

Quantikine[®] ELISA

Human Osteopontin (OPN) Immunoassay

Catalog Number DOST00

SOST00

PDOST00

For the quantitative determination of human Osteopontin (OPN) concentrations in cell culture supernates, plasma, urine, and human milk.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Osteopontin (OPN), also known as early T lymphocyte activation 1 (Eta-1), is a secreted multifunctional glyco-phosphoprotein with roles in bone metabolism, immune regulation, tissue remodeling, cell survival, and tumor progression (1-7). Gene structure and chromosomal location identify OPN as a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family that also includes bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), enamelin (ENAM), and matrix extracellular phosphoglycoprotein (MEPE) (8). Human OPN is synthesized as a 314 amino acid (aa) precursor protein with a predicted 16 aa signal peptide and a highly unusual mature protein sequence, containing 75 acidic aa and 33 potential Ser/Thr phosphorylation sites (9, 10). Although the predicted molecular weight of OPN is 33 kDa, phosphorylation and N- and O-glycosylation may allow it to appear as large as 75 kDa. Variability in post-translational modifications can influence the activity of OPN. (11). OPN contains a classic integrin binding site (RGD) that binds integrins $\alpha\beta1$, $\alpha\beta3$, $\alpha\beta5$, $\alpha5\beta1$, and $\alpha8\beta1$ (1-7). MMP-3, MMP-7, or thrombin cleavage separates the integrin-binding domain in the N-terminal fragment and the C-terminal CD44 binding domain. Proteolytic cleavage enhances OPN adhesion by revealing an additional adhesion site (SVVYGLR in the human protein) recognized by integrins $\alpha4\beta1$, $\alpha4\beta1$, and $\alpha9\beta1$ (12, 13).

Osteopontin, meaning "bone bridging", is highly expressed in mineralized tissues. It is also expressed in other tissues including cartilage, kidney, vascular tissues, activated macrophages, lymphocytes, and epithelia. In addition to being incorporated in the matrix of mineralized connective tissues, secreted OPN is found in various biological fluids including blood, milk, urine, and seminal fluid. A portion of cell expressed OPN is also retained intracellularly (14). *In vitro*, OPN stimulates the adhesion of osteoclasts to bone, and bone resorption is blocked by inhibition of this interaction (15, 16). Knockout mice have outwardly normal bone development but exhibit deficient postnatal bone resorption in several contexts, supporting a role for OPN in osteoclast function (17). In kidney epithelia, OPN is upregulated by high concentrations of oxalate and inhibits calcium oxalate crystal nucleation and growth (2, 18). In endothelial and smooth muscle cells, OPN is upregulated by high phosphate concentration and during atherosclerosis; binding of OPN to hydroxyapatite inhibits calcification of blood vessels and heart valves (7, 19, 20).

OPN expression by macrophages and T cells is upregulated by inflammatory mediators including LPS, NO, IL-1 β , and TNF- α (1, 2, 5). OPN regulates macrophage differentiation and recruitment (21, 22). It also functions as a chemotactic factor and co-stimulator of T cells and may act as a Th1 cytokine, stimulating IL-12 production (13, 23). OPN knockout mice exhibit deficient Th1 responses and are susceptible to bacterial and viral infection (24). OPN production by macrophages is upregulated at sites of tissue remodeling including the placenta, endometrium, and myocardium post-infarction (18, 25-27). OPN is expressed by many tumor types and plays a role in tumor progression and metastasis. In cancer patients, increased serum OPN concentration has been associated with increased tumor burden (3, 5, 28, 29). Binding of OPN to specific variants of the hyaluronan receptor CD44, possibly mediated by integrins, can stimulate cell migration and metastatic potential of tumors (30). OPN may also be upregulated during autoimmune processes such as rheumatoid arthritis (6, 31) or in granulomatous lesions in response to infections such as tuberculosis (3, 32).

The Quantikine Human OPN Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human OPN in cell culture supernates, plasma, urine, and human milk. It contains NS0-expressed recombinant human OPN and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human OPN showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human OPN.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for OPN has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any OPN present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for OPN is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of OPN bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DOST00	CATALOG # SOST00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
OPN Microplate	892816	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against OPN.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
OPN Standard	892818	1 vial	6 vials	200 ng/vial of recombinant human OPN in a buffered protein base with preservatives; lyophilized.	Aliquot and store at ≤ -20 °C for up to 1 month in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
OPN Conjugate	892817	1 vial	6 vials	21 mL/vial of a polyclonal antibody against OPN conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-6	895158	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives. <i>May contain a precipitate. Mix well before and during use.</i>	
Calibrator Diluent RD5-24	895325	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

DOST00 contains sufficient materials to run an ELISA on one 96 well plate.

SOST00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDOST00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for dilution of standards and samples.
- Human OPN Controls (optional; available from R&D Systems).

PRECAUTIONS

Assay Diluent RD1-6 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Note: *Although serum has been validated for use in this assay, it is not a recommended sample type because of proteolytic cleavage by thrombin during the clotting process. Serum values are approximately 50% of the plasma values. See references 32, 77-78 for more details.*

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for a total of 3 cycles. Assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Plasma samples require at least a 25-fold dilution. A suggested 25-fold dilution is 10 μL of sample + 240 μL of Calibrator Diluent RD5-24.

Urine and human milk samples require dilution in Calibrator Diluent RD5-24. Refer the Sample Values section for a range of expected values.

Cell culture supernate samples may require dilution.

REAGENT PREPARATION

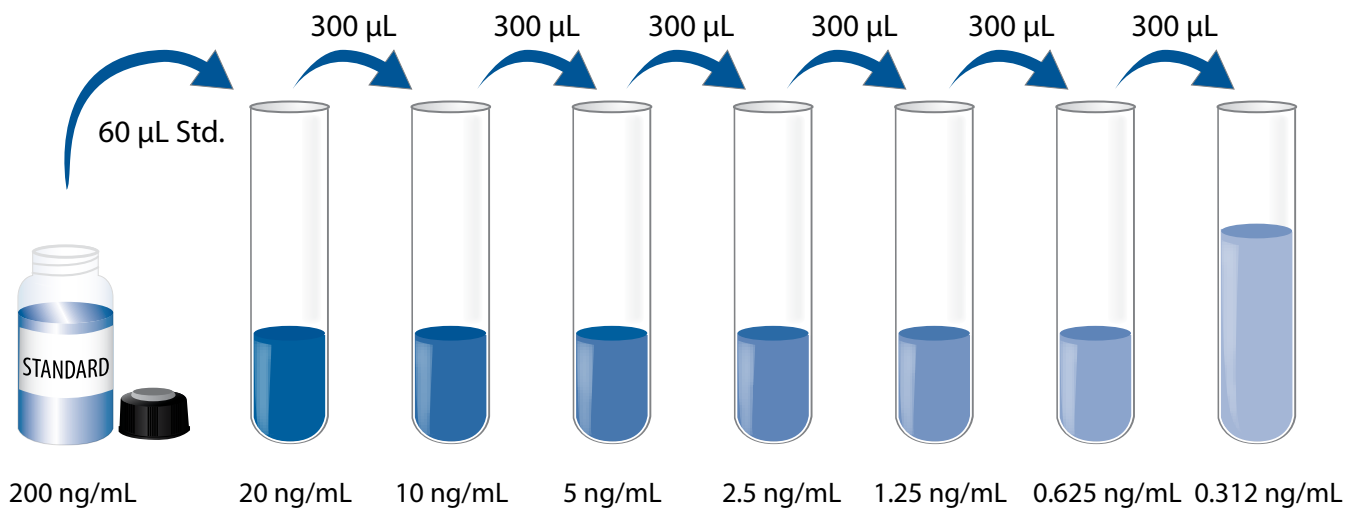
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

OPN Standard - Reconstitute the OPN Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 540 μL of Calibrator Diluent RD5-24 into the 20 ng/mL tube. Pipette 300 μL of Calibrator Diluent RD5-24 into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. Calibrator Diluent RD5-24 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 μL of Assay Diluent RD1-6 to each well. *Assay Diluent RD1-6 may contain a precipitate. Mix well before and during use.*
4. Add 50 μL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 μL of OPN Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

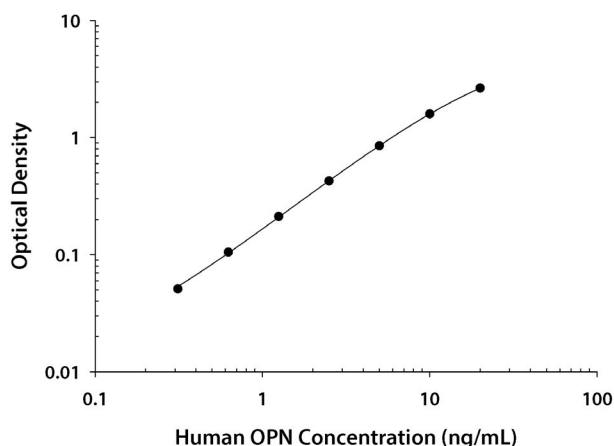
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the OPN concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.010 0.010	0.010	—
0.312	0.060 0.062	0.061	0.051
0.625	0.113 0.117	0.115	0.105
1.25	0.219 0.225	0.222	0.212
2.5	0.434 0.438	0.436	0.426
5	0.849 0.875	0.862	0.852
10	1.580 1.620	1.600	1.590
20	2.624 2.680	2.652	2.642

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	2.30	4.90	9.30	2.36	4.81	9.17
Standard deviation	0.092	0.126	0.267	0.155	0.274	0.499
CV (%)	4.0	2.6	2.9	6.6	5.7	5.4

RECOVERY

The recovery of OPN spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	104	86-113%
EDTA plasma* (n=4)	102	92-114%
Heparin plasma* (n=4)	101	95-112%

*Samples were diluted as directed in the Sample Preparation section.

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of OPN were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=5)	Human milk (n=4)
1:2	Average % of Expected	109	103	103	99	105
	Range (%)	98-113	102-104	98-110	95-102	100-108
1:4	Average % of Expected	106	104	104	99	110
	Range (%)	99-114	103-105	102-105	97-100	108-112
1:8	Average % of Expected	104	105	104	100	113
	Range (%)	97-110	104-108	102-107	98-105	111-114
1:16	Average % of Expected	105	106	105	100	114
	Range (%)	100-108	103-109	101-112	97-103	113-115

SENSITIVITY

Fifty-seven assays were evaluated and the minimum detectable dose (MDD) of OPN ranged from 0.006-0.024 ng/mL. The mean MDD was 0.011 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human OPN (amino acids 17-314) produced at R&D Systems.

SAMPLE VALUES

Although serum has been validated for use in this assay, it is not a recommended sample type because of proteolytic cleavage by thrombin during the clotting process. Serum values are approximately 50% of the plasma values. See references 32, 77, and 78 for more details.

Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of OPN in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
EDTA plasma* (n=40)	94.8	49.2-175	24.9
Heparin plasma* (n=40)	96.2	53.4-195	26.7
Urine* (n=21)	2044	122-8796	1951

*Samples were diluted as directed in the Sample Preparation section prior to assay.

Cell Culture Supernates:

Human peripheral blood cells (1×10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 5 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 μ g/mL PHA. Aliquots of the cell culture supernate were removed and assayed for levels of natural OPN.

Condition	Day 1 (ng/mL)	Day 6 (ng/mL)
Unstimulated	0.675	1.85
Stimulated	1.50	2.67

HepG2 human hepatocellular carcinoma cells were cultured in MEM supplemented with 5% fetal calf serum until confluent. Cells were then stimulated with 50 ng/mL PMA for 24 hours. An aliquot of the cell culture supernate was assayed for levels of natural OPN and measured 790 ng/mL.

A431 human epithelial carcinoma cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. An aliquot of the cell culture supernate was assayed for levels of natural OPN and measured 8.54 ng/mL.

Human Milk - Three human milk samples were assayed for levels of natural OPN and ranged from 35,600-248,000 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human OPN.

The factors listed below were prepared at 200 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human OPN control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Enterokinase
MMP-3
MMP-7
Thrombin

Recombinant mouse:

OPN

Natural bovine:

OPN

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