Product Manual

Osteopontin (human), ELISA kit

Catalog #: ADI-900-142

96 Well Kit

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Product Manual

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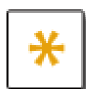
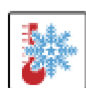


Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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DESCRIPTION

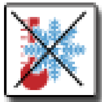
The Osteopontin (human), ELISA kit is a complete kit for the quantitative determination of OPN in human biological fluids. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human OPN immobilized on a microtiter plate to bind the human OPN in the standards or sample. A recombinant human OPN Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to human OPN is added. This antibody binds to the human OPN captured on the plate. After a short incubation, the excess antibody is washed out and Streptavidin conjugated to Alkaline Phosphatase is added, which binds to the biotinylated monoclonal human OPN antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 405nm. The measured optical density is directly proportional to the concentration of human OPN in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

INTRODUCTION

Osteopontin (OPN) is an acidic extracellular matrix cell adhesion protein that is relatively abundant not only in bone matrix, plasma, urine, and milk, but is also found in malignant and atherogenic tissues. Phosphorylation, glycosylation and calcium modifications allow intact and fragmented OPN to direct a variety of diverse responses including tissue remodeling, inflammation and cell survival[3]. Plasma OPN has been shown to be a positive indicator of colon and lung cancers as well as metastatic carcinomas[4-9]. The notable presence of OPN in a variety of tumors is strongly correlated to pathological stage, suggesting its critical role in tumor invasiveness, progression and metastasis[10, 11]. In addition, OPN inhibits inducible nitric oxide synthase activity, thereby protecting tumor cells from NO-mediated macrophage cytotoxic attack[12]. OPN is found in atherosclerotic plaques and may drive a number of diabetic vascular pathologies[13].

SAFETY WARNINGS & PRECAUTIONS

Handle with care



Avoid freeze / thaw cycles

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- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
- The activity of the Alkaline Phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10mM) such as EDTA and EGTA.
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
- The human OPN Standard provided, Catalog No. 80-1348, should be handled with care because of the known and unknown effects of human OPN.
- The human OPN standard should be stored at or below -20°C.
- Standards and samples must be prepared in polypropylene tubes. Preparation in glass will result in decreased protein stability.

MATERIALS SUPPLIED

1. **human OPN Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1344**

   A plate using break-apart strips coated with a mouse monoclonal antibody specific to OPN. The epitope includes the SVVYGLRSKSK sequence of human OPN.

2. **human OPN Antibody, 11ml, Catalog No. 80-1343**

   A yellow solution of biotinylated monoclonal antibody to OPN. The antibody epitope is located after the thrombin cleavage site.

3. **human OPN Conjugate, 11ml, Catalog No. 80-1349**

   A blue solution of Streptavidin conjugated to Alkaline Phosphatase.

4. **Assay Buffer 10 Concentrate, 100ml, Catalog No. 80-1400**

   Tris buffered saline containing detergents.

5. **Wash Buffer Concentrate, 100ml, Catalog No. 80-1287**

   Tris buffered saline containing detergents.

6. **human OPN Standard, 2 vials, Catalog No. 80-1348**

   Two vials each containing 16 ng of lyophilized recombinant human OPN.

7. **pNpp Substrate, 23ml, Catalog No. 80-0075**

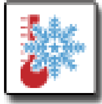
   A solution of p-nitrophenyl phosphate in buffer. Ready to use.

8. **Stop Solution, 6ml, Catalog No. 80-0247**

   A solution of trisodium phosphate in water. Keep tightly capped. Caution: Caustic.

9. **human OPN Assay Layout Sheet, 1 each, Catalog No. 30-0220**

10. **Plate Sealer, 3 each, Catalog No. 30-0012**



Reagents require separate storage conditions.

STORAGE

All components of this kit, except the standard and conjugate, are stable at 4°C until the kit's expiration date.

The Standard must be stored at or below -20°C. The conjugate may be stored at 4°C for up to 6 months, but is best stored at -20°C for long-term storage.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma # P7626 or equivalent.
3. Protease Inhibitor Cocktail (PIC), Sigma # P1860 or equivalent.
4. Precision pipets for volumes between 100µl and 1,000µl.
5. Repeater pipet for dispensing 25µl and 100µl.
6. Disposable beakers for diluting buffer concentrates.
7. Graduated cylinders.
8. A microplate shaker.
9. Adsorbent paper for blotting.
10. Microplate reader capable of reading at 405nm, preferably with correction between 570nm and 590nm.
11. Polypropylene test tubes.

SAMPLE HANDLING

The OPN ELISA is compatible with human OPN samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer 10 plus Inhibitors can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 14 for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their samples. Note that samples containing high concentrations of OPN may need to be further diluted to read in the range of the standard curve. Culture fluids, urine, breast milk, and plasma-EDTA are suitable for use in the assay. Samples, such as milk, containing a visible precipitate must be clarified prior to use in the assay. A suitable procedure is outlined below. Do not use grossly hemolyzed or lipemic samples. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay if diluted into Assay Buffer 10 plus Inhibitors. Users should only use standard curves generated in Assay Buffer 10 plus Inhibitors to calculate concentrations of OPN. To avoid cleavage of OPN in urine, breast milk, and plasma samples, PMSF should be added to a final concentration of 1mM. It is not recommended to use serum or heparin plasma in this assay as the OPN is likely to be cleaved in these matrices.

Procedure for clarification of milk

1. Centrifuge sample at 10,000rcf for 15 minutes.
2. Using a syringe, carefully pierce through the top layer and aspirate the lower layer of supernatant.
3. Centrifuge supernatant at 10,000rcf for 15 minutes.
4. Repeat #2 and centrifuge resulting supernatant at 10,000rcf for 15 minutes. Sample should now be ready to assay, following dilution in Assay Buffer 10 plus Inhibitors.

PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards and samples must be made up in plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

REAGENT PREPARATION

1. **Wash Buffer**

   Prepare the Wash Buffer by diluting 50ml of the supplied concentrate with 950ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **Assay Buffer 10 plus Inhibitors**

   Prepare the Assay Buffer 10 by diluting 100ml of the supplied concentrate with 900ml of deionized water. This can be stored at room temperature until the kit's expiration, or for 3 months, whichever is earlier. Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P1860, add 0.5µl/ml PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1mM. This modified Assay Buffer 10 must be used for all sample and standard dilutions to ensure optimal integrity of human OPN. Fresh Assay Buffer 10 plus Inhibitors must be made for each assay.

3. **human OPN Standards**

   Add 500µl of Assay Buffer 10 plus Inhibitors to one vial of human OPN Standard. Let it sit at room temperature for 5 minutes. Mix gently. This solution contains 32ng/ml human OPN and is tube #1. Label four polypropylene tubes #2 through #5. Pipet 250µl of Assay Buffer 10 plus Inhibitors into tubes #2 through #5. Pipet 250µl of the 32ng/ml standard to tube #2. Vortex. Add 250µl of tube #2 into tube #3 and vortex thoroughly. Continue this for tubes #4 and #5. The concentration of human OPN in standards #1 through #5 will be 32, 16, 8, 4 and 2ng/ml, respectively. See human OPN Assay Layout Sheet for dilution details.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards, controls and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100µl of Assay Buffer 10 plus Inhibitors into the S0 (0pg/ml standard) wells.
3. Pipet 100µl of Standards #1 through #5 into the appropriate wells.
4. Pipet 100µl of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500rpm.
7. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100µl of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500rpm.
10. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100µl of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500rpm.
13. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100µl of Substrate Solution into each well.

