Quantikine™ ELISA

Mouse Osteoprotegerin/TNFRSF11B Immunoassay

Catalog Number MOP00

For the quantitative determination of mouse Osteoprotegerin (OPG) concentrations in cell culture supernates, serum, and plasma.

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

SECTION PAGE

Manufactured and Distributed by:

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

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

INTRODUCTION

Mouse osteoprotegerin (OPG)/TNFRSF11B (also known as OCIF or osteoclastogenesis inhibitory factor) is a member of the TNF receptor superfamily that plays a key role in bone remodeling (1- 3). Unlike most members of the TNF receptor superfamily, OPG apparently exists only in a soluble form as a decoy receptor for its ligands, RANKL/TNFSF11/OPG-L/ODF/TRANCE and TRAIL. Mouse OPG is a 380 amino acid (aa) secreted glycoprotein that exists naturally as either a 120 kDa disulfide-linked homodimer, or a 60 kDa monomer (2, 4-6). The mature molecule contains four cysteine-rich domains in the N-terminal 175 aa residues, and these are followed by two potential death domains (DDs) in the next 160 amino acids, plus a C-terminal heparin-binding domain which occupies the last 50 amino acids (4-6). Each cysteine-rich domain has two intra-domain disulfide bonds, both of which are needed for activity (4, 7). The two DDs are functional when expressed cytoplasmically as an artificial transmembrane molecule (6). Since OPG is a secreted soluble protein, the role of the DDs is unclear. The C-terminus will allow heparin-binding, and interchain dimerization occurs through this region's last cysteine. OPG polymorphisms exist in mice, with five aa differences noted between NIH Swiss and Balb/c mice (4, 5). Mouse OPG shares 86% and 95% aa identity to human and rat OPG, respectively (4). Monomeric OPG is generated from the homodimeric OPG by limited proteolysis which removes several C-terminal aa residues, including the cysteine residue involved in intermolecular disulfide bonds. Depending on the cell source, the ratio of monomeric to dimeric OPG may vary. In circulation, monomeric OPG predominates. OPG is active as either a monomer or dimer, with the dimer showing more potent bioactivity (8, 9). In humans, total blood OPG increases markedly with age. Cells reported to express OPG include fibroblasts (10), ameloblasts and odontoblasts (11), osteoblasts (12, 13), follicular dendritic cells (14), smooth muscle cells (14), and B cells (14). Human OPG is known to be active in mice (12).

In general, M-CSF and RANKL act in a cooperative manner to induce bone resorption. It has been suggested that M-CSF induces CFU-Macrophage precursors to proliferate and differentiate into tartrate-resistant acid phosphatase-negative ($TRAP^-$) mononuclear osteoclast precursors (mOC). When exposed to both M-CSF and RANKL, mOC differentiates into $TRAP^+$ mOC, which fuses under the influence of M-CSF, IL-1, and RANKL to become mature $TRAP^+$ multi-nuclear osteoclasts. Although potentially bone resorptive, these mature osteoclasts do not become active (or pit forming) unless acted on by IL-1 or RANKL (1, 3, 15, 16). Both M-CSF and RANKL are essential in this process, as each activates necessary, yet distinct, signal transduction pathways (3, 17). It was suggested that RANKL is the more critical molecule since it is subject to hormone and cytokine upregulation and downregulation (2, 18-21). Any of the above events which involve RANKL can be interrupted by OPG (2). Thus, OPG is a natural antagonist to RANKL-induced osteoclastogenesis and bone resorption. Molecules known to increase OPG expression include TGF-β, IL-13, and IFN-γ (21). Notably, OPG and RANKL are made by the same cell types in bone, and it appears that the local ratio of OPG to RANKL is critical to the outcome of bone resorption (16, 20, 22).

There are two TNF superfamily ligands that bind OPG. The first is RANKL, which can exist as either a type II transmembrane glycoprotein, or a soluble trimer (23-25). The second ligand is TRAIL, otherwise known as TNFSF10 and Apo2-Ligand. TRAIL is an apoptosis-inducing TNFSF member that also exists as either a membrane-bound molecule or a soluble trimer (26-28). Besides OPG, TRAIL has four additional known signaling and decoy receptors.

The Quantikine™ Mouse Osteoprotegerin/TNFRSF11B Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse OPG in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse OPG and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant mouse OPG accurately. Results obtained using natural mouse OPG showed dose response 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 natural mouse OPG.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse OPG has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse OPG present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse OPG 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 OPG 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 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.
- It is recommended that samples be pipetted within 15 minutes.
- 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 OPG Microplate	890966	96 well microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse OPG.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse OPG Standard	890968	2 vials of recombinant mouse OPG in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard and control for each assay.
Mouse OPG Control	890969	2 vials of recombinant mouse OPG in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse OPG Conjugate	890967	12 mL of a polyclonal antibody specific for mouse OPG conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	2 vials (21 mL/vial) of a buffered protein base 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
- **Polypropylene** 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 a preservative 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. Refer to the SDS 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 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: *Heparin and citrate plasma have not been validated for use in this assay. Grossly hemolyzed or lipemic samples are not suitable for use in this assay.*

SAMPLE PREPARATION

Use polypropylene tubes.

Cell culture supernates may require dilution.

Mouse serum and plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 40 µL of sample + 160 µL of Calbrator Diluent RD5-3.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

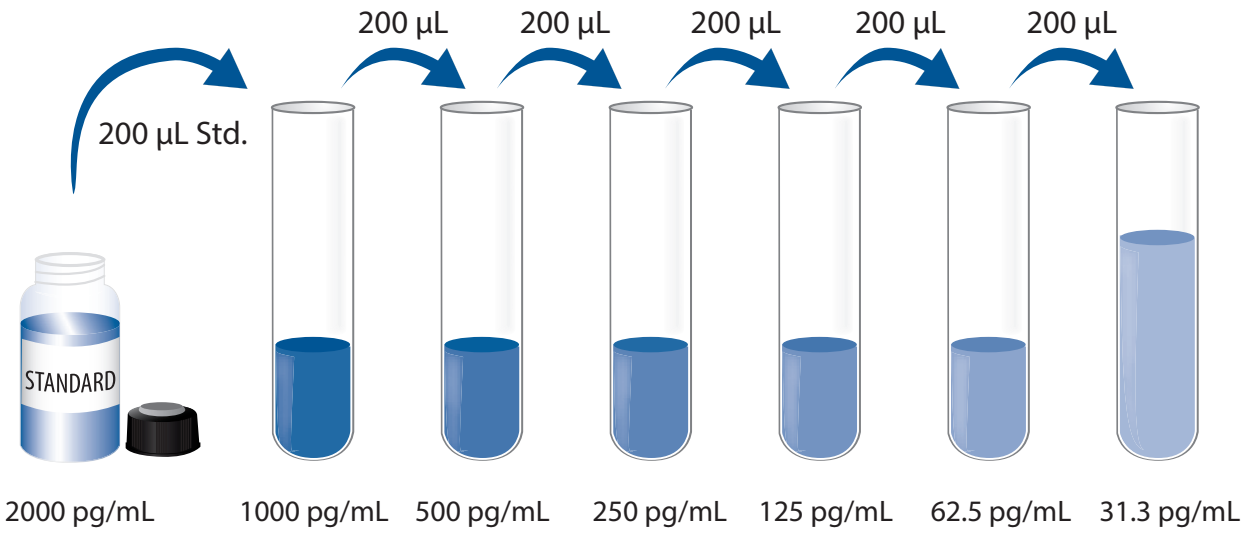
Mouse OPG Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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 480 mL of 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 OPG Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse OPG Standard with Calibrator Diluent RD5-3. Do not substitute other diluents. This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse OPG Standard (2000 pg/mL) serves as the high standard. Calibrator Diluent RD5-3 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 standards, controls, and samples be assayed in duplicate.

1. Prepare reagents and standard dilutions as directed by the previous section.
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 RD1-21 to each well.
4. Add 50 µL of standard, control or sample* per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.**
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 OPG 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. **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.

*Serum and plasma samples require dilution. See Sample Preparation section.

**For best precision, incubate the plate in a dark environment with minimum temperature fluctuation (*e.g.* room temperature incubator, desk drawer, or box).

CALCULATION OF RESULTS

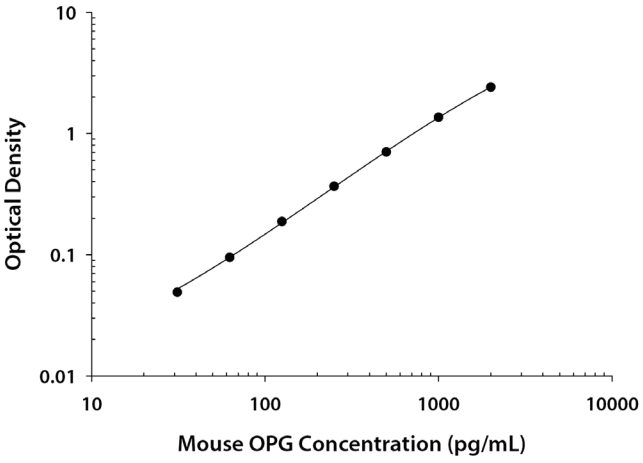
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 OPG 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	0.040 0.050	0.045	—
31.3	0.092 0.096	0.094	0.049
62.5	0.139 0.141	0.140	0.095
125	0.229 0.236	0.233	0.188
250	0.402 0.419	0.411	0.366
500	0.718 0.779	0.749	0.704
1000	1.359 1.450	1.405	1.360
2000	2.456 2.460	2.458	2.413

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 twenty 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	20	20	20
Mean (pg/mL)	69.9	311	650	68.0	332	741
Standard deviation	5.50	13.4	35.6	5.00	23.0	54.0
CV (%)	7.9	4.3	5.5	7.4	6.9	7.3

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture supernates (n=9)	101	85-116%
Serum* (n=11)	93	83-108%
EDTA plasma* (n=9)	97	88-107%

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

SENSITIVITY

Thirty-one assays were evaluated and the minimum detectable dose (MDD) of mouse OPG ranged from 1.0-6.9 pg/mL. The mean MDD was 2.8 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse OPG produced at R&D Systems®.

LINEARITY

To assess the linearity of the assay, five or more samples containing and/or spiked with high concentrations of mouse OPG were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Results from typical sample dilutions are shown.

Samples	Dilution	Observed (pg/mL)	Expected (pg/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
Cell culture supernates	1:2	1045		
	1:4	536	523	102
	1:8	258	261	99
	1:16	134	131	102
	1:32	66	65	102
Serum	1:2	1976		
	1:4	993	988	100
	1:8	519	494	105
	1:16	256	247	104
	1:32	127	124	102
EDTA plasma	1:2	1988		
	1:4	990	994	100
	1:8	532	497	107
	1:16	274	249	110
	1:32	139	124	112

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse OPG in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=37)	2.5	1.2-4.6	0.82
EDTA plasma (n=14)	2.2	1.7-3.9	0.63

Cell Culture Supernates:

Lungs from two mice were cultured in 40 mL RPMI containing 10% fetal bovine serum for 6 days. An aliquot of the cell culture supernate was removed, assayed for mouse OPG, and measured 4 ng/mL.

Mouse splenocytes (1 x 10^6 cells/mL) were cultured in RPMI containing 10% fetal bovine serum supplemented with 50 µM β-mercaptoethanol and 10 ng/mL of rhIL-2 for 3 days. An aliquot of the cell culture supernate was removed, assayed for mouse OPG, and measured 800 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse OPG.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. The same factors at 50 ng/mL were assayed for interference in the presence of mouse OPG. No significant cross-reactivity and interference was observed.

Recombinant mouse:

C10
CD40
Eotaxin
Fas
Fas Ligand
Flt-3 Ligand
G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-10 R
IL-12/IL-23 p40
IL-12 p70
IL-13
IL-17
IL-18
JE/MCP-1
KC
Leptin
LIF
M-CSF
MARC
MCP-5
MIP-1α
MIP-1β
MIP-1γ
MIP-2
OSM
PlGF-2
RANK
RANTES
SCF
TNF-α
TNF RI
TNF RII
TARC
Tpo
VEGF
VEGF R1

Recombinant human:

DcR3
DR6
APRIL
LIGHT

Recombinant human OPG cross-reacts approximately 11% in this assay.

Recombinant mouse RANKL and recombinant mouse TRAIL interfere at concentrations ≥ 1 ng/mL.

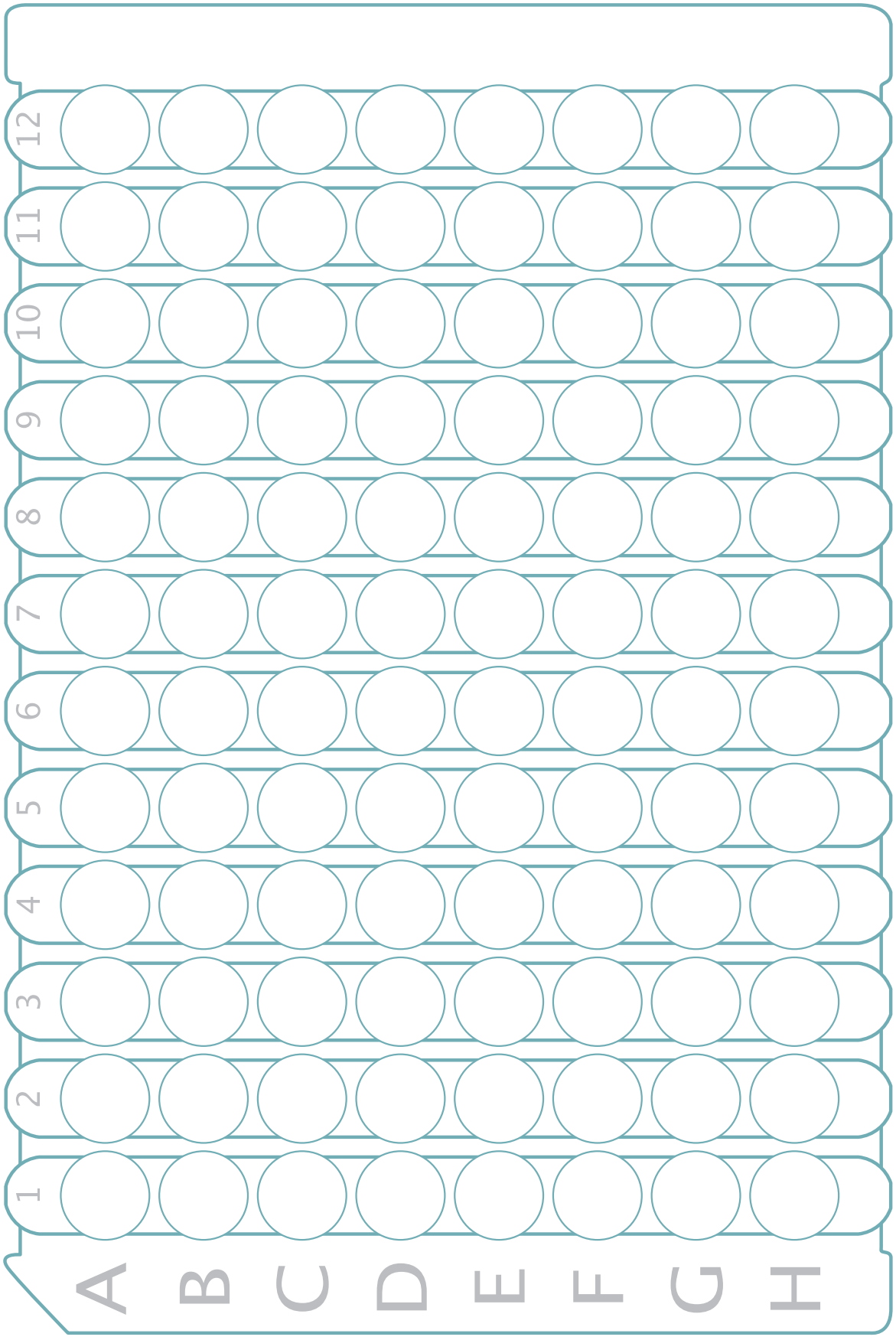
Normal rat and porcine serum samples were evaluated and measured less than the lowest standard, 31.3 pg/mL.

REFERENCES

1. Takahashi, N. *et al.* (1999) Biochem. Biophys. Res. Commun. **256**:449.
2. Yasuda, H. *et al.* (1999) Bone **25**:109.
3. Jimi, E. *et al.* (1999) J. Immunol. **163**:434.
4. Simonet, W.S. *et al.* (1997) Cell **89**:309.
5. Mizuno, A. *et al.* (1998) Gene **215**:339.
6. Yamaguchi, K. *et al.* (1998) J. Biol. Chem. **273**:5117.
7. Morinaga, T. *et al.* (1998) Eur. J. Biochem. **254**:685.
8. Tomoyasu, A. *et al.* (1998) Biochem. Biophys. Res. Commun. **245**:382.
9. Yano, K. *et al.* (1999) J. Bone Miner. Res. **14**:518.
10. Sakata, M. *et al.* (1999) J. Bone Miner. Res. **14**:1486.
11. Rani, C.S.S. and M. MacDougall (2000) Mol. Cell Biol. Res. Commun. **3**:145.
12. Udagawa, N. *et al.* (2000) Endocrinology **141**:3478.
13. Hofbauer, L.C. *et al.* (1998) Biochem. Biophys. Res. Commun. **250**:776.
14. Yun, T.J. *et al.* (1998) J. Immunol. **161**:6113.
15. Shalhoub, V. *et al.* (1999) J. Cell. Biochem. **72**:251.
16. O'Brien, E.A. *et al.* (2001) Bone **28**:208.
17. Lacey, D.L. *et al.* (2000) Am. J. Pathol. **157**:435.
18. Takai, H. *et al.* (1998) J. Biol. Chem. **273**:27091.
19. Kanzawa, M. *et al.* (2000) Eur. J. Endocrinol. **142**:661.
20. Horwood, N.J. *et al.* (1998) Endocrinology **139**:4743.
21. Nakashima, T. *et al.* (2000) Biochem. Biophys. Res. Commun. **275**:768.
22. Takami, M. *et al.* (2000) Endocrinology **141**:4711.
23. Yasuda, H. *et al.* (1998) Proc. Natl. Acad. Sci. USA **95**:3597.
24. Lacey, D.L. *et al.* (1998) Cell **93**:165.
25. Anderson, D.M. *et al.* (1997) Nature **390**:175.
26. Truneh, A. *et al.* (2000) J. Biol. Chem. **275**:23319.
27. Wiley, S.R. *et al.* (1995) Immunity **3**:673.
28. Emery, J.G. *et al.* (1998) J. Biol. Chem. **273**:14363.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES