Step 1. Required Equipment and Materials

- ☐ Microplate reader (450 nm filter)
- ☐ Pipettes (2-20 µl, 20-200 µl, 100-1000 µl)
- ☐ Multi-channel pipette
- ☐ 96-well microplate
- ☐ Incubator (37℃)
- ☐ Disposable syringe (1 ml)

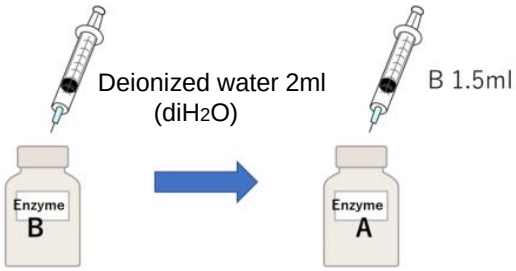
Step 2. Preparation of Working Solution and Sample

< Kit Contents >

- Substrate Buffer x1
- Enzyme A x1
- Enzyme B x1
- Enzyme C x1
- Coenzyme x1
- Indicator Solution x1

< Preparation of Working Solution >

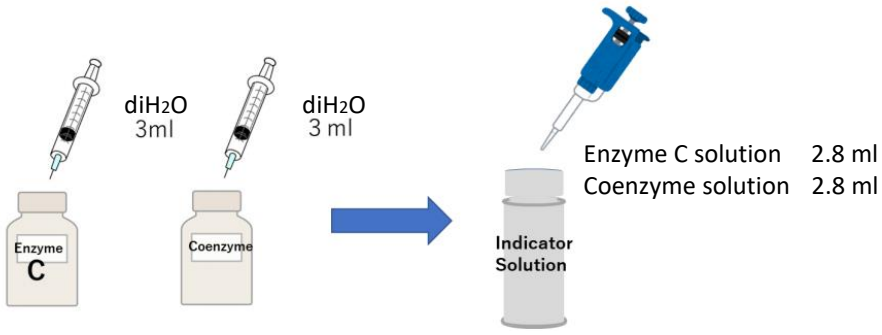
- **Enzyme Working Solution**



1) Add 2 ml of deionized water to Enzyme B vial to prepare Enzyme B solution.
2) Add 1.5 ml of Enzyme B solution to Enzyme A vial to prepare Enzyme Working Solution.

❖ Enzyme A and B vials are capped under vacuum pressure.
❖ If septum is removed without adding the solution, contents will disperse due to vacuum pressure. Please add deionized water or solution through a rubber septum with a syringe, and then remove the septum.
❖ Please follow the order or the absorbance will be lower.
❖ The Enzyme working solution is stable at -20℃ for 2 weeks.
If store in a refrigerator, stable for 3 days.

- **Indicator Working Solution**



1) Add 3 ml of deionized water to Enzyme C vial.
2) Add 3 ml of deionized water to Coenzyme vial.
3) Add 2.8 ml of Enzyme C solution and 2.8 ml of Coenzyme solution to Indicator Solution to prepare Indicator Working Solution.

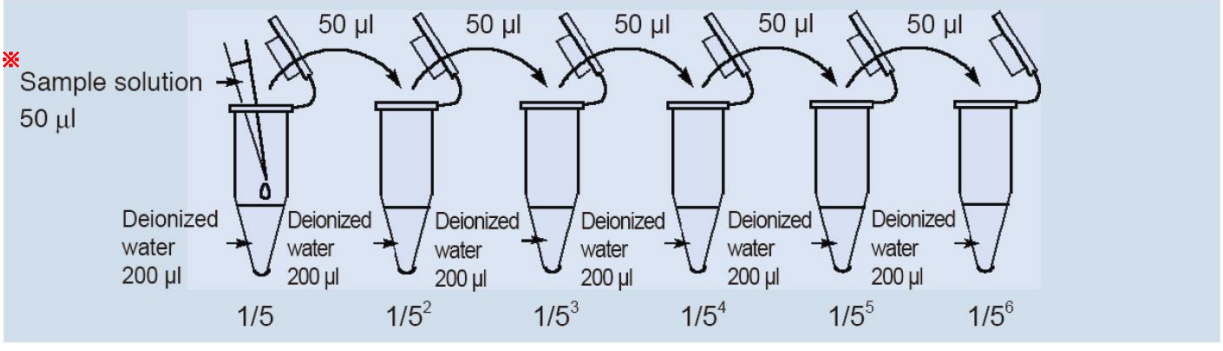
❖ If the septum is removed without adding the solution, contents of the vial will disperse due to vacuum pressure. Enzyme C and Coenzyme vials are capped under vacuum pressure. Add deionized water through a rubber septum with a syringe, and then remove the septum.

