FUJIFILM	**Amino acid Derivative Reactivity Assay (ADRA) Standard Operating Procedure**	Version 2.0 Pages:31 Annexes:0

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

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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.
Ver.2.0	August 27, 2021	1) Changed the concentration of the test chemical solution from 1 mM to 4 mM and added the description about the 4 mM concentration. 2) Added a 0.5 mg/ml concentration of the test chemical solution for the unknown molecular weight. 3) Added a description about fluorescence detection in the HPLC analysis. 4) Added squaric acid diethyl ester as another positive control. 5) Changed the range of the NAC/NAL depletion for the criteria of positive control in section 8-1. 6) Added a description about the ADRA kit. 7) Updated references to the latest information. 8) Updated apparatus and reagents to the latest information in section 1. 9) Added caution about a sterilized 96 well plate in section 1-2. 10) Added caution about the quality of NAC and NAL in section 1-3.

		11) Added caution about the preparation of the reaction fixing solution in section 2-1-4 12) Added a note regarding filtration to remove the precipitate in section 5-1.

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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 or fluorescence detection with an excitation wavelength of 284 nm and an emission wavelength of 333 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 4 mM or 0.5 mg/ml (for test chemicals of unknown molecular weight) concentration.

REFERENCES

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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)
Fluorescence detector[*2]	Fluorescence detector (for example, Shimadzu RF-20A)
pH meter with electrode and calibration	Capable of reading ±0.01 pH
HPLC Column	FUJIFILM Wako Pure Chemical Corporation, Wakopak® Core C_{18} ADRA (2.6 µm, 3.0 × 150 mm) [Catalog # 233-63991] *Alternative Column* The follow three columns have been confirmed to yield results for five test chemicals that are essentially the same as the FUJIFILM Wako column. OSAKA SODA, CAPCELL CORE C_{18} column (2.7 µm, 3.0 × 150 mm) [Cat. 51112] ChromaNik, SunShell C18 (2.6 µm, 3.0 × 150 mm) [Catalog # CB6371] 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[*3]	Column: OSAKA SODA EXP GUARD CARTRIDGE CAPCELL CORE C_{18} S-2.7 2.1×5mm [Cat. 3643] Holder: OSAKA SODA 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] Although a fluorescence detector is not always needed, it is useful when co-elution is observed in UV detection.

[*3] 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	Non-sterile polypropylene U bottom 96-well Microwell Plate[*1] 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 analysis	Use the seal having sealing and solvent-resistant performance[*2] Shimadzu GLC TORAST™ 96well Seal E Type [Catalog # 370-04030-01]

[*1] Sterilized plates should be avoided because changes in antioxidants contained in polypropylene due to heat, gamma rays, etc. may affect the reaction and/or be detected as contaminated peaks in HPLC.

[*2] 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	Water sampling bottle (SANSEI MEDICAL) [Catalog # 07-004-01]
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**
ADRA kit	FUJIFILM Wako [Catalog # 296-80901]
Trifluoroacetic Acid (TFA) (MW=114.02) CASRN 76-05-1	FUJIFILM Wako [Catalog #204-02743] 98.0%

Sodium Phosphate, Monobasic, Anhydrous (NaH2PO4, MW=119.98) CASRN 7558-80-7	FUJIFILM Wako [Catalog # 197-09705]
Sodium Phosphate, Dibasic, Anhydrous (Na2HPO4, MW=141.96) CASRN 7558-79-4	FUJIFILM Wako [Catalog # 197-02865]
0.1M Sodium Hydroxide Solution CASRN 1310-73-2	FUJIFILM Wako [Catalog # 196-02195]
Acetonitrile, HPLC Grade CASRN 75-05-8	FUJIFILM Wako [Catalog # 015-08633]
Distilled water	Distilled water or equivalent may be used.
Acetone, special grade CASRN 67-64-1	FUJIFILM Wako [Catalog # 016-00346]
Dimethyl sulfoxide (DMSO), special grade CASRN 67-68-5	FUJIFILM 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) [*1] MW=289.35	FUJIFILM Wako [Catalog # 294-83001]
NAL (refrigeration storage) [*1] MW=314.38	FUJIFILM Wako [Catalog # 290-83101]
Phenyl acetaldehyde (Positive control) MW=120.15 CASRN 122-78-1	SIGMA [Catalog # 107395] Alfa Aesar [Catalog # A14263] 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.
Squaric acid diethyl ester (Positive control) MW=170.16 CASRN 5231-87-8	Tokyo Chemical Industry [Catalog # D3253] SIGMA [Catalog # 107395] (1) Store away from high temperature or humidity.

[*1] The following qualities are required for NAC and NAL

1) Purity: Both NAC and NAL should exhibit at least 98% purity.

2) Stability: Using NAC and NAL stock solution, prepare a reference control that is free of any test chemical and quantify the residual levels of both NAC and NAL immediately after preparation (0 hours) and after a 24-hour incubation. Residual levels of NAC and NAL should be at a minimum of 90% in either case. The residual level of NAC is calculated as a percentage of the sum of NAC and

the residual level of NAC dimers.

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances described in APPENDIX II of OECD TG442C and should satisfy the requirement given therein.

2. PRE-WORK

Prepare the reagents according to the attached instructions when using the ADRA kit for the test.

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 a temperature below −75°C.

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 a temperature below −75°C.

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]

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] *Since the peak shape of NAC may be bimodal when the TFA concentration in the reaction solution during HPLC measurement is low, be sure to measure the reagents accurately.*

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 a temperature below −75°C. 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 a temperature below −75°C. 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 a temperature below −75°C for later use. Typically, cryopreserve 1 ml of stock solution in a 1.5 ml tube at a temperature below −75°C.*

*[*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 -80ºC. We recommend that the NAL stock solution also be stored at a temperature below −75°C, 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 a temperature below −75°C.

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 a temperature below −75°C.

[*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 4 mM, 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 4 mM test chemical solution is prepared by 5-fold dilution of the 20 mM solution. For a solvent of DMSO, the test chemical solution is set to 80 mM. This solution should then be diluted 20-fold with acetonitrile to prepare 4 mM test chemical solution. For test chemicals of unknown molecular weight, the initial concentration of the test chemical solution is set to 10 mg/ml, and the final 0.5 mg/ml test chemical solution is prepared by a 20-fold dilution of the 10 mg/ml 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 80 mM or 10 mg/ml solution is prepared using DMSO (4), use acetonitrile to prepare the 20-fold dilution down to a 4 mM solution.

Note 2: If none of these solvents are suitable for a given test chemical in a 20 mM or 10 mg/ml solution, assess the solubility of the test chemical in 4 mM or 0.5 mg/ml solution using solvents (1)–(3). Do not use DMSO to prepare a 4 mM or 0.5 mg/ml solution. DMSO must always be diluted 20 fold with acetonitrile to prepare a 4 mM or 0.5 mg/ml 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, 25 ml of a 4 mM test chemical solution or 5 ml of an 80 mM test chemical solution. If no purity information is available, assume 100% purity.

Preparation of the 20 mM solution (5 ml) or 4 mM solution (100 ml)

$$\mathrm{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{20\ \mathrm{mM}}{1000} \times 5\ \mathrm{ml} \quad \text{or} \quad \mathrm{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{4\ \mathrm{mM}}{1000} \times 25\mathrm{ml}$$

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

Preparation of the 80 mM solution (5 ml)

$$\mathrm{MW} \times \frac{100}{\text{Purity (\%)}} \times \frac{80\ \mathrm{mM}}{1000} \times 5\ \mathrm{ml} = \mathrm{MW} \times \frac{40}{\text{Purity (\%)}} = \text{Required quantity (mg)}$$

2. Weigh the required quantity of test chemical directly into a centrifuge tube or other suitable labware and record the actual weight. For test chemicals of unknown molecular weight, weigh more than 10 mg of test chemical solution directly into a centrifuge tube or another suitable labware and record the actual weight.
3. Calculate the required quantity of solvent using the following formula, and assess the solubility for the appropriate solvent.

Preparation of the 20 mM solution

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

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

Preparation of the 4 mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{4\ \text{mmol}} \times 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 2.5 = \text{Volume of solvent (ml)}$$

Preparation of the 80 mM solution

$$\text{Test chemical (mg)} \times \frac{1}{\text{MW}} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{80\ \text{mmol}} \times 1000\ \text{ml}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{\text{MW}} \times 0.125 = \text{Volume of solvent (ml)}$$

Preparation of the 10 mg/ml solution

$$\text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{10\ \text{mg}}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{1000} = \text{Volume of solvent (ml)}$$

Preparation of the 0.5 mg/ml solution

$$\text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{100} \times \frac{1}{0.5\ \text{mg}}$$

$$= \text{Test chemical (mg)} \times \frac{\text{Purity (\%)}}{50} = \text{Volume of solvent (ml)}$$

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 using the following formula. If specific gravity of test chemical solution is not known, assume it as 1.0.

