

For Research Use Only
Reagent for assay of kinin

MARKIT-M Bradykinin

O Introduction

Kallidinogenase as a cardiovascular active substance is one of kininogenases which release kinins by specifically acting on kininogens. It has been widely used in the medical treatment as a cardiovascular drug such as an antihypertensive drug. It is generally acknowledged that the measurement of kinin-degrading enzymes (kininases) and kinin-releasing activity in the bulk substance or preparations is important in the process and quality control in the manufacture of kallidinogenase preparations. Kininases are determined by the bioassay method with bradykinin (BK) as a substrate using the rat uterus or guinea pig ileum. On the other hand, for the measurement of kinin-releasing activity, the bioassay method is used in which the formed kinin is determined using kininogen as a substrate. However, these bioassay methods are complicated in procedure and require a great deal of labor. Therefore, we developed the kit for the assay of BK, MARKIT-A Bradykinin, based on enzyme immunoassay (EIA). However, as this method uses test tubes and requires two centrifugation steps, an improvement of the assay method has been awaited. After intensive studies, we have now developed **MARKIT-M Bradykinin**, a new kit for the assay of BK, which uses microstrip wells.

O Characteristics

- 1) MARKIT-M Bradykinin is a kit for the determination of BK based on EIA using microstrip wells.
- 2) MARKIT-M Bradykinin permits a more convenient assay using a usual ELISA instrumental system (Microplate reader, Microplate washer, etc).
- 3) MARKIT-M Bradykinin is highly efficient in determining multiple samples.
- 4) The sensitivity (lower limit of detection) of MARKIT-M Bradykinin is 0.173 ng/ml (7.2pg/well).
- 5) MARKIT-M Bradykinin can determine BK concentration in samples specifically and accurately.

O Contents

Each kit (96 assays) contains the following reagents:

Bradykinin Standard (Lyophilized)	: 1 vial (For 1 ml)
One vial contains : Bradykinin 100 ng	
Buffer solution A	: 1 bottle (30 ml)
Deproteinizing reagent	: 1 bottle (15 ml)
Each ml contains : Trichloroacetic acid 200 mg	
Buffer solution B	: 1 bottle (30 ml)
Bradykinin antibody (Lyophilized)	: 1 vial (For 15 ml)
One vial contains : Anti-Bradykinin antibody (rabbit) concentrate 0.15 ml	
Anti-rabbit IgG coated wells	: 1 plate (96 wells)
12H8 microtiter strips with Anti-rabbit IgG antibody (goat) precoated on each well	
Wash buffer concentrate	: 2 bottles (30 ml each)
Bradykinin enzyme conjugate (Lyophilized)	: 1 vial (For 8 ml)
One vial contains : Peroxidase (horseradish) labeled Bradykinin concentrate 0.08 ml	
Substrate tablet	: 1 pack (2 tablets)
Each tablet contains : <i>o</i> -Phenylenediamine dihydrochloride 13 mg	
Substrate diluent buffer	: 2 bottles (15 ml each)
Each bottle contains : Hydrogen peroxide 9.75 :1	
Stop reagent	: 1 bottle (15 ml)

Two sheets of graph paper are appended for preparing standard curves.

O Application

Determination of Bradykinin

O Principle

- 1) BK in a sample and peroxidase labeled BK are allowed to react competitively to Anti-BK antibody (rabbit) captured by Anti-rabbit IgG antibody (goat) coated on microstrip well.
- 2) The BK concentration is determined from the enzyme activity of peroxidase labeled BK bound to Anti-BK antibody.

O Assay Method

1. Instruments and equipments required

Pipettes, Multichannel pipettes and Reservoirs, Measuring pipettes (1~10 ml), Graduated cylinder (500 ml), Centrifuge (centrifugal force : more than 1,000 x g), Microplate mixer, Microplate reader, Microplate washer.

Others : Plastic tubes for pretreatment, Stopwatch, Paper towel, Aluminum foil, etc.