❖ The Indicator working solution is stable at -20℃ for 2 weeks. If stored in a refrigerator, it is stable for 3 days.

< Preparation of Sample Solution >

Dilute the Sample solution with deionized water.

Dilution ratio : 1(without dilution), 1/5, $1/5^2$, $1/5^3$, $1/5^4$, $1/5^5$, $1/5^6$



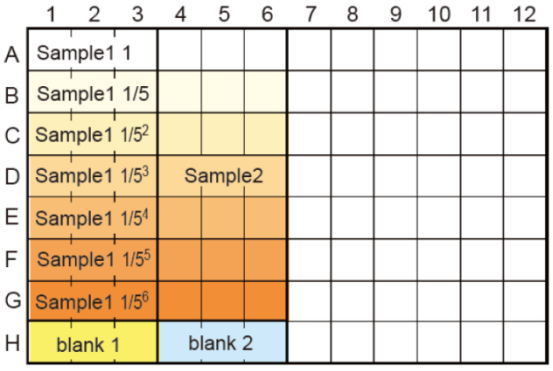
※ If the sample is not water soluble, please use DMSO or ethanol to prepare the Sample solution. Then, add buffer to perform serial dilution. Please make the initial Sample solution's DMSO or ethanol under 1%.

❖ If the sample is high in acidity due to such as citric acid or acetic acid, the content in the Substrate Buffer will precipitate and there will be no absorbance. Please adjust the pH to 5 or above for measurement.

❖ Sample with ascorbic acid will reduce Indicator solution, so there will be discrepancy. Please have less than 0.01% ascorbic acid.

Step 3. Measurement

❖ For 50 tests unit size, 2 samples can be tested in triplicate as illustrated in the following plate layout



Example of the Plate Layout (n=3)

Table. Addition Sequence & Amount of Each Solution

Addition Sequence

	Sample	blank 1	blank 2
Sample solution	20 µl	-	-
Deionized water	-	20 µl	40 µl
Substrate buffer	20 µl	20 µl	20 µl
Enzyme working solution	20 µl	20 µl	
Indicator working solution	200 µl	200 µl	200 µl

blank 1: positive control (without ACE inhibition)
blank 2: reagent blank

❖ If the solution has a strong color, such as yellow to red, that may affect the 450 nm absorption measurement reading. If so, please prepare a sample blank (sample 20 ul + deionized water 240 ul) for each serial dilution. After measuring both the sample blank and the sample absorption for each serial dilution, please subtract sample blank absorption from the sample absorption.

※ Please refer to the figure (Example of the Plate Layout) and the table (Addition Sequence & Amount of Each Solution).

1) Add 20 µl of sample solution to a sample well and 20 µl of deionized water to blank 1 and blank 2 wells.
2) Add 20 µl of Substrate buffer to each well.
3) Add 20 µl of deionized water to blank 2 well.
4) Add 20 µl of Enzyme working solution to each sample well and blank 1 well.

* Since the enzymatic reaction starts immediately after the addition of the Enzyme working solution, use a multichannel pipette to minimize the well-to-well time lag.

5) Incubate at 37 °C for 1 hour.
6) Add 200 µl of Indicator working solution to each well.
7) Incubate at room temperature for 10 minutes.
8) Read the absorbance at 450 nm with a microplate reader.
9) ACE inhibitory activity can be calculated by the following equation.

ACE inhibitory activity (inhibition rate %) = [($A_{blank\,1} - A_{sample}$) / ($A_{blank\,1} - A_{blank\,2}$)] X 100

Step 4. Determination of IC50 (50% inhibitory concentration)

1) Prepare an inhibition curve using the sample concentration for X axis and the ACE inhibitory activity for Y axis. A typical inhibition curve is shown below.
2) Determine the concentration of the sample solution that gives 50% ACE inhibitory activity as indicated in the figure.
3) Since the total volume of the inhibition assay is 60 ul (first step of the assay), the original sample is diluted 3 times in the reaction. Therefore, the concentration of the sample at 50% inhibition is one third of the concentration, which is determined by the inhibition curve.