

## Hexanoyl-Lys adduct (HEL) ELISA kit

Read entire insert before use.

Hexanoyl-Lys adduct (HEL) is formed by the reaction of linoleic acid hydroperoxide and Lysine, and a biomarker for oxidative stress. HEL ELISA kit is a competitive enzyme-linked immunosorbent assay for quantitative measurement of hexanoyl-Lys adduct. This kit is based on the monoclonal antibody clone 5H4, which is specific for HEL. Suitable for urine, serum and other biological samples. For research use only. Not for diagnostic nor medical use.

### 1. Kit contents.

|                            |   |                          |
|----------------------------|---|--------------------------|
| ①HEL Microtiter Plate      | : Precoated with HEL (8x12 wells, Split Type)   | 1 Plate                  |
| ②Primary Antibody          | : Monoclonal Antibody specific for HEL  | 1 Vial (7mL)             |
| ③Secondary Antibody        | : HRP-Conjugated Anti Mouse IgG Antibody  | 1 Vial                   |
| ④Secondary Antibody Buffer | : Phosphate Buffered Saline   | 1 Vial (12mL)            |
| ⑤Chromogen                 | : 3,3',5,5'-Tetramethylbenzidine  | 1 Vial (250 $\mu$ L)     |
| ⑥Chromogen Buffer          | : Hydrogen Peroxide/ Citrate-Phosphate Buffer   | 1 Vial (12mL)            |
| ⑦Washing Buffer (5X)       | : Concentrated Phosphate Buffered Saline  | 2 Vials (25mL X 2)       |
| ⑧Stop Solution             | : 1M Phosphoric acid  | 1 Vial (12mL)            |
| ⑨Standard A-F              | : Bz-Gly-Hexanoyl-Lys<br>( <b>A:</b> 2.6, <b>B:</b> 7.7, <b>C:</b> 22.7, <b>D:</b> 69.7, <b>E:</b> 207, <b>F:</b> 624 nmol/L) | 1 Vial each(500 $\mu$ L) |
| ⑩Plate Seal                | : Adhesive seal to prevent evaporation  | 2 Sheets                 |

\*Storage conditions: Store at 2-8°C. **Don't freeze.**

\*Expiration : 2 years. After the vials are opened, the kit should be used in one week.

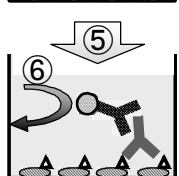
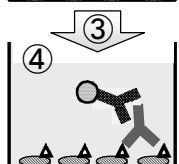
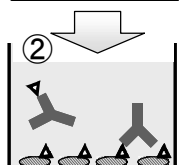
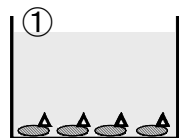
\*Measuring range : 2 – 700 nmol/L

### 2. Required but not provided.

- A) Distilled water (Preparation of washing solution)
- B) 50 micro L micropipettor and pipette tips
- C) 8-channel (50-200 micro L) micropipettor and tips.
- D) Reagent trays for 8-channel micropipettor.
- E) 4-7 degree C incubator.
- F) Microtiter plate reader (measuring wavelength 450 nm).

### 3. Principles of the procedure.

(Fig.1)



- 1) Prepare microtiter plate precoated with hexanoyl-Lys adduct (HEL).
- 2) Add HEL standard solution or sample to microtiter plate well, and subsequently add anti HEL monoclonal antibody. The HEL in the standard or sample competes with the HEL on the well surface for the anti HEL antibody. As a result, higher concentration of HEL in sample will result in reduced binding of the antibody bound to the surface of the well.
- 3) The antibody bound to the HEL in sample, is removed from the well by washing. While the antibody bound to precoated HEL remain on the surface of the well.
- 4) Peroxidase-conjugated secondary antibody is added to the well, and binds to the anti HEL antibody.
- 5) Unbound secondary antibody is removed by washing.
- 6) Addition of the chromatic reagent results in the development of color in proportion to the amount of antibody bound to the well. The reaction is terminated by stop solution. Absorbance at 450 nm is measured using microtiter plate reader.
- 7) Make a calibration curve from the absorbance data of standards, and calculate the concentration of HEL in the sample.

