Code No. 27168

Human Big Endothelin-1 Assay Kit - IBL

INTRODUCTION

Endothelins (ETs) are isopeptides produced by vascular endothelium having potent vasoconstriction activity. The peptides are encoded by three separate genes and processed to yield 39 residue Big Endothelin (Big ET) molecule, which are further processed to the 21 amino acid sequences termed Endothelin-1 (ET-1), Endothelin-2 (ET-2) and Endothelin-3 (ET-3). All of members of the endothelin family contain two essential disulfide bridges and size conserved amino acid residues at the C-terminus. The ETs are produced by a variety of issues in vivo, including lung, kidney, brain, pituitary and placenta (ref.1).

PRINCIPLE

This kit is a solid phase sandwich ELISA using 2 kinds of high specific antibodies. Tetra Methyl Benzidine (TMB) is used as coloring agent (Chromogen). The strength of coloring is in proportion to the quantities of Human Big ET-1.

MEASUREMENT RANGE

0.78 ~ 100 pg/mL

INTENDED USE

The IBL's Human Big ET-1 EIA Kit is a complete kit for the quantitative determination of Human Big ET-1 in serum, EDTA-plasma, supernatant of cell culture media and extract from tissue.

KIT COMPONENT

1	Precoated plate	: Anti- Big ET$^{22-38}$ Rabbit IgG Affinity Purify	96Well x 1
2	Labeled antibody Conc.		
		: (30X) HRP conjugated Anti-ET-1 Rabbit IgG Fab' Affinity Purify	0.4mL x 1
3	Standard	: Human Big ET-1 (Peptide)	0.5mL x 2
4	EIA buffer		30mL x 1
5	Solution for Labeled antibody		12mL x 1
6	Chromogen	: TMB solution	15mL x 1
7	Stop solution		12mL x 1
8	Wash buffer Conc.		50mL x 1

OPERATION MANUAL

1. Materials needed but not supplied

- Plate reader (450nm)
- Micropipette and tip
- Graduated cylinder and beaker
- Distilled water
- Refrigerator(as 4℃)
- Graph paper (log/log)
- Paper towel
- Tube for dilution of Standard
- Plate washer or washing bottle*
- Disposable test tube for "2, Labeled antibody Conc." and "6, Chromogen"

2. Preparation

1) Preparation of wash buffer
"8, Wash buffer Conc." is a concentrated (X40) buffer. The temperature of "8, Wash buffer Conc." shall be adjusted to room temperature and then, mix it gently and completely before use. Dilute 50mL of "8, Wash buffer Conc." with 1,950mL of distilled water and mix it. This is the wash buffer for use. This prepared wash buffer shall be stored in refrigerator and used within 2 weeks after dilution.

2) Preparation of Labeled antibody
"2, Labeled antibody Conc." is a concentrated (X30). Dilute "2, Labeled antibody Conc." with "5, Solution for Labeled antibody" in 30 times according to required quantity into a disposable test tube. Use this resulting solution as Labeled antibody.
Example)
In case you use one slit (8 well), the required quantity of Labeled antibody is 800 μL. (Dilute 30 μL of "2, Labeled antibody Conc." with 870 μL of "5, Solution for Labeled antibody" and mix it. And use the resulting solution by 100 μL in each well.)
<u>This operation should be done just before the application of Labeled antibody.</u>
The remaining "2, Labeled antibody Conc." should be stored at 4℃ in firmly sealed vial.

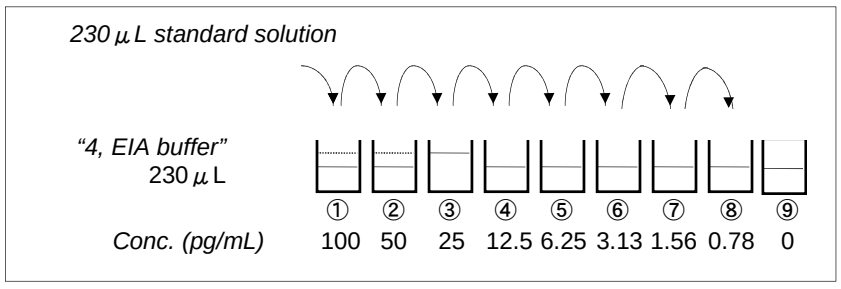
3) Preparation of Standard
Put just 0.5mL of distilled water into the vial of "3, Standard" and mix it gently and completely. This solution is 200 pg/mL Human Big ET-1 standard.

