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Significance of measurement

Reactive oxygen species (ROS) are produced during the processes of energy production in living organisms. ROS injure DNA, cells, etc., causing various organ disorders. To eliminate ROS, our body is able to synthesize antioxidants to capture ROS. Bilirubin is an antioxidant and biopyrrin is the final oxidation metabolite generated when bilirubin reacts with ROS. Unlike other antioxidants such as vitamins C and E, biopyrrin is not reduced to bilirubin in the redox environment but is excreted into urine. For this reason, an increase of bilirubin consumed by oxidative stress results in an increase in urinary biopyrrin. Urinary biopyrrin was reported to be elevated by speech stress, and our research data suggest that bilirubin eliminates ROS generated by psychological stress. Biopyrrin is also correlated with operation duration in surgical patients. Ischemia-reperfusion elevates ROS and biopyrrin production as well. Based on above information, biopyrrin is expected as a potential real-time oxidative stress marker in response to surgical stress, ischemic heart disease, sepsis, psychosomatic disorder and psychological/social stress.

Measurement principle

This product is a competitive ELISA (Enzyme-Linked Immuno Sorbent Assay) kit using anti-bilirubin monoclonal antibody (24G7) which is specific to bilirubin and its oxidative metabolite, biopyrrin.

1. Mix test samples with horseradish Peroxidase (HRP)-conjugated antibodies to allow antibodies react with biopyrrin.
2. Reaction solution is transferred from reaction well to antigen-coated well. HRP-conjugated antibodies which do not react with biopyrrin will bind to antigens on the well.
3. Wash away unbound antibodies.
4. Add chromogenic substrate to the well. The enzyme-substrate reaction is terminated by adding a sulfuric acid solution and the absorbance is then measured spectrophotometrically.

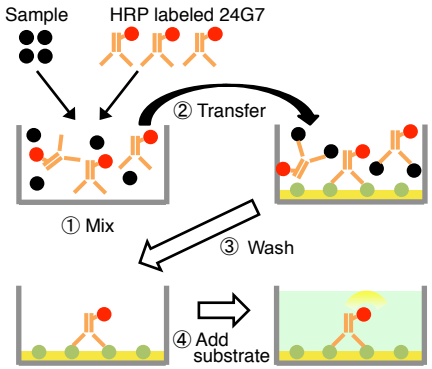


Figure 1: Schematic of the assay

Kit contents

	96 tests (product code: BP 01 DE)		
1.	Antigen-coated microplate	×1	Light shielding bag
2.	Pretreatment microplate	×1	
3.	HRP-conjugated Antibody (24G7) (100×)	0.15 mL×1	
4.	Antibody Dilution Buffer	15 mL×1	●
5.	Chromogenic Substrate (TMB)	12 mL×1	Light shielding bottle
6.	Standard Sample: bilirubin 6.4 µmol / L (Equivalent to 6.4 u / L of antibody 24G7 epitope*)	×1	Light shielding vial
7.	Sample Dilution Buffer	8 mL×1	●
8.	Washing Buffer (20×)	37.5 mL×1	●
9.	Stop Reagent (1 mol / L sulfuric acid)	12 mL×1	●
10.	Microplate sealing film	×1	
11	Brown sample tubes for serial dilutions of standard	×7	

※1 u equals to 1 µmol of anti-bilirubin monoclonal antibody 24G7 epitope.
Both Bilirubin and biopyrrin have a 24G7 epitope. Therefore, we use bilirubin which is reliable in traceability as a standard sample.

Materials Required but not supplied

- Microplate reader (Wavelength 450 nm)
- Micropipette and tip
- Multichannel pipette
- Graduated cylinder
- Sample tube
- Microplate shaker
- Paper towels
- Reservoir for multichannel pipette
- Distilled water

Handling test samples

- Use fresh samples or samples stored at -20 ºC or less. Do not use preservatives.
- If suspended matter or precipitate is observed in a test sample, centrifuge it and use the supernatant for this assay.
- Bilirubin in the test sample will cause a false positive error.
- This method does not guarantee the numerical value obtained.

Assay Protocol

※Do not expose the standard sample, antigen-coated microplate, chromogenic substrate and microplate to light during reaction

1. Reagents Preparation

(1) Standard

・Dissolve standard powder
Add the amount of sample dilution buffer indicated on the label of the standard sample bottle. Let stand for 30 minutes until the powder is completely dissolved. After dissolved, store it in a refrigerator or in a freezer if you do not use it up on the same day.

・The serial dilutions for standard curve (use supplied brown sample tubes)
The standard curve is prepared using serial dilutions of 2-fold (3.2 u / L), 4-fold (1.6 u / L), 8-fold (0.8 u / L), 16-fold (0.4 u / L), 32-fold dilution (0.2 u / L) and 64-fold dilution (0.1 u / L).

