BioLegend®

Enabling Legendary Discovery™

LEGEND MAX™

ELISA Kit with Pre-coated Plate

Human Osteocalcin (Uncarbox.)

Cat. No. 446707

ELISA Kit for Accurate Quantitation of Human Osteocalcin (Uncarboxylated) from Cell Culture Supernatant, Serum, Plasma and Other Biological Fluids

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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Introduction:

Osteocalcin is the bone's most abundant non-collagenous protein. It is produced by osteoblasts as a 49 amino acid protein with 3 glutamine acid residues (Glu 17, 21, and 24) that are gamma carboxylated in a vitamin K dependent manner. The fully carboxylated protein has a high affinity for the bone extracellular matrix. Bone resorption results in a decrease in pH, causing the decarboxylation of osteocalcin. This generates partially decarboxylated (undercarboxylated), or fully decarboxylated (uncarboxylated) osteocalcin. These forms of osteocalcin have decreased affinity for the bone extracellular matrix and are released into the bloodstream.

Fully carboxylated osteocalcin requires high calcium concentrations to be fully folded. However, under- and un-carboxylated osteocalcin forms do not require calcium to maintain their structure, and are fully folded within the blood. These forms can bind the receptor GPCR6a and act as hormones promoting insulin sensitivity, glucose tolerance, and testosterone biosynthesis. In patients with diabetes, reduced serum levels of osteocalcin are negatively correlated with obesity and insulin resistance. Furthermore, studies in mice have supported potential therapeutic applications for osteocalcin in both obesity and insulin resistance.

BioLegend's LEGEND MAX™ Osteocalcin (Uncarbox.) ELISA kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that recognizes the uncarboxylated form of osteocalcin, allowing for specific study of this hormonal form. The plate is pre-coated with a monoclonal mouse capture antibody. The detection antibody is a biotinylated monoclonal mouse antibody. This kit is specifically designed for the accurate quantitation of human osteocalcin from cell culture supernatant, serum, plasma and other biological fluids. It is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume	Part #
Anti-Human Osteocalcin Pre-coated 96-well Strip Microplate	1 plate		750000209
Human Osteocalcin Detection Antibody	1 bottle	12 mL	750000210
Human Osteocalcin Standard	1 vial	lyophilized	750000214
Avidin-HRP	1 bottle	12 mL	77897
Assay Buffer B	1 bottle	25 mL	79128
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution F	1 bottle	12 mL	76335
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

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Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Polypropylene tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate wells</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Detection Antibody</td><td rowspan="6">Store opened reagents between 2°C and 8°C and use within one month.</td></tr>
<tr><td>Avidin-HRP</td></tr>
<tr><td>Assay Buffer B</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution F</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.

3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.
4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at < -70°C. Avoid repeated freeze-thaw cycles. Serum free media is recommended where possible. Test any new lots of FBS for lot to lot variation in background levels of osteocalcin. Background signal caused by FBS in media must be tested if used.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x *g*. Remove serum layer and assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in citrate, heparin or EDTA containing tubes. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the lyophilized Human Osteocalcin (Uncarboxylated) Standard by adding the volume of Assay Buffer B to make the 20 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/ LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.
3. In general, serum and plasma samples require a 4-fold dilution, but

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samples can be diluted 2-32 fold to fit the standard range of the assay. Tissue culture supernatants and cell culture samples do not require dilution, but background signal of media used must be tested. If dilution is required, use Assay Buffer B. Lower or higher dilutions can be used as needed, but must be quantified to fit the assay range by the end user.

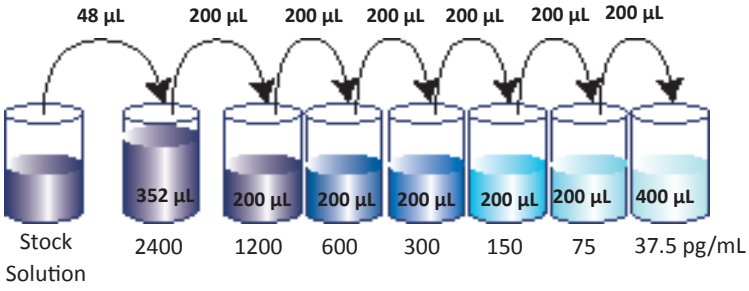
Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Add 48 µL of the 20 ng/mL stock solution to 352 uL of Assay Buffer B in a separate test tube to generate the top standard of 2400 pg/mL. Perform six two-fold serial dilutions of the 2400 pg/mL top standard using Assay Buffer B as the diluent. Thus, the Human Osteocalcin (Uncarboxylated) standard concentrations in the tubes are 2400 pg/mL, 1200 pg/mL, 600 pg/mL, 300 pg/mL, 150 pg/mL, 75 pg/mL, and 37.5 pg/mL, respectively. Assay Buffer B serves as the zero standard (0 pg/mL).