2. Preparation of reagents

(1) Standard solutions

- 1) Provide seven test tubes.
- 2) Place exactly 1.0 ml of purified water in a vial of Bradykinin Standard, mix gently to dissolve the contents completely, and transfer into a test tube to use as Standard solution 5,000 (pg/well).
- 3) Transfer 1.5 ml of Buffer solution A to a test tube, add 0.5 ml of Standard solution 5,000, then mix to prepare Standard solution 1,250.
- 4) With similar dilution procedures, prepare Standard solution 313 from 1.5 ml of Buffer solution A and 0.5 ml of Standard solution 1,250, Standard solution 78 from 1.5 ml of Buffer solution A and 0.5 ml of Standard solution 313, Standard solution 19.5 from 1.5 ml of Buffer solution A and 0.5 ml of Standard solution 78, and Standard solution 4.9 from 1.5 ml of Buffer solution A and 0.5 ml of Standard solution 19.5.
- 5) Transfer 1 ml of Buffer solution A to a test tube, and use it as Standard solution 0.
- 6) Keep frozen these Standard solutions when preserved.

(2) Bradykinin antibody solution

Place exactly 15 ml of purified water to a vial of Bradykinin antibody and dissolve the contents completely. (This Bradykinin antibody solution is stable for at least 1 week when stored frozen.)

(3) Wash buffer

Transfer the whole volume of Wash buffer concentrate to a 500 ml graduated cylinder and add purified water to make 300 ml. (Store this Wash buffer in a refrigerator and use within 1 week.)

(4) Buffer solution C

Place 1.5 ml of purified water and 0.3 ml of Deproteinizing reagent in a test tube, mix, then add 1.8 ml of Buffer solution B and mix to prepare Buffer solution C.

(5) Bradykinin enzyme conjugate solution

Place exactly 8 ml of purified water in a vial of Bradykinin enzyme conjugate and dissolve the contents completely. (Store this Bradykinin enzyme conjugate solution in a refrigerator and use within 1 week.)

(6) Substrate solution

Place one Substrate tablet in one bottle of Substrate diluent buffer and dissolve the tablet to prepare a Substrate solution. Prepare immediately before use and protect from light after preparation. (Discard the residue after use. Preservation is not allowed.)

3. Pretreatment of samples

The following methods are recommended.

(1) Urine Sample

Transfer 500 μ l of a urine sample to a plastic tube, add 100 μ l of Deproteinizing reagent, and mix, then centrifuge at 3,000 rpm (1,000-1,500 x g) for 10 minutes at 4°C. Transfer 250 μ l of the resulting supernatant into another plastic tube, then add 250 μ l of Buffer solution B and mix to prepare a pretreatment sample.

(2) Blood Sample

Aspirate 5 ml of venous blood from medial cubital vein or arterial blood from brachial artery with plastic syringe without anti-coagulant, and immediately (within 10 sec.) add this collected blood to a plastic tube containing 20 ml of ice-cooled absolute ethanol (HPLC grade) after removal of needle from the plastic syringe and mix well for 1 min. by shaking. Centrifuge at 1,500xg at 4°C for 30 min and collect the supernatant. Re-extract the pellet with 5 ml of 80% ethanol and collect the supernatant after centrifugation. Evaporate the collected supernatant of the sample (extracts) under the reduced pressure, and add 1 ml of distilled water to resolve the residue. Adjust the pH of this solution to 2~3 with 0.1N HCl, and wash the acidified solution twice with 3 ml of diethyl ether to remove the lipids containing in the sample. After removal of diethyl ether by aspiration, evaporate the water phase under the reduced pressure to dryness. Dissolve the residue in appropriate volume of Buffer solution C, then centrifuge at 10,000xg at 4°C for 30 min.. Use the supernatant as a sample for the assay of BK.