(2) Preparation of samples
Dilute samples more than 4 times with sample dilution buffer.

(3) Preparation of HRP-conjugated antibody (24G7) reagent
Add the total amount of HRP-conjugated antibody (24G7) (100 ×) to the bottle of antibody dilution buffer.

(4) Preparation of washing buffer
Dilute the supplied washing buffer (20 ×) 1:20 with distilled water.

2. Measurement method

(1) Add 20 µL of the serial dilutions for standard curve and test samples to pretreatment microplate (2 wells for each sample).

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	▲	▲	○	○	○	○	○	○
B	○	○	○	○	❶	❶	○	○	○	○	○	○
C	○	○	○	○	❷	❷	○	○	○	○	○	○
D	○	○	○	○	❸	❸	○	○	○	○	○	○
E	○	○	○	○	❹	❹	○	○	○	○	○	○
F	○	○	○	○	❺	❺	○	○	○	○	○	○
G	○	○	○	○	❻	❻	○	○	○	○	○	○
H	○	○	○	○	❼	❼	○	○	○	○	○	○

▲ : Sample dilution buffer
● : Standard (Bilirubin concentration)
❶ : 0.1 u/L
❷ : 0.2 u/L
❸ : 0.4 u/L
❹ : 0.8 u/L
❺ : 1.6 u/L
❻ : 3.2 u/L
❼ : 6.4 u/L
○ : Sample

Figure 2 : Microplate layout (example)

(2) Add 120 µL of HRP-conjugated antibody (24G7) reagent to each well of (1) and mix well by pipetting. Attach a microplate sealing film and incubate for 1hour at room temperature in the dark.

(3) Just before the end of the reaction time in (2), remove the package of antigen-coated microplate and wash it with washing buffer (1 ×).

※ Wash steps
A) Add 300 µL of washing buffer to each well.
B) Discard the washing buffer.
C) Repeat A) and B) twice (3 times in total).
D) Drain the solution completely by tapping the microplate on a stack of paper towels.

(4) Add 100 µL of the reaction solution from (2) to the washed (3) microplate. Shake it on a microplate shaker and let it reacts for 30 minutes at room temperature.

(5) Discard the reaction solution from (4) and wash the well as described in (3).

(6) Add 100 µL of chromogenic substrate to each well of (5). Shake it on a microplate shaker and let it reacts for 30 minutes at room temperature.

(7) Add 100 µL of stop reagent to each well of (6).

(8) Measure the absorbance at 450 nm.

3. Calculation of measured value

(1) Calculate the average value of absorbance for each test sample.

(2) Plot the absorbance against the biopyrrin concentration of the standard samples and create a standard curve.

(3) Read the biopyrrin concentration of test samples from the standard curve.

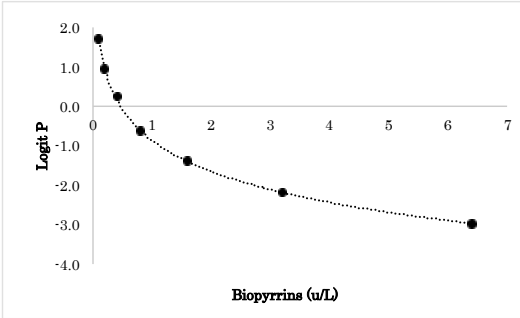
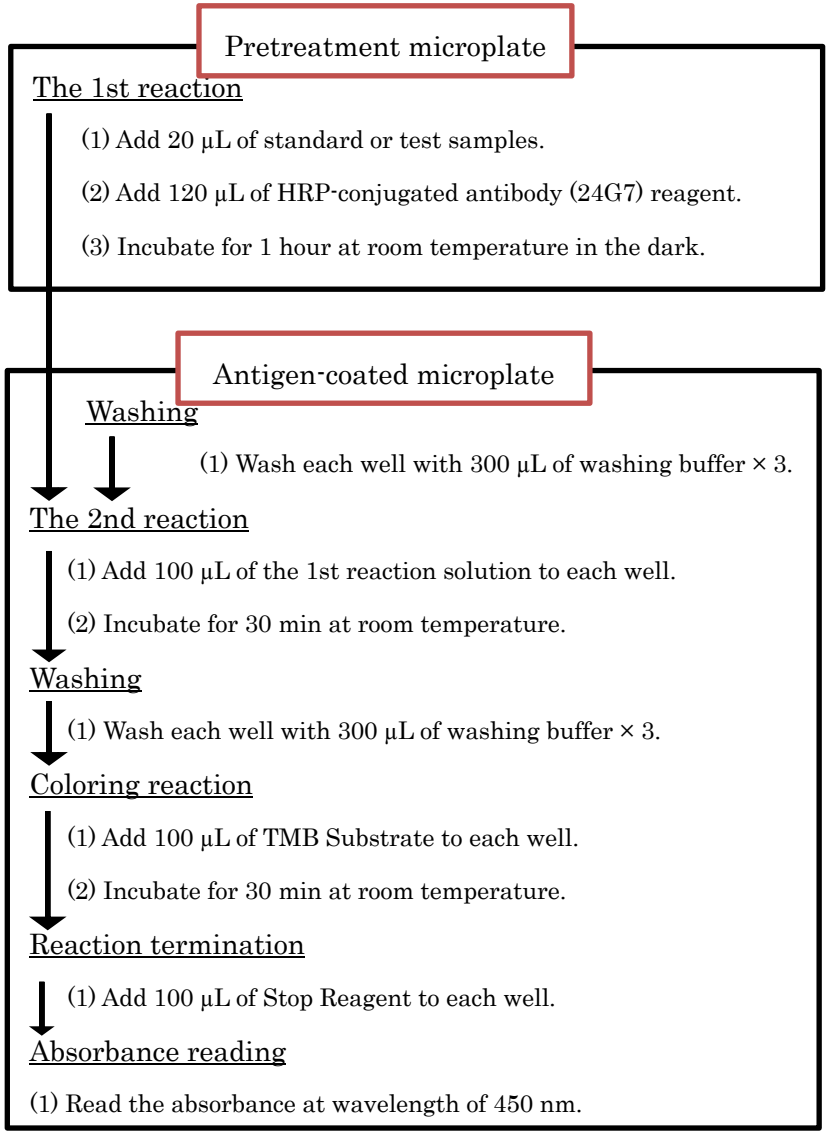


