21	Formaldehyde	0.61	Strong	S	S		S			S
22	4-(Methylamino) phenol hemisulfate salt (Metol)	0.8	Strong	S	S			S		S
23	Iodopropynyl butylcarbamate	0.9	Strong						S	
24	Squaric acid diethylester	0.9	Moderate						S	
25	Isoeugenol	1.2	Moderate							S
26	2-Hydroxyethyl acrylate	1.4	Moderate	S	S					S
27	Glyoxal	1.4	Moderate	S	S		S			S
28	Vinyl pyridine	1.6	Moderate	S	S					
29	2-Mercaptobenzothiazole	1.7	Moderate	S	S			S		S
30	Nonanoyl chloride	1.8	Moderate	S	S					
31	2-Methyl-2H-isothiazol-3-one	1.9	Moderate	S	S				S	
32	1,2-Benzisothiazoline-3-one	2.3	Moderate	S	S					S
33	Methyl-2-nonynoate	2.5	Moderate	S	S					
34	Benzylsalicylate	2.9	Moderate						NS	
35	Cinnamaldehyde	3	Moderate	S	S	S		S		
36	Phenylacetaldehyde	3	Moderate	S	S					
37	1-Thioglycerol	3.6	Moderate							NS
38	Benzylideneacetone	3.7	Moderate	S	S		S			
39	2,4-Heptadienal	4	Moderate	S	S					
40	Squaric acid	4.3	Moderate	NS	NS					
41	Citral	13 / 6.3 / 4.6 / 5.3	Moderate						S	S
42	Trans-2-hexenal	5.5	Moderate	S	S					
43	Resorcinol	5.5	Moderate	S	NS				NS	NS
44	Diethyl maleate	5.8	Moderate	S	S	S				S
45	2-phenylpropionaldehyde	6.3	Moderate	S	S					
46	Dihydroeugenol	6.8	Moderate						NS	
47	Perillaldehyde	8.1	Moderate	S	S					
48	Palmitoyl Chloride	8.8	Moderate	S	S				S	
49	1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	NS	NS					
50	α-Hexylcinnnamaldehyde	11	Weak	NS	NS					NS
51	α-Amylcinnamaldehyde	11	Weak	NS	NS					NS
52	2,3-Butanedione	11	Weak	S	S				S	
53	Farnesal	12	Weak	S	S				S	
54	Eugenol	12.9	Weak						S	S
55	Oxalic acid	15	Weak	NS	NS					
56	Benzyl benzoate	17	Weak	NS	NS					
57	4-Allylanisole	18	Weak	S	S	S				
58	Benzylcinnamate	18.4	Weak						NS	
59	Lilial	19	Weak	S	S				S	
60	Cyclamen aldehyde	22	Weak	S	NS		NS			
61	Imidazolidinyl urea	24	Weak	S	S			S		
62	5-Methyl-2,3-hexanedione	26	Weak	S	S					S
63	2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	NS	NS					
64	Ethyleneglycol dimethacrylate	28	Weak	S	NS					NS
65	Ethyl acrylate	28	Weak	S	S			S		S
66	Farnesol	28	Weak							S
67	Penicilln G	30	Weak						NS	
68	Hydroxycitronellal	33	Weak	S	S				NS	S
69	R(+)-Limonene	69	Weak							NS
70	Methylmethacrylate	90	Weak							NS
71	Xylene	95.8	Weak							NS
72	Fumaric acid	>25	NS						NS	
73	Glycerol	NC [a]	NS [b]	NS	NS			NS		NS
74	Hexane	NC	NS	NS	NS					NS
75	Diethyl phthalate	NC	NS	NS	NS				NS	NS
76	Octanoic acid	NC	NS	NS	NS					NS
77	2-Hydroxypropyl methacrylate	NC	NS	NS	NS					
78	1-Butanol	NC	NS	NS	NS		NS			NS
79	4-Hydroxybenzoic acid	NC	NS	NS	NS					
80	6-Methyl coumatrin	NC	NS	NS	NS		NS			
81	Methyl salicylate	NC	NS	NS	NS					NS
82	Chlorobenzene	NC	NS	NS	NS					
83	Lactic acid	NC	NS	NS	NS		NS			NS
84	1-Bromobutane	NC	NS	NS	NS					
85	2-Acethylcyclohexanone	NC	NS	NS	NS					
86	4'-Methoxyacetophenone	NC	NS	NS	NS		NS			
87	Ethyl benzoylacetate	NC	NS	NS	NS					
88	Ethyl vanillin	NC	NS	NS	NS					
89	Isopropanol	NC	NS	NS	NS			NS		NS
90	Propylene glycol	NC	NS	NS	NS					NS
91	Sulfanilamide	NC	NS	NS	NS				NS	
92	Isopropyl myristate	NC	NS	NS	NS					NS
93	Benzaldehyde	NC	NS	S	S					S
94	Methylparaben	NC	NS	NS	NS					
95	Nonanoic acid	21 (False +)	NS	NS	NS					
96	Propyl paraben	NC	NS	NS	NS				NS	NS
97	Salicylic acid	NC	NS	NS	NS			NS		
98	Sulphanilic acid	NC	NS	NS	NS					
99	Vanillin	NC	NS	NS	NS	NS				
100	Coumarin	NC	NS	NS	NS				NS	NS
101	Vinylidene dichloride	NC	NS	NS	NS					
102	Benzyl alcohol	NC	NS						NS	
103	Methylsalicylate	NC	NS						NS	
104	Dimethylisophthalate	NC	NS						NS	
105	4-Aminobenzoic acid	NC	NS						NS	
106	Dextran	NC	NS						NS	
107	4-Aminobenzoic acid	NC	NS						NS	

[a]Not calculated., [b]Non-sensitizer., [c]Sensitizer

1) Results in the condition that EDTA was not included in NAC solution. (Yamamoto *et al*., 2014), 2) Results in the condition that EDTA was included in NAC solution. (Fujita et al., 2018), 3) Result obtained by lead laboratory, 4) Unpublished data of lead laboratory

Appendix 17

Test chemicals that exhibited significant differences in NAL depletion and lysine peptide depletion

Appendix 17: Test chemicals that exhibited significant differences in NAL depletion and lysine peptide depletion

ADRA and DPRA are both based on the same scientific principle, and although they utilize different nucleophilic reagents, we do not anticipate that the reactivity of these reagents would differ significantly. Nevertheless, there were some test chemicals that exhibited significant differences in depletion of the NAL used in ADRA and of the lysine peptide used in DPRA. The following discussion provides an analysis of the factors that could cause a test chemical to exhibit significant differences in NAL depletion and lysine peptide depletion.

Results

Table 1 below compares NAL depletion in ADRA with lysine depletion in DPRA for the 82 chemicals used in the development of ADRA. The column labeled Δ (delta) gives the difference in percentage obtained by subtracting the NAL depletion value from the lysine peptide value. The cells in this column that are highlighted in pink indicate a chemical for which the NAL depletion value was at least 30 percentage points lower than the lysine peptide depletion value, while those that are highlighted in blue indicate a chemical for which the NAL depletion value was at least 30 percentage points higher than the lysine peptide depletion value. Additionally, the columns to the far right indicate whether the chemical in question contains aldehydes, acyl chlorides, or acrylates in its chemical structure.

Of these 82 chemicals, there were seven for which the NAL depletion value was significantly lower than the lysine peptide depletion value, and six for which the NAL depletion value was significantly higher than the lysine peptide depletion value.

There were 17 chemicals that contain aldehydes in their chemical structure, of which three exhibited significantly lower NAL depletion values and one exhibited a significantly higher NAL depletion value than the lysine peptide depletion value. There were two chemicals that contain acyl chlorides, both of which exhibited a NAL depletion value significantly higher than the lysine peptide depletion value. And there were four chemicals that contained either acrylates or methacrylates, two of which exhibited a NAL depletion value significantly lower than the lysine peptide depletion value.

Other considerations

Reactivity assays like ADRA or DPRA measure the reactivity of a nucleophilic reagent with a test chemical, but reactivity is subject to variability. As explained in the validation study report, even when the same test chemical is tested multiple times, it is not uncommon to observe variations as large as 20% or 30%. In this case, however, we looked for test chemicals for which the NAL depletion value was at least 30 percentage points higher or lower than the lysine peptide depletion value.

