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ProductInformation

Osteocalcin ELISA, Human

Product Number **CS0670**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

Osteocalcin ELISA, Human is a solid phase enzyme amplified sensitivity immunoassay performed on a multiwell plate. The assay uses a mixture of monoclonal antibodies (MAbs) directed against distinct epitopes of human osteocalcin. Standards and samples react with the capture monoclonal antibody (MAb 1) coated on the multiwell plate and with a monoclonal antibody (MAb 2) conjugated to horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAb 1 - human osteocalcin - MAb 2 - HRP, the multiwell plate is washed to remove unbound enzyme conjugated antibody. Bound enzyme conjugated antibody is measured through a chromogenic reaction. Chromogenic solution (TMB ready for use) is added and incubated. The reaction is stopped with the addition of Stop Solution and the multiwell plate is then read at 450 nm wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance, which is directly proportional to the concentration of osteocalcin. We recommend the use of a plate reader with a linearity up to 3 OD units and a data reduction method which result in high sensitivity in the low range and in an extended standard range.

Osteocalcin, γ-carboxyglutamic acid-containing protein or bone Gla-protein (BGP), is the major non-collagenous protein of the bone matrix, which constitutes 1-2% of the total bone protein. It binds strongly to apatite and calcium. γ-carboxyglutamate residues are formed by vitamin K dependent carboxylation. These residues are essential for the binding of calcium. Osteocalcin belongs to the osteocalcin/matrix Gla-protein family.

Osteocalcin has a molecular weight of 58 kDa and contains 49 amino acids, including 3 residues of γ-carboxyglutamic acid. Osteocalcin is synthesized in the bone by the osteoblasts. After production, it is partly incorporated in the bone matrix and the rest is found in the blood circulation. The exact physiological function of osteocalcin is still unclear. Studies show that the circulating levels of osteocalcin reflect the rate of bone formation. This ELISA is designed to measure intact human osteocalcin (hOST). The determination of the blood levels of osteocalcin is valuable for:

- The identification of women at risk of developing osteoporosis.
- monitoring bone metabolism during perimenopause and postmenopause.
- monitoring bone metabolism during hormone replacement therapy and treatment of premenopausal women with LH-RH agonists.
- Monitoring bone metabolism in patients with growth hormone deficiency, hypothyroidism, hyperthyroidism, and chronic renal failure.

Reagents

- **Monoclonal-Anti-Human Osteocalcin Coated 96 well plate, 1EA, Product No. O 3889 -** A plate using break-apart strips coated with monoclonal antibody (MAb 1).
- **Plate Covers, adhesive strips, 3 EA. Product No. P 4870**
- **Standard, 0 ng/mL, lyophilized, 1 VL, Product No. S 8069 –** contains osteocalcin-free human serum, with preservative and protease inhibitors.
- **Osteocalcin, Standard 1, lyophilized, 1 VL, Product No. O 6389 -**native human osteocalcin in osteocalcin-free human serum, with preservatives and protease inhibitors
- **Osteocalcin, Standard 2, lyophilized, 1 VL, Product No. O 6514 -**native human osteocalcin in osteocalcin-free human serum, with preservatives and protease inhibitors
- **Osteocalcin, Standard 3, lyophilized, 1 VL, Product No. O 6639 -**native human osteocalcin in osteocalcin-free human serum, with preservatives and proteas e inhibitors

- **Osteocalcin, Standard 4, lyophilized, 1 VL, Product No. O 6764 -**native human osteocalcin in osteocalcin-free human serum, with preservatives and protease inhibitors
- **Osteocalcin, Standard 5, lyophilized, 1 VL, Product No. O 6889 -**native human osteocalcin in osteocalcin-free human serum, with preservatives and protease inhibitors
- **Control 1, lyophilized, 1 VL, Product No. C 1616 -** in human plasma with preservative.
- **Control 2, lyophilized, 1 VL, Product No. C 1741-** in human plasma, with preservative.
- **Monoclonal Anti-Human Osteocalcin-HRP Conjugate, 1 VL, Product No. O 4014 -** in buffered solution with preservative and animal protein
- **Wash Buffer Concentrate (200X), 10 mL, Product No. W 4015**
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.

