ELISA Kit

Catalog # KAQ1381 (96 tests)

Human
Osteocalcin

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Contents and Storage

Storage

Store at 2 to 8°C.

Contents

Reagent	Quantity	Color Code	Reconstitution
OST antibody-coated microtiter strip-well plate.	96 wells	blue	Ready to use.
Calibrators (standard), 0 ng/mL in human serum, protease inhibitors and benzamidin; lyophilized.	1 vial	yellow	Add 1 mL distilled water.
Calibrators (standards), N=1-5 in human serum, protease inhibitors and benzamidin; lyophilized.	5 vials	yellow	Add 0.5 mL distilled water.
Controls 1 and 2 in human serum, protease inhibitors, benzamidin, and thymol; lyophilized.	2 vials	silver	Add 0.5 mL distilled water.
Anti-OST-HRP Conjugate in stabilizing buffer; 0.4 mL per vial.	1 vial	red	Dilute 50 x with conjugate buffer.
Conjugate buffer: Tris-HCl buffer with bovine serum albumin, bovine casein, EDTA, gentamycin and thymol; 12mL per vial.	1 vial	red	Ready to use.
Wash Solution (200x); 10 mL per vial.	1 vial	brown	Dilute 2 mL in 400 mL distilled water.
Chromogen, TMB (Tetramethylbenzidine); 12 mL per vial.	1 vial	black	Ready to use.
Stop Reagent, 1N HCl; 12 mL per vial.	1 vial	white	Ready to use.

Note: Standard 0 ng/mL is recommended for sample dilutions.
Standard is calibrated on a synthetic peptide (Peninsula 6045).

Disposal Note

This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

Safety

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Introduction

Purpose

The Invitrogen Osteocalcin (OST) EASIA is an immunoenzymatic assay for the quantitative measurement of intact human Osteocalcin (Hu OST) in human serum and plasma.

For Research Use Only. CAUTION: Not for human or animal therapeutic or diagnostic use.

Principle of the Method

The Invitrogen human Osteocalcin EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on a microtiter plate. The assay uses monoclonal antibodies (Mabs) directed against distinct epitopes of human osteocalcin. Standards and samples react with the capture monoclonal antibody (Mab 1) coated on the microtiter well and with a monoclonal antibody (Mab 2) labeled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated Mab 1 - human osteocalcin - Mab 2 - HRP, the microtiter plate is washed to remove unbound enzyme labeled antibody. Bound enzyme labeled 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 microtiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to the human osteocalcin concentration. A standard curve is plotted and human osteocalcin concentration in a sample is determined by interpolation from the standard curve.

Background Information

Osteocalcin or bone Gla protein (B.G.P.) is the major non-collagenous protein of the bone matrix. It has a molecular weight of 5800 Da and contains 49 amino acids, including 3 residues of gamma carboxy glutamic 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.

Methods

Materials Needed But Not Provided

- Microtiter plate reader (at or near 450 nm) with software
- Calibrated adjustable precision pipettes
- Distilled or deionized water
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Glass or plastic tubes for diluting solutions
- Absorbent paper towels
- Calibrated beakers and graduated cylinders
- Trasylol® at 10000IU/mL

Procedural Notes

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. **Do not mix or interchange different reagent lots from various kit lots**.
9. Do not use reagents after the kit expiration date.
10. Absorbances should be read immediately, but can be read up to 2 hours after assay completion. For best results, keep plate covered in the dark.
11. In-house controls or kit controls, if provided, should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
12. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
13. Because Stabilized *Chromogen* is light sensitive, avoid prolonged exposure to light. Avoid contact between chromogen and metal, or color may develop.

Directions for Washing

- **Incomplete washing will adversely affect the test outcome**. All washing must be performed with the Wash Buffer provided.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of each well. Take care not to scratch the inside of the well. After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, and then aspirate the liquid. Repeat as directed under Assay Procedure. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- If using an automated washer, follow the washing instructions carefully.