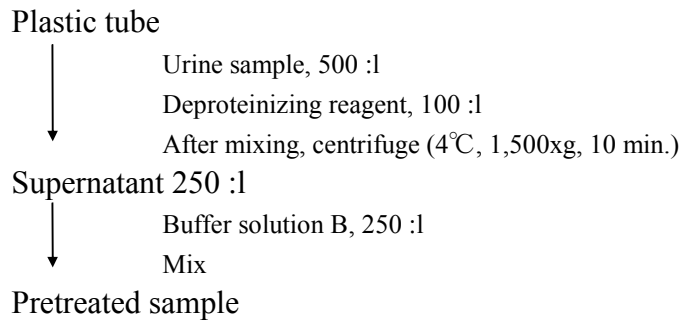
4. Procedure

- Duplicate determinations of Standard solutions are preferable.
 - Allow the wells and the reagents to come to room temperature before starting assay.
 - Keep the plate in the horizontal position during the reaction.
- (1) Take out a necessary number of Anti-rabbit IgG coated wells for determination, pipette 100 μ l of Bradykinin antibody solution into these wells, stir with a microplate mixer, then allow to stand at room temperature. After 1 hour, remove the reaction mixture with a Microplate washer and wash the wells with 300 μ l of Wash buffer. Repeat this procedure three times. Immediately proceed to the next procedure without drying the wells. (Preparation of Reaction wells)
 - (2) Add 100 μ l of Buffer solution C and 50 μ l of each Standard solution for preparing a standard curve, while 50 μ l of Buffer solution A and 100 μ l of pretreatment sample for the determination of the sample, to each Reaction well, respectively. Stir with a microplate mixer, then allow to stand at room temperature for 1 hour.
 - (3) Add 50 μ l of Bradykinin enzyme conjugate solution to each well, stir with a microplate mixer, then allow to stand overnight at 4 EC .
 - (4) Remove the reaction mixture with a Microplate washer and wash each well with 300 μ l of Wash buffer. Repeat this procedure four times. After washing, remove the residue of solution by putting the plate upside down and tapping on a paper towel. Never dry the wells completely.
 - (5) Add 100 μ l of Substrate solution to each well and allow to stand at room temperature for 30 minutes. (During this step, protect from light, for example by wrapping the plate with aluminum foil.)
 - (6) To stop the enzymatic reaction, add 100 μ l of Stop reagent at the same time interval as addition of Substrate solution.
 - (7) Stir with a microplate mixer.
 - (8) Measure the absorbance of each well at 492 nm with a Microplate reader. (It is preferable to measure at two wavelengths, i.e. primary wavelength of 492 nm and reference wavelength of 620 nm.)

