Code No. 17178

Endothelin-2 (1-31) Assay Kit - IBL

INTRODUCTION

Recently, novel, smooth muscles constricting 31-amino acid endothelins (ETs), ETs (1-31) were discovered. ETs (1-31) are generated from big endothelins through the specific cleavage of the Tyr31 – Gly32 bond by human chymase. In addition, it may transiently be generated by other chymotrypsin-type proteases, such as human cathepsin G in granulocytes and rat mast cell chymases. ETs (1-31) exhibit equivalent or lower contractile potencies in comparison with the 21-amino acid endothelins, ETs (1-21), and the effects are dependent on species, vessel type and vessel size.

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 Endothelin-2 (ET-2) (1-31).

MEASUREMENT RANGE

3.91~ 500 pg/mL

INTENDED USE

The IBL's ET-2 (1-31) EIA Kit is a complete kit for the quantitative determination of ET-2 (1-31) in serum, EDTA-plasma, supernatant of cell culture media and extract from tissue.
This assay is specific for ET-2 (1-31), which does not cross-react with ETs (1-21) and big ETs. This assay will recognize both native and synthetic peptide of ET-2 (1-31).

KIT COMPONENT

1	Precoated plate	: Anti-ET-$2^{25\text{-}31}$ Rabbit IgG Affinity Purify	96Well x 1
2	Labeled antibody	: HRP conjugated Anti- ET-2 Rabbit IgG Fab' Affinity Purify	10.5mL x 1
3	EIA buffer	: 1% BSA, 0.05% Tween 20 in PBS	30mL x 1
4	Standard	: ET-2 (1-31)	0.5mL x 1
5	Substrate Buffer	: ① White cap /Solution for (7) Chromogen TMB	5mL x 1
		: ② Pink cap /0.01% H_2O_2 solution	5.5mL x 1
6	Stop solution	: 1N H_2SO_4	11mL x 1
7	Chromogen	: Tetra Methyl Benzidine (TMB)	1mg x 2
8	Wash buffer Conc.	: 0.05% Tween20 in phosphate buffer (X40)	50mL x 1
9	Solution for Labeled antibody	: 1% BSA, 0.05%Tween20 in PBS	10.5mL x 1

OPERATION MANUAL

1. Materials needed but not supplied

- Plate reader (450nm)
- Micropipette and tip
- Graduated cylinder and beaker
- Distilled water
- Incubator (37℃±1℃)
- Refrigerator (as 4℃)
- Graph paper (log/log)
- Paper towel
- Tube for dilution of Standard
- Washing bottle for precoated plate

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) Solution of labeled antibody
Put 10.5mL of "9, Solution for Labeled antibody" into the vial of "2, Labeled antibody" and place it at room temperature for 5 minutes. Then mix it gently and completely.
This operation should be done <u>just before the application of labeled antibody.</u>

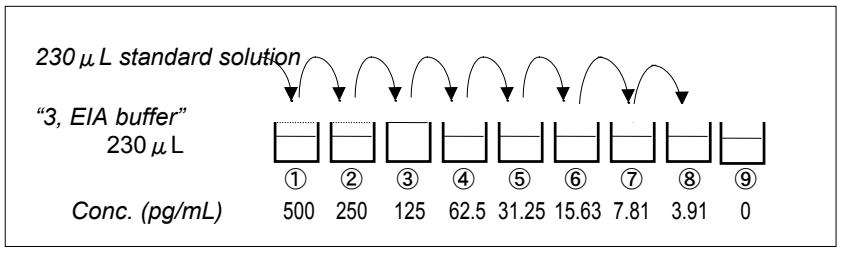
3) Preparation of TMB buffer
Put two tablets of TMB ("7, Chromogen") into the vial of "5-①, Substrate buffer" and mix it gently and completely. This prepared mixture is a white colored suspension. Then put "5-②, Substrate buffer" into this mixture and mix it gently and completely. This is TMB buffer for use. This operation should be done <u>just before the application of TMB buffer.</u>

4) Preparation of Standard
Put just 0.5mL of distilled water into the vial of "4, Standard" and mix it gently and completely. This solution is 1,000 pg/mL ET-2 (1-31) standard.

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

Tube-1	500 pg/mL	
Tube-2	250 pg/mL	
Tube-3	125 pg/mL	
Tube-4	62.5 pg/mL	
Tube-5	31.25 pg/mL	
Tube-6	15.63 pg/mL	
Tube-7	7.81 pg/mL	
Tube-8	3.91 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 500 pg/mL and 3.91 pg/mL. "3, EIA buffer" is the test sample blank as 0 pg/mL.
See following picture.



6) Dilution of test sample
Test sample may be diluted with "3, 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				
Washing 7 times				
Labeled Antibody	100 μL	100 μL	100 μL	-
Incubation for 30 minutes at 37℃ with plate lid				
Washing 9 times				
TMB buffer	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 within 30 minutes after application of Stop solution.				