Preparation of Reagents

1. Standards and controls: reconstitute the lyophilized standards and controls to the volume specified on the vial label with distilled water (1 mL for the zero standard and 0.5 mL for the other standards and controls). Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion. After reconstitution, standards and controls are very unstable. Use them immediately after reconstitution. For longer storage periods, aliquots should be made and kept at -20°C for maximally 6 weeks. Freezing should be performed immediately after use, do not wait for freezing until all the samples are pipetted. Avoid subsequent freeze-thaw cycles.
2. Working anti-OST-HRP conjugate: Prepare an adequate volume of conjugate solution by adding 40 µL of the concentrated anti-OST-HRP conjugate to 2 mL of conjugate buffer. Use a vortex to homogenize. Prepare quantity needed just at time of use.
3. Washing solution: add 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to mix. Prepare quantity needed just at time of use.

Sample Preparation

- Collect blood by venipuncture, taking care to avoid hemolysis. The samples must be kept in an ice bath. Separate the serum from the cells within 3 hours, the use of a refrigerated centrifuge is recommended. Add 100 µL Trasylol® (10,000IU/mL) to the serum immediately after centrifugation (to obtain 1000 IU Trasylol® per mL sample). With this treatment, samples are stable for 3 days at 2-8°C.
- Samples held for a longer time should be frozen at -20°C prior to assaying and can only be thawed once. For repeat testing, freeze the samples in aliquots and discard each sample after the first thawing.
- Prior to use, all the samples should be at room temperature. Vortex the samples before use.

Assay Procedure

Be sure to read the *Procedural Notes* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator at 2-8°C for future use.)
2. Add 25 µL of each standard, control or sample into the appropriate wells.
3. Add 100 µL of working *Anti-OST-HRP conjugate* into all wells.
4. Cover the plate with a plate cover and incubate for **2 hours at room temperature**.
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 3 times. See **Directions for Washing.**
6. Add 100 µL of *Chromogen Solution* into each well within 15 minutes following the washing step.
7. Incubate the plate for **30 minutes at room temperature and in the dark. *Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceeds the limits of the instrument. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

8. Add 100 µL of *Stop Solution* into each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
9. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 1 hour after adding the *Stop Solution*.
10. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.
11. Read the concentrations for unknown samples and controls from the standard curve. (Samples producing signals greater than that of the highest standard should be diluted in calibrator (0ng/mL) and reanalyzed, multiplying the concentration found by the appropriate dilution factor.) If Trasylol® is added to the samples (100 µL/mL), sample values have to be multiplied by 1.1.

Typical Data (Example)

The following data were obtained for the various standards over the range of 0 to 75 ng/mL Human OST.

Standard OST (ng/mL)	Optical Density (450 nm)
75	2.415
31.5	1.420
12.7	0.641
4.1	0.229
1.56	0.118
0	0.033

Performance Characteristics

Sensitivity

The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.08 ng/mL.

Precision

1. Intra-Assay Precision

Samples of known Hu OST concentration were assayed in replicates of 20 to determine precision within an assay.

	Sample 1	Sample 2
Mean (ng/mL)	11.4	28.2
SD	0.5	0.28
%CV	4.7	3.1

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 10 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2
Mean (ng/mL)	11.8	27.7
SD	0.4	1.55
%CV	3.5	5.6

SD = Standard Deviation
CV = Coefficient of Variation

Linearity of Dilution

Sample	Dilution	Theoretical Concent. (ng/mL)	Measured Concent. (ng/mL)
Serum 1	1/1	-	28.6
	1/2	14.3	14.2
	1/4	7.1	7.1
	1/8	3.6	3.4
	1/16	1.8	1.4
Serum 2	1/1	-	30.8
	1/2	15.4	15
	1/4	7.7	7.7
	1/8	3.8	3.7

Samples were diluted with zero calibrator.

