Cat. # MK150

For Research Use

TaKaRa

Acrolein-Lysine Adduct Competitive EIA Kit

Product Manual

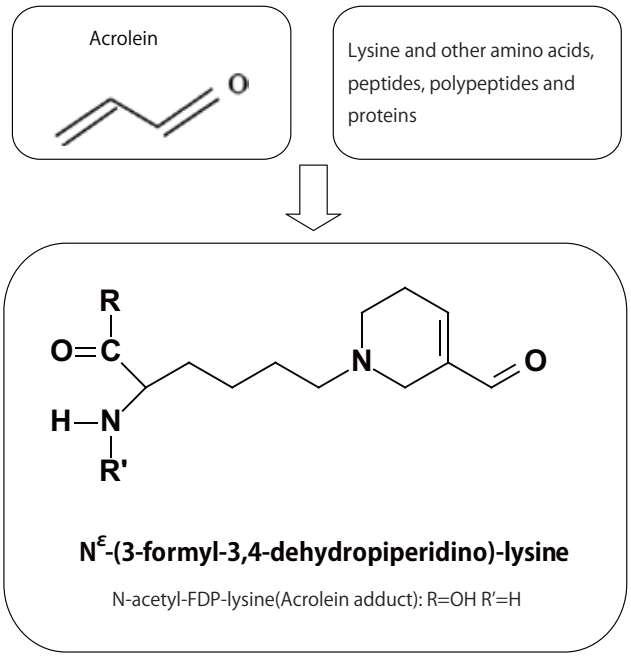
v201608

Table of Contents

I. Description

Acrolein (CH_2 =CHCHO) is a chemical substance that is produced by burning of petroleum, coal, wood, and plastic, and also by cigarette smoke, exhaust gas, and the heating of cooking oil. Acrolein is highly cytotoxic and is a by-product of lipid peroxidation in the human body. The biological and physiological effects of acrolein are being actively investigated.[1) - 5)]

The Acrolein-Lysine Adduct Competitive EIA Kit can be used for measurement of acrolein-protein adducts in blood, urine, or tissue samples. The kit is a competitive EIA that uses a monoclonal antibody that reacts specifically with the formyl-dehydropiperidino (FDP)-lysine (Lys) structures that are created when acrolein binds to the lysine residues of proteins. Quantitative measurement of acrolein adducts in a sample is calculated using the quantity of FDP-Lys.



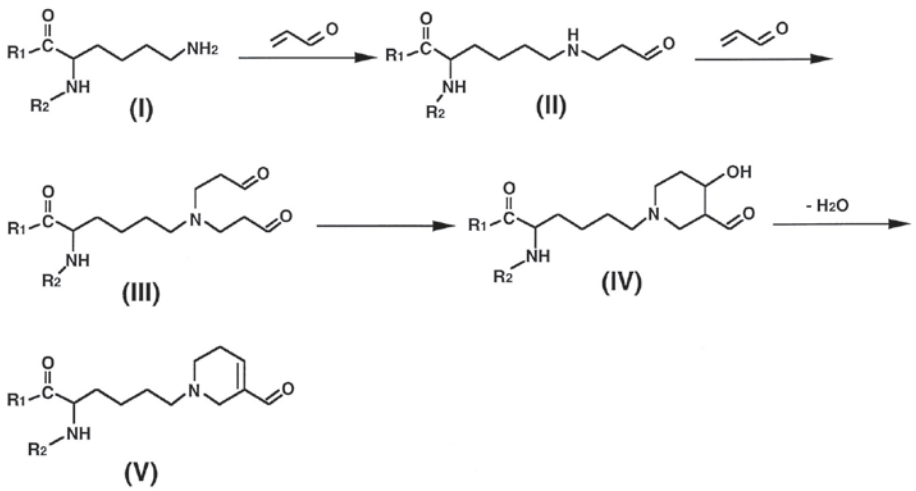


Figure 1. Mechanism of adduct formation. (From reference 2)

