Quantikine® ELISA

Mouse/Rat Osteopontin (OPN) Immunoassay

Catalog Number MOST00

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

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

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

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 αvβ1, αvβ3, αvβ5, α5β1, and α8β1 (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 α4β1, α4β1 and α9β1 (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-1β, 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 Mouse/Rat OPN Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse or rat 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 or rat 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 mouse or rat OPN.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse/rat OPN has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse or rat OPN present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat 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 or rat 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 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse/Rat OPN Microplate	893133	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse/rat 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.*
Mouse/Rat OPN Standard	893135	12.5 ng of recombinant OPN in a buffered protein base with preservatives; lyophilized.	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Mouse/Rat OPN Control	893136	Recombinant OPN in a buffered protein base with preservatives; lyophilized. The assay value of the Control should be within the range specified on the label.	
Mouse/Rat OPN Conjugate	893134	12 mL of a polyclonal antibody specific for mouse/rat OPN conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-12	895214	2 vials (21 mL/vial) of diluted animal serum with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* 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.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

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

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

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum 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 20 minutes at 2000 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 - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °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. The suggested dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD6-12. Complete the 100-fold dilution by adding 20 µL of this solution to 180 µL 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 µL of sample to 180 µL of Calibrator Diluent RD6-12.

Mouse urine samples require at least a 500-fold dilution. The suggested dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD6-12. Complete the 500-fold dilution by adding 10 µL of this solution to 490 µL of Calibrator Diluent RD6-12.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

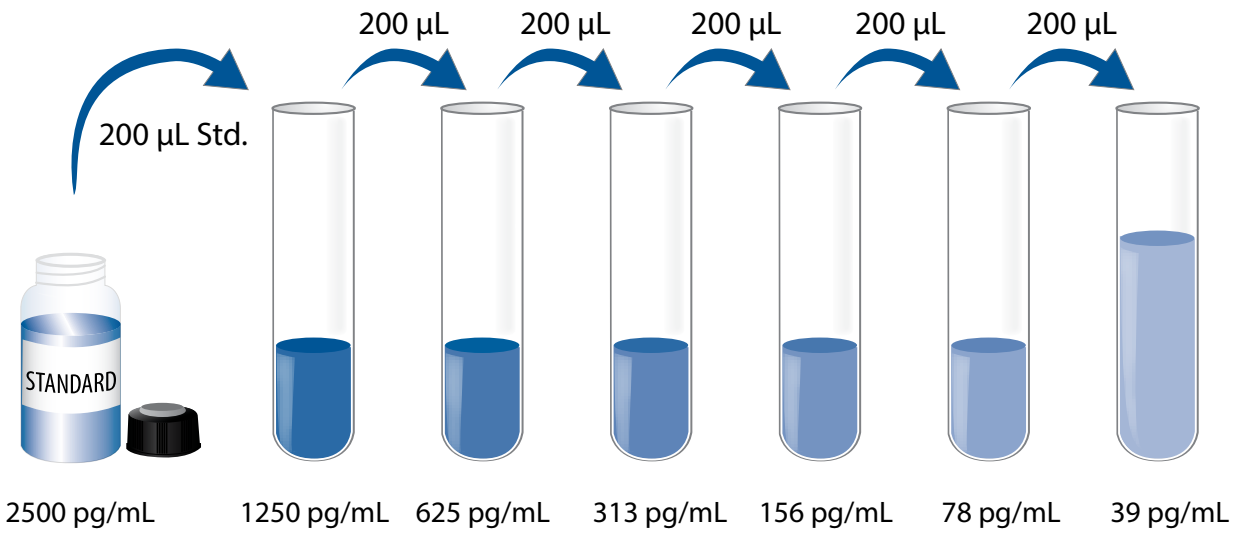
Mouse/Rat OPN Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 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. 100 µL of the resultant mixture is required per well.

Mouse/Rat OPN Standard - Reconstitute the Mouse/Rat 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 µL 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/Rat 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 µL of Assay Diluent RD1W to each well.
4. Add 50 µL 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 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 100 µL of Mouse/Rat 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 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 100 µL 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 may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

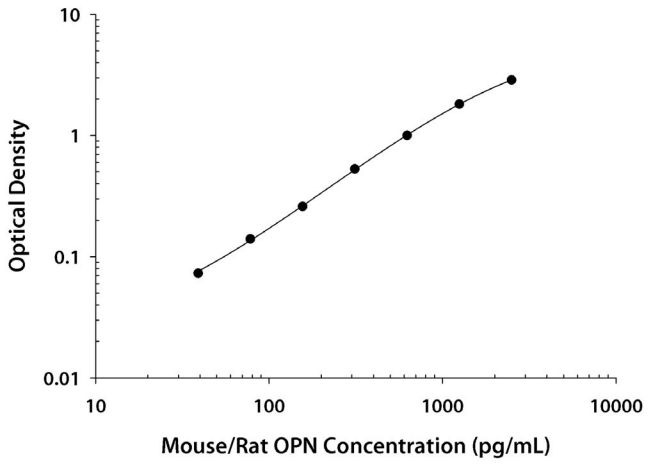
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse/rat 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.