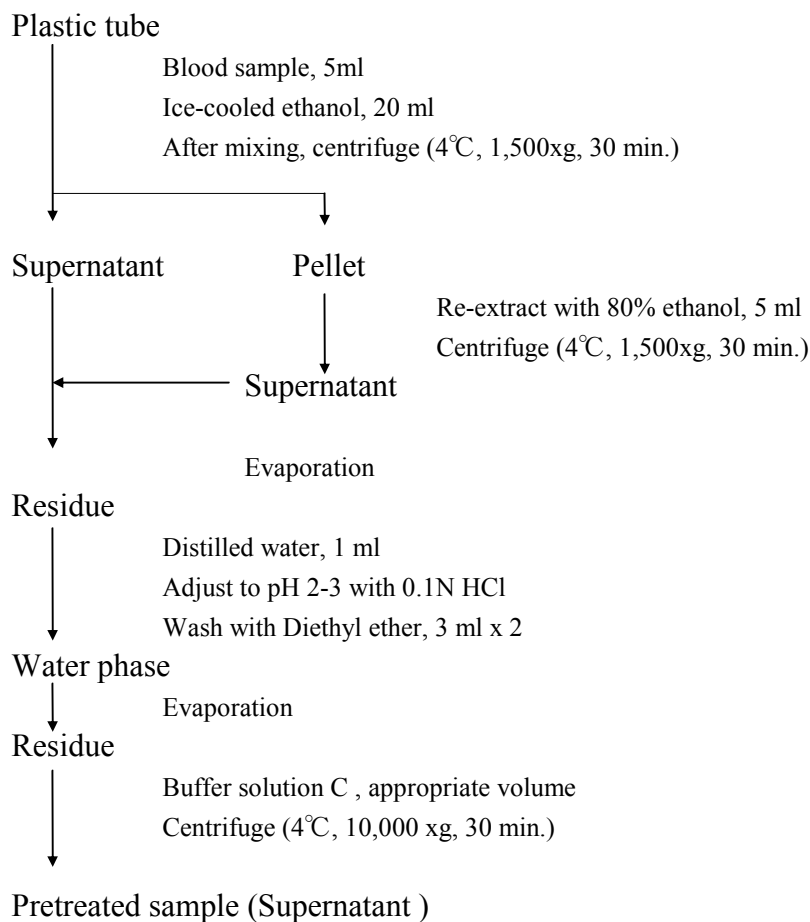
5. Outline of assay procedures

(1) Pretreatment procedure of samples

1) Urine sample



2) Blood sample



(2) Assay procedure

1) Preparation of Reaction wells

Anti-rabbit IgG coated well

9 BK antibody solution, 100 :l

After stirring, allow to stand at room temperature for 1 hour

9

Wash the well three times with 300 :l of Wash buffer

9

Reaction well

2) Assay method

	(For preparing standard curve)	(For determining sample)
Reaction well	1 well	1 well
Buffer solution C	100 :l	—
Standard solution	50 :l	—
Buffer solution A	—	50 :l
Pretreated sample	—	100 :l

↓

After stirring, allow to stand for 1 hour at room temperature (primary antigen-antibody reaction)

↓

BK enzyme conjugate solution 50 :l

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After stirring, allow to stand overnight at 4EC (secondary antigen-antibody reaction)

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Wash each well four times with 300 :l of Wash buffer

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Substrate solution 100 :l

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Allow to stand at room temperature for 30 minutes (enzymatic reaction)

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Stop reagent 100 :l

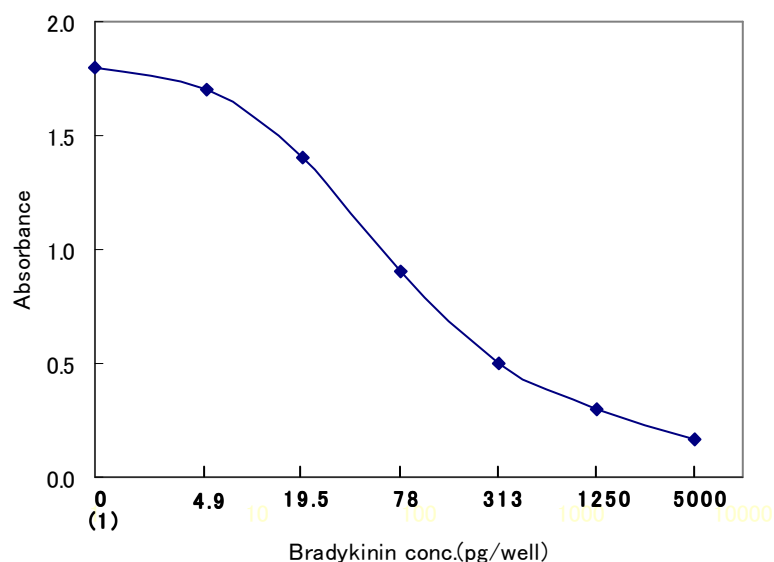
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Measure the absorbance at 492 nm

6. Preparation of a standard curve and reading of BK concentration

- (1) The abscissa (logarithmic scale) and the ordinate (uniform scale) of the graph paper (semilogarithmic paper) attached to a kit represent the concentration of Standard solution and the absorbance, respectively. Plot the absorbance of each Standard solution and draw a smooth curve nearly fitting the points.
- (2) By using the standard curve obtained, read the BK concentration corresponding to the absorbance of a pretreated sample. The BK concentration is expressed as the weight (pg) per well of BK in 100 :l of the pretreated sample. (unit : pg/well)
- (3) To calculate the BK concentration (pg/ml) of the original samples, multiply the reading values as expressed in pg/well by the following coefficient.
 - 1) Urine Sample : 24
 - 2) Blood Sample : (volume of Buffer solution C / volume of the treated blood sample) x 10
- (4) For high-concentration (more than 5,000 pg/well) samples, it is necessary to dilute the pretreated sample with Buffer solution C appropriately or to dilute the original sample with purified water appropriately for pretreatment and then to perform the procedure (1) and subsequent procedures and to convert by multiplying the reading by a dilution factor. Incidentally, you must not use the pretreated sample diluted with purified water for determination.

Typical standard curve



O Cross reactivity

Compounds	%	Compounds	%
Bradykinin (BK)	100	[1-7]-BK	<0.1
Lys-BK	100	[1-6]-BK	<0.1
Met-Lys-BK	100	[1-5]-BK	<0.1
T-Kinin	100	BK potentiator B	<0.1
Tyr-BK	100	BK potentiator C	<0.1
(Hyp ³)-BK	79	Angiotensin I	<0.1
Des-Arg ¹ -BK	45	Angiotensin II	<0.1
Des-Arg ⁹ -(Leu ⁸)-BK	<0.1	LMW-Kininogen	<0.1
[1-8]-BK	0.2	Kininogen (Bovine Plasma)	<0.1

O Precautions for use or in handling

1. General precautions

- (1) In order to obtain reliable and consistent results, the instruction in this booklet must be strictly adhered to.
- (2) Do not use the kit reagents after the date of expiry.
- (3) Do not mix the BK enzyme conjugate, the BK antibody and the Anti-rabbit IgG coated wells from different lot kits.
- (4) White powder may adhere on the Anti-rabbit IgG coated wells. This is no influence to get the results.
- (5) Be sure to handle the Deproteinizing reagent because its main ingredient, trichloroacetic acid, is very corrosive to the skin.
- (6) Be sure to handle the Stop reagent. It contains sulfuric acid.
- (7) If the kit reagents contacted with skin, mouse or eyes, irrigate with water and seek medical attention immediately.
- (8) Read well the instruction of the instruments or equipments required for the assay.
- (9) Use the kit carefully under the supervision of specialists or leaders who have enough knowledge of safety regarding biological researches and experiments.

2. Procedure cautions

- (1) It is preferable to determine samples in duplicate until you are accustomed to the procedure.
- (2) Add the reagents in the order as directed in the Assay method. Proceed with samples and Standard solutions simultaneously under the same conditions.
- (3) When two or more Substrate tablets are used simultaneously, dissolve each tablet separately in each bottle of Substrate diluent buffer, then mix in another container before use.
- (4) Do not damage or soil the bottom of the Anti-rabbit IgG coated wells. The wells are cuvettes to measure the absorbance.
- (5) Do not foam or scatter working solution in the well to prevent from contamination among the wells.
- (6) Avoid contamination among the kit reagents or contamination of microbes in the sample and the kit reagents

3. Safety cautions

(1) Virus :

As no human serum or plasma is used in this kit, the kit reagents may have no infection ability of HBV, HIV and HCV. But the kit reagents should be handled with the same precautions at any other potentially biohazardous materials.

Inactivate virus in samples, reagents and used apparatus by one of the following methods when the determination was completed.

- 1) Autoclave (121°C, 20 min./ 115°C, 30 min.)
- 2) Sink in sodium hypochlorite (available chlorine at 25,000 ppm for 30 min. to 1 hr./ at 10,000 ppm for a night)
- 3) Sink in glutaraldehyde (2% for over 1 hr.)

(2) Pipetting :

Do not use your mouth for pipetting the reagents and samples at any time.

4. Cautions for waste

The waste solution after the assay should be treated by the same method as shown in above 3.(1) and discarded with large quantities of water.

O Storage and expiry period

Storage : Store in a cool place (2-10°C) and protect from light.
Expiry period : 1 year

O Package size

MARKIT-M Bradykinin : 1 kit (for 96 assays)

O References

- 1) Uchida Y., Katori M. : Jpn. J. Clin. Med., 40 (special fall issue): p772, 1982
- 2) Uchida Y., Katori M. : Acta Haematol. Jpn., 45: p498, 1982
- 3) Tanimoto T., Fukuda H. et al. : Iyakuin Kenkyu, 16:839, 1985
- 4) Majima M., Katori M., et al. : Kinin 93 Brazil, Abstract book p62, 1993
- 5) Majima M., Katori M., et al. : Jpn. J. Pharmacol., 65: p79, 1994
- 6) Sakata Y., Akaike T., et al. : Microbiology & Immunology, 40: p415, 1996

* 1) - 3) : Available only in Japanese.

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