II. Components

1.	Antigen Coated Microtiter Plate Acrolein-Lysine Adduct Immobilized plates (96 wells: 8 wells x 12 strips)	1 plate
2.	Sample Diluent Buffer solution for sample dilution	50 ml
3.	Standard N-acetyl-FDP-lysine (7 different concentrations)	350 µl x 7
4.	Anti Acrolein Monoclonal Antibody (Lyophilized) Primary antibody for acrolein adduct	For 10 ml
5.	Antibody Diluent Solution for reconstituting the primary antibody	11 ml
6.	Wash Buffer (20X) Buffer solution with Tween 20	50 ml
7.	POD-Labeled Anti Mouse IgG Conjugate (100X conc.) POD-labeled mouse IgG secondary antibody	120 µl
8.	Conjugate Diluent Diluent for labeled secondary antibody	12 ml
9.	Substrate Solution (TMBZ) 3,3',5,5'-Tetramethylbenzidine solution	12 ml
10.	Stop Solution without Sulfuric Acid Reaction stop solution (Contains no sulfuric acid)	12 ml
11.	Plate Seal	2 pieces

III. Materials Required but not Provided

- Pipettes, micropipettes, and tips
- Tubes or flasks for preparation of buffers
- Plate-washing device; dispensing burette and aspirator for manual washing
  Note: Personal Microplate Washer (Cat. #MK950)＊ is recommended.
- Plate mixer
- Temperature-controlled incubator (20 - 30℃)
- Microplate reader (capable of measuring absorbance of up to 3.5 when set to 450 nm)

＊: Not available in all geographic locations. Check for availability in your area.

IV. Storage 4℃

V. Intended Use

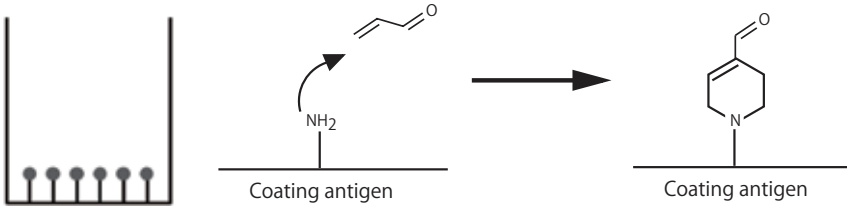
- Detection of acrolein adducts in environmental samples
- Detection of acrolein adducts produced as a by-product of industrial product manufacturing
- Detection of acrolein adducts in biological samples

Note: This kit is for research use only. It cannot be used for diagnostic purposes.

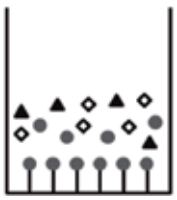
VI. Principles

This kit is a single-antibody competitive EIA. The quantity of antigen in the sample is measured by adding the primary antibody in the presence of both solid-phase antigen (acrolein-lysine adduct) and the test sample. An enzyme-labeled secondary antibody detects primary antibody bound on the solid-phase plate. When there is a large amount of antigen (acrolein adduct) in the sample, the majority of the primary antibody will be bound in solution, resulting in a decrease in the amount of the primary antibody bound to the solid-phase antigen and a proportional decrease in the absorbance.

1. Bring the Antigen Coated Microtiter Plate to room temperature.

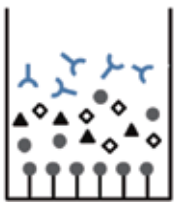


2. Dilute the Standard or sample appropriately and add to the Antigen Coated Microtiter Plate.



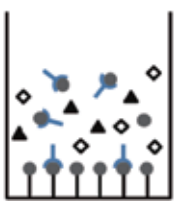
Add 50 μl of the sample or Standard per well.

3. Add the reconstituted Anti Acrolein Monoclonal Antibody.



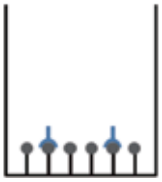
Add 50 μl of the Anti Acrolein Monoclonal Antibody per well.

4. The antibody will react with acrolein adduct in the sample and bound to the plate. (If there is a large quantity of acrolein adduct in the sample, less of the primary antibody will bind to the solid phase.)



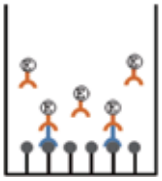
Allow to stand for 30 minutes at room temperature (20 - 30℃).

5. Wash the plate to remove excess reactants.



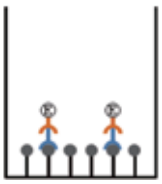
Wash 4 times (200 - 300 μl/well).

