Osteocalcin (1-43/49) ELISA

For the quantitative determination of human carboxylated osteocalcin (1-43) (N-terminal or mid-regional) and human carboxylated osteocalcin (1-49) (intact) in serum or plasma.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 43-OSNHU-E01
Size: 96 wells
Version: V9/RUO/2020-02- ALPCO 2.1

INTENDED USE

This ELISA (enzyme-linked immunosorbent assay) kit is intended for the quantitative determination of both human carboxylated osteocalcin (1-49) and carboxylated osteocalcin (1-43) (also referred as N-terminal and mid-regional osteocalcin) levels in serum or plasma samples. This assay is useful for researching the bone formation or osteoblast activity associated with changes in the rate of bone turnover in metabolic bone disease. This kit is for research use only.

INTRODUCTION

Osteocalcin [also known as bone Gla protein (BGP)] is a major noncollagenous protein found in bone and dentin. Synthesis of osteocalcin involves vitamin K and vitamin D_3. Freshly synthesized osteocalcin is part released into the bloodstream and partly incorporated into the bone matrix. Both osteocalcin (1-49) and its fragments, including osteocalcin (1-43), are released into the blood stream. Serum osteocalcin (1-43) is also generated by catabolic breakdown of osteocalcin (1-49) in circulation, the liver and kidneys, as well as by degradation during storage of samples, because of a labile six-amino acid C-terminal sequence that, at room temperature, is easily cleaved off. Several studies have confirmed that the measurement of the much more stable N-terminal and mid-regional osteocalcin [osteocalcin (1-43/49)] is useful and may contribute to more accurate assessments of the bone turnover rate.

As osteocalcin is manufactured by osteoblasts, it is often used as a biochemical marker, or biomarker, for the bone formation process. It has been routinely observed that higher serum-osteocalcin levels are relatively well correlated with increases in bone mineral density (BMD) during treatment with anabolic bone formation drugs for osteoporosis, such as Forteo. In many research studies, osteocalcin is used as a preliminary biomarker for the effectiveness of a given drug on bone formation.

PRINCIPLE OF THE ASSAY

The assay utilizes the two-site “sandwich” technique with two selected antibodies that bind to different epitopes of human osteocalcin. Assay standards, controls, and samples are added directly to wells of a streptavidin coated microtiter plate. Subsequently, a mixture of biotinylated human osteocalcin N-terminal region specific polyclonal antibody and a peroxidase-labeled human osteocalcin 20 – 43 region specific monoclonal antibody is added to each well. After the first incubation period, a “sandwich” of “biotinylated antibody – human osteocalcin – HRP-monoclonal antibody” is formed and this immunocomplex is captured to the wall of the microtiter plate via streptavidin-biotin affinity binding. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the wells are then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human osteocalcin in a test sample. A standard curve is generated by plotting the absorbance versus the respective human osteocalcin concentration for each standard on point-to-point or 4 parameter curve fit. The concentration of human osteocalcin in test samples is interpolated from this standard curve.

REAGENTS

Preparation and Storage

This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit, refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

Reagents Provided

1. Streptavidin Coated Microplate
One microplate coated with anti-human FGF-21 antibody.
Qty: 1 x 96 well microplate
Storage: 2 – 8°C
Preparation: Ready to use

2. HRP-Conjugated Osteocalcin Antibody
HRP-conjugated monoclonal anti-human osteocalcin (20-43) antibody in stabilized protein matrix.
Qty: 1 x 1.2 mL
Storage: 2 – 8°C
Preparation: 21X Concentrate. Reagent must be diluted with Biotinylated Osteocalcin Antibody.

3. Biotinylated Osteocalcin Antibody
Biotinylated anti-human osteocalcin N-terminal region specific antibody in stabilized protein matrix.
Qty: 2 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to use

4. Wash Concentrate
Surfactant in a phosphate buffered saline with non-azide preservative.
Qty: 1 x 30 mL
Storage: 2 – 25°C
Preparation: 30X Concentrate. Dilute contents with 870 mL distilled water and mix well.

5. HRP Substrate
Tetramethylbenzidine (TMB) with stabilized hydrogen peroxide.
Qty: 1 x 22 mL
Storage: 2- 8°C
Preparation: Ready to use.

6. Stop Solution
1.0 M sulfuric acid
Qty: 1 x 12 mL
Storage: 2 -25 2 – 8°C
Preparation: Ready to use

7. Human Osteocalcin Standards
Human osteocalcin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. **Refer to vials for exact concentration for each standard.**
Qty: 6 vials
Storage: 2 – 8°C (Lyophilized), <-20°C (Reconstituted) Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of deionized water, allowed to sit 10 minutes, then mixed by inversions or gentle vortexing. Ensure solids are dissolved completely prior to use.