Preparation of the 20 mM solution (5 ml)

$$\frac{100}{\text{Concentration (w/w\%)}} \times \frac{1}{\text{Specific gravity (g/ml)} \times 1000 \text{ ml}} \times \text{MW} \times \frac{20 \text{ mM} \times 5 \text{ ml}}{1000} \times 1000 \text{ (µl)}$$

$$= \frac{10}{\text{Concentration (w/w\%)}} \times \frac{\text{MW}}{\text{Specific gravity (g/ml)}} = \text{Volume of test chemical (µl)}$$

$$5000 \text{ µl} - \text{Volume of test chemical (µl)} = \text{Solvent volume (µl)}$$

Preparation of the 4 mM solution (25 ml)

$$\frac{100}{\text{Concentration (w/w\%)}} \times \frac{1}{\text{Specific gravity (g/ml)} \times 1000 \text{ ml}} \times \text{MW} \times \frac{4 \text{ mM} \times 25 \text{ ml}}{1000} \times 1000 \text{ (µl)}$$

$$= \frac{10}{\text{Concentration (w/w\%)}} \times \frac{\text{MW}}{\text{Specific gravity (g/ml)}} = \text{Volume of test chemical (µl)}$$

$$25 \text{ ml} - \frac{\text{Volume of test chemical (µl)}}{1000} = \text{Solvent volume (ml)}$$

Preparation of the 10 mg/ml solution (1 ml)

$$\frac{100}{\text{Concentration (w/w\%)}} \times \frac{10 \text{ mg/ml} \times 1 \text{ ml}}{\text{Specific gravity (g/ml)} \times 1000 \text{ (mg)}} \times 1000 \text{ (µl)}$$

$$= \frac{1000}{\text{Concentration (w/w\%)}} \times \frac{1}{\text{Specific gravity (g/ml)}} = \text{Volume of test chemical (µl)}$$

$$1000 \text{ µl} - \text{Volume of test chemical (µl)} = \text{Solvent volume (µl)}$$

Preparation of the 0.5 mg/ml solution (20 ml)

$$\frac{100}{\text{Concentration (w/w\%)}} \times \frac{0.5 \text{ mg/ml} \times 20 \text{ ml}}{\text{Specific gravity (g/ml)} \times 1000 \text{ (mg)}} \times 1000 \text{ (µl)}$$

$$= \frac{1000}{\text{Concentration (w/w\%)}} \times \frac{1}{\text{Specific gravity (g/ml)}} = \text{Volume of test chemical (µl)}$$

$$1000 \text{ µl} - \text{Volume of test chemical (µl)} = \text{Solvent volume (µl)}$$

2-5. Preparation of HPLC System

Prepare an HPLC system with a 281-nm UV detector and/or a 284-nm/333 nm (Ex/Em) FL detector. Prepare mobile phases 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, 4 mM, 80 mM, 10 mg/ml, or 0.5 mg/ml 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, 4 mM, 80 mM, 10 mg/ml, or 0.5 mg/ml solution directly in a 15 ml or other appropriate tube. Then calculate the required quantity of solvent needed based on the actual weight of the test chemical. In the above operation, use the formula described in section "2-4. Solubility Assessment of Test Chemicals" to calculate the required quantities of the test chemical and of the solvent.
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, 80 mM, or 10 mg/ml of test chemical solution to the final 4 mM or 0.5mg/ml solution with each appropriate solvent in the 1.4 ml Test chemical solution Storage Tube or other appropriate tube (For example, add 200 µl of 20 mM test chemical solution to 800 µl of appropriate solvent, add 50 µl of 80 mM or 10 mg/ml test chemical solution to 950 µl of appropriate solvent).[*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) or squaric acid diethyl ester (molecular weight = 170.16) 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%.

$$\text{MW} \times \frac{10}{\text{Purity}(\%)} = 120.15 \times \frac{10}{90} = 13.35\ (\text{mg}) = \text{Phenyl acetaldehyde (mg)}$$

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 the positive control with acetonitrile in the quantities calculated above (20 mM).
5. Dilute 20 mM of positive control solution 5-fold with acetonitrile in the 1.4 ml Test chemical solution Storage Tube or other appropriate tube. (final concentration or 5 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 when using frozen stock solutions. 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 1:200 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 281 nm and/or fluoresces at 284 nm/333 nm (Ex/Em) 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 = 1:200 5 µM NAC, 1 mM test chemical	**Ratio of NAL to test chemical = 1: 200** 5 µM NAL, 1 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. When co-elution of the test chemical with NAC or NAL is observed, fluorescence detection (284 nm for excitation and 333 nm for emission) is available.
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 1: If NAC and NAL peaks are saturated due to the performance of the fluorescence detector, each solution may be diluted 2–10 fold before measurement. However, the solution composition (concentration of buffer and TFA) after dilution should be the same as that before dilution since changes of the pH in the solution may affect measurement results.

Note 2: 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	FUJIFILM Wako, Wakopak® Core C_{18} ADRA (2.6 µm, 3.0 × 150 mm) [Catalog # 233-63991] *Alternative Column* The follow three columns have been confirmed to yield results for five test chemicals that are essentially the same as the FUJIFILM Wako column. OSAKA SODA, CAPCELL CORE C_{18} column (2.7 µm, 3.0 × 150 mm) [Cat. 51112] ChromaNik, SunShell C18 (2.6 µm, 3.0 × 150 mm) [Catalog # CB6371] 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	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector (281 nm)
Fluorescence detector	Fluorescence detector (for example, Shimadzu RF-20A)
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		

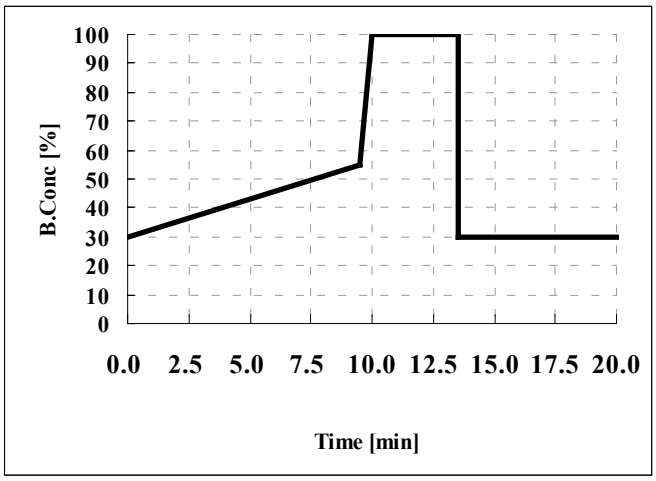
Note 1: The mixer quantity should be verified and adjusted in advance because the appropriate elution pattern of NAC/NAL peak will not be shown if the mixer quantity for mixing each mobile phase is not appropriate (For example, 0.5 ml mixing quantity is appropriate for Shimadzu prominence HPLC system).

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

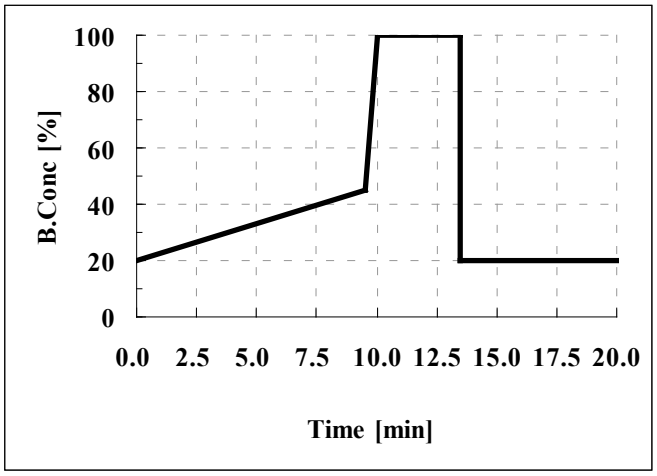
Note 3: If more than one wavelength is detected, also 291 nm besides 281 nm should be detected to check out peak purities of NAC/NAL.

Graphical Display of Gradient for NAC/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. If precipitation removal is difficult to achieve by centrifugation or recovery of the supernatant, filtration is available. In this case, it is necessary to confirm in advance that there is no change in peak area of NAC and NAL before and after filtration using a reference control or standard solution. 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[§†] 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[§†] 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[§†] 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 and/or fluorescence at Ex 284 nm/Em 333 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 and CV for the three Reference Controls C. The CV must be less than 10%.

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 for absorbance and/or Ex 284 nm/Em 333 nm for fluoresence 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 for absorbance and/or Ex 284 nm/Em 333 nm for fluoresence of Reference Control B and C replicate.
- Mean NAC/NAL peak area at 281 nm for absorbance and/or Ex 284 nm/Em 333 nm for fluoresence 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 for absorbance and/or Ex 284 nm/Em 333 nm for fluoresence 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 for absorbance and/or Ex 284 nm/Em 333 nm for fluoresence 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 for absorbance and/or Ex 284 nm/Em 333 nm for fluoresence 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 or squaric acid diethyl ester 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	30	80	70	100
Squaric acid diethyl ester	30	80	70	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.

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%.

8-2. 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%

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

If co-elution is observed in either the UV detector or fluorescence detector, the depletion measured by the detector in which co-elution is not observed can be adopted. If co-elution is observed in both detectors or if the fluorescence detector is not ready, handling of co-elution is described below.

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/or fluoresces at 284 nm/333 nm (Ex/Em) and has a similar retention time as a NAC or NAL (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
Less than 4.9%	Non-sensitizer	Inconclusive	Apply NAC-only prediction model
4.9% or higher	Sensitizer	Sensitizer	Apply NAC-only prediction model

NAC only prediction model

NAC Depletion	**Judgement**
less than 5.6%	Non-sensitizer
5.6% or higher	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.