4. Add 50 µL of Assay Buffer B to wells that will contain standard and sample dilutions. Then add 50 µL of standard dilutions or samples to the appropriate wells.

5. Seal the plate with a Plate Sealer included in the kit and incubate on plate shaker 30-60 seconds at low speed (150 -240 rpm), then incubate the plate overnight (23-25 hrs) at 4°C without shaking. (NOTE: do not over shake plate as this will greatly reduce assay signal)

6. Discard the contents of the plate into a sink. Wash the plate 4 times with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

7. Add 100 µL of Human Osteocalcin (Uncarboxylated) Detection Antibody solution to each well. Seal the plate and incubate at room temperature 1 hr with for 30-60 seconds on shaker at low speed, then the remaining time sitting.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 6.

9. Add 100 µL of Avidin-HRP solution to each well, seal the plate and incubate at room temperature 30 min with 30-60 seconds on shaker at low speed, then remaining time sitting.

10. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 6. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

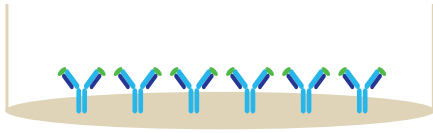
11. Add 100 µL of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells containing Human Osteocalcin (Uncarboxylated) should turn blue in color with intensity proportional to its concentration. It is not necessary to seal the plate during this step.

12. Stop the reaction by adding 100 µL of Stop Solution to each well. The solution color should change from blue to yellow.

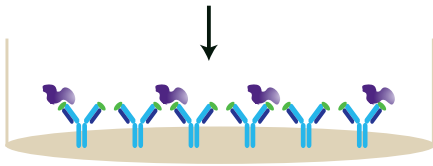
13. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Assay Procedure Summary

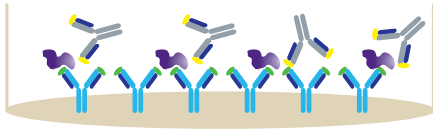
1. Add 50 µL Assay Buffer B to standard wells and sample wells



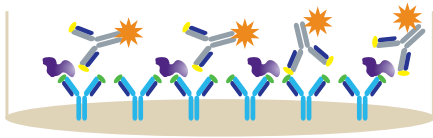
2. Add 50 µL of standard or sample
Incubate 30-60 seconds shaking, then overnight (23-25 hr) sitting at 4°C



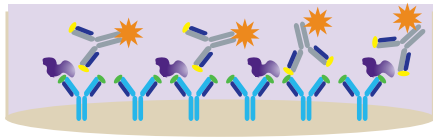
3. Wash 4 times
Add 100 µL Detection Antibody solution
Incubate 30-60 seconds shaking
1 hr, RT, sitting



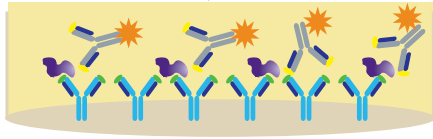
4. Wash 4 times
Add 100 µL Avidin-HRP solution
Incubate 30-60 seconds shaking
30 min, RT, sitting



5. Wash 5 times
Add 100 µL Substrate Solution F
Incubate 15 min, RT, in the dark



6. Add 100 µL Stop Solution



7. Read absorbance at 450 nm and 570 nm

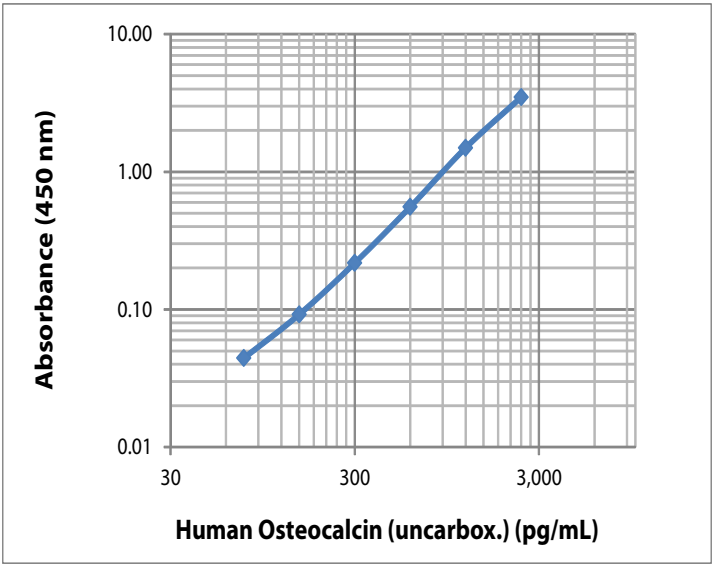
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics:

Specificity: This kit recognizes natural and synthetic Human Osteocalcin (Uncarboxylated). No cross reactivity was observed when this kit was used to analyze the following recombinant proteins at 50 ng/mL.

Human	MGP, BMP-2, GMCSF, Osteopontin, CCL1, CCL2, CCL3, CCL5, CCL8, CCL11, CCL13, CCL14, CCL19, CXCL9, CXCL10, CXCL11, IL-8, IL-10, IL-12(p70), IL-15, IL-17A, IL-18, Cystatin C, EGF, EPO, GCSF, FASL, FGF-basic, IFN-γ, IGF-1, LT-α, MMP-2, NGAL, Paxillin, PDGF-BB, PLGF-1, PTH, RBP4, Resistin, SCF, TGF-α, TGF-β3, and TNF-α

No detection in mouse, rat, cynomologus, or rhesus macaque serum. Bovine osteocalcin has 97% homology with human, and has high cross reactivity. FBS causes background in this ELISA in a lot specific manner. Some lots of FBS have very high background in osteocalcin, and others do not. Heat treatment will decrease FBS background. It is recommended that serum free media be used were possible or that each new lot of FBS be tested for acceptable background levels prior to use.

Cross reactivity with fully carboxylated osteocalcin is <4.0% and undercarboxylated <5.2%.

Sensitivity: The minimum detectable concentration of human osteocalcin (uncarboxylated) is 14.72 ± 5.0 pg/mL (n=10).

Recovery: Synthetic Uncarboxylated Osteocalcin (1500, 375, and 93.8 pg/mL) was spiked into five different human samples, and then analyzed with the LEGEND MAX™ Human Osteocalcin (Uncarbox.) ELISA Kit.

Sample Type	N	% Recovery
Serum	5	95.3
Citrate Plasma	5	106.5
EDTA Plasma	5	94.1
Heparin Plasma	5	101.1

Linearity: Natural human samples were diluted 2 fold to produce samples within the dynamic range of the kit. Samples were then assayed to determine the dilutional linearity.

Sample Type	N	% Linearity
Serum	5	100.1
Citrate Plasma	5	93.6
EDTA Plasma	5	105.5
Heparin Plasma	5	110.7

Intra-Assay Precision: Two samples containing different Human Osteocalcin (Uncarboxylated) concentrations were tested on one plate with 16 replicates.

Concentration	Sample 1	Sample 2
Number of Replicates	15	16
Mean Concentration (pg/mL)	1312	258
Standard Deviation	54.4	8.1
%CV	4.1	3.1

Inter-Assay Precision: Two samples containing different Human Osteocalcin (Uncarboxylated) concentrations were tested in four independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	4	4
Mean Concentration (pg/mL)	1269	247.9
Standard Deviation	102.9	17.5
%CV	8.1	7.1

Normal Biological Samples:
Human serum and plasma samples (n = 28) were assayed for natural human osteocalcin (uncarboxylated). Serum and plasma range detected was 1,100 - 11,600 pg/mL. Serum, Heparin, and Citrate plasma samples gave very similar readings in paired sample tests, but EDTA plasma had somewhat higher concentrations detected.

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN3)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template

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

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9727 Pacific Heights Blvd.
San Diego, CA 92121
Tel: 1.858.768.5800
Tel: US & Canada Toll-Free: 1.877.Bio-Legend (1.877.246.5343)
Fax: 1.877.455.9587
Email: info@biolegend.com
biolegend.com

For a complete list of world-wide BioLegend offices and distributors, please visit our website at: biolegend.com

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