

Quantikine[®]

Human Osteopontin (OPN) Immunoassay

Catalog Number DOST00
SOST00
PDOST00

For the quantitative determination of human OPN concentrations in cell culture supernates, plasma, urine, and breast 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 glycoprotein. Its putative functions include roles in bone metabolism, immune regulation, wound healing, cell survival, and tumor progression (1 - 5). Based on gene structure and chromosomal location, OPN is 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) (6). Human OPN cDNA encodes a 314 amino acid (aa) precursor protein with a predicted 16 aa signal peptide that is cleaved to yield the 298 aa mature protein. It is a highly acidic, multi-domain protein with a predicted molecular weight of approximately 33 kDa, although it may range up to 75 kDa due to extensive glycosylation and phosphorylation (7 - 12).

OPN is expressed mainly by bone, kidney, and epithelial tissues but can also be found in endometrial tissues, endothelial cells, T cells, macrophages, smooth muscle cells, and many tumor types (1, 5, 10, 13 - 20). It is upregulated in tissues during several pathological processes including atherosclerosis, valve stenosis, myocardial infarction, and rheumatoid arthritis (21 - 25). OPN is found in several biological fluids including human plasma, serum, breast milk, and urine, and is upregulated in certain cancers, fulminant hepatitis, tuberculosis, and autoimmune diseases such as multiple sclerosis and lupus erythematosus (5, 26 - 38).

OPN activities are mediated by an array of receptors including many integrins and the hyaluronan receptor, CD44. The OPN protein contains multiple domains responsible for integrin binding. It has a typical Arg-Gly-Asp (RGD) site that binds several integrins of the α_V class ($\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$), $\alpha_5\beta_1$, and $\alpha_8\beta_1$, a lopinavir (LPV)-containing domain that binds $\alpha_4\beta_1$, and an SVVYGLR site that binds $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_9\beta_1$ (39 - 47). OPN may also associate with variants of CD44, potentially with integrin cooperation, to stimulate cell migration (48 - 50). An intracellular pool of OPN may also enhance cell migration through interaction with CD44 and ERM (ezrin/radixin/moesin) proteins, and/or via the regulation of CD44 cell surface expression (51 - 53).

The activities of OPN and proteases are reciprocally modulated. Cleavage by thrombin, MMP-3, MMP-7, or MMP-12 can produce OPN fragments that have biological activity different from the whole protein (54 - 57). For instance, thrombin, MMP-3, or MMP-7 cleavage may enhance integrin-dependent cell adhesion and/or migration (57, 58). In turn, OPN is shown to activate MMP-2 or -3 by mechanisms that may include conversion/activation of the pro-form or reactivation of TIMP-inhibited enzyme (59, 60).

As the name implies, OPN may have a role in bone metabolism. *In vitro*, it stimulates the adhesion of osteoclasts to bone, and bone resorption is blocked by inhibition of this interaction (61, 62). Knockout mice have outwardly normal bone development, but do exhibit deficient postnatal bone resorption in several contexts, supporting a role for OPN in osteoclast function (63 - 66). OPN may also contribute directly to the regulation of mineral crystal formation and growth. It binds hydroxyapatite and suppresses crystal formation both *in vitro* and *in vivo* (65, 67 - 69). OPN is also a regulator of inflammation (1, 70). Inflammatory mediators including LPS, NO, IL-1 β , and TNF- α , stimulate OPN expression (71). OPN regulates macrophage differentiation and recruitment (72, 73). It also functions as a chemotactic factor and co-stimulator of T cells and may act as a Th1 cytokine, stimulating IL-12 production (58, 74).

OPN knockout mice exhibit deficient Th1 responses and are susceptible to bacterial and viral infection (75). In contrast, OPN may have context-dependent anti-inflammatory effects as well. For instance, it suppresses the release of several inflammatory mediators from osteoarthritic chondrocytes *in vitro* (76).

The Quantikine OPN Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human OPN in cell culture supernates, plasma, urine, and breast 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 the Quantikine OPN kit can be used to determine relative mass values for naturally occurring 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.
- 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 in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Description	Part #	Cat. # DOST00	Cat. # SOST00
OPN Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against OPN.	892816	1 plate	6 plates
OPN Conjugate - 21 mL/vial of polyclonal antibody against OPN conjugated to horseradish peroxidase with preservatives.	892817	1 vial	6 vials
OPN Standard - 200 ng/vial of recombinant human OPN in a buffered protein base with preservatives, lyophilized.	892818	1 vial	6 vials
Assay Diluent RD1-6 - 11 mL/vial of a buffered protein base with preservatives. May contain a precipitate. Mix well before and during use.	895158	1 vial	6 vials
Calibrator Diluent RD5-24 - 21 mL/vial of a buffered protein base with preservatives.	895325	1 vial	6 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
Plate Covers - Adhesive strips.	—	4 strips	24 strips

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.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-6	
	Calibrator Diluent RD5-24	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store at ≤ -20° C for up to 1 month in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
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.
- 500 mL graduated cylinder.
- 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 eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles. For more information on collecting and handling urine specimens, refer to the National Committee for Clinical Laboratory Standards (NCCLS) publication GP16-T.

Breast Milk - Centrifuge for 15 minutes at 10,000 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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

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

Urine and breast 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

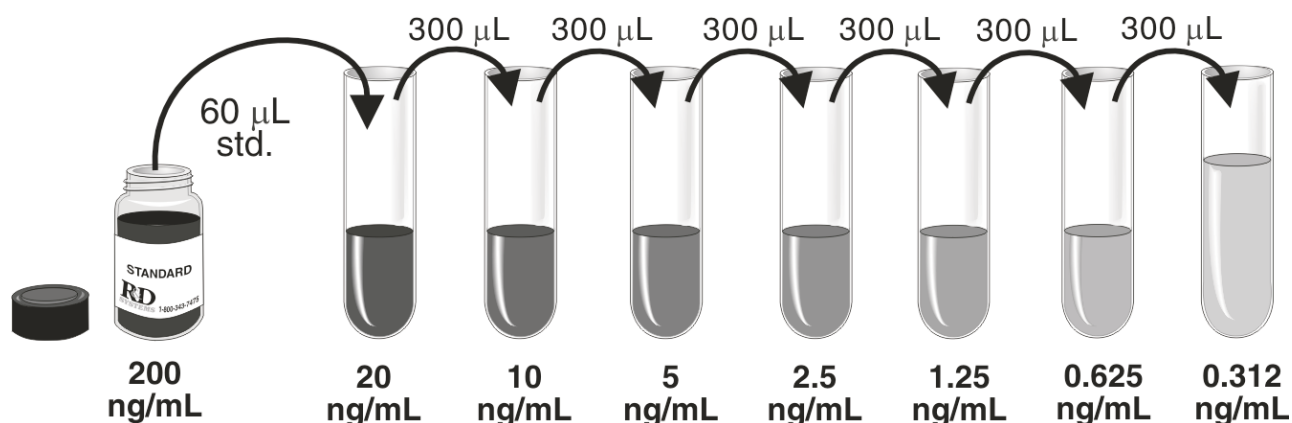
Bring all reagents to room temperature before use.

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 samples, controls, and standards 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, 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.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.



2. Add 100 μL Assay Diluent RD1-6 to each well.



3. Add 50 μL Standard, control or sample* to each well. Incubate 2 hrs. at RT.



4. Aspirate and wash 4 times.



5. Add 200 μL Conjugate to each well. Incubate 2 hrs. at RT.



6. Aspirate and wash 4 times.



7. Add 200 μL Substrate Solution to each well. Incubate 30 min. **Protect from light.**



8. Add 50 μL Stop Solution to each well. Read at 450 nm within 30 min.
 λ correction 540 or 570 nm

*Samples may require dilution. See Sample Preparation.

CALCULATION OF RESULTS

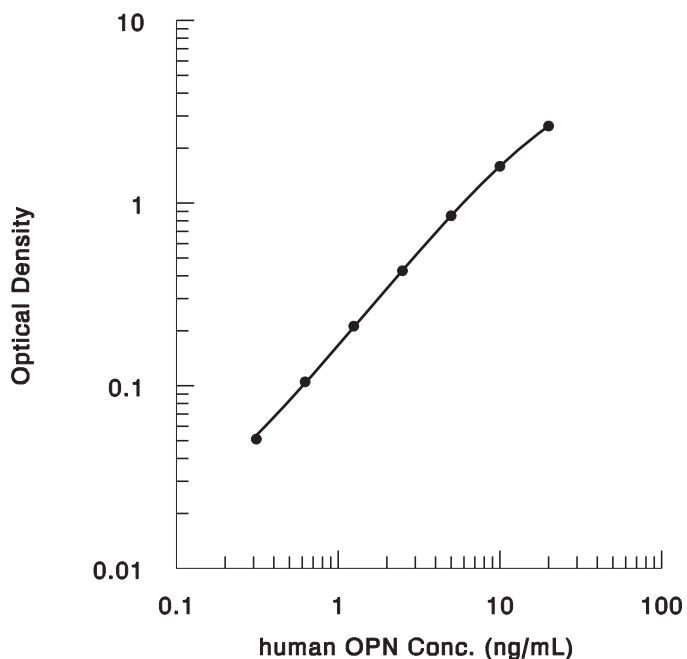
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 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	0.062 0.113	0.061	0.051
0.625	0.117 0.219	0.115	0.105
1.25	0.225 0.434	0.222	0.212
2.5	0.438 0.849	0.436	0.426
5	0.875 1.580	0.862	0.852
10	1.620 2.624	1.600	1.590
20	2.680	2.652	2.642

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

		Cell culture media (n=4)	Heparin plasma (n=4)	EDTA plasma (n=4)	Urine (n=5)	Breast milk (n=4)
1:2	Average % of Expected	109	103	103	99	105
	Range (%)	98 - 113	98 - 110	102 - 104	95 - 102	100 - 108
1:4	Average % of Expected	106	104	104	99	110
	Range (%)	99 - 114	102 - 105	103 - 105	97 - 100	108 - 112
1:8	Average % of Expected	104	104	105	100	113
	Range (%)	97 - 110	102 - 107	104 - 108	98 - 105	111 - 114
1:16	Average % of Expected	105	105	106	100	114
	Range (%)	100 - 108	101 - 112	103 - 109	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 - 78 for more details.

Plasma/Urine - Plasma and urine samples were evaluated for the presence of OPN in this assay.

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

*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 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 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.

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

SPECIFICITY

This assay recognizes recombinant and natural human OPN. The factors listed below were prepared at 200 ng/mL in Calibrator Diluent RD5-24 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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PLATE LAYOUT

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

	A	B	C	D	E	F	G	H
1								
2								
3								
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