Code No. 27258

Human Osteopontin N-Half Assay Kit - IBL

INTRODUCTION

Osteopontin (OPN) is a secretory glycoprotein first isolated from the bone. At present, it is known as a highly acidic calcium-binding phosphorylated protein with sugar chain bonds, that is secreted from diverse cells, including osteoblasts, renal tubular cells, macrophages, activated T lymphocytes and vascular smooth muscle cells. Its molecular weight can vary depending on the sugar chain formation and phosphorylation, and has been reported to range from 44 to 66 kDa.
One major characteristic of OPN is that its molecule contains an Arg-Gly-Asp (RGD) amino acid sequence. This motif is also seen in fibronectin, vitronectin and other extracellular proteins. It is known that OPN binds through this motif to members of the integrin family (e.g., $\alpha v\beta 3$) of cell surface receptors.
Another unique characteristic of OPN is that it can assume various molecular forms in vivo by undergoing RNA splicing, saccharification, phosphorylation, sulfation, degradation by protease, etc. OPN is considered to coexist with thrombin locally in tissues such as injured tissue, inflamed tissue, vascularized tissue, and tumor tissue. Coexistence with thrombin increases the likelihood of proteolysis, and this process may have an important physiological role. Some investigators have reported evidence of enhancement of the cell adhesion of OPN by thrombin treatment. This suggests that cleavage of OPN with thrombin causes the hidden sequence of the adhering sites to be exposed. It has also been reported that the fragments of OPN produced following cleavage by thrombin react with $\alpha 9\beta 1$ integrin. Furthermore, in view of the finding that OPN has many cell-binding sites and can react with various receptors, interactions between OPN and these cells or receptors may play some unknown roles.
This kit can specifically measure the N-terminal OPN fragment (hereinafter called "OPN N-Half") cleaved by thrombin. OPN molecules without thrombin cleavage, on the other hand, are hardly detected by the kit.

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 Osteopontin N-Half.

MEASUREMENT RANGE

6.25 - 400 pmol/L

INTENDED USE

- This kit is to be used for the in-vitro quantitative determination of OPN N-Half in EDTA plasma, urine, synovial fluid or cell culture media. Please store all samples at -80°C before use because OPN molecule is unstable protein. Since measured value falls by being left in room temperature or repetition of freeze/thaw cycles, cautions are required.
- The recommend dilution for human EDTA plasma samples is about 2 - 8 fold by EIA buffer or PBS. However, in many cases of normal persons, it is recognized that OPN N-Half is present in minute amounts in their plasma. Therefore, the concentration of OPN N-Half in human plasma is expected to be below the detection sensitivity of this kit.
- The assay by serum samples give any values, but it might be not reflected correct values, because OPN is unstable and is easily cleaved by thrombin.
- The recommend dilution for urine samples is about 5 - 10 fold by EIA buffer or PBS, but the dilution rate should be optimized by each laboratories. Since it is easy to decompose a urine sample, we recommend to add PMSF (protease inhibitor) etc. Moreover, when it cannot measure immediately after extraction, please store at -80°C or less. Since measured value falls by repetition of freeze/thaw cycles, cautions are required. The amount of Human OPN N-Half in urine has report of being in inverse proportion to urine volume. We recommend to carry out Creatinine compensation in the case of measurement.
- The recommend dilution for cell culture media samples is various by using cells, therefore, the dilution rate should be optimized by each laboratories.
- Both recombinant and native forms of Human OPN N-Half can be detected with the kit.

KIT COMPONENT

1	Precoated plate: Anti-OPN N-Half (34E3) Mouse IgG MoAb Affinity Purify	96Well x 1
2	Labeled antibody Conc. : (30X) HRP conjugated Anti-Human OPN (O-17) Rabbit IgG Fab' Affinity Purify	0.4mL x 1
3	Standard : Recombinant Human OPN N-Half	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
- Deionized water
- Refrigerator (as 4°C)
- Graph paper (log/log)
- Incubator (37°C±1°C)
- Tube for dilution of Standard
- Paper towel
- Washing bottle for precoated plate
- Disposable test tube for "2, Labeled antibody Conc." and "6, Chromogen"
- PBS

2. Preparation

1) Preparation of wash buffer
"8, Wash buffer Conc." is a concentrated (40X) buffer. Adjust the temperature of "8, Wash buffer Conc." to room temperature and then, mix it gently and completely before use. Dilute 50mL of "8, Wash buffer Conc." with 1,950mL of deionized 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 (30X). 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.)
This operation should be done just before the application of Labeled antibody.
The remaining "2, Labeled antibody Conc." should be stored at 4°C in firmly sealed vial.

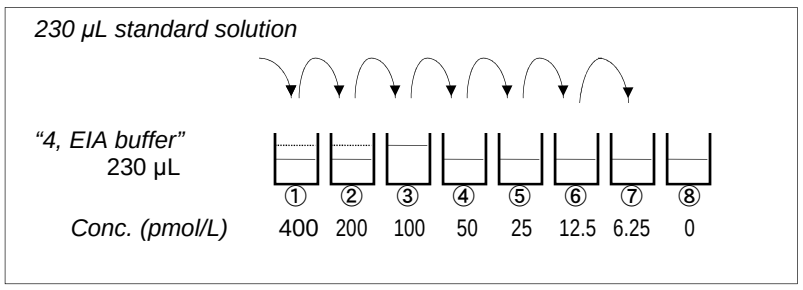
3) Preparation of Standard
Put just 0.5 mL of deionized water into the vial of "3, Standard" and mix it gently and completely. This solution is 800 pmol/L Human OPN N-Half Standard.

4) Dilution of Standard
Prepare 8 tubes for dilution of "3, Standard". Put 230µL each of "4, EIA buffer" into the tube.
Specify the following concentration of each tube.

Tube-1	400 pmol/L
Tube-2	200 pmol/L
Tube-3	100 pmol/L
Tube-4	50 pmol/L
Tube-5	25 pmol/L
Tube-6	12.5 pmol/L
Tube-7	6.25 pmol/L
Tube-8	0 pmol/L (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 7 points of diluted standard between 400 pmol/L and 6.25 pmol/L. Tube-8 is the test sample blank as 0 pmol/L.

See following picture.



5) Dilution of test sample
Test sample may be diluted with "4, EIA buffer" or PBS if the need arises.
If the concentration of Human OPN N-Half in samples may not be estimated in advance, the pre-assay with several different dilutions will be recommended to determine the proper dilution of samples.

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 1-7) 100 µL	EIA buffer (Tube-8) 100 µL	EIA buffer 100 µL
Incubation for 60 minutes at 37°C with plate lid				
4 times (wash buffer more than 350 µL)*				
Labeled Antibody	100 µL	100 µL	100 µL	-
Incubation for 30 minutes at 4°C with plate lid				
5 times (wash buffer more than 350 µL)*				
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-8), test sample and dilutions of standard (tube-1-7) into the appropriate wells.
3) Incubate the precoated plate for 60 minutes at 37°C after covering it with plate lid.
4) Wash the plate with the prepared wash buffer and remove all liquid.*
5) Pipette 100µL 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 4°C after covering it with plate lid.
7) 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 100µL from the test tube into the wells. Please do not return the rest of the test tube in "6, Chromogen" bottle to avoid 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".

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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 against a reagent blank.
The measurement shall be done within 30minutes after the addition of “7, Stop solution”.

SPECIAL ATTENTION

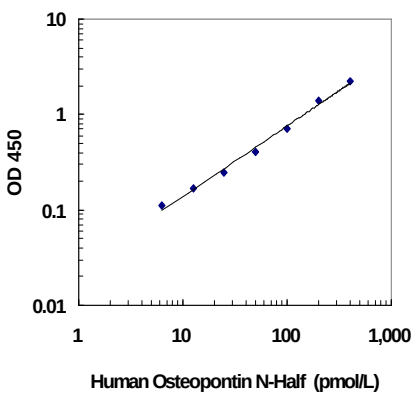
1) Test samples should be measured soon after collection. For the storage of test samples, store them frozen and do not repeat freeze/thaw cycles. Thaw the test samples at low temperature and mix them completely before measurement.
2) Test samples should be diluted with “4, EIA buffer” or PBS if the need arises.
3) Duplicate measurement of test samples and standard 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) “6, Chromogen” should be stored in the dark due to its sensitivity against light. “6, Chromogen” should be avoided contact with metals.
8) Measurement should be done within 30 minutes after addition of “7, Stop solution”.
9) Adding PMSF (protease inhibitor) to urine sample is recommended to avoid cleavage of OPN. Moreover, when it cannot measure immediately after collection, please store at -80°C or less. Since measured value falls by repetition of freeze/thaw cycles, cautions are required.
10) Please perform plasma by EDTA blood collecting. Moreover, when it cannot measure immediately after collection, please store at -80°C or less. Since measured value falls by repetition of freeze/thaw cycles, cautions are required.

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. (pmol/L)	Absorbance (450nm)
400	2.313
200	1.437
100	0.775
50	0.469
25	0.303
12.5	0.225
6.25	0.169
0 (Test Sample Blank)	0.059



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 (pmol/L)	Theoretical Value (pmol/L)	%
Human Urine	4	186.29	201.41	92.5
	8	100.70	100.00	100.7
	16	47.32	50.00	94.6
	32	25.23	25.00	100.9
	64	13.54	12.50	108.4
Human Plasma (EDTA)	2	291.53	400.86	72.7
	4	189.64	200.36	94.6
	8	93.95	100.00	93.9
	16	46.23	50.00	92.5
	32	21.72	25.00	86.9
10%FCS added TIL Media※	2	299.45	400.00	74.9
	4	184.69	200.00	92.3
	8	96.99	100.00	97.0
	16	47.57	50.00	95.1
	32	25.82	25.00	103.3

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2. Added Recovery Assay

Specimen	Theoretical Value (pmol/L)	Measurement Value (pmol/L)	%
Human Urine (x10)	399.05	350.71	87.9
	249.05	230.54	92.6
	174.05	167.92	96.5
	136.55	132.25	96.9
	117.80	115.29	97.9
Human Plasma (EDTA) (x4)	301.40	291.96	96.9
	151.40	161.79	106.9
	76.40	81.05	106.1
	38.90	37.73	97.0
	20.15	19.75	98.0
10%FCS added TIL Media※ (x2)	300.00	260.89	87.0
	150.00	123.73	82.5
	75.00	59.15	78.9
	37.50	32.50	86.7
	18.75	18.50	98.7

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3. Intra - Assay

Measurement Value (pmol/L)	SD value	CV value (%)	n
24.51	2.39	9.8	14
80.05	1.87	2.3	14
267.40	4.47	1.7	14

4. Inter - Assay

Measurement Value (pmol/L)	SD value	CV value (%)	n
21.76	3.08	14.2	14
83.82	3.42	4.1	14
304.02	15.18	5.0	14

5. Sensitivity

3.09 pmol/L
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°C. 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 have your skin and clothes contact “7, Stop solution” and pay attention to the disposal of “7, Stop solution”.
4. “1, Precoated plate” and “3, Standard” contain sodium azide. Therefore, dispose these materials after diluting them with large quantity of water to avoid production of explosive metallic azide.
5. Precipitation may occur 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 a different lot or kit.
8. Do not use the expired reagents.
9. This kit is for research purpose only. Do not use for clinical diagnosis.

REFERENCES

1. Hasegawa M, Nakoshi Y, Iino T, Sudo A, Segawa T, Maeda M, Yoshida T, Uchida A. Thrombin-cleaved osteopontin in synovial fluid of subjects with rheumatoid arthritis. J Rheumatol. 2009 Feb;36(2):240-5.

STORAGE AND THE TERM OF VALIDITY

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

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