CERI

Receipt Number	822-16-D-4124
Study Number	G21-0014

FINAL REPORT

Direct Peptide Reactivity Assay of APFHx (C-1500N)

April, 2017

Chemicals Evaluation and Research Institute, Japan, Hita

GLP STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor DAIKIN INDUSTRIES, LTD.

Title Direct Peptide Reactivity Assay of APFHx (C-1500N)

Study Number G21-0014

The study was conducted in compliance with the following GLP principles.

OECD Principles of Good Laboratory Practice, November 26, 1997, ENV/MC/CHEM (98)17

This final report accurately reflects the raw data and the test data are valid.

Study Director: April 3, 2017
Date

TABLE OF CONTENTS

1. TITLE

Direct Peptide Reactivity Assay of APFHx (C-1500N)

2. SPONSOR

Name DAIKIN INDUSTRIES, LTD.

Address 1-1, Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

3. TESTING FACILITY

Name Chemicals Evaluation and Research Institute, Japan, Hita (CERI Hita)

Address 3-822, Ishii-machi, Hita-shi, Oita 877-0061, Japan

4. OBJECTIVE

The objective of this study is to predict the skin sensitivity of the test substance by evaluation of the reactivity of test substance to cysteine peptide and lysine peptide.

5. TEST METHOD

OECD Guideline for the Testing of Chemicals, No. 442C, *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA), February 4, 2015

6. GLP PRINCIPLE

OECD Principles of Good Laboratory Practice, November 26, 1997, ENV/MC/CHEM (98)17

7. DATES

Study initiation	February 20, 2017
Experiment start	March 1, 2017
Experiment completion	March 3, 2017
Study completion	April 3, 2017

8. STUDY DIRECTOR

9. PERSONNEL CONCERNED WITH STUDY

(Preparation of peptide standard solution, reference control, positive control solution, test substance solution and reaction solution)

(Analysis of peptide standard solution, reference control and reaction solution)

10. RETENTION OF TEST SUBSTANCE, RAW DATA, ETC.

The original study plan, original final report, raw data, study contract documents, test substance information and other record documents will be retained in the testing facility. The remaining test substance will be returned to the sponsor. The retention period is 10 years after the completion of the study. After the termination of the retention period, any measures (continuous storage, disposal or return) will be done with the approval of the sponsor.

11. APPROVAL OF FINAL REPORT

Study Director: April 3, 2017

Date

12. SUMMARY

The study was performed according to OECD Guideline for the Testing of Chemicals, No. 442C to predict the skin sensitivity of APFHx (C-1500N).

The test substance dissolved in acetonitrile was mixed with cysteine peptide solution or lysine peptide solution and incubated at 25°C for 24 hours and more. The reaction solution was analyzed by high performance liquid chromatography and peak area for each peptide was determined. Percent cysteine peptide depletion and percent lysine peptide depletion, and the mean value of the percent cysteine and percent lysine depletion were calculated.

Consequently, the percent peptide depletions were 1.3% for the cysteine peptide and 100.0% for the lysine peptide. The mean value of the percent cysteine and lysine depletion was 50.7%. Therefore, the reactivity class of the test substance was classified to "High reactivity", and the skin sensitivity was predicted as "Positive" in this testing condition.

13. MATERIALS

13.1 Test substance

a) Chemical name, etc. (information provided by the sponsor)

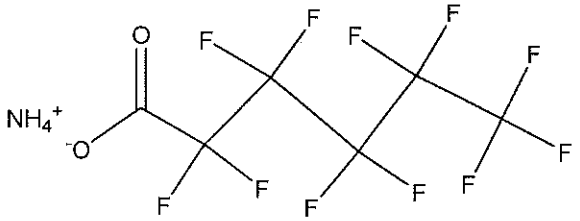
Chemical name 2,2,3,3,4,4,5,5,6,6,6-Undecafluorohexanoic acid, ammonium salt
Other name APFHx (C-1500N)
CAS number 21615-47-4

b) Supplier and lot number (information provided by the sponsor)

Supplier DAIKIN INDUSTRIES, LTD.
Lot number C150E57002

c) Structural formula, etc. (information provided by the sponsor)

Structural formula



Molecular formula $C_6H_4F_{11}NO_2$
Molecular weight 331.08

d) Purity, etc. (information provided by the sponsor)

Purity 99.8%
Impurity Water 0.2%
The test substance was treated as 100% in purity.

e) Physicochemical properties (information provided by the sponsor)

Appearance at ordinary temperature White powder
Water solubility >500 g/L

f) Storage conditions

The test substance was put into a shaded and air-tight container and stored in a desiccator in the test substance storage room at room temperature (acceptable range: from 10°C to 30°C).

g) Handling

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, a mask, a head cap, safety glasses and a lab coat were worn when handling the test substance.