## 4. Sample pretreatment.

### A. Urine sample

Dilution of samples for at least 4 times using phosphate buffered saline (PBS at pH7.4). For urine sample from experimental animal such as dogs and cats, 10 times or 20 times dilution is recommended. If insoluble materials are observed, remove them by centrifugal machine. If the urine contains proteins, treat the urine by the same procedure as for serum sample.

### B. Serum sample

1) Prepare "Enzyme reagent", by dissolving 14 mg/mL of alpha-chymotrypsin in PBS (pH7.4).  
2) Dilute the serum sample for at least 2 times using PBS (pH7.4).  
3) Mix 300 micro L of diluted sample and 60 micro L of "Enzyme reagent", and incubate at 37 degree C for over night.

4) Filtrate using ultra filter with cut-off molecular weight 10kDa (for example Microcon YM-10, Millipore), and remove enzymes. Apply the filtrate to ELISA.

\*This is an example of procedure. Please investigate optimum condition depending on the sample.

## 5. Procedure.

Bring all reagents, samples and microtiter plate to room temperature before use.

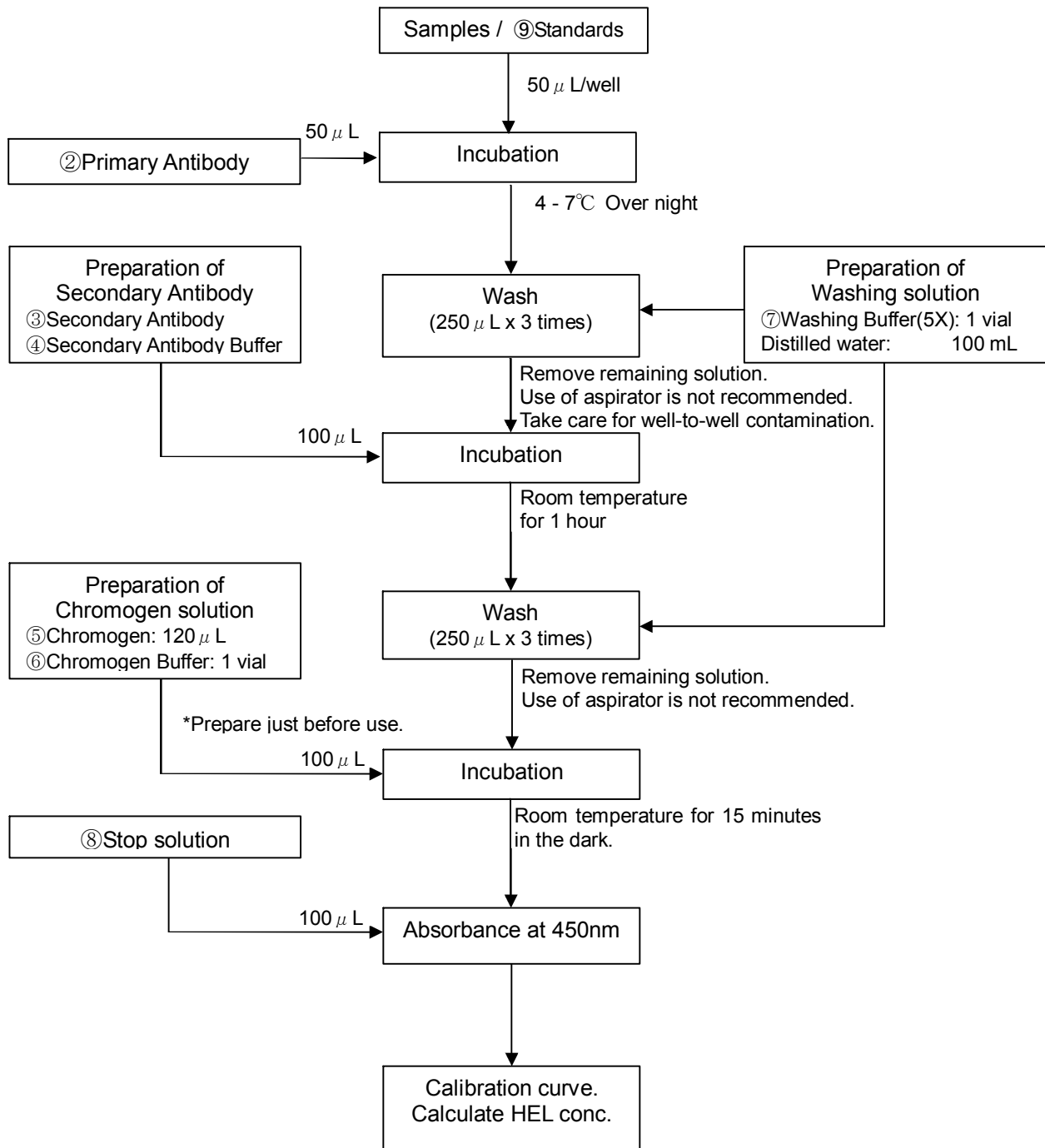
- A)** Pick out ① *Microtiter plate* from the bag. To use some wells at the next experiment, remove the well splits from the frame, put it into the bag, and store at 4 degree C. It will be available for 1 week.
- B)** Prepare *Washing solution* by mixing one bottle of ⑦ *Washing buffer(x5)* and 100 mL of distilled water.
- C)** Add 50 micro L of ⑨ *Standards(A ~F)* or sample per well. For the *Blank well*, add 100 micro L of *Washing solution*. The typical layout of microtiter plate is shown in Fig.2.
- D)** Add 50 micro L of ② *Primary Antibody* to all well except *Blank well*. Seal the microtiter plate tightly with ⑩ *Plate Seal*. Mix gently by shaking the microtiter plate horizontally. Incubate at 4 - 7 degree C for over night.
- E)** Reconstitute ③ *Secondary Antibody* with one bottle of ④ *Secondary Antibody Buffer*. This is stable for 1 week at 4 degree C.
- F)** Remove the plate seal, and pour off the contents of microtiter plate by turn the plate upside down. The use of aspirator is not recommended. Remove the remaining solution by blotting the plate against clean paper towel. Add 250 micro L of *Washing solution* to each well, mix gently by horizontal shaking, and remove the contents similarly. Repeat washing procedure twice and remove the remaining solution of the well.
- G)** Add 100 micro L of *Secondary Antibody* to all well. Seal the microtiter plate tightly with ⑩ *Plate Seal*. Mix gently by shaking the microtiter plate horizontally. Incubate at room temperature (20-24 degree C) for 1 hour.
- H)** Prepare *Chromogen solution*. Add 120 micro L of ⑤ *Chromogen* to ⑥ *Chromogen Buffer* bottle. Please note that Chromogen solution should be prepared just before use. Alternatively, dilute ⑤ *Chromogen* with 100 volumes of ⑥ *Chromogen Buffer*.
- I)** Remove the plate seal, and wash the plate as mentioned at STEP F for 3 times. Remove the remaining solution of the well.
- J)** Add 100 micro L of *Chromogen solution* to all well, and incubate at room temperature for 15 minutes in the dark.
- K)** Add 100 micro L of ⑧ *Stop Solution* to all well, mix gently, wait for 3 minutes, and measure the absorbance at 450 nm.