15. Incubate for 30 minutes at room temperature on a plate shaker at ~500rpm.
16. Pipet 25µl Stop Solution to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 405nm, preferably with correction between 570 and 590nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of human OPN in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of human OPN can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Plot the Average Net OD for each standard versus human OPN concentration in each standard. Approximate a straight line through the points. The concentration of human OPN in the unknowns can be determined by interpolation.

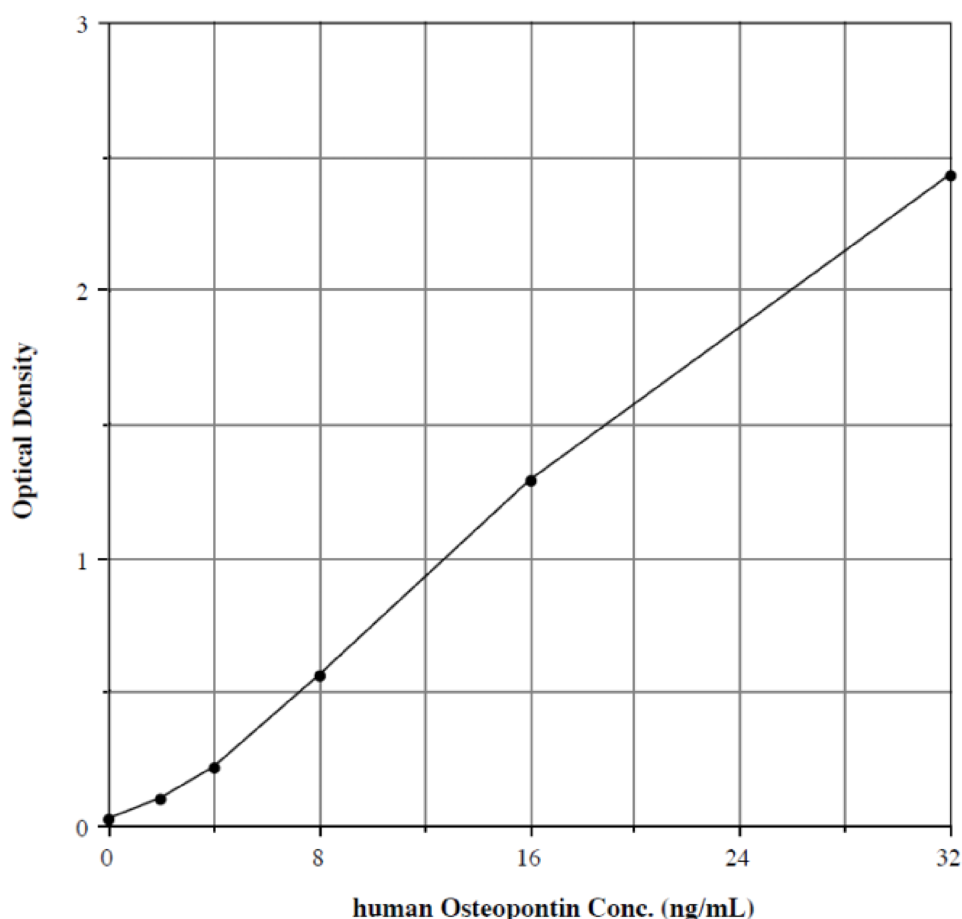
TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Average OD	Net OD	human OPN (ng/mL)
Blank	(0.067)		
S0	0.093	0.026	**0**
S1	2.494	2.427	**32**
S2	1.355	1.288	**16**
S3	0.628	0.561	**8**
S4	0.283	0.216	**4**
S5	0.170	0.103	**2**
Unknown 1	2.057	1.990	**24.9**
Unknown 2	0.319	0.252	**4.4**

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve must not be used to calculate human OPN concentrations; each user must run a standard curve for each assay.



PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[14]. All assay development and validation was performed using Assay Buffer 10 plus Inhibitors.

Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty (20) wells run with 0pg/ml Standard, and comparing to the average optical density for twenty (20) wells run with Standard #5. The detection limit was determined as the concentration of human OPN measured at two (2) standard deviations from the 0pg/ml Standard along the standard curve.