1) Determine wells for reagent blank. Put 100 μL each of "3, 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) Wash each well of the precoated plate vigorously with wash buffer using washing bottle. Then, fill each well with wash buffer and place the precoated plate for 15~30 seconds. Remove wash buffer completely from the precoated plate by snapping.
<u>This procedure must be repeated more than 7 times.</u>
Then, remove the remaining liquid from all wells completely by snapping the precoated plate onto paper towel.
In case of using plate washer, after 4 times washing with plate washer, washing with above washing bottle must be repeated 3 times.
5) Pipette 100 μL of labeled antibody solution into the wells of test samples, diluted standard and test sample blank.
6) Incubate the precoated plate for 30 minutes at 37℃ after covering it with plate lid.
7) Wash the precoated plate 9 times in the same manner above 4).
8) Pipette 100 μL TMB buffer into the wells.
9) Incubate the precoated plate for 30 minutes at room temperature in the dark. The liquid will turn blue by the addition of TMB buffer.
10) Pipette 100 μL of "6, Stop solution" into the wells. Mix the liquid by tapping the side of precoated plate. The liquid will turn yellow by the addition of "6, 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 "6, Stop solution" .

SPECIAL ATTENTION

1. Test samples should be measured soon after the collection. In case of the storage of test samples, they should be stored under frozen conditions and do not repeat freeze/thaw cycles. Thaw the test samples at low temperature and mix them completely before measurement.
2. Test samples should be diluted with "3, EIA buffer", if the need arises.
3. The measurement of test samples and standard in duplicate is recommended.
4. Use test samples in neutral pH range. The contaminations of organic solvent may affect the measurement.
5. Use only wash buffer contained in this kit for washing the precoated plate. Insufficient washing may lead to the failure in measurement.
6. Remove the wash buffer completely by tapping the precoated plate on paper towel.
Do not wipe wells with paper towel.
7. TMB buffer should be prepared just before use. Do not use the colored TMB buffer. TMB tablets must be put to only "5-①, Substrate buffer". TMB tablets can not be dissolved in "5-②, Substrate buffer" buffer. TMB buffer should be stored in the dark due to its sensitivity against light. TMB buffer should be

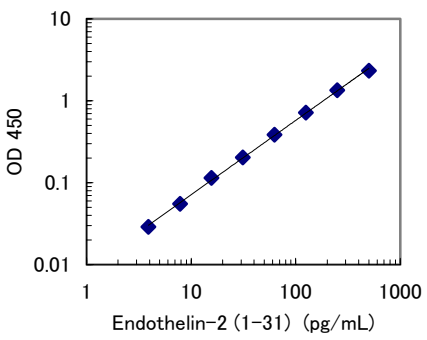
avoided contact with metals.
8. Measurement should be done within 30 minutes after addition of “6, Stop solution” .
9. Storage of HRP conjugated antibody is not recommended. However, if the HRP conjugates do not use at one time, please store it at below -20℃.

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)
500	2.487
250	1.512
125	0.882
62.5	0.549
31.25	0.366
15.63	0.276
7.81	0.217
3.91	0.191
0 (Test Sample Blank)	0.162



＊ 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

Specimen	Titer (X)	Measurement Value (pg/mL)	Theoretical Value (pg/mL)	%
10% FCS added RPMI-1640	2	124.5	125	99.6
	4	56.9	62.5	91.0
	8	28.9	31.3	92.3
	16	14.3	15.6	91.0
Human Serum	2	120.7	125	96.6
	4	57.5	62.5	92.0
	8	30.8	31.3	98.4
	16	12.9	15.6	82.7

2. Added Recovery Assay

Specimen	Theoretical Value (pg/mL)	Measurement Value (pg/mL)	%
10% FCS added RPMI-1640	125	124.0	99.2
	62.5	60.8	97.3
	31.3	31.1	99.4
	15.6	14.6	93.6
Human Serum (x4)	125	117.3	93.8
	62.5	57.4	91.8
	31.3	31.2	99.7
	15.6	15.2	97.4

3. Inter – Assay

Measurement Value (pg/mL)	SD value	CV value (%)	n
295.55	5.99	2.0	8
69.80	2.06	3.0	8
14.59	1.04	7.1	8

4. Intra – Assay

Measurement Value (pg/mL)	SD value	CV value (%)	n
284.72	12.38	4.3	16
66.03	6.47	9.8	16
14.72	1.11	7.5	16

5. Specificity

Compound	Cross Reactivity
Endothelin-2 (1-31)	100.0%
Endothelin-1 (1-31)	1.08%
Endothelin-3 (1-31)	≦0.1%
Endothelin-1 (1-21)	≦0.1%
Endothelin-2 (1-21)	≦0.1%
Endothelin-3 (1-21)	≦0.1%
Big Endothelin-1	0.17%
Big Endothelin-2	7.98%
Big Endothelin-3	≦0.1%
VIC (Mouse Endothelin-2)	≦0.1%
Rat Big Endthelin-1	0.20%

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. HRP conjugated antibody and standard are lyophilized products. Be careful to open these vials.
3. “6, Stop solution” is a strong acid substance. Therefore, be careful not to contact your skin and clothes with “6, Stop solution” and pay attention to the disposal of “6, Stop solution”.
4. “1, Precoated plate” and “4, Standard” contain sodium azide. Therefore, dispose these materials after diluting them with large quantity of water to avoid the production of explosive metallic azide.
5. Cetylpyridinium chloride is used as preservative for “2, Labeled antibody”, “3, EIA buffer” and “9, Solution for Labeled antibody”.
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 – 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 “3, 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℃
The term of validity : 6 months
(The expiry date is specified in 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

Version	
990301	*Established*
000601	*Revised (Up-dated layout)*
001024	*Revised*
010820	*Revised*
020311	*Revised*
	(Addition of "Attention for sample handling" at INTEND USE)
030415	*Revised*
040201	*Revised (Up-dated layout)*
040501	*Revised*

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