13.2 Positive control substance

a) Name, etc.

Chemical name Cinnamaldehyde
CAS number 104-55-2
Molecular weight 132.16

b) Purity, etc.

Purity 99.1%

The positive control substance was treated as 100% in purity.

c) Physicochemical properties

Appearance at ordinary temperature Yellow and clear liquid

d) Manufacturer, grade and lot number

Manufacturer Wako Pure Chemical Industries

Grade Special grade

Lot number ECL6837

e) Storage conditions

The positive control substance was put into a shaded and air-tight container and stored in test substance storage room at room temperature (acceptable range: from 10°C to 30°C).

f) Handling

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, a mask, a head cap, safety glasses and a lab coat were worn when handling the test substance. The positive control substance and the preparation including the positive control substance were treated under yellow light.

13.3 Peptide

a) Cysteine peptide

Manufacturer Scrum

Lot number 15611

Purity 92.81%

Cysteine peptide was treated by correcting in purity.

b) Lysine peptide

Manufacturer Scrum

Lot number 16335

Purity 93.21%

Lysine peptide was treated by correcting in purity.

c) Storage conditions

Place Clinical examination room 2

Temperature -30°C to -10°C

13.4 Solvent

a) Name, etc.

Chemical name Acetonitrile

CAS number 75-05-8

b) Purity

100.0%

c) Manufacturer, grade and lot number

Manufacturer	Wako Pure Chemical Industries
Grade	For high performance liquid chromatography
Lot number	KPE0556

d) Storage conditions

Place	Analytical test room
Temperature	Room temperature

e) Reason of selection

For the positive control substance, the solvent is prescribed in the test method. For the test substance, the test substance was dissolved at the concentration of 100 mmol/L (mM) in acetonitrile which is recommended as a solvent in OECD TG442C.

f) Confirmation of effect of acetonitrile on stability of peptide

The lot of the acetonitrile used in this study was confirmed not to affect the stability of the peptides.

14. METHOD

14.1 Preparation of peptide standard solution, positive control solution and test substance solution

a) Preparation of peptide standard solution

1) Cysteine peptide

On the experiment day, 10.70 mg of the cysteine peptide was weighed and 19.8 mL of 100 mM phosphate buffer (pH 7.5) was added to prepare 0.667 mM cysteine peptide standard stock solution. The 0.667 mM standard stock solution was diluted with acetonitrile to prepare 0.534 mM standard solution. The 0.534 mM standard solution was serially-diluted with the solution of phosphate buffer/acetonitrile (8/2 v/v) to prepare 0.267, 0.134, 0.0667, 0.0334 and 0.0167 mM standard solution.

2) Lysine peptide

On the experiment day, 11.00 mg of the lysine peptide was weighed and 19.8 mL of 100 mM ammonium acetate buffer (pH 10.2) was added to prepare 0.667 mM lysine peptide standard stock solution. The 0.667 mM standard stock solution was diluted with acetonitrile to prepare 0.534 mM standard solution. The 0.534 mM standard solution was serially-diluted with the solution of ammonium acetate buffer/acetonitrile (8/2 v/v) to prepare 0.267, 0.134, 0.0667, 0.0334 and 0.0167 mM standard solution.

b) Preparation of positive control solution

On the experiment day, cinnamaldehyde (26.09 mg for cysteine peptide; 26.60 mg for lysine peptide) was weighed and dissolved to 2 mL of acetonitrile to prepare 100 mM positive control solution.

c) Preparation of test substance solution

On the experiment day, test substance (62.90 mg for cysteine peptide; 69.38 mg for lysine peptide) was weighed and dissolved to 2 mL of acetonitrile to prepare 100 mM

test substance solution.

14.2 Analytical condition

a) Instruments (HPLC12)

Pump A and B	L-2130 (Hitachi High-Technologies)
Auto sampler	L-2200 (Hitachi High-Technologies)
UV-VIS detector	L-2400 (Hitachi High-Technologies)
Column oven	L-2300 (Hitachi High-Technologies)
Data processor	EZChrom Elite (Hitachi High-Technologies)

b) Analytical condition

Column: L-column2 ODS (2.1 mm I.D. × 100 mm, CERI)

Column oven temperature: 30°C

Mobile phase: A: 0.1% Trifluoroacetic acid aqueous solution

B: 0.085% Trifluoroacetic acid acetonitrile solution

Gradient condition

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	90	10
10	75	25
10.5	10	90
12.5	10	90
13	90	10
20	90	10

Flow rate: 0.35 mL/min

Wavelength: 220 nm

Injection volume: 3 μL

Autosampler temperature: 25°C

Autosampler rinse solution: Acetonitrile/distilled water (1/1 v/v)

14.3 Preparation of calibration curve

Standard solutions of each peptide and the solution of each buffer/acetonitrile (8/2 v/v) were analyzed according to the condition described in 14.2 and a calibration curve for each peptide was made by the concentration of standard solution and the peak area (Figs. 1 and 2). The coefficients of determination (r^2) of both cysteine peptide and lysine peptide were 1.000, which satisfied the acceptance criteria. The concentrations for each peptide described below were calculated by the calibration curves.

14.4 Verification of suitability

For each peptide, 750 μL of the 0.667 mM standard stock solution was mixed with 250 μL of acetonitrile to prepare reference control A (n=3). Reference control A was analyzed according to the condition described in 14.2. Consequently, the mean concentrations of reference control A were 0.498 and 0.504 mM for cysteine peptide and lysine peptide,

respectively, which satisfied the acceptance criteria (Tables 1 and 2).

14.5 Verification of retention time of test substance

Verification of the retention time of the test substance was performed under non-GLP.

a) Cysteine peptide

To prepare co-elution control, 750 µL of the phosphate buffer was mixed with 200 µL of acetonitrile, and then mixed with 50 µL of the test substance solution. The co-elution control was left at 25°C for 24 hours, and then analyzed according to the condition described in 14.2. Consequently, no peaks derived from the test substance were detected at the retention time of the peptide.

b) Lysine peptide

To prepare co-elution control, 750 µL of the ammonium acetate buffer was mixed with 250 µL of the test substance solution. The co-elution control was left at 25°C for 24 hours, and then analyzed according to the condition described in 14.2. Consequently, no peaks derived from the test substance were detected at the retention time of the peptide.

14.6 Preparation of reference control B and C, and each reaction solution

For each peptide, reference control B (n=6), reference control C (n=3), positive control reaction solution (n=3) and test substance reaction solution (n=3) were prepared and left at 25°C.

a) Preparation of reference control B

For each peptide, 750 µL of the 0.667 mM standard stock solution was mixed with 250 µL of acetonitrile to prepare reference control B.

b) Preparation of reference control C

For each peptide, 750 µL of the 0.667 mM standard stock solution was mixed with 250 µL of acetonitrile to prepare reference control C.

c) Preparation of positive control reaction solution

1) Cysteine peptide

To prepare positive control reaction solution, 750 µL of the 0.667 mM standard stock solution was mixed with 200 µL of acetonitrile, and then mixed with 50 µL of the positive control solution.

2) Lysine peptide

To prepare positive control reaction solution, 750 µL of the 0.667 mM standard stock solution was mixed with 250 µL of the positive control solution.

d) Preparation of test substance reaction solution

1) Cysteine peptide

To prepare test substance reaction solution, 750 µL of the 0.667 mM standard stock solution was mixed with 200 µL of acetonitrile, and then mixed with 50 µL of the test substance solution.

Test substance reaction solution was visually inspected immediately after the preparation and 23 hours after the preparation. Consequently, no suspension or

precipitation were observed.

2) Lysine peptide

To prepare test substance reaction solution, 750 μL of the 0.667 mM standard stock solution was mixed with 250 μL of the test substance solution.

Test substance reaction solution was visually inspected immediately after the preparation and 22 hours after the preparation. Consequently, no suspension or precipitation were observed.

14.7 Analysis of reference control B and C, and each reaction solution

For each peptide, the reference control B and C, positive control reaction solution and test substance reaction solution were analyzed according to the condition described in 14.2. The analysis of the positive control reaction solution and test substance reaction solution was conducted 24 hours after the preparation or after.

Consequently, the coefficients of variation (CV) of the peak area of the reference control B and C were 1.1% and 1.8% for cysteine peptide and lysine peptide, respectively, which satisfied the acceptance criteria (Tables 3 and 4). The mean concentrations of the reference control C were 0.479 mM and 0.484 mM for cysteine peptide and lysine peptide, respectively, which satisfied the acceptance criteria (Tables 5 and 6).

14.8 Evaluation of result

a) Calculation of the percent peptide depletion

The percent peptide depletion was calculated according to the equation shown below.

$$\text{Percent peptide depletion (\%)} = \left(1 - \frac{\text{Peptide peak area of each reaction solution}}{\text{Mean peptide peak area in reference control C}}\right) \times 100$$

For each peptide, the mean value of the percent peptide depletion was calculated and regarded as percent peptide depletion for each peptide.

b) Evaluation method

The mean value of the percent cysteine and lysine depletion was calculated for the test substance. From the mean value of the percent cysteine and lysine depletion, the reactivity class was classified and the skin sensitivity was predicted.

Mean value of cysteine and lysine depletion	Reactivity class	Prediction
0% ≤ Mean depletion ≤ 6.38%	No or Minimal reactivity	Negative
6.38% < Mean depletion ≤ 22.62%	Low reactivity	Positive
22.62% < Mean depletion ≤ 42.47%	Moderate reactivity	
42.47% < Mean depletion ≤ 100%	High reactivity	

14.9 Acceptance criteria

When the following criteria i) to v) are satisfied, this study is judged as valid.

i) The calibration curve has an r^2>0.990.

ii) The mean percent peptide depletions for the positive control are between 60.8% to 100% for the cysteine peptide and between 40.2% to 69.0% for the lysine peptide.

iii) The standard deviations (SD) of the percent peptide depletion of the positive control and test substance are <14.9% for the cysteine peptide and <11.6% for the lysine peptide.

iv) The mean peptide concentrations of the reference control A and C are 0.50±0.05 mM.

v) The CV of peptide peak area for the reference control B and C is <15.0%.

15. DEVIATION FROM STUDY PLAN

No deviation from the study plan occurred.

16. RESULT

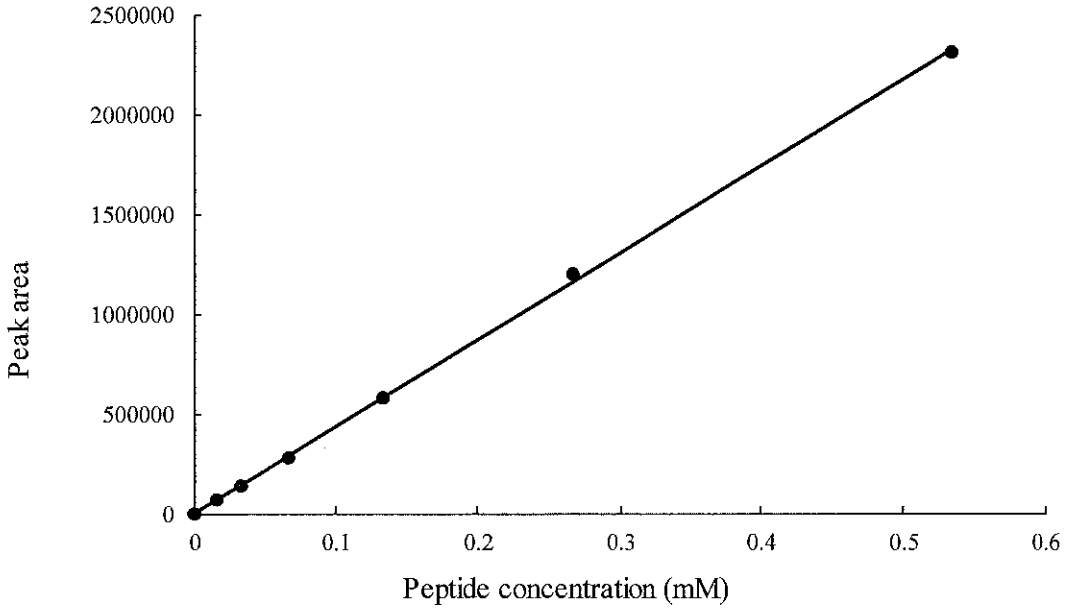
The percent peptide depletions of the test substance were 1.3% for the cysteine peptide and 100.0% for the lysine peptide (Tables 7 and 8). The mean value of the percent cysteine and lysine depletion was 50.7% (Table 9). The SDs of the percent peptide depletion were 0.7% for the cysteine peptide and 0.0% for the lysine peptide, which satisfied the acceptance criteria.

The percent peptide depletions of the positive control were 74.5% for the cysteine peptide and 52.8% for the lysine peptide (Tables 7 and 8). The SDs of the percent peptide depletion were 0.3% for the cysteine peptide and 1.7% for the lysine peptide, which satisfied the acceptance criteria.

17. DISCUSSION AND CONCLUSION

Because the mean value of the percent cysteine and lysine depletion was 50.7%, the reactivity class of the test substance was classified to "High reactivity" and the skin sensitivity was predicted as "Positive".

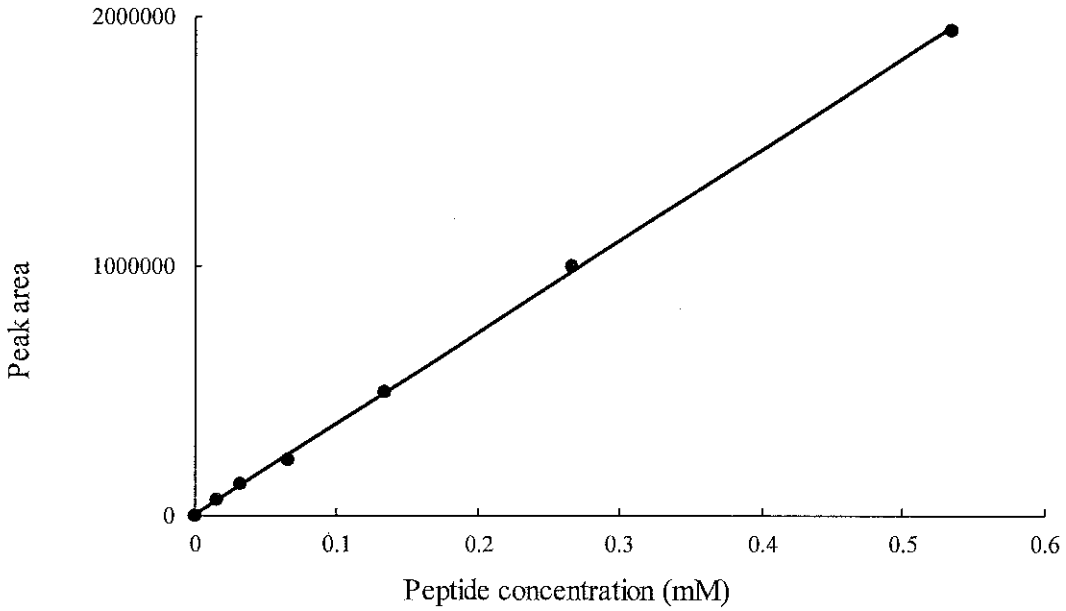
FIGURES



Peptide concentration (mM)	Peak area
0	0
0.0167	67306
0.0334	139614
0.0667	276727
0.134	577038
0.267	1194723
0.534	2305877

Regression equation	y = 4349751x-1969
r^2	1.000

Fig. 1 Calibration curve of the cysteine peptide



Peptide concentration (mM)	Peak area
0	0
0.0167	59503
0.0334	124598
0.0667	219831
0.134	492933
0.267	995677
0.534	1939986

Regression equation	y = 3653422x-1449
r^2	1.000

Fig. 2 Calibration curve of the lysine peptide

TABLES

Table 1 System suitability test for the cysteine peptide analysis

Reference control A	Peak area	Peptide concentration (mM)			
		Individual	Mean	SD	CV (%)
1	2173813	0.500	0.498	0.006	1.2
2	2182010	0.502			
3	2134880	0.491			