Mean OD for S0 = 0.024 ± 0.002 (8.3%)

Mean OD for Standard #5 = 0.097 ± 0.005 (5.2%)

Delta Optical Density (2 - 0ng/ml) = 0.097 - 0.024 = 0.073

2 SD's of 0pg/ml Standard = 2 x 0.002 = 0.004

Sensitivity = $\frac{0.004}{0.073}$ x 2ng/ml = **0.110ng/ml**

Linearity

A sample containing 23.287ng/ml human OPN was serially diluted 3 times 1:2 in the Assay Buffer 10 plus Inhibitors supplied in the kit and measured in the assay. The data was plotted graphically as actual human OPN concentration versus measured human OPN concentration. The line obtained had a slope of 0.955 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human OPN and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of human OPN in multiple assays (n=12). The precision numbers listed below represent the percent coefficient of variation for the concentrations of human OPN determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	human OPN (ng/mL)	Intra-assay % CV	Inter-assay % CV
Low	4.36	3.2	
Medium	11.36	3.7	
High	30.55	5.0	
Low	5.05		8.3
Medium	12.27		9.2
High	26.00		10.1

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactant in Assay Buffer 10 plus Inhibitors. These samples were then measured in the human OPN assay and the measured human OPN concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
recombinant human OPN	100%
native human OPN	111%
native bovine OPN	<0.1%
recombinant mouse OPN	<0.1%

SAMPLE RECOVERIES

Please refer to pages 6-8 for Sample Handling recommendations and Standard preparation. Human OPN concentrations were measured in a variety of different samples including tissue culture media, human urine, plasma and milk. Human OPN was spiked into these matrices which were then diluted with Assay Buffer 10 plus Inhibitors and assayed in the kit. Note that a different dilution may be required due to varying concentrations of OPN in samples. The dilutions shown below were those required for normal samples to fall within the standard range of this assay.

Tissue Culture media	% Recovery	Recommended Dilution*
MDA-MB-435 Conditioned Media	86.0	≥1:2
human Urine	113.4	≥1:32
human EDTA Plasma	106.6	≥1:8
human milk	109.7	≥1:6400

* See Sample Handling instructions on page 6 for details.

human OPN Sample Values

The following samples were tested for the presence of OPN:

Sample Type	# of Samples Tested	Range (ng/mL)	Mean (ng/mL)
plasma-EDTA	30	14.0 - 45.3	
urine	1		1,894
milk	1		55,466

REFERENCES

1. T. Chard, “An Introduction to Radioimmunoassay & Related Techniques 4th Ed.”, (1990) Amsterdam: Elsevier.
2. P. Tijssen, “Practice & Theory of Enzyme Immunoassays”, (1985) Amsterdam: Elsevier.
3. D.T. Denhardt, et al., J. Clin. Invest., (2001) 107: 1055-1061.
4. D. Agrawal, et al., J. Natl. Cancer Inst., (2002) 94: 513-521.
5. S.B. Hotte, et al., Cancer, (2002) 95: 506-512.
6. K.A. Furger, et al., Curr. Mol. Med., (2001) 1: 621-632.
7. H. Singhal, et al., Clin. Cancer Res., (1997) 3: 605-611.
8. A.F. Chambers, H. Singhal, et al., Lung Cancer, (1996) 15: 311-323.
9. L.F. Brown et al., Am. J. Pathol., (1994) 145: 610-623.
10. D. Coppola, et al., Clin. Cancer Res., (2004) 10: 184-190.
11. A.B. Tuck, et al., Arch. Pathol. Med., (1997) 121: 578-584.
12. J.A. Scott, et al., Am. J Physiol., (1998) 275 (Heart Circ. Physiol. 44): H2258-H2265.
13. M. Takemoto, et al., Atherioscler, Thomb. Vasc. Biol., (2000) 20: 624-628.
14. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

NOTES

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GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
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