

Human Osteocalcin IRMA Kit

Immutopics

Immutopics, Inc.

Immunoradiometric Assay (IRMA) for the
Quantitative Determination of Human Osteocalcin
Levels in Serum or Plasma

100 Test Kit

For RESEARCH Use Only
Not for use in diagnostic procedures

Cat. # 50-1000

CAUTION: Radioactive Materials
Not for Internal or External Use in Humans or Animals

Store at 2 - 8° Upon Receipt

INTENDED USE

This kit is intended for research use only and therefore **not** for use in diagnostic procedures and **not** for use in establishing the safety or effectiveness of the device. For use only by distributors, laboratories and researchers certified by Immutopics, Inc.

INTRODUCTION

Osteocalcin, a 49 amino acid peptide, is the major noncollagen protein of bone. It contains three gamma-carboxyglutamic acid (GLA) residues at positions 17, 21, and 24 and is, therefore, also known as bone gla-protein or BGP. The exact biological function of osteocalcin is not known but the three gamma-carboxyglutamic acid residues confer on it a very strong ability to bind to hydroxyapatite and calcium.

Vitamin K is essential for the biosynthesis of osteocalcin which is stimulated by 1,25-dihydroxyvitamin D. Osteocalcin is synthesized by osteoblasts during the process of bone formation and mostly incorporated into bone matrix with some escaping into the blood. The circulating level of total osteocalcin is primarily composed of a large N-terminal midregion fragment resulting from the cleavage of the intact molecule and the intact molecule itself. Since the half-life in blood is relatively short (about 5 minutes) the osteocalcin level in blood reflects new protein synthesis and therefore its measurement provides a valuable tool for assessing skeletal metabolism. As a product unique to the osteoblast, it also represents the activity of the cell responsible for the formation of bone.

Serum osteocalcin concentrations are higher in infants and children than in adults. Most adult normal ranges show higher levels in men than in pre-menopausal women. Following menopause, osteocalcin levels increase. Concentrations are increased in patients with various bone diseases characterized by increased osteoblastic activity such as Paget's disease, osteomalacia, osteitis fibrosa, and renal osteodystrophy. Increased levels have also been observed in patients with increased concentrations of thyroid hormone, growth hormone, vitamin D and parathyroid hormone. Disease states characterized by decreases in levels of these hormones have shown decreased concentrations of osteocalcin.

TEST PRINCIPLE

This Human Osteocalcin IRMA Kit is a two-site immunoradiometric assay for the measurement of both intact osteocalcin and its large N-terminal midregion fragment in serum or plasma. Two different polyclonal goat antibodies to human osteocalcin have been purified by affinity chromatography. The antibody which recognizes the 20-36 region of the peptide is immobilized onto plastic beads to capture the osteocalcin molecules and the other antibody which recognizes the 1-19 region of the peptide is radiolabeled for detection.

A sample containing human osteocalcin is incubated simultaneously with an antibody coated bead and the ¹²⁵I labeled antibody. The

osteocalcin contained in the sample is immunologically bound by both the immobilized antibody and labeled antibody to form a "sandwich" complex:

Bead/Anti-Osteocalcin -- Osteocalcin -- Acridinium Anti-Osteocalcin
(20-36) (1-19)

At the end of the incubation period, the bead is washed to remove any unbound labeled antibody and other components. The radioactivity bound to the bead is then measured in a gamma counter. The radioactivity of the antibody complex bound to the bead is directly proportional to the amount of osteocalcin in the sample. A standard curve is generated by plotting the CPM versus the respective osteocalcin concentration for each standard on logarithmic scales. The concentration of osteocalcin in the sample is determined directly from this curve.

REAGENTS: Preparation and Storage

Store the kit at 2-8°C upon receipt. **Store the standards and controls at -20°C or below after reconstitution.** 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 and mix by gentle swirling and inversion. Reagents from different kit lot numbers should not be combined or interchanged.

- 1. HUMAN OSTEOCALCIN ANTIBODY COATED BEADS (40-1010)**
One container of 100 polystyrene beads (8 mm diameter) coated with antibody to human osteocalcin 20-36 plus desiccant.
- 2. ¹²⁵I LABELED HUMAN OSTEOCALCIN ANTIBODY (40-1020)**
Two vials each containing 10.5 mL of ¹²⁵I labeled anti-human osteocalcin 1-19 in 0.25M phosphate buffered saline with 0.025M EDTA, with protein stabilizers and 0.1% sodium azide. Each vial contains less than 10 uCi (370 kBq) of radioactivity.
- 3. HUMAN OSTEOCALCIN STANDARDS (40-1031 to 40-1036)**
Six vials each containing synthetic human osteocalcin (1-49) lyophilized in a protein matrix with 0.1% sodium azide. **Refer to vial label for exact concentration.** Before use reconstitute the vial with the osteocalcin concentration of 0 ng/mL with 2.0 mL of deionized water. Before use reconstitute each of the other five vials of standards with 1.0 mL of deionized water. Allow the vials to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use.