**(Fig.2 Typical layout of microtiter plate for triplicate assay.)**

To avoid edge effects, the use of outmost wells (Rows A and H, marked as "X") is not recommended. 54 wells(18 samples x 3) are applicable for test samples.

|          | 1                | 2 | 3 | 4        | 5 | 6 | 7         | 8 | 9 | 10        | 11 | 12 |
|----------|------------------|---|---|----------|---|---|-----------|---|---|-----------|----|----|
| <b>A</b> | Blank wells(n=3) |   |   | X        | X | X | X         | X | X | X         | X  | X  |
| <b>B</b> | ⑨Standard-A      |   |   | Sample-1 |   |   | Sample-7  |   |   | Sample-7  |    |    |
| <b>C</b> | ⑨Standard-B      |   |   | Sample-2 |   |   | Sample-8  |   |   | Sample-8  |    |    |
| <b>D</b> | ⑨Standard-C      |   |   | Sample-3 |   |   | Sample-9  |   |   | Sample-9  |    |    |
| <b>E</b> | ⑨Standard-D      |   |   | Sample-4 |   |   | Sample-10 |   |   | Sample-10 |    |    |
| <b>F</b> | ⑨Standard-E      |   |   | Sample-5 |   |   | Sample-11 |   |   | Sample-11 |    |    |
| <b>G</b> | ⑨Standard-F      |   |   | Sample-6 |   |   | Sample-12 |   |   | Sample-12 |    |    |
| <b>H</b> | X                | X | X | X        | X | X | X         | X | X | X         | X  | X  |

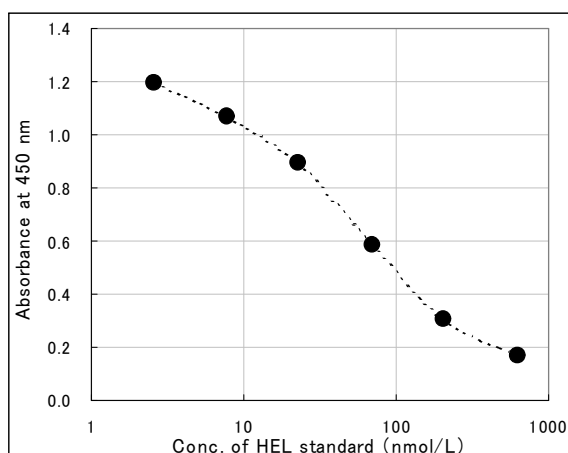
## 6. Procedure flow chart.



## 7. Standard curve and calculations.

Generate the standard curve by plotting absorbance vertical axis and log of concentration as the horizontal axis. An example is shown in Fig.3. Any smooth curve fit may be applicable. Please note that the standard curve should be established for every assay.

[Fig.3] A standard curve example.



|                 | HEL conc. (nmol/L) | Absorbance(450nm) |
|-----------------|--------------------|-------------------|
| ⑨HEL Standard-A | 2.6                | 1.197             |
| -B              | 7.7                | 1.070             |
| -C              | 22.7               | 0.895             |
| -D              | 69.7               | 0.586             |
| -E              | 207                | 0.305             |
| -F              | 624                | 0.166             |

## 8. References.

- 1) Yoji Kato, Yoko Mori, Yuko Makino, Yasujiro Morimitsu, Sadayuki Hiroi, Toshitsugu Ishikawa, Toshihiko Osawa.  
Formation of N<sup>ε</sup>-(hexanonyl) lysine in protein exposed to lipid hydroperoxide.  
Journal of Biological Chemistry, Vol. 274, No. 29, pp. 20406 – 20414, 1999
- 2) Yoji Kato, Yoshiaki Miyake, Kanefumi Yamamoto, Yoshiharu Shimomura, Hiroto Ochi, Yoko Mori, Toshihiko Osawa.  
Preparation of a monoclonal antibody to N<sup>ε</sup>-(hexanonyl) lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle.  
Biochem. Biophys. Res. Commun., Vol. 274(2), pp389-393, 2000

## 9. Technical support.

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