(pg/mL)	O.D.	Average	Corrected
0	0.042 0.050	0.046	—
39	0.118 0.120	0.119	0.073
78	0.185 0.187	0.186	0.140
156	0.297 0.316	0.306	0.260
313	0.567 0.584	0.576	0.530
625	1.046 1.051	1.048	1.002
1250	1.841 1.883	1.862	1.816
2500	2.905 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. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	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 OPN spiked to levels throughout the range of the assay in 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%

SENSITIVITY

Forty-one assays were evaluated and the minimum detectable dose (MDD) of 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.

LINEARITY

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

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

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

*Samples were screened and diluted dependent upon sample values.

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

SAMPLE VALUES

Serum/Plasma/Urine - Samples were evaluated for detectable levels of OPN in this assay.

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

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

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

Cell Culture Supernates:

J774A.1 mouse reticulum cell sarcoma macrophage cells (1 x 10^6 cells/mL) were cultured for 3 days in RPMI containing 10% fetal bovine serum. An aliquot of the cell culture supernate was removed, assayed for detectable levels of OPN, and measured 266 ng/mL.

L-929 mouse fibroblast cells (0.5 x 10^5 cells/mL) were cultured for 3 days in MEM containing 10% equine serum and stimulated with 2.5 ng/mL LPS. An aliquot of the cell culture supernate was removed, assayed for detectable levels of 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 µg/mL ConA. An aliquot of the cell culture supernate was removed, assayed for detectable levels of 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 µg/mL ConA. An aliquot of the cell culture supernate was removed, assayed for detectable levels of OPN, and measured 347 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat OPN.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range mouse/rat 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-β R1
TSG

Recombinant human:
Enterokinase
Fibronectin
Thrombin
Vitronectin

Other recombinants:
bovine Osteopontin
bovine Enterokinase

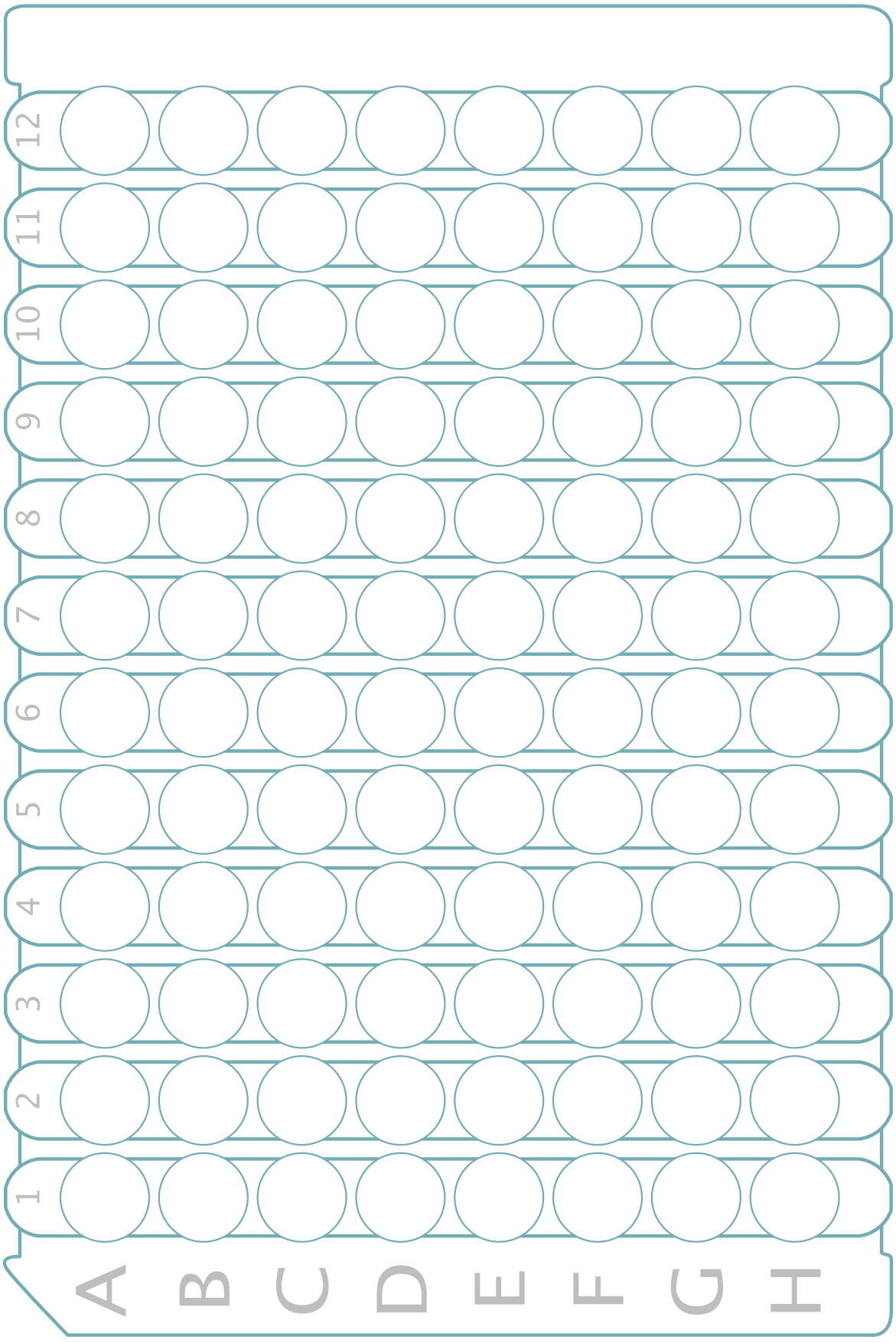
In this assay, NS0-expressed recombinant human Osteopontin shows 8.2% cross-reactivity at a concentration of 0.62 ng/mL.