6. Add the POD-Labeled Anti Mouse IgG Conjugate to the plate.



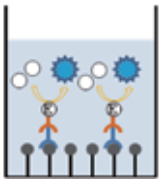
Add 100 μl of the POD-Labeled Anti Mouse IgG Conjugate per well.
Incubate at room temperature (20 - 30℃) for 60 minutes.

7. Wash the plate to remove unbound POD-labeled Anti Mouse, IgG Conjugate.



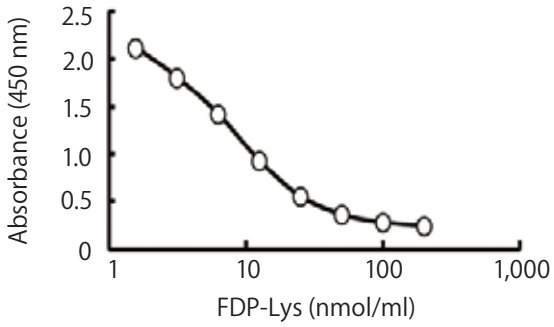
Wash 4 times (200 - 300 μl/well).

8. Add the Substrate Solution and allow color to develop (blue).



Add 100 μl of the Substrate Solution (TMBZ) per well.
Incubate at room temperature (20 - 30℃) for 5 - 15 minutes to allow the substrate color-development reaction.

9. Add 100 μl of the Stop Solution per well. The color of the solution will change from blue to yellow. Measure absorbance at 450 nm.



VII. Protocol

1. Samples

- Urine, blood serum, blood plasma, ascitic fluid, or cell supernatant/extracts, from any source can be used for measurement. In addition, intermediate products can be measured.
- Store samples at 2 - 10℃; store frozen if measurement will take place >12 hours after sample collection.
- If dilution is necessary, dilute using Sample Diluent.
- When measuring biological specimens (i.e., urine), dilute at least 10 times using Sample Diluent before measurement.
- For blood specimens, measurement of concentrated specimens may result in poor linearity. Preliminary testing of the dilution ratio is necessary when measuring blood samples.
- The POD-labeled secondary antibody is an anti-mouse IgG conjugate. When measuring mouse blood samples, non-specific background may be obtained. Therefore, it is necessary to dilute at least 10 times with Sample Diluent, and to perform relative evaluation using samples from a control group.
- Because this measurement with this kit relies on an antigen/antibody reaction, the pH of the reaction system should be maintained within a neutral range. In addition, protein denaturants may inhibit the reaction.
- Impurities and turbidity in the sample should be removed before measurement by centrifugation (3,000 rpm, 10 minutes) or filtration.
- Hemolyzed blood serum may affect the results.

2. Reagent Preparation

- Antigen Coated Microtiterplate
  Prior to use, bring to room temperature and open.
  The Plate Seal may be used to prevent drying.

- Primary Antibody Solution
  Add 10 ml of Antibody Diluent to Anti Acrolein Monoclonal Antibody (Lyophilized) and dissolve completely for use.
  The reagent is stable for 2 weeks at 4℃ after preparation.
  Roughly 5.5 ml of solution is needed per plate.

- POD-Labeled Secondary Antibody Solution
  Dilute POD-Labeled Anti Mouse IgG Conjugate (100X conc.) 100 times with Conjugate Diluent.
  11 ml of the solution is needed per plate. Diluted POD-labeled secondary antibody solution should be used within 24 hours after preparation.

- Wash Solution
  Dilute the Wash Buffer (20X) with distilled water to obtain a 1X solution.
  The resulting solution will be PBS with 0.05% Tween 20.

- N-acetyl-FDP-lysine Standard Solution (Standard)
  These consist of seven concentrations standards (200, 100, 50, 25, 12.6, 6.25, and 3.13 nmol/ml), and can be used as is.
  To avoid loss due to non-specific adsorption, do not transfer to a different tube or container.
  Use Sample Diluent for blank measurements.
  Store at 4℃ protected from light in the glass container provided.

- Substrate Solution (TMBZ)
  Bring to room temperature before use. Prior to use, confirm that the Substrate Solution has not turned dark blue. Avoid mixing with tap water, as reaction with metal ions may change the color.
  When only using a partial amount, aliquot the necessary quantity.
- Stop Solution without Sulfuric Acid
  This is a peroxidase reaction stop solution that contains no sulfuric acid. Because this is a highly-concentrated solution, mix well using a plate mixer after adding to the plate.

3. Procedure

Assay samples in duplicate. Allow reagents in the kit and samples to return to room temperature and mix all solutions uniformly without creating bubbles before use.

(1) Use a separate 96 well plate to prepare dilutions of the sample and add 50 µl to each well of the Antigen Coated Microtiter plate using an 8-channel pipette. Also, directly add 50 µl of each concentration of Standard into the plate from the specialized glass containers (avoid transferring the standard to different plates/tubes to avoid adsorption). Then add 50 µl of Anti Acrolein Monoclonal Antibody solution to each well. Complete addition of the primary antibody solution within 5 minutes using equipment such as a continuous dispensing burette or an 8-channel pipette. Lightly agitate the entire plate to ensure that the solution uniformly mixed. Seal the plate and allow to stand for 30 minutes at room temperature (20 - 30℃). Perform the reaction at room temperature (20 - 30℃). Incubation at 37℃ may compromise antigenicity. [First reaction]

(2) Discard the reaction solution, and wash 4 times with the Wash Solution (PBS with 0.05% Tween20). Then add 100 µl of the POD-Labeled Anti Mouse IgG Conjugate to each well using an 8-channel pipette, seal the plate, and allow to stand at room temperature (20 - 30℃) for 60 minutes. [Second reaction]

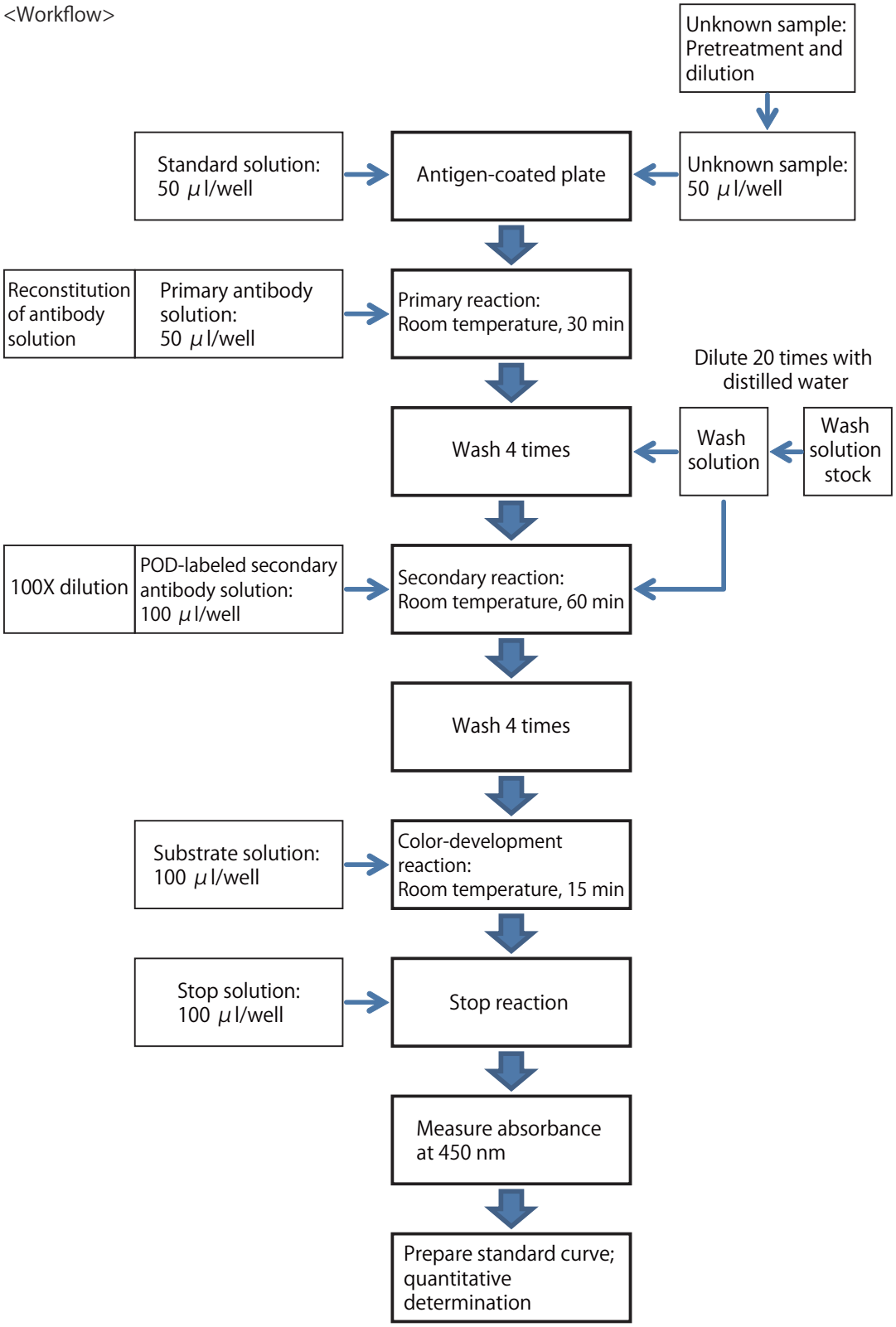
(3) Discard the reaction solution, wash 4 times with the Wash Solution (PBS with 0.05% Tween20), and completely remove the all of the liquid. Then, add 100 µl of the Substrate Solution (TMBZ) to each well using an 8-channel pipette and allow to stand at room temperature (20 - 30℃) for approximately 5 - 15 minutes. [Third reaction]