8. Human Osteocalcin Controls
Human osteocalcin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. **Refer to vials for exact concentration range for each control.**
Qty: 2 Vials
Storage: 2 – 8°C (Lyophilized), <-20°C (Reconstituted) Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of deionized water, allowed to sit 10 minutes, then mixed by inversions or gentle vortexing. Ensure solids are dissolved completely prior to use.

SAFETY PRECAUTIONS

Reagents must be used in a professional laboratory environment and are for research use only. Source material containing bovine serum was derived in the contiguous 48 United States from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear disposable gloves while performing this assay and handle reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB is an irritant to skin and mucous membranes and may cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid is corrosive, avoid skin contact. Do not get in eyes, on skin, or clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 25 µL, 100 µL, 200 µL, and 1000 µL, etc.
2. Repeating dispenser suitable for delivering 100 µL and 200 µL.
3. Disposable pipette tips suitable for dispensing the volumes above.
4. Disposable 12 x 75 mm or 13 x 100 plastic test tubes.
5. Disposable plastic 1000 mL bottle with cap.
6. Aluminum foil.
7. Deionized or distilled water.
8. Plastic microplate cover or polyethylene film.
9. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
10. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.
11. ELISA plate shaker
12. Timer

SAMPLE COLLECTION

Only 50 µL of human serum or plasma sample is required for human osteocalcin measurement in duplicate. Samples should not be taken from individuals taking biotin-containing multivitamins or dietary supplements at least 48 hours prior to sample collection. Whole blood should be collected by venipuncture and must be allowed to clot for a minimum of 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1500xg for 10 minutes). The serum should be separated from the clot within three hours of blood collection and transferred to a clean test tube. Serum samples can be stored at 2-8°C or room temperature for up to 6 days until measurement. Samples should be stored frozen (< -20°C) for longer storage. Avoid more than three freeze-thaw cycles of samples. Hemolyzed samples should be avoided.

ASSAY PROCEDURE

1. Reagent Preparation

(1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

(2) Wash Concentrate must be diluted to working strength prior to use. Please see REAGENTS section for details.

(3) Reconstitute all assay standards and controls by adding **0.5 mL** of distilled or deionized water to each vial. Allow the standards and controls to sit undisturbed for 5 minutes, and then mix well by inversion or gentle vortexing. Ensure all solids are dissolved completely prior to use. Reconstituted standards and controls must be stored at - 18°C or below. Do not exceed 3 freeze-thaw cycles.

2. Assay Procedure

(1) Place enough streptavidin-coated microwell strips in a holder to run human osteocalcin standards, controls, and unknown samples in duplicate. Unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.

(2) Plate Map

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	SAMPLE 1
B	STD 1	STD 5	SAMPLE 1
C	STD 2	STD 6	SAMPLE 2
D	STD 2	STD 6	SAMPLE 2
E	STD 3	C 1	SAMPLE 3
F	STD 3	C 1	SAMPLE 3
G	STD 4	C 2	…
H	STD 4	C 2	…

(3) *Antibody working solution*- Prepare working HRP-conjugated Osteocalcin Antibody and Biotinylated Osteocalcin Antibody by 1:21 fold dilution of the conjugation antibody with the biotinylated antibody solution. For each strip, mix 2 mL of the Biotinylated Antibody Solution with 100 µL of the HRP-Conjugated Osteocalcin Antibody.

Note: This antibody mixture should be freshly prepared.

(4) Add **25 µL** of standards, controls, and serum/plasma samples into the designated microwell.

(5) Add **200 µL** of the *antibody work solution* into each well.

(6) Cover the plate with a plate sealer and aluminum foil to avoid exposure to light.

(7) Incubate the plate at room temperature (20- 25°C), shaking at 350 rpm ± 100 rpm for **1 hour.**

(8) Remove the aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.

(9) Add **200 µL** of HRP Substrate into each well.

(10) Cover the plate with a new plate sealer and aluminum foil to avoid exposure to light.

(11) Incubate plate at room temperature static for 20 minutes.

(12) Remove the aluminum foil and plate sealer. Add 50 µL of Stop Solution into each well. Mix gently.

(13) Read the absorbance using a microplate reader at 450 nm within 10 minutes.

PROCEDURAL NOTES

1. It is recommended that all standards, controls, and unknown samples be assayed in duplicate. The average absorbance readings of each duplicate should be used for data reduction and the calculation of results.
2. Keep light-sensitive reagents in the original amber bottles.
3. Store unused well strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
8. Prepare a calibration curve for each run, do not use data from previous runs.
9. To avoid cross-contamination, use a clean disposable pipette tip for addition of each reagent and sample.

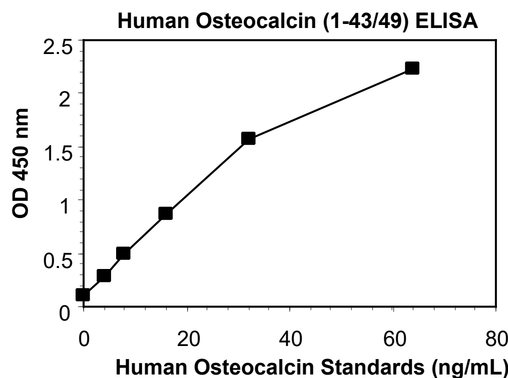
INTERPRETATION OF RESULTS

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the zero standard from the average absorbance of all other readings to obtain the corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs (e.g. Point-to-Point, 4-Parameter) may also be used for the calculation of results.
4. The human osteocalcin concentrations for the controls and the samples are read directly from the standard curve using their respective corrected absorbance.

EXAMPLE DATA AND STANDARD CURVE

Typical absorbance data and the resulting standard curve from human osteocalcin ELISA are represented. **This curve should not be used in lieu of the standard curve run with each assay.**

Well I.D.	Results		
	Average	Corrected	
0 ng/mL	0.112	0	
4 ng/mL	0.279	0.167	
8 ng/mL	0.494	0.382	
16 ng/mL	0.866	0.754	
32 ng/mL	1.570	1.458	
64 ng/mL	2.232	2.120	
Control 1	0.363	0.251	5.26 ng/mL
Control 2	0.663	0.551	11.69 ng/mL



LIMITATIONS OF THE PROCEDURE

1. Research suggests that an abnormally high osteocalcin value is likely to indicate a more significant bone turnover condition. For sample values reading greater than the highest standard, it is recommended to re-assay sample with dilution.
2. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity

The analytical sensitivity of this human osteocalcin ELISA as determined by the 95% confidence limit on 8 replicate determinations of both zero and level 2 standards is approximately 0.31 ng/mL.

Analytical Specificity

This assay shows less than 15% cross-reactivity to uncarboxylated osteocalcin.

High Dose "hook" effect

No "hook" effect was observed for osteocalcin levels up to 1,250 ng/mL.

Precision

The intra-assay precision was validated by measuring two samples in a single assay with 16 replicate determinations.

Mean Osteocalcin Value (ng/mL)	CV (%)
11.9	4.7
40.2	5.0

The inter-assay precision is validated by measuring two control samples in duplicate in 6 individual assays.

Mean Osteocalcin Value (ng/mL)	CV (%)
5.6	8.3
11.9	5.7

Linearity

Two serum samples from dialysis subjects were diluted with a BSA-based 0.01M phosphate, 0.15M sodium chloride buffer matrix and assayed. The results reported as ng/mL are as follows:

#	DILUTION	OBSERVED VALUE	EXPECTED VALUE	RECOVERY
1	Neat	69.6	-	-
	1:2	34.5	34.8	99%
	1:4	15.1	17.4	87%
2	Neat	42.1	-	-
	1:2	21.4	21.1	101%
	1:4	10.4	10.5	99%

Recovery

Two serum samples were spiked with three assay standards in equal volume (1 vol. + 1 vol. mixture) and assayed. The results reported as ng/mL are as follows:

#	Orig. Value	Spiked Sample Value	Observed Value	Expected Value	Recovery %
Sample 1					
1	33.4	8	18.5	20.7	89
2	33.4	16	23.8	24.7	96
3	33.4	32	30.4	32.7	93
Sample 2					
4	15.7	8	11.4	11.9	96
5	15.7	16	15.3	15.9	96
6	15.7	32	24.4	23.9	102

Interfering Substances

Substance	Test Osteocalcin (ng/mL)	Control Osteocalcin (ng/mL)	Dcut (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
Lipids 3000 mg/mL	42.6	42.6	3.2	0	0
	13.1	12.1	1	1	8.2
	8.8	8.2	0.6	0.6	7.3
Hemoglobin 200 mg/mL	42	41.3	3.1	0.7	1.7
	13.6	13.4	1	0.2	1.5
	9.7	9.1	0.7	0.6	6.6
Hemoglobin 66.6 mg/mL	42.3	41.3	3.1	1	0.2
	13.5	13.4	1	0.1	0.7
	9.4	9.1	0.7	0.3	3.3
Bilirubin 20 mg/mL	22.2	22.9	1.7	0.7	3.1
	7.4	7.5	0.6	0.1	1.3
	4.7	4.6	0.3	0.1	2.2

Short Assay Procedure:

1. Add 25 µL of standards, controls and samples into the designated microwells.
2. Add 200 µL of antibody mixture to each well.
3. Cover with plate sealer and aluminum foil and incubate for 1 hour at RT, shaking at 350 rpm.
4. Wash each well 5 times with 350-400 µL of working wash solution.
5. Add 200 µL of HRP Substrate into each well.
6. Cover with plate sealer and aluminum foil and incubate for 20 minutes at RT, static.
7. Add 50 µL of Stop Solution into each well.
8. Read the absorbance at 450 nm within 10 min.

REFERENCES

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