Table 2 System suitability test for the lysine peptide analysis

Reference control A	Peak area	Peptide concentration (mM)			
		Individual	Mean	SD	CV (%)
1	1841606	0.504	0.504	0.001	0.2
2	1838438	0.504			
3	1843538	0.505			

Table 3 Reference controls of the cysteine peptide for stability over analysis time

Group	No.	Peak area			
		Individual	Mean	SD	CV(%)
Reference control B	1	2061097	2059348	23383	1.1
	2	2077245			
	3	2034235			
	4	2054550			
	5	2019503			
	6	2048103			
Reference control C	1	2064295			
	2	2081734			
	3	2093368			

Table 4 Reference controls of the lysine peptide for stability over analysis time

Group	No.	Peak area			
		Individual	Mean	SD	CV(%)
Reference control B	1	1830500	1804431	33315	1.8
	2	1824837			
	3	1853413			
	4	1804290			
	5	1822849			
	6	1804184			
Reference control C	1	1752492			
	2	1758608			
	3	1788710			

Table 5 Reference controls C of the cysteine peptide for calculation of percent peptide depletion

Group	No.	Peak area		Concentration (mM)			
		Individual	Mean	Individual	Mean	SD	CV(%)
Reference control C	1	2064295	2079799	0.475	0.479	0.004	0.8
	2	2081734		0.479			
	3	2093368		0.482			

Table 6 Reference controls C of the lysine peptide for calculation of percent peptide depletion

Group	No.	Peak area		Concentration (mM)			
		Individual	Mean	Individual	Mean	SD	CV(%)
Reference control C	1	1752492	1766603	0.480	0.484	0.005	1.0
	2	1758608		0.482			
	3	1788710		0.490			

Table 7 Percent cysteine peptide depletion

Group	No.	Peak area				Peptide depletion (%)			
		Individual	Mean	SD	CV (%)	Indivisual	Mean	SD	CV (%)
Positive control (Cinnamaldehyde)	1	534167	530888	6728	1.3	74.3	74.5	0.3	0.4
	2	535349				74.3			
	3	523149				74.8			
APFHx (C-1500N)	1	2067534	2053358	15204	0.7	0.6	1.3	0.7	53.8
	2	2037301				2.0			
	3	2055238				1.2			

Table 8 Percent lysine peptide depletion

Group	No.	Peak area				Peptide depletion (%)			
		Individual	Mean	SD	CV (%)	Indivisual	Mean	SD	CV (%)
Positive control (Cinnamaldehyde)	1	806013	834673	30312	3.6	54.4	52.8	1.7	3.2
	2	866403				51.0			
	3	831603				52.9			
APFHx (C-1500N)	1	0	0	0	-	100.0	100.0	0.0	0.0
	2	0				100.0			
	3	0				100.0			

Table 9 Mean value of percent cysteine and lysine depletion

Chemical name	Cysteine depletion (%)	Lysine depletion (%)	Mean of cysteine and lysine depletion (%)	Reactivity class	DPRA prediction
APFHx (C-1500N)	1.3	100.0	50.7	High reactivity	Positive

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor: DAIKIN INDUSTRIES, LTD

Title: Direct Peptide Reactivity Assay of APFHx (C-1500N)

Study Number: G21-0014

I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study. The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows.

Item of inspection	Date of inspection	Date of report
Study plan	February 20, 2017	February 20, 2017
Study plan amendment No. 1	February 20, 2017	February 20, 2017
Preparation of test substance solution	March 1, 2017	March 1, 2017
Preparation of positive control solution	March 1, 2017	March 1, 2017
Preparation of peptide standard solution, positive control solution, and each reaction solution (cysteine)	March 1, 2017	March 1, 2017
Preparation of peptide standard solution, positive control solution, and each reaction solution (lysine)	March 2, 2017	March 2, 2017
Analysis of reference control B and C, and each reaction solution	March 2, 2017	March 2, 2017
Raw data and draft final report	April 3, 2017	April 3, 2017
Final report	April 3, 2017	April 3, 2017

Date: April 3, 2017

Quality Assurance Manager: ____