(4) Add 100 µl of Stop Solution to each well in the same order that the Substrate Solution (TMBZ) was added, and agitate well using a plate mixer after the reaction has been stopped.

(5) Measure absorbance at a wavelength of 450 nm.
The color is stable for at least 6 hours after the reaction is stopped.

(6) Prepare a standard curve by plotting the concentration of each Standard on the x-axis and the corresponding absorbance on the y-axis. Use the absorbance of the specimen to calculate the corresponding concentration of acrolein adduct.

<Workflow>


Unknown sample: Pretreatment and dilution
Standard solution: 50 μl/well
Antigen-coated plate
Unknown sample: 50 μl/well
Reconstitution of antibody solution
Primary antibody solution: 50 μl/well
Primary reaction: Room temperature, 30 min
Dilute 20 times with distilled water
Wash 4 times
Wash solution
Wash solution stock
100X dilution
POD-labeled secondary antibody solution: 100 μl/well
Secondary reaction: Room temperature, 60 min
Wash 4 times
Substrate solution: 100 μl/well
Color-development reaction: Room temperature, 15 min
Stop solution: 100 μl/well
Stop reaction
Measure absorbance at 450 nm
Prepare standard curve; quantitative determination

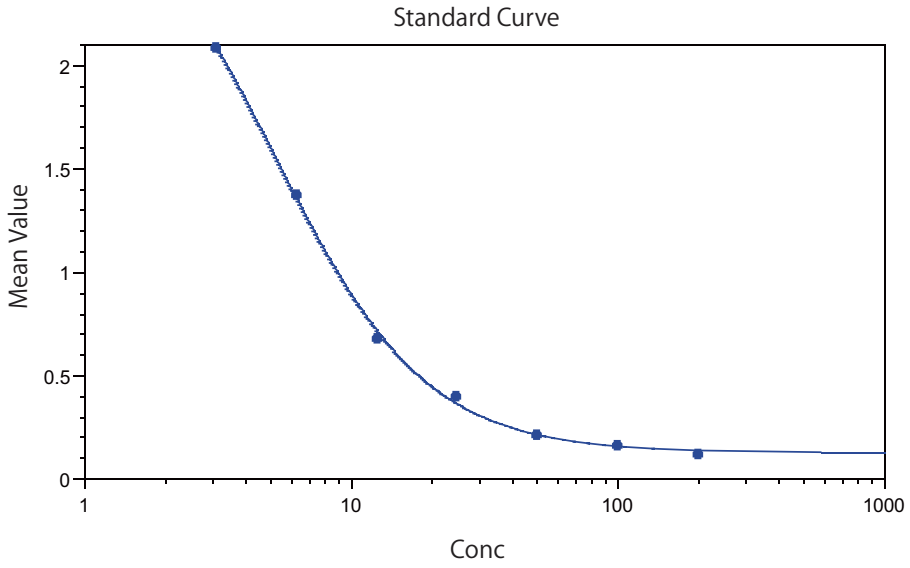
VIII. Performance

1. Standard Curve (Acrolein-Lysine Adduct Competitive EIA Kit)

The following is a typical standard curve. Prepare a standard curve for each experiment.

Range of Measurement: 3.13 - 200 nmol/ml
Range of Confidence: 3.13 - 100 nmol/ml
Limit of Detection: 3.13 nmol/ml

(Curve Fit : 4-Parameter)



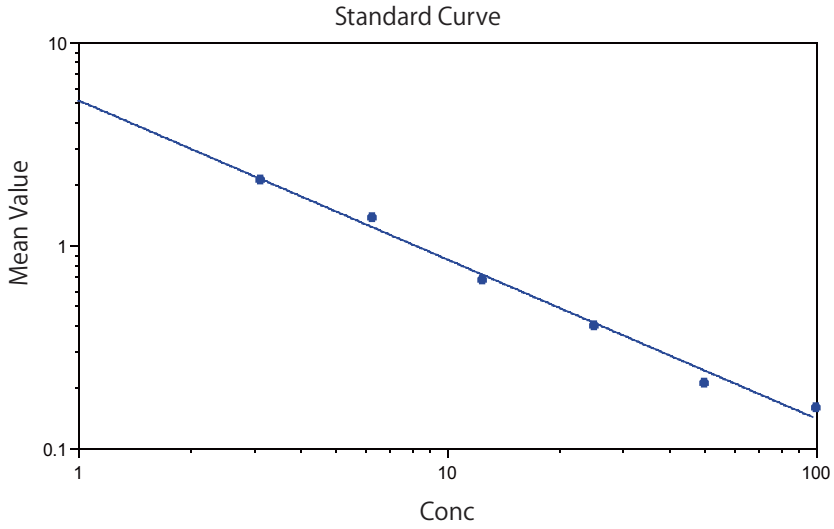
4-P Fit: $y = (A - D)/(1 + (x/C)^B) + D$:

A	B	C	D	R^2
2.99	1.53	5.2	0.125	0.999

N-acetyl-FDP-Lysine (nmol/ml)	200.0	100.0	50.0	25.0	12.5	6.25	3.13
A_{450}	0.120	0.160	0.210	0.401	0.679	1.375	2.083

Color development: 15 minutes

(Curve Fit : Log-Log)



Log-Log Fit: Log(y) = A + B * Log(x) : A 0.713 B -0.783 R^2 0.99

N-acetyl-FDP-Lysine (nmol/ml)	100.0	50.0	25.0	12.5	6.25	3.13	0.0
A_{450}	0.160	0.210	0.401	0.679	1.375	2.083	2.303

2. Reproducibility

<Intra-assay precision test>
Reproducibility testing was performed with three concentrations of a control containing N-acetyl-FDP-Lysine.

Sample (n=4)	Mean (nmol/ml)	SD	CV (%)
Control A	10.491	0.472	4.5
Control B	7.367	0.470	6.4
Control C	5.777	0.401	6.9

<Inter-assay precision test (n=3)>
Three concentrations of a control were assayed on three separate days.

Sample	Mean (nmol/ml)	SD	CV (%)
Control D	10.457	0.842	8.1
Control E	7.349	0.431	5.9
Control F	5.226	0.395	7.6

<Spike and Recovery Test>

Equal amounts of various concentrations of acrolein-containing samples were mixed and the recovery rate was calculated by comparing the anticipated theoretical value with the actual measurement.