Of the 82 chemicals used in the development of ADRA and DPRA, were seven for which the NAL depletion value was significantly lower than the lysine peptide depletion value, and six for which the NAL depletion value was significantly higher than the lysine peptide depletion value. Thus, there does not appear to be any significant difference between these two test methods based on the number of test chemicals alone.

In comparing these differences for each individual test chemical, we looked to see if any particular class of chemical exhibited significant differences in depletion, and identified acyl chlorides, acrylates,

and aldehydes as chemical structures that did.

Aldehydes were the most commonly represented chemical structure in the 82 test chemicals, and although three aldehydes exhibited significantly lower and one exhibited significantly higher levels of depletion, 13 of 17 aldehydes exhibited no significant difference. From this we concluded that aldehydes do not in general exhibit a significant difference in NAL depletion and lysine peptide depletion.

There were two acyl chlorides that exhibited significantly higher levels of NAL depletion, which is to say that, relative to NAL, their reactivity with lysine peptide was extremely low. One factor in this is that both these substances are highly hydrophobic. The cLogP value of nonanoyl chloride is 3.8, while that of palmitoyl chloride is 7.5, which presents the possibility that these two test chemicals precipitated in the reaction solution and did not react sufficiently with a nucleophilic reagent. (See Appendix 7.) Also, acyl chlorides are known to react immediately with NH_2 and OH groups. In DPRA, the lysine peptide reaction solution contains ammonium phosphate buffer solution with a pH of 10.2, and while NH_2 groups are present in the lysine peptide reaction solution at a ratio of 1:50 with the test chemical, NH_3 also exists at this same ratio. Because of this, acyl chlorides have a higher probability of colliding with the NH_3 in the buffer solution than of reacting with lysine peptide, and we consider it likely that partial or significant reactivity with NH_3 results in reduced reactivity with lysine peptide. Based on these two factors, we speculate that, relative to NAL, the reactivity of lysine peptide was extremely low.

There were two acrylates and two methacrylates, two of which exhibited significantly lower levels of NAL depletion, which is to say that, relative to lysine peptide, their reactivity with NAL was extremely low. The factors contributing to this are unclear. In general, SH groups are known to be more nucleophilic than NH_2 groups and therefore react more easily with acrylates. (B.D. Mather *et al*., 2006) In fact, the two test chemicals in question had extremely high rates of NAC depletion—namely, 100% for 2-hydroxyethyl acrylate and 87.8% for ethyl acrylate. Because of this, the mean % depletion values used to predict sensitization potential were quite distant from the threshold value, and both test chemicals were correctly predicted to be sensitizers. Based on this, and irrespective of the fact that the factors contributing to low reactivity with NAL remain unclear, the reactivity of acrylates in general is largely dependent on reactivity with NAC, which means that low reactivity with NAL is unlikely to affect predictions of skin sensitization potential.

Reference

Brian D. Mather, Kalpana Viswanathan, Kevin M. Miller, Timothy E. Long, (2006), Michael addition reactions in macromolecular design for emerging technologies, *Progress Polymer Sciences*. **31**, 487–531.

PART II:

Amino acid Derivative Reactivity Assay (ADRA) Using a Chemical Solution Prepared at 4 mM; ADRA (4 mM) JaCVAM Ring Study Report

Version 1.0

September, 2021

Index

Summary

The results of an alternative method to determine the skin sensitization potential, namely, the amino acid derivative reactivity assay (ADRA) using a test chemical solution prepared at 4 mM (ADRA 4 mM), are described in this report. ADRA (4 mM) was performed at 5 participating laboratories to validate the within- and between-laboratory reproducibility to evaluate whether ADRA (4 mM) can yield accurate results for the test chemicals showing different results with ADRA 1 and 4 mM.

A modular approach to the European Centre for the Validation of Alternative Methods (ECVAM) principles was used for testing the validity (OECD Series on Testing and Assessment, Number 34, 2005, Hartung *et al.*, 2004), and the ring study team (RST) empirically evaluated modules 1–4 (test definition, within-laboratory reproducibility, transferability, and between-laboratory reproducibility); in addition, the RST used these results to evaluate modules 5 and 6 (predictive capacity and applicability domain).

Each of the 5 participating laboratories performed 3 test runs of identical sets of 8 coded test chemicals (proficiency substances) to validate within- and between-laboratory reproducibility. In addition, to validate whether accuracy could be improved by ADRA (4 mM), 4 test chemicals which were judged as "false negative" by ADRA (1 mM) and as "positive" by ADRA (4 mM) were encoded, and the validity of a total of 12 test chemicals together with 8 proficiency substances was evaluated using ADRA (4 mM).

The results for both within- and between-laboratory reproducibility were all 100% (8/8) at all the 5 participating laboratories. In addition, the 4 test chemicals, which were judged as "false negative" by ADRA (1 mM) and as "positive" by ADRA (4 mM) were correctly judged as "positive" using ADRA (4 mM) at the 5 participating laboratories. Thus, on the basis of these results, the RST concluded that the ADRA (4 mM) test method can be easily transferred to general laboratories.

Background

The different methods that address the elements of the adverse outcome pathway (AOP) as endpoints when testing skin sensitization potential include: (i) the direct peptide reactivity assay (DPRA) and ADRA (OECD TG442C, 2021), which address the key event 1 (KE1) of covalent binding with proteins (haptenization); (ii) the ARE-Nrf2 Luciferase KeratinoSens^{TM} and ARE-Nrf2 Luciferase LuSens tests (OECD TG442D, 2018), which address the KE2 of keratinocyte activation; and (iii) the human cell line activation test (h-CLAT), the U937 cell line activation test (U-Sense^{TM}), and the IL-8 Luc assay (OECD TG442E, 2018), which address the KE3 of dendritic cell activation.

Compared with cell-based *in vitro* test methods, the DPRA is an easy-to-use method and has excellent predictive capacity. The DPRA, however, has some significant limitations. Therefore, the ADRA was developed as an improvement over the DPRA to evaluate the KE1 in sensitization mechanisms and to reduce the limitations of DPRA (Fujita *et. al.*, 2014). Since then, ADRA has been used for determining the sensitization potential using various concentrations of the test chemical solution. Since ADRA was developed in reference to DPRA, the sensitization potential was initially evaluated by preparing the test chemical solutions at 100 mM, the same concentration as that used in the DPRA (Fujita *et al*., 2014). However, because the nucleophilic reagent of ADRA has a high detection sensitivity, when using 100 mM of the test chemical solution (ADRA (100 mM)), after the reaction, the sample was diluted and subjected to high-performance liquid chromatography (HPLC). On the basis of these results, the ADRA (1 mM) method was established by preparing test chemical solutions at a concentration of 1 mM, which reduced the concentration of the test chemical to 1/100 of the amount used previously for the test chemical and nucleophilic reagent (Yamamoto *et al.*, 2015); in addition, the precipitation frequency of the test chemical in the reaction solution was reduced (Yamamoto *et al*., 2019a). These results may be attributed to the fact that in the DPRA, high concentrations of test chemicals were used in the reaction solution, such as 5 mM in cysteine peptide (Cys-peptide) reaction buffer and 25 mM in Lys-peptide reaction buffer, whereas low concentrations of test chemicals such as 0.25 mM in both *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine (NAL) reaction buffer were used in the ADRA (1 mM). Therefore, we began a validation study from 2016 to include the ADRA (1 mM) method in the OECD test guideline (TG), and this method was approved as OECD TG in 2019 (OECD ADRA validation report, 2018, Fujita *et al*., 2019). In the ADRA, similar to DPRA, the molecular weight of the test chemical is required to evaluate the sensitization, and evaluation of the sensitization of the mixture or of chemicals with an unknown molecular weight is not possible. Therefore, in ADRA, to find the optimum weight concentration of the test chemical solution, the test chemicals were prepared at concentrations of 0.5, 0.2, 0.1, and 0.05 mg/mL, and ADRA was performed using the above 82 test chemicals (Yamamoto *et al*., 2019b). Compared with DPRA, ADRA (1 mM) and ADRA (0.5 mg/mL) showed similar accuracy to that obtained with the other methods, and the accuracy and

sensitivity decreased as the concentration of the test chemical solution decreased. In addition, the accuracy of ADRA (0.5 mg/mL) was higher than that of ADRA (1 mM) because the false-negatives decreased, whereas the false-positives increased. Thus, ADRA (0.5 mg/mL) was an optimal method for testing chemicals and mixtures of unknown molecular weight by using a test chemical solution prepared at 0.5 mg/mL.