Note: See vial labels for exact concentrations and reconstitution volumes of controls and standards

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm and 650 nm, or capable of reading at 405 nm and 650 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. The human blood components included in this kit have been tested by European approved and USA FDA approved methods and found negative for HBsAg, anti-HCV and anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum, or plasma specimens should be in accordance with appropriate safety procedures. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

1. Heparin or EDTA plasma, and serum are the recommended samples.
   1.1. Collect blood by venipuncture, taking care to avoid hemolysis. The samples must be kept in an ice bath.
   1.2. Separate the plasma or serum from the cells within 3 hours. The use of a refrigerated centrifuge is recommended.
   1.3. Add Trasylol® to the plasma or serum immediately after centrifugation: add 100 µL Trasylol per mL of plasma or serum to obtain 1000 IU Trasylol per mL sample.
2. Sampling conditions can affect values measured in serum or plasma, therefore, strict precautions have to be taken during sampling to avoid impurities contained in sampling materials that would stimulate osteocalcin production by blood cells and thus falsely increase plasma osteocalcin values.
3. Remove serum from the clot after centrifugation as soon as possible and store at 2-8 °C.
4. With this treatment the samples are stable for 3 days at 2-8 °C.
5. For a longer delay the samples must be frozen (–20°C); however, the samples can only be thawed once.
6. For repeat testing, freeze the samples in aliquots and discard each sample after the first thawing.
7. Do not use citrate plasma.
8. Do not use hemolyzed or lipemic samples.
9. If a specimen is expected or known to have a concentration above the highest standard, dilute the sample with the zero standard to fall within the dynamic range of the assay.

Reagent Preparation

Standards and Controls

1. Reconstitute the lyophilized standards and controls to the volume specified on the vial label with distilled water (1 mL for standard 0 and 0.5 mL for the controls and standards 1-5).
2. Allow to remain undisturbed until completely dissolved, and then mix well by gentle inversion.
3. For long-term storage, aliquots should be frozen at –20 °C (maximum 2 months) or at –70 °C for the shelf life duration.

Wash Buffer

1. Dilute 2 mL of *Wash Buffer Concentrate* in 400 mL distilled water or all the contents of the *Wash Buffer Concentrate* vial in 2000 mL distilled water (use a magnetic stirrer).
2. The *Wash Buffer Concentrate* is stable at room temperature until expiration date.
3. In order to avoid precipitation, it is recommended to prepare a fresh diluted Wash Buffer each day.

Quality Control

1. The two controls provided in the kit can be used as internal laboratory controls.
2. **Note**: Other controls, which contain azide will interfere with the enzymatic reaction and cannot be used.
3. Each laboratory is advised to run internal controls.
4. Collect and aliquot serum, EDTA plasma or heparin plasma pools to serve as controls.
5. Freeze immediately. Repeated freezing and thawing are not recommended.
6. Record keeping: it is good laboratory practice to record the kit lot numbers and date of reconstitution for the reagents in use.
7. Controls should be routinely assayed as unknown samples to measure assay variability.
8. It is recommended that quality control charts be maintained to monitor the performance of the kits. Control ranges are indicated on vial labels.
9. Out of range control results indicate the assay must be repeated.
10. Repeat patient samples may also be used to measure inter-assay precision.
11. Data reduction: it is good practice to construct a standard curve for each run to check visually the curve fit selected by the computer program.

Storage/Stability

All components of this kit are stable at 2-8 °C. Any unused reconstituted standard or control, should be discarded or frozen at –20 °C for 6 weeks. Standards and controls can be frozen and thawed one time only without loss of immunoreactivity. Conjugate vial should be stored at 2-8 °C.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- The Stabilized Chromogen is ready to use and is stable
- Do not use reagents beyond the kit shelf life.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 3 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of samples.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle three times, blotting as dry as possible after the 3rd wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

Osteocalin Assay Summary

1. **Add 25 µL of Standards, Controls or Samples**
   Add 100 µL Monoclonal Anti-Osteocalcin-HRP Conjugate

   Incubate 2 hours at RT on shaker
   Aspirate and wash 3X

2. **Add 100 µL Stabilized Chromogen within 15 minutes following final wash above**

   Incubate 30 minutes at RT on shaker (avoid direct sunlight)

3. **Add 200 µL of Stop Solution**

   Read at 450nm

Total Assay Time - 2.5 hours

1. Determine the number of wells for the assay run, including: 2 zero standard wells, 10 standard wells, 2 Control 1 and 2 Control 2 wells.
2. Add 2 wells for each sample to be assayed.
3. Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

1st incubation

a Add 25 µL Standard 0, Standards 1 to 5, Control 1 and Control 2 to designated wells.
b Add 25 µL of each sample to be tested to the appropriate duplicate wells.
c Add 100 µL Monoclonal Anti-Osteocalcin-HRP Conjugate to all wells.
d Tap gently on the plate to mix, cover with Plate Cover and incubate two hours at room temperature. on a horizontal shaker set at 700 ± 100 rpm
e Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 3 times following washing instructions.