Sample A	Sample B	Theoretical Value (A+B)/2	Assay Result	Recovery Rate (%)
11.471	11.471	11.471	7.874	68.6
11.471	7.868	9.670	7.860	81.3
11.471	5.028	8.250	7.515	91.1
11.471	1.637	6.554	7.254	110.7
7.868	5.028	6.448	5.911	91.7
7.868	1.637	4.753	4.570	96.2
12.500	6.250	9.375	8.444	90.1
6.250	3.130	4.690	5.141	109.6

Units: nmol/ml

IX. Experimental Examples

1. Dilution Linearity of Biological Samples

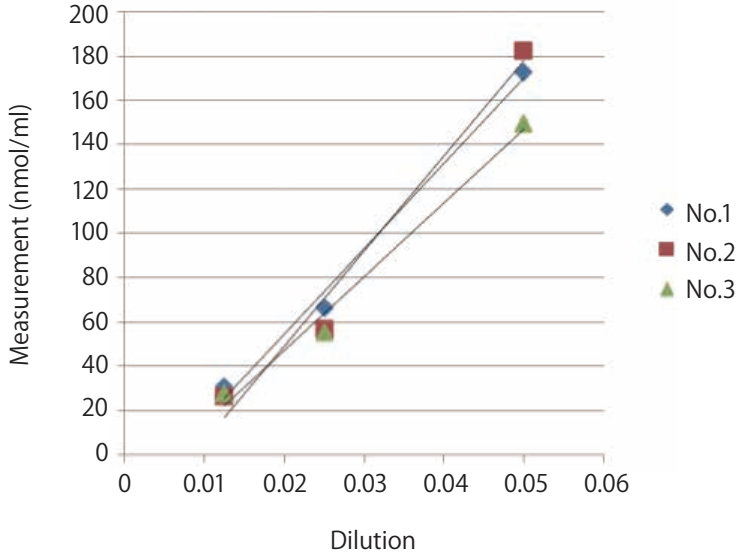
Acrolein-lysine adducts were measured in urine and stool (water extracted) samples from 8 - 9 week old mice (BALB/c and C57BL6). For stool samples, 500 μl of Sample Diluent and 1 standard-sized fecal pellet were homogenized with the TaKaRa BioMasher Standard (Sterile) (Cat. #9791). The samples were centrifuged and the supernatant was used for analysis. For urine samples, spot urine was used. When performing comparative evaluation between individuals, it is advisable to use pooled urine from a metabolic cage. Dilution by at least 10 - 20 fold is necessary to obtain a dilution curve from urine samples.

Measured Concentration of Acrolein-Lysine Adducts

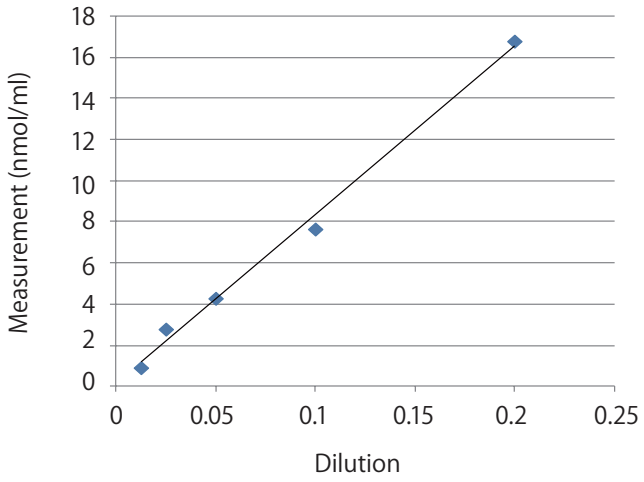
Mouse Strain	Sample	Dilution Ratio				
		5X	10X	20X	40X	80X
BALB/c No.1	Urine	over	over	172.7	66.5	30.5
BALB/c No.2	Urine	over	over	182.5	56.6	26.3
BALB/c No.3	Urine	over	over	149.6	55.1	27.6
C57BL6 No.1	Urine	over	over	over	84.3	35.8
C57BL6 No.2	Urine	over	over	150.1	47.8	20.5
C57BL6 No.3	Stool Water Extracted	16.8	7.7	4.3	2.8	1.0

Units: nmol/ml

< BALB/c No. 1 - 3, Urine>



< C57BL6 No. 3, Stool >



2. Relative Measurement of Urine Protein

Acrolein and IgG were measured in the urine from normal (female) mice.
Kits: Acrolein-Lysine Adduct Competitive EIA Kit (Cat. #MK150) and Mouse IgG EIA Kit (Cat. #MK137; discontinued)
Samples: Spot urine from 8 - 9 week old mice (BALB/c and C57BL6 strains)
For acrolein, 40X sample dilution was used.
For mouse IgG, 20X sample dilution was used.