Therefore, in order to determine whether the ADRA (0.5 mg/mL) method can actually be included in the OECD TG, validation tests for 10 proficiency substances were performed at 5 participating laboratories. The concordance rate of the within and between-laboratory reproducibility was good at 100% and 100%, respectively (Yamamoto, 2022, in press).

The accuracy of ADRA (1 mM) in assessing the 82 test chemicals was similar to that of DPRA; however, in the dataset of 137 test chemicals including pre/pro-haptens and met, ADRA (1 mM) had a lower accuracy than DPRA (Yamamoto *et al*., 2019b). These results may be because chemicals with depletion values closer to the criteria in ADRA (1 mM) tend to be false negatives. Therefore, when the optimum molar concentration of the test chemical solution in ADRA was examined, the accuracy of ADRA (4 mM) using a chemical solution prepared at 4 mM was the highest and the number of false negatives was the lowest (Imamura *et al*., 2021).

Management of the Study

1. Study objectives

An ADRA for assessing the skin sensitization potential was developed by Fujifilm Corporation as an alternative to *in vivo* testing. ADRA is an alternative method evaluating KE1 in the skin sensitization mechanism included in the OECD TG442C. Recently, we found that ADRA (4 mM) using the chemical solution prepared at 4 mM had a higher accuracy and lower frequency for false negative chemicals than ADRA (1 mM) mentioned in the TG. However, the current OECD TGs stipulate that the test chemical solution is prepared at 1 mM for ADRA; therefore, we aimed to alter the concentration of the test chemical solution from 1 mM to 4 mM to evaluate the skin sensitization for chemicals with known molecular weights.

Therefore, the ADRA (4 mM) ring study was initiated in 2020. The primary objective of the study was to evaluate the reliability (based on within- and between-laboratory reproducibility) of ADRA (4 mM) with an intention of evaluating the potential suitability of ADRA (4 mM) for future use in combination with other test methods such as animal and *in vitro* tests that are currently used.

A secondary objective was to evaluate whether ADRA (4 mM) can accurately detect 4 chemicals with different results at 1 mM and 4 mM in concentration of test chemical solution (1 mM, false negative and 4 mM, positive) in any laboratory. Furthermore, since ADRA's nucleophilic reagents have fluorescence intensity as well as Ultraviolet (UV) absorption, ADRA-FL (fluorescence) method with using FL detector as an HPLC detector almost eliminates co-elution with the test chemicals and is very useful. Therefore, in this ring study, in order to verify the identity between UV detection and FL detection, FL detection was performed for all samples at the same time as conventional UV detection.

The results of testing performed at 5 independent laboratories to accurately validate the reliability of ADRA (4 mM) are presented in this report. Since this study differs from the previous validation study in terms of organizational structure and the number of test chemicals, it is referred to as “ring study” to distinguish it from the validation study (OECD ADRA validation report ver1.2, 2018, Fujita *et al*., 2019).

2. Study Plan

The study plan was drafted, approved, and issued by the ring study team (RST) before initiation of testing. The study plan is included in Appendix 1.

2-1. Study coordinator and sponsor

The study coordinator and sponsor of this ring study and the developer of this test is Fujifilm Corporation. On behalf of Fujifilm Corporation, Dr. Toshihiko Kasahara prepared the draft study plan, study protocol, test chemicals, and study report.

2-2. Consultant

This ring study was supported by Dr. Atsushi Ono of Okayama University and Dr. Hajime Kojima of JaCVAM as consultants. In addition, Dr. Takashi Sozu of Tokyo University of Science supported this study as a biostatistician.

2-3. Laboratories

The following 5 laboratories participated in the testing of the chemicals for the ADRA ring study.

Laboratory A
Lion Corporation
Human & Environmental Safety Evaluation Center
Kanagawa, Japan
Shinichi Watanabe, Study Director

Laboratory B
Mitsui Chemicals, Inc.
Chemical Safety Department
Responsible Care & Quality Assurance Division
Chiba, Japan
Koji Wakabayashi, Study Director

Laboratory C
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Osaka, Japan
Keiichi Fujimoto, Study Director

Laboratory D
Nissan Chemical Industries, Ltd.
Toxicology & Environmental Science Department
Biological Research Laboratories
Saitama, Japan
Kazuya Takeuchi, Study Director

Laboratory E
Fujifilm Corporation
Safety Evaluation Center
Ecology & Quality Management Div.
ESG Div.
Kanagawa, Japan
Toshihiko Kasahara, Study Director

2-3-1. Why was the study conducted at 5 participating laboratories

Ring study testing is normally performed at a minimum of 3 participating laboratories. Since all the 5 laboratories participating in this ring study had participated in the ADRA (1 mM) validation study performed in 2016, the operation of the HPLC-fluorescent device alone was examined at each laboratory and the technology transfer step was omitted.

We allowed all the 5 participating laboratories that volunteered to participate in the technology. Further, since all 5 participating laboratories wished to continue to participate until the end of the ring study, the ring study testing was conducted at the 5 participating laboratories.

The higher the number of participating laboratories in a ring study, the easier it becomes to identify issues that arise from differences in ambient conditions at each laboratory, which according to us is a rapid and effective means for establishing a universally suitable test method. Because of time and cost considerations, a validation study is performed at only 3 participating laboratories, which is the minimum number necessary to ensure a valid statistical analysis. For example, according to the DPRA validation study report, testing was conducted only at 3 participating laboratories, one of which was the lead laboratory, Proctor & Gamble. Although the initial ADRA validation study, which was performed in 2016, was conducted at 4 participating laboratories, and this ring study testing was conducted at 5 participating laboratories, the calculations of within-laboratory reproducibility, between-laboratory reproducibility, and predictive capacity were based on the results from sets of 3 participating laboratories (See 7. Statistical Analysis of Test Data, 7-1. Data analysis.) to enable direct comparison of these results with those of DPRA and other test methods.

2-3-2. Explanation of why a lead laboratory participates in the ring study

The objective of the testing performed during this ring study is to demonstrate that the participating laboratories are able to replicate to a high degree of certainty the same accurate test results obtained and published by the lead laboratory. Considering this objective and the fact that the lead laboratory is experienced in this technology, out of fairness alone, the lead laboratory itself would ordinarily not participate in validation testing. In the event, however, that the lead laboratory were to participate in ring study under the same conditions as the participating laboratories, test results and comments from the

lead laboratory would be useful in reviewing the results of the participating laboratories, and in cases where problems were encountered during the ring study, could be considered helpful in finding solutions. Thus, Fujifilm, the lead laboratory, mirrored the ADRA validation, testing the encoded chemicals, and undergoing quality control (QC) checks under the same conditions as the other participating laboratories.

3. Test Design

Reference document: Study plan (Appendix 1)

Study stages

The ring study comprised and was implemented in 2 distinct stages:

Transferability: Since all 5 laboratories that wished to participate in this ring study had previously participated in the validation study of ADRA (1 mM) performed in 2016, only the operation of the HPLC-fluorescent device was examined at each laboratory and the technology transfer step was omitted.

Reliability: Evaluation of test performance on the basis of the results of testing performed with coded chemicals at the participating laboratories.