4) Dilution of Standard
Prepare 9 tubes for dilution of "3, Standard". Put 230 μL each of "4, EIA buffer" into the tube.
Specify the following concentration of each tube.

Tube -1	100 pg/mL
Tube -2	50 pg/mL
Tube -3	25 pg/mL
Tube -4	12.5 pg/mL
Tube -5	6.25 pg/mL
Tube -6	3.13 pg/mL
Tube -7	1.56 pg/mL
Tube -8	0.78 pg/mL
Tube -9	0 pg/mL (Test Sample Blank)

Put 230 μL of Standard solution into tube–1 and mix it gently. Then, put 230 μL of tube-1 mixture into tube-2. Dilute two times standard solution in series to set up 8 points of diluted standard between 100 pg/mL and 0.78 pg/mL. "4, EIA buffer" is the test sample blank as 0 pg/mL.

See following picture.



5) Dilution of test sample
Test sample may be diluted with "4, EIA buffer" if the need arises.
It is necessary to pre-extraction procedure by Sep-Pak C-18 column if you would like to apply serum, plasma or tissue samples. (see "Attention for sample handling" at the next page).

3. Measurement procedure

All reagents shall be brought to room temperature approximately 30 minutes before use. Then mix it gently and completely before use. Confirm no change in quality of the reagents. Standard curve shall be prepared simultaneously with the measurement of test samples.

Reagents	Test Sample	Standard	Test Sample Blank	Reagent Blank
	Test sample 100 μL	Diluted standard (Tube 1~8) 100 μL	EIA buffer (Tube -9) 100 μL	EIA buffer 100 μL
Incubation for overnight at 4℃ with plate lid				
4 times (wash buffer more than 350 μL) (Refer to No. 8 and 9 described in OPERATING PRECATION.)*				
Labeled Antibody	100 μL	100 μL	100 μL	-
Incubation for 30 minutes at 4℃ with plate lid				
5 times (wash buffer more than 350 μL) (Refer to No. 8 and 9 described in OPERATING PRECATION.)*				
Chromogen	100 μL	100 μL	100 μL	100 μL
Incubation for 30 minutes at room temperature (shielded)				
Stop solution	100 μL	100 μL	100 μL	100 μL
Read the plate at 450nm against a Reagent Blank within 30 minutes after application of Stop solution.				

1) Determine wells for reagent blank. Put 100 μL each of "4, EIA buffer" into the wells.
2) Determine wells for test sample blank, test sample and diluted standard. Then, put 100 μL each of test sample blank (Tube-9), test sample and dilutions of standard (Tube-1~8) into the appropriate wells.
3) Incubate the precoated plate for overnight at 4℃ after covering it with plate lid.
4) Washing (Refer to No. 8 and 9 described in OPERATING PRECATION.)* Wash the plate with the prepared wash buffer and remove all liquid.
5) Pipette 100 μL of Labeled antibody into the wells of test samples, diluted standard and test sample blank.
6) Incubate the precoated plate for 30 minutes at 4℃ after covering it with plate lid.
7) Washing (Refer to No. 8 and 9 described in OPERATING PRECATION.)* Wash the plate with the prepared wash buffer and remove all liquid.
8) "6, Chromogen" should be taken the required quantity into a disposable test tube. Then, pipette 100 μL from the test tube into the wells. Please avoid to return the rest of test tube into "6, Chromogen" bottle due to avoid to cause of contamination.
9) Incubate the precoated plate for 30 minutes at room temperature in the dark. The liquid will turn blue by the addition of "6, Chromogen".
10) Pipette 100 μL of "7, Stop solution" into the wells. Mix the liquid by tapping the side of precoated plate. The liquid will turn yellow by the addition of "7, Stop solution" .
11) Remove any dirt or drop of water on the bottom of the precoated plate and confirm there is no bubble on the surface of the liquid. Then, run the plate reader and conduct measurement at 450nm.
The measurement shall be done within 30minutes after the addition of "7, Stop solution" .

OPERATING PRECATION*

1. Test samples should be measured soon after collection. For storage of samples, store them frozen and do not repeat freeze/thaw cycles. Thaw the test samples at a low temperature and mix them completely before measurement.
2 Test samples should be diluted with "4, EIA buffer" contained in this kit.
3 Duplicate measurement of test samples and standards is recommended.
4 Standard curve should run for each assay.
5 Use test samples in neutral pH range. The contaminations of organic solvent may affect the measurement.
6 All reagents should be brought to room temperature (R.T.) and mixed completely and gently before use. After mixing them, make sure of no change in quality of the reagents.
7 Use only "8, Wash buffer conc." contained in this kit for washing the precoated plate. Insufficient washing may lead to the failure in measurement.
8 Using a plate washer is recommended (wait time zero second). It should be washed by a plate washer immediately after each reaction. If you use a washing bottle instead of a plate washer, after filling wash buffer in each well, immediately turn the plate upside down and shake it off to completely remove the wash buffer. Repeat the number of times of wash defined in a table for measurement procedure described in section 3. It should be properly washed off as instructed in order to avoid any insufficient wash.

9 Carefully tap the plate against a clean paper towel without contacting with inside of each well to completely remove the washing buffer after repeated the determined number of wash.
10 “6, Chromogen - TMB solution” should be stored in the dark due to its sensitivity against light. It should be also avoided contact with metals. Required quantity should be prepared into a collecting container for each use.
11 After adding TMB solution into the wells, the liquid in the wells gradually changes the color in blue. In this process the plate should be in dark. Remained TMB solution in the collecting container should not be returned into the original bottle of TMB solution to avoid contamination.
12 Measurement of O.D. should be done within 30 minutes after addition of “7, Stop solution”.

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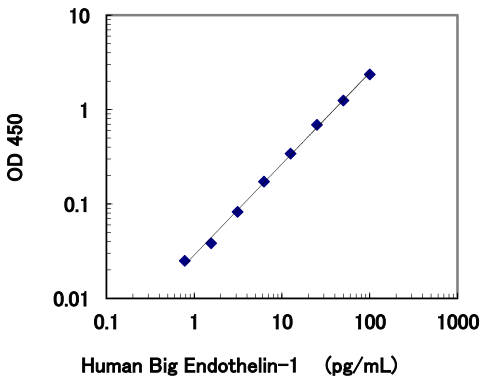
CALCULATION OF TEST RESULT

Subtract the absorbance of test sample blank from all data, including standards and unknown samples before plotting. Plot the subtracted absorbance of the standards against the standard concentration on log-log graph paper. Draw the best smooth curve through these points to construct the standard curve. Read the concentration for unknown samples from the standard curve.

Example of standard curve

Conc. (pg/mL)	Absorbance (450nm)
100	2.481
50	1.370
25	0.812
12.5	0.463
6.25	0.293
3.13	0.203
1.56	0.159
0.78	0.146
0 (Test Sample Blank)	0.121



The typical standard curve is shown above. This curve can not be used to derive test results. Please run a standard curve for each assay.

PERFORMANCE CHARACTERISTICS

1. Titer Assay (Samples with standard added are used.)

Specimen	Titer (X)	Measurement Value (pg/mL)	Theoretical Value (pg/mL)	%
10% FCS added RPMI-1640	2	43.34	50.00	86.7
	4	23.66	25.00	94.7
	8	12.73	12.50	101.8
Human Serum	4	15.78	25.00	63.1
	8	8.57	12.50	68.6
	16	5.10	6.25	81.5
Human Plasma (EDTA)	4	20.98	25.00	83.9
	8	11.90	12.50	95.2
	16	6.30	6.25	100.8

2. Added Recovery Assay

Specimen	Theoretical Value (pg/mL)	Measurement Value (pg/mL)	%
10% FCS added RPMI-1640 (x2)	50	47.02	94.0
	25	25.02	100.1
	12.5	13.14	105.1
Human Serum (x16)	50	28.88	57.8
	25	16.88	67.5
	12.5	8.74	69.9
Human Plasma (EDTA) (x8)	50	36.52	73.0
	25	21.06	84.2
	12.5	10.55	84.4

3. Intra - Assay

Measurement Value (pg/mL)	SD value	CV value (%)	n
59.35	1.15	1.9	20
15.86	0.40	2.5	20
5.44	0.25	4.6	20