		Urine Acrolein (nmol/ml)		Urine IgG (ng/ml)		Acrolein/IgG (nmol/ng)
Mouse Strain	Sample	40X	Value Converted for Undiluted Solution	20X	Value Converted for Undiluted Solution	per 1 ml
BALB/c No.1	Urine	66.5	2,661.9	62.0	1,239.6	2.1
BALB/c No.2	Urine	56.6	2,262.2	50.4	1,007.3	2.2
BALB/c No.3	Urine	55.1	2,203.4	44.2	884.0	2.5
C57BL6 No.1	Urine	84.3	3,372.8	122.5	2,450.3	1.4
C57BL6 No.2	Urine	47.8	1,913.0	100.9	2,017.2	0.9

Results: Comparative evaluation with a protein in the urine can be used to normalize acrolein measurements. Albumins, etc. may be used for normalization.

3. Measurement from Tissue Extracts

Extracts prepared from various normal mouse tissues were prepared by solubilizing with lysis buffer using a TaKaRa BioMasher Standard (Sterile) homogenizer.
Measurement was performed on serial dilutions (10, 20, 40, and 80 - fold dilutions).
The value measured for the 10 - fold dilution was used, and values were normalized to the value of lysis buffer alone.
Samples B, C, and D were isolated simultaneously from the same individual.

Sample	Tissue	Wet Weight	Buffer Volume	Measured Value (10X) (nmol/ml)	Stock Solution Converted Value (nmol/ml)	Acrolein-Lysine Adduct per mg of Tissue (nmol/mg)
A	ICR Mouse Liver	420 mg	1 ml	10.6	71	0.169
B	BALB/c Mouse Spleen	40 mg	1 ml	4.37	9	0.225
C	BALB /c Mouse Brain	250 mg	1 ml	7.75	43	0.172
D	BALB/c Mouse Kidney	50 mg	1 ml	5.5	20	0.400
E	Lysis Buffer	—	1 ml	3.5	0	—

Result: Acrolein adduct accumulation was observed in kidney tissue.
This suggests the possibility that acrolein is excreted in the urine.

X. Precautions

1. Do not mix reagents or solutions from kits with different lot numbers.
2. Do not expose reagents to strong light during storage or reaction.
3. The pipettes, etc. used for the substrate solution (TMBZ) and stop solution should not contain metal.
4. Prevent the substrate solution (TMBZ) and stop solution from coming into contact with hands and mucous membranes.
5. Do not use substrate solution (TMBZ) that has changed color.
6. Each reaction can be affected by time and temperature; prepare a standard curve for each experiment.
7. Handle blood specimens with sufficient care.

XI. References

1) K. Uchida. Production of acrolein by protein peroxidation reactions and protein modification. *Journal of Oleo Science.* (1998) **47**:1207-1215.
2) K. Uchida, *et al*. Acrolein is a product of lipid peroxidation : formation of free acrolein and its conjugate with lysine residues in oxidative low density lipoproteins. *J Biol Chem*. (1998) **273**:16058-16066.
3) K. Uchida, *et al*. Protein-bound acrolein : Potential markers for oxidative stress. *Proc Natl Acad Sci USA.* (1998) **95**:4882-4887.
4) N. Y. Calingasar, *et al*. Protein-bound acrolein : A novel marker of oxidative stress in Alzheimer' s disease. *J Neurochem*. (1999) **72**:751-756.
5) K. Sato, *et al*. A one-hour ELISA for quantitation of Acrolein and hydroxynonenal-Modified proteins by epitope-bound casein matrix method. *Anal Biochem*. (1999) **270**:323-328.

XII. Related Products

TaKaRa BioMasher Standard (Sterile) (Cat. #9791)
Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
Personal Microplate Washer (Cat. #MK950)*

ELISA kits that measure urine proteins:
Fibronectin EIA Kit (Cat. #MK115)
Laminin (LN) EIA Kit (Cat. #MK107)
E-cadherin EIA Kit (Cat. #MK117)
Human Albumin EIA Kit (Cat. #MK132)
Human IgG EIA Kit (Cat. #MK136)

* : Not available in all geographic locations. Check for availability in your area.