REFERENCES

1. Giachelli, C.M. and S. Steitz (2000) Matrix Biol. **19**:615.
2. Mazzali, M. *et al.* (2002) QJM: An International Journal of Medicine **95**:3.
3. O'Regan, A.W. (2003) Cytokine Growth Factor Rev. **14**:479.
4. Rittling, S.R. and A.F. Chambers (2004) Br. J. Cancer **90**:1877.
5. Denhardt, D.T. *et al.* (2001) J. Clin. Invest. **107**:1055.
6. Gravallese, E.M. (2003) J. Clin. Invest. **112**:147.
7. Giachelli, C.M. *et al.* (2005), Circ. Res. **96**:717.
8. Fisher, L.W. and N.S. Fedarko (2003) Connect. Tissue Res. **44**(Suppl 1):33.
9. Miyazaki, Y. *et al.* (1990) J. Biol. Chem. **265**:14432.
10. Behrend, E.I. *et al.* (1993) J. Biol. Chem. **268**:11172.
11. Singh, K. *et al.* (1990) J. Biol. Chem. **265**:18696.
12. Agnihotri, R. *et al.* (2001) J. Biol. Chem. **276**:28261.
13. O'Regan, A.W. *et al.* (1999) J. Immunol. **162**:1024.
14. Suzuki, K. *et al.* (2002) J. Bone Miner. Res. **17**:1486.
15. Reinholt, F.P. *et al.* (1990) Proc. Natl. Acad. Sci. USA **87**:4473.
16. Ross, F.P. *et al.* (1993) J. Biol. Chem. **268**:9901.
17. Asou, Y. *et al.* (2001) Endocrinology **142**:1325.
18. Brown, L.F. *et al.* (1992) Mol. Biol. Cell **3**:1169.
19. Giachelli, C.M. *et al.* (1995) Ann. N.Y. Acad. Sci. **760**:109.
20. O'Brien, K.D. *et al.* (1995) Circulation **92**:2163.
21. Weber, G.F. *et al.* (2002) J. Leukoc. Biol. **72**:752.
22. Giachelli, C.M. *et al.* (1998) Am. J. Pathol. **152**:353.
23. O'Regan, A.W. *et al.* (2000) J. Leukoc. Biol. **68**:495.
24. Ashkar, S. *et al.* (2000) Science **287**:860.
25. Johnson, G.A. *et al.* (2003) Biol. Reprod. **69**:1458.
26. Apparao, K.B. *et al.* (2001) J. Clin. Endocrinol. Metab. **86**:4991.
27. Murry, C.E. *et al.* (1994) Am. J. Pathol. **145**:1450.
28. Senger, D.R. *et al.* (1989) Anticancer Res. **9**:1291.
29. Fedarko, N.S. *et al.* (2001) Clin. Cancer Res. **7**:4060.
30. Katagiri, Y.U. *et al.* (1999) Cancer Res. **59**:219.
31. Petrow, P.K. *et al.* (2000) Arthritis Rheum. **43**:1597.
32. Koguchi, Y. *et al.* (2003) Am. J. Respir. Crit. Care Med. **167**:1355.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES