Quantikine® ELISA

Human Osteocalcin Immunoassay

Catalog Number DSTCN0

For the quantitative determination of human Osteocalcin concentrations in cell culture supernates and EDTA plasma.

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

Osteocalcin, also known as Bone Gla Protein (BGP), is a secreted protein that plays important roles in bone physiology, energy metabolism, and male fertility (1). Human Osteocalcin is synthesized by osteoblasts with a 28 amino acid (aa) propeptide and a 49 aa mature peptide (2). The propeptide is removed intracellularly to generate the mature 5-6 kDa Osteocalcin (3). Osteocalcin is modified by gamma carboxylation (Gla) on three glutamic acid residues, preferentially at Glu21 and Glu24 and less frequently at Glu17 (3, 4). The propeptide serves as a recognition domain for a Vitamin K-dependent carboxylase that generates the Gla modification (5). The anti-coagulant compound Warfarin reduces the availability of Vitamin K, thereby blocking gamma carboxylation of Osteocalcin as well as several factors in the blood coagulation cascade. The Gla-modification of Osteocalcin is required for its binding to hydroxyapatite and accumulation in mineralized bone (6, 7). Warfarin administration prevents incorporation into bone and results in elevated circulating levels of Osteocalcin (8). Osteocalcin is released from bone as the intact mature protein and peptide fragments (9). Serum levels are positively correlated with bone turnover and are decreased in osteopetrosis, a disorder of impaired bone resorption (9-12). Circulating Osteocalcin and its fragments are normally cleared by the kidney but accumulate in the serum during uremia (7, 13).

The un- or under-carboxylated forms of Osteocalcin are directly involved in glucose homeostasis (14). They induce the developmental growth of pancreatic islets, Insulin secretion, production of GLP-1, improved glucose tolerance, production of Adiponectin, and reduced fat accumulation (15-19). Insulin R signaling enhances this effect by promoting bone resorption and the synthesis of uncarboxylated Osteocalcin (12, 19). The peptide hormone Leptin inhibits Insulin secretion in part by blocking Osteocalcin bioactivity (16). Serum levels of Osteocalcin are inversely correlated with Leptin, triglyceride, and fasting glucose levels and positively correlated with Adiponectin and Insulin levels (20-22). The proportion of Gla-Osteocalcin is increased in overweight and obese humans (23).

Osteocalcin interacts with the 7-transmembrane segment protein GPRC6A which is expressed on renal proximal and distal tubules, small intestinal epithelial cells, pancreatic beta cells, and testicular Leydig cells (17, 18, 24-26). In mouse, GPRC6A deficiency leads to fatty liver, hyperglycemia, Insulin resistance, decreased bone mineralization, reduced testosterone production, and impaired renal function (17, 25). Osteocalcin released during bone resorption interacts with GPCR6A and induces the production of testosterone by Leydig cells (26, 27). Osteocalcin activity is inhibited by OST-PTP, a protein tyrosine phosphatase that limits Insulin production and sensitivity (14). OST-PTP inhibits the signaling of Insulin R and also regulates male fertility (12, 26).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Osteocalcin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Osteocalcin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Osteocalcin 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 Osteocalcin 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, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

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

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Osteocalcin Microplate	894534	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Osteocalcin.	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.*
Human Osteocalcin Conjugate	894535	21 mL of a monoclonal antibody specific for human Osteocalcin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Osteocalcin Standard	894536	Synthetic human Osteocalcin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-117	895859	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-67	895860	21 mL of diluted animal serum with preservatives. *Used undiluted for EDTA plasma samples. Used diluted 1:5 for cell culture supernate samples.*	
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	895032	6 mL of 2 N sulfuric 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.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human Osteocalcin Controls (optional; available from R&D Systems).

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. 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 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 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Hemolyzed, lipemic, or icteric samples are not suitable for use in this assay.*
Samples with abnormally high levels of Albumin interfere in this assay.
Serum, heparin plasma, and citrate plasma are not suitable for use in this assay.

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. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

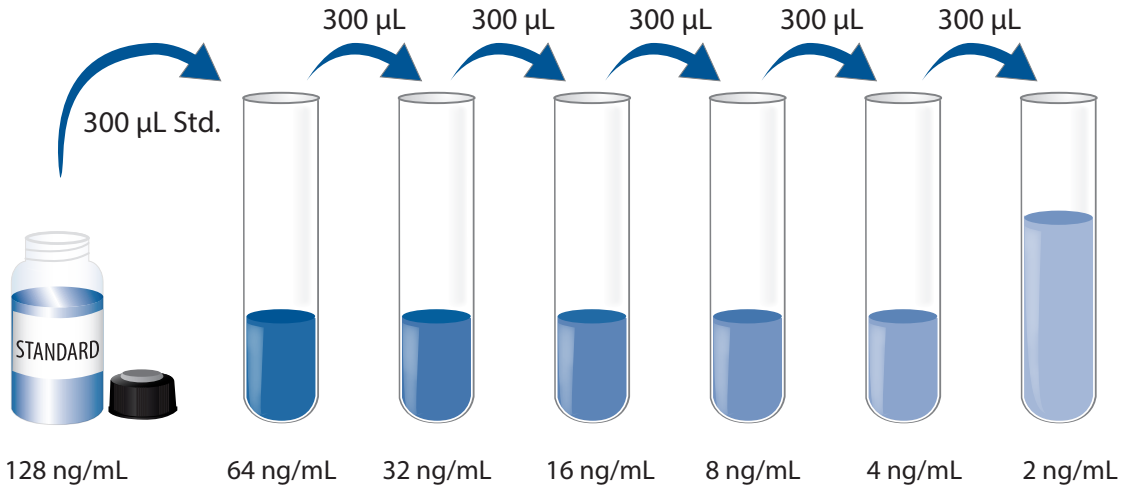
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD6-67 (diluted 1:5) - **For cell culture supernate samples only.** Add 4 mL of Calibrator Diluent RD6-67 to 16 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD6-67 (diluted 1:5).

Human Osteocalcin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Osteocalcin Standard with deionized or distilled water. This reconstitution produces a stock solution of 128 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 300 µL of Calibrator Diluent RD6-67 (diluted 1:5) (*for cell culture supernates samples*) or Calibrator Diluent RD6-67 (*for EDTA plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 64 ng/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 ng/mL).

Note: *Pipette curve within 45 minutes of preparation.*



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, and reseal.
3. Add 100 µL of Assay Diluent RD1-117 to each well.
4. Add 50 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
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 Human Osteocalcin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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.

CALCULATION OF RESULTS

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

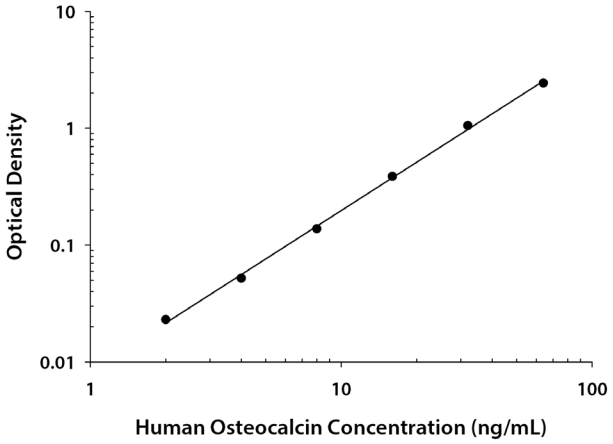
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Osteocalcin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

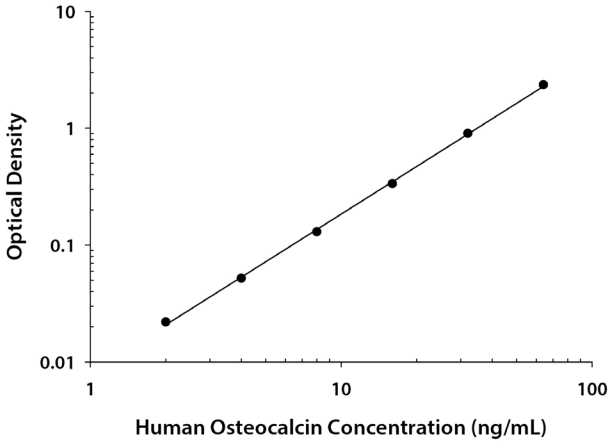
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.016 0.018	0.017	—
2	0.038 0.042	0.040	0.023
4	0.069 0.069	0.069	0.052
8	0.151 0.159	0.155	0.138
16	0.399 0.409	0.404	0.387
32	1.056 1.078	1.067	1.050
64	2.306 2.597	2.452	2.435

EDTA PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.019 0.023	0.021	—
2	0.043 0.043	0.043	0.022
4	0.072 0.073	0.073	0.052
8	0.148 0.154	0.151	0.130
16	0.352 0.361	0357	0.336
32	0.918 0.928	0.923	0.902
64	2.373 2.384	2.379	2.358

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.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	8.15	17.2	33.3	7.91	16.1	33.6
Standard deviation	0.268	0.395	0.544	0.766	0.991	1.66
CV (%)	3.3	2.3	1.6	9.7	6.2	4.9

EDTA PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	9.06	19.6	40.0	9.99	19.9	40.1
Standard deviation	0.306	0.379	0.653	0.694	1.39	2.44
CV (%)	3.4	1.9	1.6	6.9	7.0	6.1

RECOVERY

The recovery of human Osteocalcin spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	98-120%
EDTA plasma (n=4)	102	80-116%

SENSITIVITY

Fifty-seven assays were evaluated and the minimum detectable dose (MDD) of human Osteocalcin ranged from 0.071-0.898 ng/mL. The mean MDD was 0.402 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.

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human Osteocalcin were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	EDTA plasma (n=5)
1:2	Average % of Expected	102	103
	Range (%)	97-113	96-123
1:4	Average % of Expected	97	101
	Range (%)	89-108	91-132
1:8	Average % of Expected	94	100
	Range (%)	85-103	90-132
1:16	Average % of Expected	92	88
	Range (%)	80-103	81-93

CALIBRATION

This immunoassay is calibrated against a highly purified synthetic human Osteocalcin.

SAMPLE VALUES

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

Sample Type	Mean fo Detectable (ng/mL)	% Detectable	Range (ng/mL)
EDTA plasma (n=35)	19.3	97	ND-65.0

ND=Non-detectable

Cell Culture Supernates:

MG-63 human osteosarcoma cells were cultured in MEM supplemented with 10% fetal bovine serum until confluent. An aliquot of the cell culture supernate was removed, assayed for human Osteocalcin, and measured 3.65 ng/mL.

ML-1 human myeloblastic leukemia cells were cultured in IMDM supplemented with 20% fetal bovine serum until confluent. An aliquot of the cell culture supernate was removed, assayed for human Osteocalcin, and measured 13.0 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Osteocalcin. This assay also recognizes Des-γ-carboxylated-Osteocalcin.

REFERENCES

1. Booth, S.L. *et al.* (2013) Nat. Rev. Endocrinol. **9**:**43**.
2. Celeste, A.J. *et al.* (1986) EMBO J. **5**:1885.
3. Benton, M.E. *et al.* (1995) Biochemistry **34**:9541.
4. Poser, J.W. *et al.* (1980) J. Biol. Chem. **255**:8685.
5. Suttie, J.W. *et al.* (1993) FASEB J. **7**:445.
6. Price, P.A. *et al.* (1976) Proc. Natl. Acad. Sci. USA **73**:1447.
7. Price, P.A. *et al.* (1981) J. Biol. Chem. **256**:12760.
8. Price, P.A. and M.K. Williamson (1981) J. Biol. Chem. **256**:12754.
9. Ivaska, K.K. *et al.* (2004) J. Biol. Chem. **279**:18361.
10. Schafer, A.L. *et al.* (2011) J. Clin. Endocrinol. Metab. **96**:E1982.
11. Ivaska, K.K. *et al.* (2005) Clin. Chem. **51**:618.
12. Ferron, M. *et al.* (2010) Cell **142**:296.
13. Gundberg, C.M. and R.S. Weinstein (1986) J. Clin. Invest. **77**:1762.
14. Lee, N.K. *et al.* (2007) Cell **130**:456.
15. Ferron, M. *et al.* (2008) Proc. Natl. Acad. Sci. USA **105**:5266.
16. Hinoi, E. *et al.* (2008) J. Cell Biol. **183**:1235.
17. Wei, J. *et al.* (2014) Diabetes **63**:1021.
18. Mizokami, A. *et al.* (2013) PLoS ONE **8**:e57375.
19. Fulzele, K. *et al.* (2010) Cell **142**:309.
20. Saleem, U. *et al.* (2010) Arterioscler. Thromb. Vasc. Biol. **30**:1474.
21. Fernandez-Real, J.M. *et al.* (2009) J. Endocrinol. Metab. **94**:237.
22. Hamann, C. *et al.* (2011) Am. J. Physiol. Endocrinol. Metab. **301**:E1220.
23. Foresta, C. *et al.* (2010) J. Clin. Endocrinol. Metab. **95**:3502.
24. Pi, M. and L.D. Quarles (2012) Endocrinology **153**:2062.
25. Pi, M. *et al.* (2008) PLoS ONE **3**:e3858.
26. Oury, F. *et al.* (2011) Cell **144:**796.
27. Oury, F. *et al.* (2013) J. Clin. Invest. **123**:2421.