Code No. 27351

Mouse Osteopontin Assay Kit - IBL

INTRODUCTION

Osteopontin (OPN) is a secreted glycoprotein that was originally isolated from bone. At present, it is known as a highly acidic calcium-binding glycosylated phosphoprotein secreted by many cell types, including osteoblasts, kidney tubule cells, macrophages, activated T cells, and vascular smooth muscle cells. Its molecular weights have been reported in the range of 66 kDa to 44 kDa depending on glycosylation and phosphorylation.

One important feature of OPN is that it contains an Arg-Gly-Asp (RGD) amino acid sequence. This motif is present in fibronectin, vitronectin and a variety of other extra cellular proteins that bind members of the integrin family of cell surface receptors such as $\alpha v \beta 3$.

Another important of OPN is is the presence of various molecular forms in vivo due to differential RNA splicing, glycosylation, phosphorylation, sulfation, and susceptibility to proteases. Both OPN and thrombin are likely to be localized together at the site of injury, in- flammation, and angiogenesis and in tumor tissues. Osteopontin is susceptible to proteolytic fragmentation, and this process may have physiologic importance. A report demonstrated that thrombin treatment enhanced OPN cell adhesive activity, suggesting that cleavage of OPN by thrombin exposes a cryptic adhesive sequence. More recently, it was shown that an aminoterminal OPN fragment contains a cryptic binding site that can be recognized by $\alpha 9 \beta 1$ integrin. Furthermore, OPN contains multiple cell binding sites and interacts with various receptors; these interactions may have distinct functional.

PRINCIPLE

This kit is a solid phase sandwich ELISA using 2 kinds of high specific antibodies. Tetra Methyl Benzidine (TMB) is used as a coloring agent (Chromogen). The strength of coloring is in proportional to the quantities of Mouse OPN.

MEASUREMENT RANGE

1 - 64 ng/mL (15.4 – 985 pmol/L)

INTENDED USE

- This kit is to be used for the in-vitro quantitative determination of Mouse Osteopontin (Mouse OPN) in plasma (EDTA), urine, or cell culture media. Please store all samples at –80°C before use because OPN molecule is unstable protein.
- The recommend dilution for mouse EDTA plasma samples is about 100 - 6,400 fold by PBS. Please assay again with more dilution if the assay with dilution of 100 - 6,400 fold take range over the high standard value.
- The assay by serum or heparin plasma samples give any values, but it might be not reflected correct values, because OPN is unstable and is easily cleaved by thrombin. And, OPN has several heparin binding sites in the molecules, so that heparin plasma will give any effect in the assay.
- The recommend dilution for urine samples is about more than 2,000 - 64,000 fold, 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 dissolution, cautions are required.
- The recommend dilution for cell culture media samples is various by using cells, therefore, the dilution rate should be optimized by each laboratories.
- The kit can not assay thrombin-cleaved mouse OPN.
- Both recombinant and native forms of mouse OPN can be detected with the kit.

KIT COMPONENT

1	Precoated plate : Anti-Mouse OPN (O-17) Rabbit IgG Affinity Purify	96Well x 1
2	Labeled antibody Conc. : (30X) HRP conjugated Anti-Mouse OPN (O-165) Rabbit IgG Fab' Affinity Purify	0.4mL x 1
3	Standard : Recombinant Mouse OPN	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

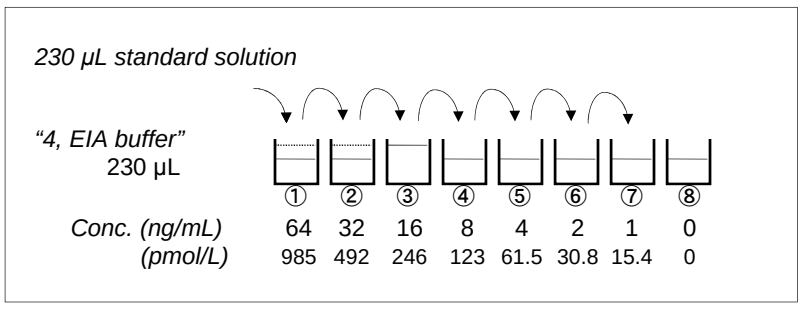
- Plate reader (450nm)
- Micropipette and tip
- Graduated cylinder and beaker
- Deionized water
- Incubator (37°C±1°C)
- Refrigerator (as 4°C)
- Graph paper (log/log)
- Paper towel
- Tube for dilution of Standard
- Washing bottle for precoated plate
- Disposable test tube for "2, Labeled antibody Conc." 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 50 mL 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.)
<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°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 Mouse OPN standard 128 ng/mL (1,970 pmol/L).
4) Dilution of Standard
Prepare 8 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	64 ng/mL	(985 pmol/L)
Tube-2	32 ng/mL	(492 pmol/L)
Tube-3	16 ng/mL	(246 pmol/L)
Tube-4	8 ng/mL	(123 pmol/L)
Tube-5	4 ng/mL	(61.5 pmol/L)
Tube-6	2 ng/mL	(30.8 pmol/L)
Tube-7	1 ng/mL	(15.4 pmol/L)
Tube-8	0 ng/mL	(Test Sample Blank)

Put 230 µL of Standard solution into tube–1 and mix it gently. Then, put 230 µL of tube-1mixture into tube-2. Dilute two times standard solution in series to set up 7 points of diluted standard between 64 ng/mL (985 pmol/L) and 1 ng/mL (15.4 pmol/L). Tube-8 is the test sample blank as 0 ng/mL.
See following picture.



5) Dilution of test sample
see p.2 DETERMINATION EXAMPLES .
Test sample may be diluted with "4, EIA buffer" or PBS if the need arises.
If the concentration of Mouse OPN 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. Make sure of 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	1100 µL	100 µL	-
Incubation for 30minutes 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 addition 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 1 hour at 37°C after covering it with plate lid.
4) 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°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 100 µL from the test tube into the wells. Please do not return the rest of test tube to "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" .
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.

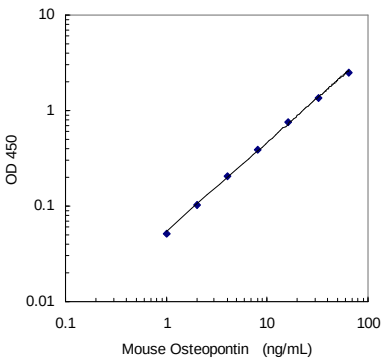
6) Remove the wash buffer completely by tapping the precoated plate on paper towel.
Do not wipe wells with paper towel.
7) "6, Chromogen" should be stored in the dark due to its sensitivity against light. "6, Chromogen" should be avoided contact with metals.
8) Measurement should be done within 30 minutes 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 dissolution, 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. (ng/mL) (pmol/L)	Absorbance (450nm)
64 (985)	2.498
32 (492)	1.377
16 (246)	0.762
8 (123)	0.393
4 (61.5)	0.206
2 (30.8)	0.106
1 (15.4)	0.055
0 (Test Sample Blank)	0.003



∗ 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 (ng/mL)	Theoretical Value (ng/mL)	%
10% FCS added RPMI-1640	2	14.10	16	88.1
	4	8.11	8	101.3
	8	3.92	4	98.0
	16	1.94	2	97.0
Mouse Plasma (EDTA) (BALB/c)	100	38.75	43.29	89.5
	200	21.31	22.31	95.5
	400	10.47	10.97	95.4
Mouse Urine (BALB/c)	2,000	66.79	67.92	98.3
	4,000	36.01	36.00	100.0
	8,000	18.83	18.45	102.1

2. Added Recovery Assay

Specimen	Theoretical Value (ng/mL)	Measurement Value (ng/mL)	%
10% FCS added RPMI-1640 (x2)	16.04	14.85	92.6
	8.04	7.47	92.9
	4.04	3.85	95.3
Mouse Plasma (EDTA) (BALB/c) (x100)	44.54	42.89	96.3
	36.54	36.45	99.8
	32.54	32.92	101.2
Mouse Urine (BALB/c) (x3,000)	52.89	50.19	94.9
	44.89	42.78	95.3
	40.89	40.45	98.9

3. Intra - Assay

Measurement Value (ng/mL)	SD value	CV value (%)	n
31.81	1.37	4.3	24
16.41	0.73	4.4	24
8.53	0.34	4.0	24

4. Inter - Assay

Measurement Value (ng/mL)	SD value	CV value (%)	n
32.63	3.02	9.2	38
16.89	1.60	9.5	38
8.75	0.77	8.7	38

5. Specificity

Compound	Cross Reactivity
mouse-OPN	100%
human-OPN	4.3%
rat-OPN	14.8%

6. Sensitivity
0.15 ng/mL (2.31 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. Dispose used materials after rinsing them with large quantity of water.
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.

DETERMINATION EXAMPLES

Mouse (BALB/c) OPN

Sample: Plasma (EDTA)

Titer (X)	Measure-ment Value (ng/mL)	Consent-ration (ng/mL)
100	27.29	2,728.80
200	14.31	2,861.80
400	6.97	2,789.20
	Average	2,793.27

Sample: Urine

Titer (X)	Measure-ment Value (ng/mL)	Consent-ration (ng/mL)
2,000	59.92	119,834.00
4,000	32.00	127,992.00
8,000	16.45	131,592.00
	Average	126,472.67

STORAGE AND THE TERM OF VALIDITY

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

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