Figure 3: Standard curve (example)

FLOWCHART OF BIOPYRRIN ELISA ASSAY



Measurement note

1. Handling of test samples
   - Samples should be measured freshly or stored frozen (-30 ºC or less, avoid from light) immediately after collection.
   - Bilirubin in the test sample will cause a false positive error.
   - If suspended matter or precipitate is observed in a test sample, centrifuge it and use the supernatant for this assay.

2. Measurement
   - Do not expose the standard sample, antigen-coated microplate, chromogenic substrate, and microplate to light during reaction.
   - Do not use reagents of different lots.
   - Create a standard curve for each measurement.
   - Do not let antibody-coated microplate dry after washing until measurement is completed.
   - The bottom surface of microplate is immobilized with antibody which might be shed by contact with the pipette. Please make sure the pipette does not touch the bottom or wall of the microplate.
   - Unevenness of temperature in microplate may cause variations in measured values.
     A) Be sure to allow the reagents and microplates to warm to room temperature (20 to 25 °C) before use.
     B) Always perform reactions at room temperature. Indoor places may also have temperature unevenness due to wind blowing or so. Please do not use this kit in places where hot air or cold air may occur, such as the vicinity of air outlet of air conditioner, places closed to windows, etc.
     C) Long time of touch may warm the microplate and results in a temperature difference in the microplate. Please do not touch the microplate as possible.
   - Please drop the reagents in the exact chronological order and take the reaction time accurately.
   - Stop solution is a strong acid. Please handle it with care.

3. Precautions when using divided kit
   - If you do not use up the standard sample on the same day, please close the cap tightly and freeze it. Please discard the diluted standard samples.
   - Reagents other than the standard sample should be tightly closed and kept in a refrigerator.
   - Please keep unused antigen-coated microplates enclosed with desiccant and store it in dark places in chuck bags.
   - Reagents other than the standard sample and antigen-coated microplate can be stored in a refrigerator for one week after opening.

Product Specifications

Measurement range: 0.1 to 6.4 u / L
(1 u equals to 1 µmol of anti-bilirubin monoclonal antibody 24G7 epitope)
Numbers of test: 96 wells/microplate
Measurement method: ELISA method
Measured wavelength: 450 nm
Test sample: urine

References

1.) Yamaguchi, T., Shioji, I., Sugimoto, A., Komoda, Y., & Nakajima, H. Epitope of 24G7 anti-bilirubin monoclonal antibody. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1289* (1), 110-114. 1996

Manufacturer and distributor

Metallogenics Co., Ltd.
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* Redox assay TM is the name of the reagent kit of Metallogenics Co., Ltd.

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※ This product is for research use. Please understand that we cannot guarantee the values perfectly.
※ The noted performance is the standard value when using a general microplate reader. Please understand that variation may occur depending on the types of equipment.
※ When inquiring about quality, please contact us after confirming the lot number affixed to the reagent kit packaging bag.
※ Product specifications · service · packaging form · measurement protocols may be changed without notice. Please follow this instruction manual properly.
※ Please follow the attached Material Safety Data Sheet (MSDS) for transportation, handling, processing, disposal of this product.