Recovery

Sample	Added OST (ng/mL)	Recovered OST (ng/mL)	Recovery (%)
Serum	1.4	1.55	111
	4.04	4	99
	8.4	8.3	99
	15	14.5	97
	31	31	100
	64.6	64.4	99

Specificity

This method detects intact human osteocalcin. N-terminal and C-terminal fragments have been tested at their maximum levels found in normal and pathological samples, were added to a low and a high value calibrator. No cross reactivity was observed at these concentrations.

Expected Values

These values are given only for guidance; each laboratory should establish its own normal range of values.

Normal values are expected between 5 to 25 ng/mL.

High Dose Hook Effect

A sample spiked with OST up to 625 ng/mL gives a result higher than the last standard point.

Limitations of the Procedure

Do not extrapolate the standard curve beyond the top standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute all samples above the top standard point; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Hu OST in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Appendix

Troubleshooting Guide

Elevated background	*Cause:* Insufficient washing and/or draining of wells after washing. Solution containing either biotin or HRP-conjugate can elevate the background if residual is left in the well. *Solution:* Wash according to the protocol. Verify the function of automated plate washer. At the end of each washing step, invert plate on absorbent tissue on countertop and allow to completely drain, tapping forcefully if necessary to remove residual fluid. *Cause:* Contamination of substrate solution with metal ions or oxidizing reagents. *Solution:* Use distilled/deionized water for dilution of wash buffer and use plastic equipment. DO NOT COVER plate with foil. *Cause:* Contamination of pipette, dispensing reservoir or substrate solution with HRP conjugate. *Solution:* Do not use chromogen that appears blue prior to dispensing onto the plate. Obtain new vial of chromogen. *Cause:* Incubation time is too long or incubation temperature is too high. *Solution:* Reduce incubation time and/or temperature.
Elevated sample/ standard ODs	*Cause:* Incorrect dilution of standard stock solution. *Solution*: Follow the protocol instructions regarding the dilution of the standard. *Cause*: Incubation times extended. *Solution:* Follow incubation times outlined in protocol. *Cause:* Incubations carried out at 37°C when RT is dictated. *Solution:* Perform incubations at RT (= 25 ± 2°C) when instructed in the protocol.
Poor standard curve	*Cause:* Reagents (lyophilized standard, etc.) from different kits, either different cytokine or different lot number, were substituted. *Solution:* NEVER substitute any components from another kit. *Cause*: Errors in pipetting the standard or subsequent steps. *Solution*: Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device.

Weak/no color develops	*Cause:* Reagents not at RT (25 ± 2°C) at start of assay. *Solution:* Allow ALL reagents to warm to RT prior to commencing assay. *Cause:* Incorrect storage of components, e.g., not stored at 2 to 8°C. *Solution:* Store all components exactly as directed in protocol and on labels. *Cause:* TMB solution lost activity. *Solution 1:* The TMB solution should be clear before it is dispensed into the wells of the microtiter plate. An intense aqua blue color indicates that the product is contaminated. Please contact Technical Support if this problem is noted. To avoid contamination, we recommend that the quantity required for an assay be dispensed into a disposable trough for pipetting. Any TMB solution left in the trough should be discarded. *Solution 2:* Avoid contact of the TMB solution with items containing metal ions. *Cause:* Attempt to measure analyte in a matrix for which the ELISA assay has not been optimized. *Solution:* Please contact Technical Support for advice when using nonvalidated sample types. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.
Poor Precision	*Cause:* Errors in pipetting the standards, samples or subsequent steps. *Solution:* Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device. Check for any leaks in the pipette tip. *Cause:* Repetitive use of tips for several samples or different reagents. *Solution:* Use fresh tips for each sample or reagent transfer. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.

Technical Support

Contact Us

For more troubleshooting tips, information, or assistance, please call, email, or go online to www.invitrogen.com/ELISA.

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Explanation of symbols

Symbol	Description	Symbol	Description
	Catalogue Number		Batch code
	Research Use Only		*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer		European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

NOTES

Human Osteocalcin Assay Summary