4. Inter - Assay

Measurement Value (pg/mL)	SD value	CV value (%)	n
57.98	2.82	4.9	28
14.86	1.50	10.1	28
4.98	0.64	12.8	28

5. Specificity

Compound	Cross Reactivity
Human Big Endothelin-1	100.0%
Rat Big Endothelin-1	100.0%
Endothelin-1	≦0.1%
Endothelin-3	≦0.1%

6. Sensitivity
0.30 pg/mL
The sensitivity for this kit was determined using the guidelines under the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols. (National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

PRECAUTION FOR INTENDED USE AND/OR HANDLING

1. All reagents should be stored at 2~8℃. All reagents shall be brought to room temperature approximately 30 minutes before use.
2. “3, Standard” is lyophilized products. Be careful to open this vial.
3. “7, Stop solution” is a strong acid substance. Therefore, be careful not to contact your skin and clothes with “7, Stop solution” and pay attention to the disposal of “7, Stop solution”.
4. Dispose used materials after rinsing them with large quantity of water.
5. The precipitation may grow in “2, Labeled antibody Conc.”, however, there is no problem in the performance.
6. Wash hands after handling reagents.
7. Do not mix the reagents with the reagents from different lot or different kit.
8. Do not use the reagents expired.
9. This kit is for research purpose only. Do not use for clinical diagnosis.

Attention for sample handling:

This kit will allow a direct assay samples containing a low concentration of protein (e.g. cell culture media, urine and so on). However, extraction and concentration of Endothelin from samples will be required for samples containing a high concentration of protein (e.g. plasma, tissue homogenates and so on). Extraction of test sample with Sep-Pak C-18 column is recommended as below:

1. Pre-treatment of Sep-Pak C-18 column (1)
   a. Washing with 4mL of pure methanol.
   b. Washing 2 times with 2mL of distilled water.
   c. Washing 2 times with 2mL of 0.1% TFA solution

2. Pre-treatment of samples
   a. Plasma (serum) – Addition of 6mL of 10% CH_3COOH to 2mL of plasma with mixing
   b. Tissue sample
      (1) Addition of 1M CH_3COOH - 20mM HCl solution to tissue sample and homogenize.
      (2) After boiling for ten minutes, centrifuge at 10,000rpm for 10min and collecting a supernatant.

3. Extraction of sample
   a. Addition of treated sample to Sep-Pak C-18 column.
   b. Washing 3 times with 3mL of distilled water.
   c. Elution with 2mL of an appropriate solution (2) and collection to vial

4. Measurement
Collected sample in vial should be lyophilized and stored under frozen condition until measurement. Stored sample should be reconstituted with 0.1mL of an appropriate solution (3) and added 0.2mL of “4, EIA buffer” and mixed. Confirm that the pH of sample is in a neutral range before measurement. There is a difference in recovery rate between samples. Please test added recovery assay in advance.

(1) Part No. 23501, manufactured by Waters Ltd. (U.S.A)
Amprep C2 column (Amersham Pharmacia Inc.) is also able to use instead of .Sep-Pak C-18
(2) 0.1% Trifluoroacetic Acid (4) plus 60% Acetonitrile in dH_2O
(3) 0.1% Trifluoroacetic Acid in DMSO
(4) No. 206-10731, manufactured by Wako Pure Chemical Industries Ltd. (Japan) is used in our protocol.

STORAGE AND THE TERM OF VALIDITY

Storage Condition : 2 - 8 °C
The expiry date is specified on outer box.

REFERENCES

1. Terui N, Suzuki H . CENTRAL NERVOUS SYSTEM AND BLOOD PRESSURE CONTROL 1992, *Proceedings of The 7th Workshop on “Brain and Blood Pressure Control”* p.141-148
2. Wakisaka *et al*., Endothelin-1 kinetics in plasma urine, and blister fluid in burn patients. *Annals of Plastic Surgery.* 37, No.3, 305-309 1996
3. Unoki H. *et al*. Low-density lipoproteins modulate endothelial cells to secrete endothelin-1 in a polarized pattern : a study using a culture model system simulating arterial intima. *Cell and Tissue Research*. 295(1) :89-99, 1999

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