4. Selection of Test Chemicals

The chemicals tested in this ring study were basically proficiency substances. In addition, since the ADRA (4 mM) method to be validated this time has an advantage of decreasing the number of chemicals that are judged as false negatives in ADRA (1mM), we also verified whether these chemicals were correctly judged as positive by ADRA (4 mM) in multiple laboratories. Furthermore, in this ring study, in addition to the conventional positive control substance; phenylacetaldehyde, a new reagent, namely, squaric acid diethylester was also evaluated using the chemical solution prepared at 4 mM. The ADRA kit was used as the nucleophilic reagent used in this ring study. The ADRA kit consists of 2 vials each for NAC and NAL, and one vial each for NAC and NAL can evaluate up to 14 samples. Therefore, in this ring study, 2 substances (*p*-benzoquinone and glycerol), which are expected to have little variation in data at multiple laboratories, were removed from the 10 proficiency substances and thus the remaining 8 substances, 4 substances, which were judged as false negatives in ADRA (1 mM), and 2 positive control substances, making it a total of 14 substances were selected by the Chemical Selection Group and Lead laboratory of the RST. Then, these substances were approved by the OECD Expert Group (Table 1).

Table 1. Test chemicals for the ADRA ring study

	Test chemicals	CAS No.	Physical State	Molecular weight	Source	Lot.	*In vivo* prediction [1]	ADRA Prediction[2]	
								1 mM	4 mM
1	Diphenylcyclopropenone	886-38-4	Solid	206.24	ALDRICH	BCCB3433	Sensitizer (extreme)	Pos.	Pos.
2	2-Methyl-2H-isothiazol-3-one	2682-20-4	Solid	115.15	ALDRICH	BCCD7472	Sensitizer (moderate)	Pos.	Pos.
3	Palmitoyl chloride	112-67-4	Liquid	274.87	FFWK	APG0787	Sensitizer (moderate)	Pos.	Pos.
4	Imidazolidinyl urea	39236-46-9	Solid	388.29	ALDRICH	BCCB8623	Sensitizer (weak)	Pos.	Pos.
5	Farnesal	19317-11-4	Liquid	220.35	Fluorochem	FCB026064	Sensitizer (weak)	Pos.	Pos.
6	*m*-Aminophenol	591-27-5	Solid	109.13	TCI	HYFKD-EH	Sensitizer (moderate)	Neg.	Pos.
7	3-Propylidenephthalide	17369-59-4	Liquid	174.20	TCI	7CR6K-RB	Sensitizer (moderate)	Neg.	Pos.
8	Ethyleneglycol dimethacrylate	97-90-5	Liquid	198.22	ALDRICH	MKCM4438	Sensitizer (weak)	Neg.	Pos.
9	n-Butyl glycidyl ether	2426-08-6	Liquid	130.19	TCI	OAJGM-RF	Sensitizer (weak)	Neg.	Pos.
10	Isopropanol	67-63-0	Liquid	60.10	FFWK	ESN2258	Non-sensitizer	Neg.	Neg.
11	Dimethyl isophthalate	1459-93-4	Solid	194.19	FFWK	SKN5952	Non-sensitizer	Neg.	Neg.
12	Propyl paraben	94-13-3	Solid	180.20	FFWK	CAE0383	Non-sensitizer	Neg.	Neg.
P.C.	Phenylacetaldehyde	122-78-1	Liquid	120.15	ALDRICH	SHBL4340	Sensitizer (moderate)	Pos.	Pos.
	Squaric acid diethylester	5231-87-8	Liquid	170.16	ALDRICH	BCBZ8850	Sensitizer (strong)	Pos.	Pos.

No.1–5 and No.10–11 are proficiency substances.

No.6–9 and No.10–11 are chemicals, which were judged as “false negative” by ADRA (1 mM) and as “positive” by ADRA (4 mM)

P.C.: Positive control, Pos.: Positive, Neg.: Negative

[1] The *in vivo* potency is referred from published data. (Basketter et al., 1992, ECETOC, 2003, Natsch et al., 2013)

[2] ADRA predictions are based on published data. (Imamura et al., 2021)

Abbreviations; ALDRICH, Sigma-Aldrich Corporation, St Louis, MO, USA; FFWK, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; Fluorochem, Fluorochem Ltd., Hadfield, UK ; TCI, Tokyo Chemical Industry Co Ltd., Tokyo, Japan

5. Acquisition, Coding, and Distribution

5-1. Acquisition, coding, and distribution of chemicals

The evaluation of within- and between-laboratory reproducibility and predictive capacity was made using the results of tests conducted at the participating laboratories using coded chemicals. The coding was supervised by JaCVAM. The JaCVAM was responsible for coding and distributing all test chemicals, reference chemicals, and control chemicals used in testing for the ring study.

5-2. Handling

JaCVAM provided the chemical master (defined as “the person responsible for the handling of the chemicals”) at each participating laboratory with essential information about the test chemicals, including physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions. This person was responsible for storing each chemical in accordance with the storage instructions and separately received sealed safety information, including the Material Safety Data Sheets (MSDS), which specified hazard identification, exposure control, and personal protection for each chemical. The test chemicals were delivered directly to these persons. The MSDS were to be accessed only in the event of a laboratory accident, and the information disclosed only to those who needed to know. No such accidents occurred during the course of the ring study, and upon completion of the study, all residual test chemicals were disposed of in compliance with the rules and regulations of the participating laboratories, and all MSDS were returned to JaCVAM still in their sealed envelopes.

6. Data management

Data collection and analysis for the ring study were performed by biostatisticians and the quality assurance group. These independent biostatisticians collected and organized data using custom data collection software, and all records were checked by the quality assurance group. Assays and quality assurance were performed in according with good laboratory practice (GLP) (Balls *et al*., 1995); however, all the participating laboratories did not routinely practice GLP. The participating laboratories were deemed to have conducted the experiments in accordance with the Study Plan and the standard operating procedure (SOP) provided by the RST. All raw data and data analysis sheets were pre-checked for quality by each laboratory and then were reviewed by the RST quality assurance team. The raw and collated data accurately reflected the test results.

7. Statistical Analysis of Test Data

7-1. Data analysis

Only test results that met the prescribed test acceptance criteria were included in the statistical analysis although records of all test results, including those that were affected by human error or were otherwise problematic, were provided.

Reproducibility of the test method was evaluated by calculating within- and between-laboratory reproducibility. Testing for the ring study was conducted at 5 participating laboratories. Between-laboratory reproducibility is normally calculated using results from 3 participating laboratories, so, on the advice of biostatisticians, the RST evaluated the results from 5 laboratories using the following method.

Calculation of between-laboratory reproducibility

Step 1. Reproducibility was calculated for each facility using test results from only 3 of the 4 participating laboratories, and between-laboratory reproducibility was calculated in this manner for all the 5 participating laboratories.

Step 2. An average value was calculated using the between-laboratory reproducibility of each of the 5 participating laboratories as determined in Step 1 (immediately above).

Table 2. Example of predictions made at the 5 participating laboratories

	Lab. A	Lab. B	Lab. C	Lab. D	Lab. E
Chemical 1					
Chemical 2					
Chemical 3					
Chemical 4					
Chemical 5					

Please note: Table 1 is an example demonstrating how data was presented for evaluation. It does not represent data collected during the ADRA ring study.

The pink cells indicate that the test chemical was predicted to be a sensitizer, the blue cells indicate the test chemical was predicted to be a non-sensitizer.

Illustrative examples of predictions for the 5 test chemicals obtained from testing at 5 participating laboratories are shown in Table 1.

Reproducibility (concordance) using results from just three of the five participating laboratories is calculated using the following method.

Reproducibility (concordance) using results from just three of the five participating laboratories is calculated by the following method.

Initially, Lab A and B are left out and concordance was calculated only for Labs C, D, and E. Since all three labs showed reproducible results for 2 (Chemicals 1 and 2) of the 5 test chemicals, the reproducibility was 2/5 × 100% or 40%. Similarly, when Lab A and C were excluded, the reproducibility was 3/5 × 100% or 60%. When Lab A and D were excluded, the reproducibility was 3/5 × 100% or 60%. When Lab A and E were excluded, the reproducibility was 3/5 × 100% or 60%. When Lab B and C were excluded, the reproducibility was 1/5 × 100% or 20%. When Lab B and D were excluded, the reproducibility was 2/5 × 100% or 40%. When Lab B and E were excluded, the reproducibility was 1/5 × 100% or 20%. When Lab C and D were excluded, the reproducibility was 2/5 × 100% or 40%. When Lab C and E were excluded, the reproducibility was 1/5 × 100% or 20%. When Lab D and E were excluded, the reproducibility was 2/5 × 100% or 40%. Using these 10 figures, the average reproducibility across all 4 participating laboratories was (40 + 60 + 60 + 60+ 20 + 40 + 20+ 40 + 20 + 40)/10 × 100% or 40%. Thus, the between-laboratory reproducibility based on the results from the 5 participating laboratories was 40%.