Substrate incubation

a Add 100 µL of Stabilized Chromogen into all wells within 15 minutes following final wash above.
b Incubate the plate for 30 min**.** at room temperature on a horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.

Stop reaction

a Add 200 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix.

Absorbance

Read absorbances within 3 hours and calculate the results as described below.

Results

- Plot the ODs on the ordinate against the standard concentrations on the abscissa using either linear- or semi-log graph paper and draw the curve by connecting the plotted points with straight lines.
- If Trasylol is added to samples (100 µL/mL), the assay sample values have to be multiplied by 1.1.
- The osteocalcin concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
- Multiply the values obtained for the samples by dilution factor of each sample
- If the sample absorbance is greater than that of the highest standard, dilute the sample and reassay.

Product Profile

Typical Results

The standard curves below are for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

hOST Standards ng/mL	**OD 450 nm**	**OD 405 nm**
0	0.025	0.011
2	0.11	0.039
5	0.37	0.11
14	1.82	0.54
35	4.47	1.3
90	8.85	2.6

Performance characteristics

Sensitivity

The Minimum Detectable Concentration (MDC) is estimated to be 0.4 ng/ml and is defined as the osteocalcin concentration corresponding to the average OD of the zero standard + 2 standard deviations.

Precision

1. Intra-Assay Precision
Samples of known concentration were assayed in replicates of 20 to determine precision within an assay.

	Sample 1	Sample 2
Mean (ng/mL)	11.5	19.6
Standard Deviation (SD)	0.3	0.4
Coefficient of Variation (%)	2.5	1.9

2. Inter-Assay Precision
Samples were assayed 10 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2
Mean (ng/mL)	9.4	20.6
Standard Deviation (SD)	0.8	1.2
Coefficient of Variation (%)	9.2	6.0

Specificity

This method detects full length human osteocalcin. N-terminal fragments and C-terminal fragments have been tested and they do not interfere with this test at their maximum levels found in normal and pathological samples.

Recovery

Added hOST ng/mL	Measured hOST ng/mL	% Recovery
1	1.09	109
2.5	2.86	114
7.0	7.44	106
17.5	19.04	108
45.0	45.45	101

Linearity of Dilution

Dilution	Serum		
	Expected ng/mL	Measured ng/mL	% Expected
Neat	59.8	-	-
1:2	29.9	31.16	104
1:4	14.95	14.77	99
1:8	7.47	7.25	97
1:16	3.73	3.25	87
1:32	1.86	1.74	94
1:64	0.93	0.84	90
1:128	0.46	0.47	102

High dose hook effect

A sample spiked with osteocalcin up to 5000 ng/mL gives a response higher than that obtained for the last standard point (90 ng/mL).

References

1. Price, P.A., Vitamin K-dependent formation of bone Gla protein (osteocalcin) and its function., Vitam. Horm. **42**, 65-108. (1985).
2. Hoang, Q. Q. et al., Bone recognition mechanism of porcine osteocalcin from crystal structure., Nature, **425**, 977-980 (2003).
3. Mitchell, B. D., et al.,Genes influencing variation in serum osteocalcin concentrations are linked to markers on chromosomes 16q and 20q., J. Clin. Endocrinol. Metab*.,* **85**, 1362-1366 (2000).
4. Roy, M.E., Correlations between osteocalcin content, degree of mineralization, and mechanical properties of C. *carpio* rib bone., J. Biomed. Mater. Res., **54,** 547-53 (2001).
5. Minisola, S. et al., Serum osteocalcin in primary hyperparathyroidism: short-term effect of surgery., Miner Electrolyte Metab., **14**, 201-207 (1988).
6. Coulton, L.A. et al., An evaluation of serum osteocalcin in Paget's disease of bone and its response to diphosphonate treatment., Arthritis Rheum., **31**, 1142-1147 (1988).

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