INTRODUCTION
Endothelins (ETs) are isopeptides produced by vascular endothelium having potent vasoconstrictor 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 members of the endothelin family contain two essential disulfide bridges and size conserved amino acid residues at the C-terminal. The ETs are produced by a variety of tissues in vivo, including lung, kidney, brain, pitharynx and placenta (ref 1).

PRINCIPLE
This kit is a solid phase sandwich ELISA using two kinds of high specific antibodies. The monoclonal antibody IgG affinity purified anti-big ET antibodies are covalently linked to the solid phase after blocking with bovine serum albumin. The other monoclonal antibody IgG affinity purified anti-big ET antibodies are immobilized on the solid phase. Three dilutions of standard and test sample are added to the precoated tube and left for 30 minutes at room temperature (shileded). After 3 washes, 100 μL of EIA buffer are filled in the wells with the precoated plate. Incubation for overnight at 4℃ with plate lid. 8 times (wash buffer more than 350 μL) (Ref to No. 8 and 9 described in OPERATING PRECAUTION.)*

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 Endothelin-1 in serum, EDTA-plasma, supernatant of cell culture media and extract from tissue.

KIT COMPONENT
1 Precocated plate : Anti- Big ET IgG Affinity Purify
2 Labeled antibody Conc. : (30X) HRP conjugated Anti-ET-1 Rabbit IgG Fab’ Affinity Purify
3 Standard : Human Big ET-1 (Peptide)
4 EIA buffer
5 Solution for Labeled antibody
6 Chromogen : TMB solution
7 Stop solution
8 Wash buffer Conc.

OPERATION MANUAL
1. Materials needed but not supplied
   - Plate reader (450nm)
   - Micropipette and tip
   - Graduated cylinder and beaker
   - Distilled water
   - Refrigerator(s) 4℃
   - Graph paper (log/log)
   - Paper towel
   - Tube for dilution of Standard
   - Plate washer or washing bottle*
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 (8well), 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 10μL in each well.)
   This operation should be done just before the application of Labeled antibody.
   The remaining "2. Labeled antibody Conc." should be stored at 4℃ in firmly sealed vial.

3) Preparation of Standard
   Put just 0.5μL 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 8 tubes for dilution of "3. Standard". Put 230 μL of 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 3.12 pg/mL
   Tube -7 1.56 pg/mL
   Tube -8 0.78 pg/mL
   Tube -9 0.39 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.

5) Dilution of test sample
   Test sample may be diluted with "4. EIA buffer" if the need arises. It is necessary to prepare procedure extraction 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
<table>
<thead>
<tr>
<th>Standard</th>
<th>Test Sample</th>
<th>Test Sample Blank</th>
<th>Reagent Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test sample 100 μL</td>
<td>Diluted standard (Tube -1~8) 100 μL</td>
<td>EIA buffer (Tube -9) 100 μL</td>
<td>EIA buffer 100 μL</td>
</tr>
</tbody>
</table>

Incubation for overnight at 4℃ with plate lid
4 times (wash buffer more than 350 μL) (Ref to No. 8 and 9 described in OPERATING PRECAUTION.)*

Labeled Antibody
100 μL | 100 μL | 100 μL | 100 μL |
Incubation for 30 minutes at 4℃ with plate lid
5 times (wash buffer more than 350 μL) (Ref to No. 8 and 9 described in OPERATING PRECAUTION.)*

Chromogen
100 μL | 100 μL | 100 μL | 100 μL |
Incubation for 30 minutes at room temperature (chilled)

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 PRECAUTION.)*
   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 PRECAUTION.)*
   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 of 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.

OPERATING PRECAUTION*
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 using "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 used 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.

Manufactured: Immuno-Biological Laboratories Co., Ltd.
URL: http://www.ibl-japan.co.jp E-mail: do-ibl@ibl-japan.co.jp
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 absorbance recovery assay in advance.

   (1) Part No. 23501, manufactured by Waters Ltd. (U.S.A)
   (2) 0.1% Trifluoroacetic Acid in DMSO
   (3) 0.1% Trifluoroacetic Acid in 0.9% NaCl
   (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

CONTACT DETAILS
Immuno-Biological Laboratories Co., Ltd.
1091-1 Naka, Fujioka-Shi, Gunma 375-0005
TEL.: 0274-22-2885
FAX: 0274-23-6055

Version 4. May 2017

For Non-Clinical Research Use Only