Calculations of reproducibility in this report are calculated on the basis of the results from sets of 5 participating laboratories as described above.

7-2. Evaluation criteria

In order to evaluate the whether the objectives of this ring study had been met, it was necessary to define in advance the minimum performance criteria to be used to evaluate the performance of the test method.

In doing this, the RST considered:

1. The background and objectives of the ring of the test method
2. The expected performance of a test method proposed for regulatory acceptance as a scientifically validated alternative to animal testing in this context
3. The utility of the test method (i.e., to be used in combination with other alternative test methods.)

On the basis of the above considerations, the RST set the target minimum performance standards for both within- and between-laboratory reproducibility for the 8 proficiency substances at 85%. For 4 chemicals, false negative at 1 mM and positive at 4 mM, the criteria was set that 2 of the 4 chemicals are judged to be positive at the all participating laboratories.

Within-/Between-Laboratory Reproducibility

Tests of 8 proficiency substances for within-/between-laboratory reproducibility were performed using the SOP. Overall results are described in Tables 3 and 4.

Table 3. Depletion of 8 proficiency substances at the 5 laboratories using UV detection

No.	Test chemical	LLNA potency	Depletion (%)	Lab.A LION						Lab.B MITSUI						Lab.C SUMITOMO						Lab.D NISSAN						Lab.E FUJI Film					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
1	**Diphenyl cyclopropenone**	Extreme	Each value	65.2	2.1	70.7	4.0	68.6	5.4	68.8	3.7	69.3	2.7	64.7	6.0	70.6	4.2	75.5	5.7	73.5	7.0	68.7	5.8	71.7	5.4	72.9	6.3	69.0	7.3	69.3	5.5	68.8	6.5
			Mean	33.6		37.4		37.0		36.3		36.0		35.3		37.4		40.6		40.2		37.3		38.6		39.6		38.2		37.4		37.6	
2	**2-Methyl-2H-isothiazol-3-one**	Moderate	Each value	100	0.0	100	0.0	100	0.0	100	0.6	100	0.0	100	0.1	100	0.0	100	4.8	100	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100	0.2
			Mean	50.0		50.0		50.0		50.3		50.0		50.1		50.0		52.4		50.0		50.0		50.0		50.0		50.0		50.0		50.1	
3	**Palmitoyl Chloride**	Moderate	Each value	8.4	82.1	7.1	87.3	8.6	82.3	7.9	89.4	10.2	94.8	9.4	100	11.0	100	20.0	100	36.6	100	10.6	100	17.7	100	19.1	100	2.7	100	7.5	100	5.6	97.9
			Mean	45.3		47.2		45.4		48.7		52.5		54.7		55.5		60.0		68.3		55.3		58.8		59.6		51.4		53.8		51.8	
4	**Imidazolidinyl urea**	Weak	Each value	58.6	3.6	58.9	3.6	60.2	6.8	64.7	4.0	65.0	7.0	58.4	8.0	61.2	4.7	65.3	11.2	61.2	6.8	59.2	2.4	61.6	3.2	60.3	3.6	61.6	6.3	62.2	7.3	61.3	8.4
			Mean	31.1		31.3		33.5		34.3		36.0		33.2		33.0		38.3		34.0		30.8		32.4		31.9		34.0		34.7		34.8	
5	**Farnesal**	Weak	Each value	86.0	16.7	85.1	21.6	81.6	23.8	89.6	11.3	94.2	11.4	92.2	18.8	97.1	14.6	100	24.2	100	30.2	33.7	12.9	64.4	16.9	30.0	17.2	81.8	12.9	83.6	14.5	89.9	15.9
			Mean	51.3		53.3		52.7		50.4		52.8		55.5		55.8		62.1		65.1		23.3		40.6		23.6		47.4		49.0		52.9	
6	**Isopropanol**	Non-sensitizer	Each value	0.0	0.0	0.4	1.2	0.2	0.2	0.4	0.5	2.5	0.1	0.0	1.1	0.7	0.0	2.9	3.5	0.0	0.3	0.0	0.0	0.2	0.0	0.0	0.0	0.7	0.0	0.0	0.4	0.0	0.4
			Mean	0.0		0.8		0.2		0.5		1.3		0.6		0.3		3.2		0.2		0.0		0.1		0.0		0.4		0.2		0.2	
7	**Dimethyl isophthalate**	Non-sensitizer	Each value	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.2	0.0	1.6	0.0	0.0	1.4	0.4	2.2	1.5	0.7	0.5	1.2	0.0	0.0	0.0	0.0	0.4	0.1	0.1	0.1	0.0	0.0	0.0
			Mean	0.0		0.0		0.3		0.1		0.8		0.0		0.9		1.8		0.6		0.6		0.0		0.2		0.1		0.1		0.0	
8	**Propyl paraben**	Non-sensitizer	Each value	0.0	0.1	0.2	0.4	0.0	0.7	0.0	0.3	0.0	1.5	0.0	0.0	1.5	0.3	2.1	1.7	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.3	0.0	0.0	0.2
			Mean	0.0		0.3		0.4		0.2		0.8		0.0		0.9		1.9		0.7		0.0		0.0		0.1		0.1		0.2		0.1	

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd. NISSAN: Nissan Chemical Corp.
FUJIFILM: Fujifilm Corp. ■Positive, ■Negative, ■Co-elution was observed. NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine

Table 4. Depletion of 8 proficiency substances at the 5 laboratories using fluorescence (FL) detection

No.	Test chemical	LLNA potency	Depletion (%)	Lab.A LION						Lab.B MITSUI						Lab.C SUMITOMO						Lab.D NISSAN						Lab.E FUJI Film					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
1	**Diphenyl cyclopropenone**	Extreme	Each value	64.1	2.6	70.0	3.5	68.1	5.2	69.9	4.1	69.6	3.3	65.9	6.5	70.5	4.2	75.2	5.1	73.5	8.0	69.5	5.5	72.1	6.1	72.5	6.4	68.2	7.4	68.8	5.2	68.4	6.3
			Mean	33.3		36.7		36.7		37.0		36.5		36.2		37.4		40.2		40.8		37.5		39.1		39.5		37.8		37.0		37.4	
2	**2-Methyl-2H-isothiazol-3-one**	Moderate	Each value	99.2	0.1	99.2	0.1	99.3	0.3	100	1.2	100	0.3	100	1.4	99.2	0.1	99.2	4.5	99.1	0.9	99.3	0.0	99.2	0.0	99.2	0.0	100	0.0	99.5	0.6	99.6	0.9
			Mean	49.6		49.7		49.8		50.6		50.1		50.7		49.7		51.9		50.0		49.7		49.6		49.6		50.0		50.0		50.3	
3	**Palmitoyl Chloride**	Moderate	Each value	8.7	81.9	7.5	86.9	8.7	82.5	8.7	89.5	10.9	94.6	9.6	99.3	11.0	100	20.6	100	37.1	100	11.4	100	16.1	99.9	19.1	100	2.8	100	7.6	99.3	5.3	97.9
			Mean	45.3		47.2		45.6		49.1		52.7		54.4		55.5		60.3		68.5		55.7		58.0		59.5		51.4		53.5		51.6	
4	**Imidazolidinyl urea**	Weak	Each value	57.9	2.8	58.3	1.8	59.1	5.6	66.1	2.0	65.3	4.0	59.1	3.9	61.4	3.7	65.4	10.7	61.5	6.2	59.3	1.4	60.6	2.6	60.0	2.4	60.9	4.6	62.4	5.4	60.8	5.5
			Mean	30.3		30.1		32.4		34.0		34.7		31.5		32.5		38.1		33.8		30.3		31.6		31.2		32.7		33.9		33.2	
5	**Farnesal**	Weak	Each value	97.9	17.7	91.9	19.9	94.8	22.9	95.7	11.8	97.9	12.4	95.5	21.0	96.6	16.3	98.9	25.3	100	30.3	83.5	19.1	83.7	18.0	81.1	17.9	80.7	13.5	82.9	13.3	88.1	14.9
			Mean	57.8		55.9		58.9		53.8		55.1		58.3		56.5		62.1		65.1		51.3		50.8		49.5		47.1		48.1		51.5	
6	**Isopropanol**	Non-sensitizer	Each value	0.0	0.1	0.6	1.4	0.0	0.3	1.5	1.0	3.6	0.0	0.8	2.3	0.7	0.0	2.8	3.3	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.3	0.0	0.4
			Mean	0.1		1.0		0.2		1.2		1.8		1.5		0.4		3.0		0.3		0.0		0.0		0.0		0.2		0.2		0.2	
7	**Dimethyl isophthalate**	Non-sensitizer	Each value	0.0	0.3	0.1	0.0	0.0	0.3	0.0	0.1	0.1	1.3	0.3	1.3	1.5	0.3	1.8	1.3	0.7	0.5	1.1	0.0	3.2	0.0	0.0	0.7	0.0	0.1	0.0	0.0	0.0	0.0
			Mean	0.2		0.0		0.2		0.0		0.7		0.8		0.9		1.6		0.6		0.5		1.6		0.4		0.1		0.0		0.0	
8	**Propyl paraben**	Non-sensitizer	Each value	0.0	0.4	0.5	0.2	0.0	0.7	0.5	0.1	0.0	1.3	0.6	0.7	1.7	0.2	2.3	1.2	1.4	0.7	0.0	0.0	0.0	0.1	0.0	0.4	0.0	0.0	0.3	0.0	0.0	0.2
			Mean	0.2		0.3		0.4		0.3		0.7		0.6		0.9		1.8		1.1		0.0		0.1		0.2		0.0		0.1		0.1	

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd. NISSAN: Nissan Chemical Corp.
FUJIFILM: Fujifilm Corp. ■Positive, ■Negative NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine

1. Solvent selection

Solvents for the 8 test chemicals used for within-/between-laboratory reproducibility were selected at each participating laboratory in accordance with the SOP. Prediction of the sensitization potential for all test chemicals was concordant at all 5 participating laboratories. Different solvents were selected for palmitoyl chloride at the 5 participating laboratories (Table 5). For this test chemical, the 3 participating laboratories—LION, MITSUI, and FUJIFILM—used acetonitrile and the two participating laboratories—SUMITOMO and NISSAN— used acetone as the solvent. Since palmitoyl chloride was predicted as a sensitizer at all participating laboratories, differences in solvent had no effect on the prediction of sensitization potential for this test chemical. On the other hand, NAC depletion was slightly different between the acetonitrile and acetone solvents. NAC yields higher depletion values in acetone solution but lower depletion values in acetonitrile solution. The reason for this difference of depletion is explained in Section 3-2 “Reproducibility in the test chemicals” below.

Table 5. Test chemicals and solvents at the 5 participating laboratories

No.	Test chemicals	Lab.A LION			Lab.B MITSUI			Lab.C SUMITOMO			Lab.D NISSAN			Lab.E FUJIFilm		
		Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
1	Diphenylcyclopropenone	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
2	2-Methyl-2H-isothiazol-3-one	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
3	Palmitoyl Chloride	AN	AN	AN	AN	AN	AN	AT	AT	AT	AT	AT	AT	AN	AN	AN
4	Imidazolidinyl urea	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
5	Farnesal	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
6	Isopropanol	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
7	Dimethyl isophthalate	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
8	Propyl paraben	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd.
NISSAN: Nissan Chemical Corp. FUJIFILM: Fujifilm Corp.
AN: Acetonitrile W: Water AT: Acetone

2. Co-elution

ADRA uses NAC and NAL, which are measured at a longer wavelength (λ_{max}) and a higher molar absorption coefficient (ε) than the peptides used in DPRA. Therefore, no measurable coelution has been observed in the results obtained using ADRA results thus far. However, coelution of farnesal and NAC was observed in LION and NISSAN. These coelutions were observed in all 3 replicate tests and not at the other 3 participating laboratories. Therefore, it is considered that this test chemical may coelute depending on the type and setting of the HPLC. On the other hand, no co-elution was observed using the FL detection method.

3. Within-Laboratory Reproducibility

3-1. Reproducibility (concordance in prediction)

Five sensitizers and 3 non-sensitizers were tested 3 times each, yielding results that were 100% concordant in prediction at all laboratories. The reproducibility was 100% for both UV and FL detection at all laboratories (Tables 5 and 6).

Table 6. Within-laboratory reproducibility of 8 proficiency substances at 5 laboratories using UV detection

Laboratory				
A	B	C	D	E
8/8 (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)

Table 7. Within-laboratory reproducibility of 8 proficiency substances at 5 laboratories by FL detection

Laboratory				
A	B	C	D	E
8/8 (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)

3-2. Reproducibility in the test chemicals

The results showed good overall reproducibility, and variability in the NAC depletion across the 3 replicate tests was ≤5% for all 8 test chemicals, except palmitoyl chloride in SUMITOMO and farnesal in NISSAN. For this chemical, SUMITOMO and NISSAN use acetone as a solvent, and a large variability was observed in the NAC depletion at these 2 laboratories compared to that at the other 3 laboratories using acetonitrile. Palmitoyl chloride is an acid chloride, which is hydrolyzed to a non-reactive palmitic acid. Therefore, it is considered that there is competition between reaction NAC and NAL and hydrolysis in the reaction solution containing a large amount of water. Thus, it is considered that there is a variability of hydrolysis for palmitoyl chloride when acetone was used as the solvent, and the reactivity of NAC also varied. On the other hand, it is considered that since the reactivity with NAL is very high, most of NAL reacts with palmitoyl chloride in a short time, and the reaction was not affected by hydrolysis. The variability in depletion for farnesal in NISSAN was observed by UV detection only. This is because the accurate NAC depletion could not be calculated because of the coelution of the test chemical and NAC. Therefore, no variation in NAC depletion was observed in the fluorescence measurement without coelution.

Similarly, variability in NAL depletion across the 3 replicate tests that met test acceptance criteria was 8% or less for all 8 test chemicals in all laboratories.

3-3. Conclusion

Within-laboratory reproducibility was 100% (8/8) at all the participating laboratories. All results met the 85% minimum target value established by the RST, indicating excellent within-laboratory reproducibility for ADRA (4 mM).

4. Between-Laboratory Reproducibility

4-1. Between-laboratory reproducibility (concordance in prediction)

Between-laboratory reproducibility was evaluated on the basis of the test results from 8 coded test chemicals. Final predictions for these test chemicals were determined by majority of the 3 independent test results.

Prediction of the sensitization potential for all test chemicals was concordant at all 5 participating laboratories. According to the formula below, the between-laboratory reproducibility was calculated to be 100% using both UV and FL detection, which exceeded the minimum target value of 85% set by the RST.

(1) BLR by HPLC-UV

$$8/8 \times 100 = 100\ (\%)$$

(2) BLR by HPLC-FL

$$8/8 \times 100 = 100\ (\%)$$

4-2. Reproducibility in the test chemicals

The results showed good overall reproducibility, and variability in the NAC depletion across the tests at 5 participating laboratory was ≤5% for all 8 test chemicals, except palmitoyl chloride and farnesal. The variability of depletion for palmitoyl chloride is considered to be due to the difference in the solvent used as described in Section 3-2 “Reproducibility in the test chemicals”. In addition to the coelution at NISSAN, farnesal is an aldehyde compound, which makes it particularly sensitive to variations in the testing conditions, which could explain the variability in depletion.

Similarly, variability in NAL depletion across the tests at the 5 participating laboratories was ≤5% for all 8 test chemicals, except palmitoyl chloride and farnesal. The reason of variability in NAL depletion for these two test chemicals is same as that for NAC mentioned above.

4-3 . Conclusion

The concordant predictions of the skin sensitization potential of the 8 test chemicals by the 5 participating laboratories are a measure of the between-laboratory reproducibility. Between-laboratory reproducibility, indicating the concordance of sensitizers and non-sensitizers, was 100%, which shows high reproducibility similar to that of ADRA (1 mM).

The RST confirmed that the results satisfied the 85% minimum target value for between-laboratory

reproducibility specified in the ADRA (4 mM) study plan. Thus, since the between-laboratory reproducibility was more than 90%, ADRA (4 mM), like ADRA (1 mM), is suitable as an alternative method to animal testing in combination with other non-animal test methods.

5. Reproducibility between ultraviolet and fluorescence detection

Prediction of the sensitization potential for all test chemicals was concordant between UV and FL detection at all 5 participating laboratories. Variability in the NAC depletion between UV and FL detection was ≤7% for all 8 test chemicals except coeluted result for farnesal. Similarly, variability in NAL depletion between UV and FL detection was ≤7% for all 8 test chemicals.

6. Explanation of retesting

There were disparate instances of retesting at the 4 of 5 participating laboratories whenever 1 of the 3 replicate tests of the 12 test chemicals (8 proficiency substances and 4 substances, which are judged as false negatives in ADRA (1 mM)) failed to satisfy the test acceptance criteria. The reasons for retesting are summarized below in Table 8. The cells highlighted in pink indicate instances in which the test acceptance criterion shown on the left was not satisfied (6 instances in total), and the cells highlighted in blue indicate instances by other reason (the test acceptance criteria were satisfied but the value for Reference Control C was so low that the apparent depletion was less than -10% (1 instance) or human error of test operation (1 instance)).

Retesting was performed on one occasion at MITSUI because the NAC sample was mistaken for palmitoyl chloride because of human error.

One retest was performed for two reasons at SUMITOMO. The first is that the SD of NAC depletion for dimethyl isophthalate was 43.5% by UV detection and 43.4% by FL detection, which falls outside the acceptance criteria of <10%. The second is that the CV of the peak area for NAL of Reference Control C in acetone solution was 10.8% by UV detection and 11.5% by FL detection, which falls outside the acceptance criteria of <10%.

Retesting was performed once for 3 reasons at NISSAN. The first is that the SD of NAC depletion for palmitoyl chloride was 46.0% by UV detection and 45.1% by FL detection, which falls outside the acceptance criteria of <10%. The second and third is that the SD of NAL depletion for palmitoyl chloride was 50.8% by UV detection and 49.2% by FL detection, which falls outside the acceptance criteria of <10%. The third is that NAC depletion of dimethyl isophthalate and propyl paraben were all less than -10%.

Retesting was performed on one occasion at FUJIFilm because the SD of NAC depletion for m-aminophenol was 12.7% by both UV and FL detection, which falls outside the acceptance criteria of <10%.

Table 8. Reasons for retesting at each participating laboratory

Reason of retest (acceptance criteria)					Lab.A LION			Lab.B MITSUI			Lab.C SUMITOMO			Lab.D NISSAN			Lab.E FUJIFilm		
					Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Calibration curve		NAC	Calibration linearity	> 0.990															
		NAL																	
Reference controls	A	NAC	Concentration	3.2 - 4.4μM															
		NAL																	
	B	NAC	Peak area	CV < 10%															
		NAL																	
	C Acetonitrile	NAC	Concentration	3.2 - 4.4μM															
			Peak area	CV < 10%															
		NAL	Concentration	3.2 - 4.4μM								not met							
			Peak area	CV < 10%															
	C Others	NAC	Concentration	3.2 - 4.4μM															
			Peak area	CV < 10%															
		NAL	Concentration	3.2 - 4.4μM															
			Peak area	CV < 10%															
Positive control		NAC	depletion	SD < 10%															
		NAL	depletion	SD < 10%															
Test chemicals		NAC	depletion	SD < 10%								not met (1 chem)		not met (2 chem)			not met (1 chem)		
		NAL	depletion	SD < 10%										not met (1 chem)					
Others										error in operation				NAC depletion <-10% (2 chem)					

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd.
NISSAN: Nissan Chemical Corp. FUJIFILM: Fujifilm Corp.

NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine, SD: Standard deviation

Predictive Capacity

1. Chemicals with different results at 1 mM and 4 mM

In this study, 4 test chemicals with different results at 1 and 4 mM were selected to confirm the predictive capacity for chemicals that indicate depletion close to the criteria. The results for these test chemicals are described in Tables 9 and 10.

The target accuracy for the 4 test chemicals is 50% at each participating laboratory. Final predictions for these test chemicals were determined by majority of the 3 independent test results. All laboratories provided 4/4 × 100 = 100 (%) accuracy for both UV and fluorescence detection, which exceeded the minimum target value of 50%.

SUMITOMO provided the inconsistent result for m-aminophenol (i.e., 2 positive results and 1 negative result). Since the chemicals that exhibit depletion near the criteria were originally selected, this inconsistent result is in the range of possible varieties.

2. Predictive capacity for published data of 136 chemicals

Results published studies indicate that ADRA (4 mM) identified sensitizers and non-sensitizers with an accuracy of 76% (104/136), a sensitivity of 76% (74/98), and a specificity of 79% (30/38) relative to LLNA results (Imamura *et al*., 2021). Compared with ADRA with 1 mM test chemical solutions, testing with ADRA (4 mM) showed an 11 percentage point increase in sensitivity (64/98 vs 74/98) and a 4 percentage point increase in accuracy (98/136 vs 104/136) but a 10 percentage point decrease in specificity (34/38 vs 30/38). It should be noted that this data set contains chemicals outside the applicability domain.

Table 9. Depletion of 4 test chemicals with different judgments at 1 and 4 mM at the 5 laboratories using UV detection

No.	Test chemical	LLNA potency	Depletion (%)	Lab.A LION						Lab.B MITSUI						Lab.C SUMITOMO						Lab.D NISSAN						Lab.E FUJI Film					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
1	**m-Aminophenol**	Moderate	Each value	32.1	0.0	33.8	0.0	30.8	0.0	24.3	0.1	27.1	0.0	22.3	0.0	14.8	0.8	2.9	2.1	10.8	0.1	24.9	0.0	28.6	0.0	32.5	0.0	12.8	0.0	18.9	0.5	23.9	0.5
			Mean	16.1		16.9		15.4		12.2		13.5		11.2		7.8		2.5		5.5		12.4		14.3		16.2		6.4		9.7		12.2	
2	**3-Propylidene phthalide**	Moderate	Each value	34.1	50.4	42.0	59.2	42.1	52.9	35.5	64.2	24.5	72.5	23.0	72.4	31.2	69.2	20.4	80.5	26.1	71.8	13.2	52.8	21.9	56.4	25.5	62.0	16.8	59.9	23.3	67.5	18.1	58.8
			Mean	42.3		50.6		47.5		49.9		48.5		47.7		50.2		50.5		49.0		33.0		39.1		43.8		38.3		45.4		38.4	
3	**Ethyleneglycol dimethacrylate**	Weak	Each value	16.6	1.0	21.0	0.3	17.6	2.4	21.6	1.0	18.2	1.5	16.5	0.3	20.3	1.5	18.7	3.8	29.1	3.0	14.0	0.2	20.1	1.3	19.9	1.5	19.0	1.6	19.3	1.2	19.4	0.7
			Mean	8.8		10.7		10.0		11.3		9.9		8.4		10.9		11.2		16.1		7.1		10.7		10.7		10.3		10.2		10.1	
4	**n-Butyl glycidyl ether**	Weak	Each value	24.6	1.2	23.2	0.0	25.7	2.1	32.3	3.2	30.7	1.6	28.0	4.8	23.6	1.0	27.2	4.8	37.3	3.8	26.2	0.1	31.9	0.7	33.7	0.0	21.0	2.5	26.0	2.9	25.7	3.1
			Mean	12.9		11.6		13.9		17.7		16.1		16.4		12.3		16.0		20.6		13.2		16.3		16.8		11.7		14.4		14.4	

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd. NISSAN: Nissan Chemical Corp.
FUJIFILM: Fujifilm Corp. ■Positive, ■Negative

NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine

Table 10. Depletion of 4 test chemicals with different results at 1 and 4 mM at the 5 laboratories using FL detection

