

# Quantikine<sup>®</sup>

## Mouse\* Osteopontin Immunoassay

Catalog Number MOST00

**For the quantitative determination of mouse Osteopontin (OPN) concentrations in cell culture supernates, mouse serum, plasma, and urine.**

**\*This assay also recognizes rat Osteopontin.**

***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). Murine OPN is synthesized as a 294 amino acid (aa) precursor protein with a predicted 16 aa signal peptide and a highly unusual mature protein sequence, containing 68 acidic aa and 23 potential Ser/Thr phosphorylation sites (9, 10). Although the predicted molecular weight of OPN is 31 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  $\alpha v\beta 1$ ,  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha 5\beta 1$ , and  $\alpha 8\beta 1$  (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 (SLAYGLR in the mouse protein) recognized by integrins  $\alpha 4\beta 1$ ,  $\alpha 4\beta 1$  and  $\alpha 9\beta 1$  (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 $\beta$ , and TNF- $\alpha$  (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 Mouse OPN Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse OPN in cell culture supernates, serum, plasma, and urine. It contains NS0-expressed recombinant mouse OPN and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse OPN showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Mouse OPN kit can be used to determine relative mass values for naturally occurring mouse OPN.

This kit demonstrates significant cross-reactivity with rat OPN and has been validated for the determination of relative mass values for natural rat OPN in cell culture supernates, rat serum, and plasma. The amount of natural rat OPN measured is expressed as mouse OPN equivalent.

## **PRINCIPLE OF THE ASSAY**

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse OPN has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse OPN present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse OPN is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse OPN bound in the initial step. The sample values are then read off the standard curve.

## **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 the Calibrator Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins and other factors present in biological samples. Until all factors have been tested, however, the possibility of interference cannot be excluded.

## **PRECAUTION**

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face and clothing protection when using this material.

## **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.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- Allow the plate to soak for at least 30 seconds between washes to improve assay performance.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

## MATERIALS PROVIDED

**Mouse OPN Microplate** (Part 893133) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse OPN.

**Mouse OPN Conjugate** (Part 893134) - 12 mL of a polyclonal antibody specific for mouse OPN conjugated to horseradish peroxidase with preservatives.

**Mouse OPN Standard** (Part 893135) - 12.5 ng of recombinant mouse OPN in a buffered protein base with preservatives; lyophilized.

**Mouse OPN Control** (Part 893136) - 1 vial of recombinant mouse OPN in a buffered protein base with preservatives; lyophilized. The concentration range of mouse OPN after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

**Calibrator Diluent RD6-12** (Part 895214) - 2 vials (21 mL/vial) of diluted animal serum with preservatives.

**Assay Diluent RD1W** (Part 895038) - 12 mL of a buffered protein solution with preservatives.

**Wash Buffer Concentrate** (Part 895024) - 50 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

**Color Reagent A** (Part 895000) - 12 mL of stabilized hydrogen peroxide.

**Color Reagent B** (Part 895001) - 12 mL of stabilized chromogen (tetramethylbenzidine).

**Stop Solution** (Part 895174) - 23 mL of diluted hydrochloric acid.

**Plate Covers** (Part 640197) - 4 adhesive strips.

## STORAGE

<b>Unopened Kit</b>	Store at 2 - 8° C. Do not use past kit expiration date.	
<b>Opened/ Reconstituted Reagents</b>	Mouse OPN Conjugate	May be stored for up to 1 month at 2 - 8° C.*
	Diluted Wash Buffer	
	Stop Solution	
	Calibrator Diluent RD6-12	
	Assay Diluent RD1W	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
	Mouse OPN Standard (2500 pg/mL)	
	Mouse OPN Control	
	Microplate Wells	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.*

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

## 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.
- 100 mL and 1000 mL graduated cylinders.
- Test tubes for dilution of standards.

## SAMPLE COLLECTION AND STORAGE

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Serum** - Allow blood samples to clot for 2 hours at room temperature or overnight at  $2 - 8^{\circ}$  C before centrifuging. Centrifuge for 20 minutes at  $2000 \times g$ . Remove serum and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at  $2000 \times g$  within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Urine** - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

## SAMPLE PREPARATION

Mouse serum and plasma samples require a 100-fold dilution into Calibrator Diluent RD6-12. The suggested dilution can be achieved by adding  $10 \mu\text{L}$  sample to  $90 \mu\text{L}$  of Calibrator Diluent RD6-12. Complete the 100-fold dilution by adding  $20 \mu\text{L}$  of this solution to  $180 \mu\text{L}$  of Calibrator Diluent RD6-12.

Rat serum and plasma samples require a 10-fold dilution into Calibrator Diluent RD6-12. The suggested 10-fold dilution can be achieved by adding  $20 \mu\text{L}$  sample to  $180 \mu\text{L}$  of Calibrator Diluent RD6-12.

Cell culture supernate samples may require dilution. The dilution needed is dependent upon sample values.

Mouse urine samples require at least a 200 to 800-fold dilution.

## REAGENT PREPARATION

Bring all reagents to room temperature before use.

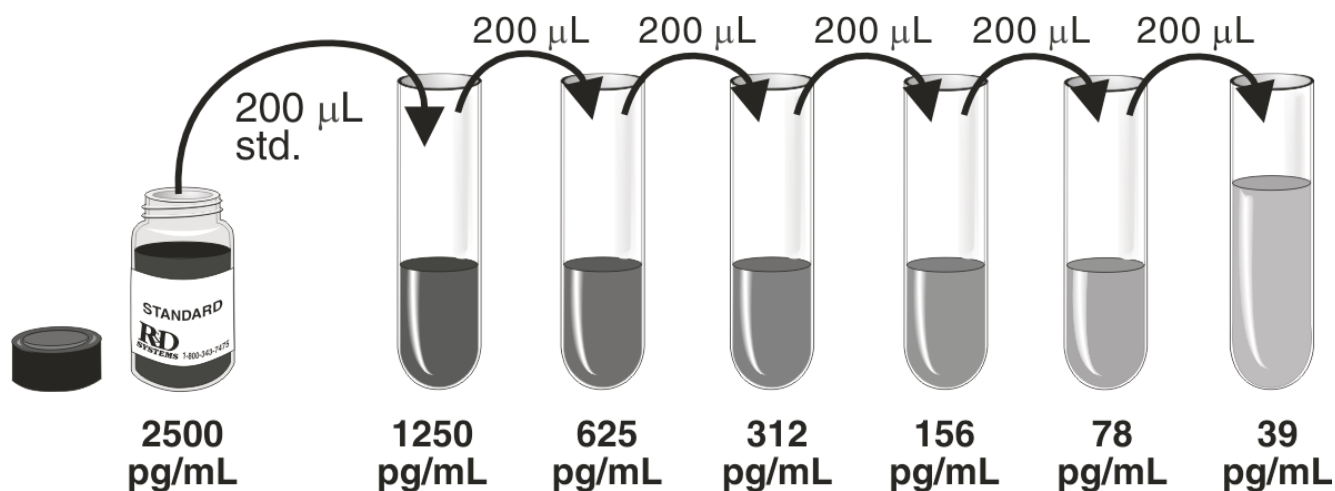
**Mouse OPN Kit Control** - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 625 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. 100  $\mu$ L of the resultant mixture is required per well.

**Mouse OPN Standard** - Reconstitute the mouse OPN Standard with 5.0 mL of Calibrator Diluent RD6-12. Do not substitute other diluents. This reconstitution produces a stock solution of 2500 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200  $\mu$ L of Calibrator Diluent RD6-12 into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted mouse OPN Standard serves as the high standard (2500 pg/mL). Calibrator Diluent RD6-12 serves as the zero standard (0 pg/mL).



## ASSAY PROCEDURE

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

1. Prepare all reagents, standard dilutions, control, 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 50  $\mu\text{L}$  of Assay Diluent RD1W to each well.
4. Add 50  $\mu\text{L}$  of Standard, Control, or sample\* per well. Mix by tapping the plate frame for 1 minute. 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 four times for a total of five washes. Wash by filling each well with Wash Buffer (400  $\mu\text{L}$ ) 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 100  $\mu\text{L}$  of Mouse 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 100  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 100  $\mu\text{L}$  of Stop Solution to each well. 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 require dilution. See the Sample Preparation section.

## PROCEDURE SUMMARY AND CHECKLIST

1.  Bring all reagents to room temperature.  
 Prepare reagents and samples as instructed.  
 Return unused components to storage temperature as indicated in the instructions.
  
2.  Add 50  $\mu\text{L}$  Assay Diluent RD1W to each well.
  
3.  Add 50  $\mu\text{L}$  Standard, Control, or sample\* to each well.  
 Tap plate gently for one minute.  
 Cover the plate and incubate for 2 hours at room temperature.
  
4.  Aspirate and wash each well five times.
  
5.  Add 100  $\mu\text{L}$  Conjugate to each well.  
 Cover the plate and incubate for 2 hours at room temperature.
  
6.  Aspirate and wash each well five times.
  
7.  Add 100  $\mu\text{L}$  Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
  
8.  Add 100  $\mu\text{L}$  Stop Solution to each well.
  
9.  Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

\*Samples require dilution. See Sample Preparation section.

## CALCULATION OF RESULTS

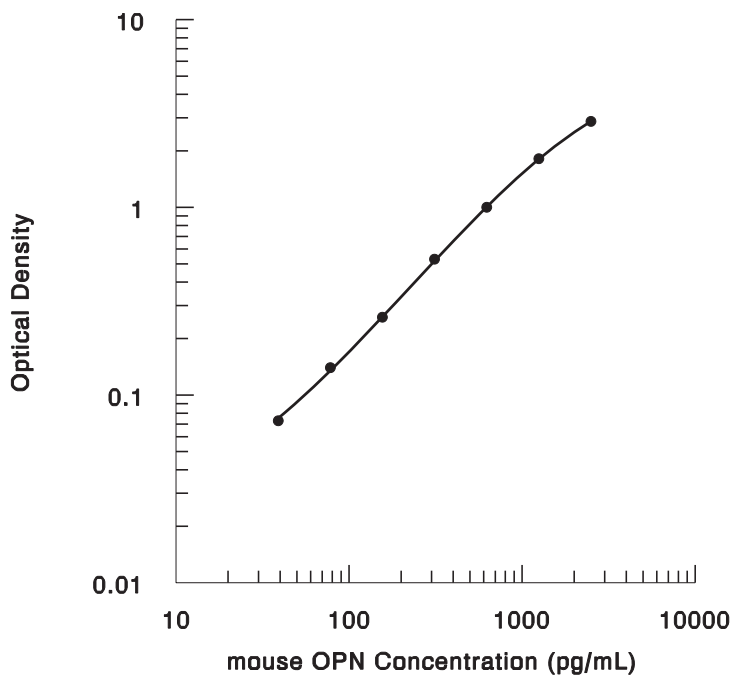
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 mouse 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.

Because samples have been diluted prior to assay, 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.



pg/mL	O.D.	Average	Corrected
0	0.042 0.050 0.118	0.046	—
39	0.120 0.185	0.119	0.073
78	0.187 0.297	0.186	0.140
156	0.316 0.567	0.306	0.260
312	0.584 1.046	0.576	0.530
625	1.051 1.841	1.048	1.002
1250	1.883 2.905	1.862	1.816
2500	2.931	2.918	2.872

## 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 separate assays to assess inter-assay precision.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	62	67	59
Mean (pg/mL)	96.0	236	1085	116	279	1076
Standard deviation	7.3	13.8	43.4	9.8	22.5	90.0
CV (%)	7.6	5.8	4.0	8.4	8.1	8.4

## RECOVERY

The recovery of mouse OPN spiked to three levels throughout the range of the assay in the cell culture supernate samples was evaluated.

Sample Type	Average % Recovery	Range
Mouse cell culture supernates (n=7)	98	87 - 106%
Rat cell culture supernates (n=7)	93	82 - 107%

## LINEARITY

To assess the linearity of the assay, samples containing mouse or rat OPN were diluted with Calibrator Diluent RD6-12 and then assayed.

<b>Mouse Samples</b>		Cell culture supernates* (n=13)	Serum** (n=6)	Heparin plasma** (n=5)	EDTA plasma** (n=6)	Urine** (n=6)
1:2	Average % of Expected	95	99	102	102	100
	Range (%)	84 - 102	94 - 104	99 - 107	97 - 108	93 - 105
1:4	Average % of Expected	95	96	98	103	108
	Range (%)	83 - 101	93 - 97	93 - 102	97 - 108	102 - 120
1:8	Average % of Expected	100	101	99	104	111
	Range (%)	86 - 109	92 - 111	92 - 107	100 - 109	103 - 120
1:16	Average % of Expected	104	101	97	108	112
	Range (%)	87 - 117	92 - 108	92 - 112	101 - 115	101 - 129

<b>Rat Samples</b>		Cell culture supernates* (n=9)	Serum** (n=5)	Heparin plasma** (n=5)	EDTA plasma** (n=5)
1:2	Average % of Expected	98	100	99	98
	Range (%)	93 - 103	94 - 104	97 - 102	95 - 101
1:4	Average % of Expected	98	101	98	100
	Range (%)	86 - 108	96 - 107	95 - 101	98 - 101
1:8	Average % of Expected	101	102	98	98
	Range (%)	87 - 119	96 - 108	95 - 102	94 - 101
1:16	Average % of Expected	104	109	104	104
	Range (%)	83 - 117	103 - 116	98 - 110	102 - 108

\*Mouse and rat cell culture supernate samples were screened and diluted dependent upon sample values.

\*\*Mouse and rat serum/plasma and mouse urine samples were diluted as directed in the Sample Preparation section.

## SENSITIVITY

Forty-one assays were evaluated and the minimum detectable dose (MDD) of mouse OPN ranged from 3.2 - 8.5 pg/mL. The mean MDD was 5.7 pg/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 mouse OPN produced at R&D Systems. The recombinant protein contains 284 amino acid residues and has a predicted molecular mass of approximately 31.5 kDa. As a result of glycosylation, it migrates as an approximately 60 kDa protein.

Based on the total amino acid analysis, the absorbance of a 1 mg/mL solution of the NS0-expressed recombinant mouse OPN at 280 nm was determined to be 0.61 A.U.

## SAMPLE VALUES

**Serum/Plasma** - Individual mouse and rat serum and plasma samples were evaluated for detectable levels of mouse OPN in this assay. Serum and plasma samples are not matched.

Mouse Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Mouse serum* (n=20)	96	55 - 140	24
Mouse heparin plasma* (n=20)	79	37 - 142	22
Mouse EDTA plasma* (n=10)	110	73 - 155	30
Mouse urine** (n=6)	988	549 - 1814	466

\*Samples were diluted 100-fold prior to assay.

\*\*Mouse urine samples were diluted as directed in the Sample Preparation section.

Rat Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Rat serum* (n=20)	8.3	4.6 - 13.2	2.0
Rat heparin plasma* (n=20)	10.3	1.9 - 14.9	3.3
Rat EDTA plasma* (n=20)	7.7	2.4 - 14.5	3.4

\*Samples were diluted 10-fold prior to assay.

## Cell Culture Supernates -

J774 cells, a mouse macrophage cell line, ( $1 \times 10^6$  cells/mL) were cultured for 3 days in RPMI containing 10% fetal bovine serum. The cell culture supernate was assayed for detectable levels of mouse OPN and measured 266 ng/mL.

Mouse L929 cells ( $0.5 \times 10^5$  cells/mL) were cultured for 3 days in MEM containing 10% equine serum, stimulated with 2.5 ng/mL LPS. The cell culture supernate was assayed for detectable levels of mouse OPN and measured 70 ng/mL.

Rat lung conditioned media (2 lungs, 1 - 2 mm pieces) was cultured for 3 days in 30 mL of RPMI supplemented with 10% fetal bovine serum and stimulated with 10  $\mu$ g/mL ConA. The cell culture supernate was assayed for detectable levels of rat OPN and measured 2666 pg/mL.

Rat heart conditioned media (1 heart, 1 - 2 mm pieces) was cultured for 3 days in 30 mL RPMI, supplemented with 10% fetal bovine serum, and stimulated with 10  $\mu$ g/mL ConA. The cell culture supernate was assayed for detectable levels of rat OPN and measured 347 pg/mL.

## SPECIFICITY

This assay recognizes both recombinant and natural mouse and rat OPN. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6-12 and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range mouse OPN control were assayed for interference. No significant cross-reactivity or interference was observed.

### **Recombinant mouse:**

Adiponectin  
BMPR-1A  
Chordin  
MMP-3  
MMP-7  
Noggin  
TGF- $\beta$  R1  
TSG

### **Recombinant human:**

Enterokinase  
Fibronectin  
Thrombin  
Vitronectin

### **Others:**

bovine Osteopontin  
bovine Enterokinase

In this assay, NS0-expressed recombinant human Osteopontin shows 8.2% cross-reactivity at a concentration of 0.62 ng/mL.

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# PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>H</b>			

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