No.	Test chemical	LLNA potency	Depletion (%)	Lab.A LION						Lab.B MITSUI						Lab.C SUMITOMO						Lab.D NISSAN						Lab.E FUJI Film					
				Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3		Set1		Set2		Set3	
				NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL	NAC	NAL
1	**m-Aminophenol**	Moderate	Each value	31.5	0.4	32.9	0.0	30.7	0.3	24.0	1.8	26.4	0.3	23.2	1.8	14.1	0.6	2.9	1.6	11.2	0.2	24.6	0.0	29.5	0.0	32.8	0.0	12.6	0.1	18.6	0.6	23.8	1.0
			Mean	15.9		16.5		15.5		12.9		13.4		12.5		7.4		2.2		5.7		12.3		14.7		16.4		6.4		9.6		12.4	
2	**3-Propylidene phthalide**	Moderate	Each value	33.6	50.8	41.5	59.4	41.7	53.3	33.2	64.1	25.3	72.6	24.4	73.4	31.4	70.0	20.2	81.2	25.9	71.7	17.7	52.9	22.9	56.2	26.9	61.8	16.6	60.1	23.9	66.7	18.4	58.0
			Mean	42.2		50.4		47.5		48.7		48.9		48.9		50.7		50.7		48.8		35.3		39.6		44.4		38.4		45.3		38.2	
3	**Ethyleneglycol dimethacrylate**	Weak	Each value	15.7	1.6	20.5	0.3	17.6	2.5	23.2	1.4	19.1	2.1	18.3	2.1	20.7	1.5	18.8	3.5	29.7	2.7	13.9	0.6	20.8	2.9	20.4	3.2	18.9	4.2	19.5	1.3	19.0	0.8
			Mean	8.6		10.4		10.0		12.3		10.6		10.2		11.1		11.2		16.2		7.2		11.8		11.8		11.6		10.4		9.9	
4	**n-Butyl glycidyl ether**	Weak	Each value	24.0	1.3	23.2	0.3	25.7	2.5	33.3	3.0	31.0	2.8	29.5	4.5	23.7	1.3	26.9	5.6	37.2	4.0	26.4	0.0	31.6	1.1	34.0	0.0	20.3	2.4	25.7	3.0	25.4	3.2
			Mean	12.7		11.7		14.1		18.2		16.9		17.0		12.5		16.3		20.6		13.2		16.4		17.0		11.4		14.4		14.3	

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd. NISSAN: Nissan Chemical Corp.
FUJIFILM: Fujifilm Corp. ■Positive, ■Negative

NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine

Positive control (results and establishing the control range)

1. Reproducibility of positive control

In this ring study, 2 kinds of positive controls were used, namely, phenylacetaldehyde and squaric acid diethylester. These positive controls were tested 3 times at each participating laboratory.

NAC depletion was 25.1–70.6% for phenylacetaldehyde and 18.9–65.6% for squaric acid diethylester, both of which showed high variability (Tables 11 and 12). The minimum depletion was observed at SUMITOMO for both phenylacetaldehyde (25.1%) and squaric acid diethylester (18.9%). These depletion values are less than half of the mean depletion (27.7% for phenylacetaldehyde and 25.9% for squaric acid diethylester) and less than mean-3SD, excluding this minimum depletion (31.1% for phenylacetaldehyde and 33.7% for squaric acid diethylester). Although not clear from these results, it is possible that the reactivity of the positive control to NAC was underestimated in the first SUMITOMO.

On the other hand, NAL depletion was 80.8–100% for phenylacetaldehyde and 99.5–100% for squaric acid diethylester, both of which showed good reproducibility.

2. Establishing the control range

Data on depletion of the positive control reagent from each of the participating laboratories was used to calculate the mean ± 3 standard deviation (SD), which was used as a control criterion. Since phenylacetaldehyde and squaric acid diethylester have moderate levels of reactivity and can easily exhibit variability in reactivity with even a slight change in the reaction conditions can cause variability in depletion, the mean ± 3SD for each participating laboratory was used as a control criterion. As mentioned above, however, since NAC depletion in the first set at SUMITOMO may be underestimated for some reason, the mean ± 3SD was calculated from the data excluding this. Thus, the control range for positive control reagents at 4 mM was established as follows:

- **NAC depletion**
  Phenylacetaldehyde: 30–80%; Squaric acid diethyl ester: 30–80%
- **NAL depletion**
  Phenylacetaldehyde: 70–100%; Squaric acid diethyl ester: 70–100%

Table 11. Depletion of 2 positive controls at 5 laboratories using UV detection

No.	Positive control	Depletion (%)	Lab.A LION			Lab.B MITSUI			Lab.C SUMITOMO			Lab.D NISSAN			Lab.E FUJIFilm		
			Set1	Set2	Set3	Set1	Set2	Set3	Set1	Set2	Set3	Set1	Set2	Set3	Set1	Set2	Set3
1	Phenylacetaldehyde	NAC	56.4	60.5	70.6	64.4	55.7	54.4	25.1	67.6	66.4	39.6	60.6	65.8	45.5	51.1	52.0
		NAL	87.6	87.6	81.6	100	100	100	100	100	100	88.8	82.8	85.3	99.7	80.8	96.9
2	Squaric acid diethylester	NAC	52.7	50.1	50.0	52.7	42.0	44.2	18.9	54.8	65.4	41.1	45.7	44.6	51.8	52.1	50.7
		NAL	100	100	100	100	100	100	94.9	100	100	100	100	100	100	100	100

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NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine

Table 12. Depletion of 2 positive controls at 5 laboratories using FL detection

No.	Positive control	Depletion (%)	Lab.A LION			Lab.B MITSUI			Lab.C SUMITOMO			Lab.D NISSAN			Lab.E FUJIFilm		
			Set1	Set2	Set3	Set1	Set2	Set3	Set1	Set2	Set3	Set1	Set2	Set3	Set1	Set2	Set3
1	Phenylacetaldehyde	NAC	55.3	59.6	69.6	62.8	51.5	54.4	25.1	67.5	66.2	39.6	60.4	65.9	44.7	50.7	51.3
		NAL	100.0	100.0	100.0	100	100	100	100	100	100	99.8	99.8	99.7	100	100	100
2	Squaric acid diethylester	NAC	59.8	57.6	57.5	62.2	50.4	53.5	22.5	64.7	65.6	53.2	57.2	55.5	59.5	60.6	58.7
		NAL	100	100	100	100	100	100	94.8	100	100	100	100	100	100	100	100

LION: Lion Corp. MITSUI: Mitsui Chemicals, Inc. SUMITOMO: Sumitomo Chemical Co. Ltd. NISSAN: Nissan Chemical Corp. FUJIFILM: Fujifilm Corp.

NAC: *N*-(2-(1-naphthyl)acetyl)-l-cysteine, NAL: α-*N*-(2-(1-naphthyl)acetyl)-l-lysine

RST overall conclusions and recommendations

1. Overall conclusions

The object of this ring study was to assess the within-laboratory reproducibility and between-laboratory reproducibility of ADRA (4 mM) using coded test chemicals and to determine whether ADRA (4 mM) can accurately identify 4 chemicals with different results at 1 and 4 mM concentration of test chemical solution. Thus, the results of the RST can provide sufficient evidence for and against ADRA (4 mM) and deem it as a scientifically validated and appropriate method to be included in the OECD TG.

The RST has concluded that the results of this ring study show that ADRA (4 mM) satisfies the requirements of Modules 1 to 4 of the ECVAM Modular Approach to Validation—namely, for test definition, within-laboratory reproducibility, between laboratory reproducibility, and transferability. Additionally, based on these results and published data, the RST considers that requirements of Module 5, predictive capacity, and Module 6, applicability domain, have also been satisfied when assessed against other validated test methods for assessing the skin sensitization potential of chemicals.

The RST considers the results of this ring study to demonstrate conclusively that ADRA (4 mM) is a scientifically sound a highly reliable *in chemico* alternative to testing using laboratory animals.

2. Recommendations

ADRA (4 mM) is recommended for use as an alternative to existing *in vivo* tests within an integrated testing strategy that utilizes multiple alternative testing methods to conduct a comprehensive assessment of the skin sensitization potential rather than as a standalone test. ADRA (4 mM) is more suitable than ADRA (1 mM) for determining the skin sensitization potential.

List of Appendices

Appendix 1: Study Plan
Appendix 2: Standard Operating Procedure (SOP)

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