Use the standards immediately after reconstitution; freeze the unused portion for later use. After reconstitution the standards are stable until the expiration date on the kit box when stored at -20°C or below with up to 3 freeze/thaw cycles.

4. HUMAN OSTEOCALCIN CONTROLS I & II (40-1041 & 40-1042)

Two vials each containing human osteocalcin (1-49) lyophilized in a protein matrix with 0.1% sodium azide. **Refer to vial label for control ranges.** Before use reconstitute each control with 1.0 mL of deionized water. Allow the vials to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use.

Use the controls immediately after reconstitution; freeze the unused portion for later use. After reconstitution the controls are stable until the expiration date on the kit box when stored at -20°C or below with up to 3 freeze/thaw cycles.

5. WASH CONCENTRATE (40-0050)

One bottle containing 30 mL of a 30 fold concentrate. Before use dilute the contents to 900 mL with deionized water and mix well. Upon dilution this yields a working wash solution containing a surfactant in 0.01 M phosphate buffered saline with 0.05% sodium azide. The diluted wash solution should be stored at room temperature and is stable until the expiration date on the kit box.

6. HUMAN OSTEOCALCIN SAMPLE DILUENT (Optional reagent, must be ordered separately using catalog # 30-1031)

One bottle containing 50 mL of a protein matrix with 0.1% sodium azide in liquid, ready-to-use form. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the bottle.

SAFETY PRECAUTIONS

Some of the reagents in this kit contain sodium azide. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up (Manual Guide-Safety Management No. CDC-22 Center for Disease Control, Atlanta, Georgia, April 30, 1976).

For practitioners or institutions receiving radioisotopes under a general license:

This radioactive material may be received, acquired, possessed and used only by physicians, veterinarians in the practice of veterinary medicine, clinical laboratories or hospitals, and only for in vitro laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and the general license of the U.S. Nuclear Regulatory Commission or of the state with which the Commission has entered into an agreement for the exercise of regulatory authority.

1. Storage of radioactive material should be limited to a specifically designated area.
2. Access to radioactive material must be limited to authorized personnel only.
3. Do not pipette radioactive material by mouth.
4. Do not eat or drink within designated radioactive work area.
5. Areas where spills occur should be wiped up and then washed with an alkali detergent or radiological decontamination solution. Any glassware used must be rinsed completely with water before washing with other laboratory glassware.

For practitioners or institutions receiving radioisotopes under a specific license:

The receipt, use, transfer and disposal are subject to the regulations and conditions of your specific license.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Polystyrene or polypropylene tubes, 12 x 75 mm.
2. Test tube rack.

3. Marking pen for labeling tubes.
4. 1.0 mL and 2.0 mL volumetric pipets for reconstituting standards and controls.
5. Precision pipets capable of delivering 10 µL and 200 µL.
6. Forceps or suitable bead dispenser.
7. Parafilm® or equivalent for covering tubes.
8. Repeating dispenser suitable for delivering 2.0 mL.
9. Aspiration device or suitable bead washer.
10. Container for storage of wash solution.
11. Gamma counter.
12. Deionized water.
13. Vortex mixer.
14. Timer.
15. Horizontal rotator capable of maintaining 180-220 RPM.

SPECIMEN COLLECTION

Measurement of the human osteocalcin concentration may be made using serum or plasma. Twenty microliters of serum or plasma are required to assay the sample in duplicate. To obtain serum, collect blood by venipuncture and allow it to clot at room temperature. Centrifuge the sample and separate the serum or plasma from the cells. If the assay is to be performed within 4 hours the samples must be refrigerated at 2-8°C, otherwise the samples should be stored frozen at -20°C or below. EDTA or heparinized plasma are acceptable samples.

ASSAY PROCEDURE

1. Pipet 10 µL of standard, control or patient sample into appropriately labeled tubes. **(see Limitations, #2)**
2. Pipet 200 µL of ¹²⁵I Labeled Human Osteocalcin Antibody into all tubes.
3. Vortex all tubes.
4. Using forceps or appropriate bead dispenser, add one bead to each tube. Tilt tube rack to approximately a 30 degree angle to prevent splashing. Cover tube rack with Parafilm® or equivalent.
5. Incubate tubes at room temperature for 3 hours on a horizontal rotator set at 180-220 RPM.
6. Aspirate the contents of each tube. Wash beads three times by dispensing 2 mL of wash solution into each tube and then completely aspirating the contents.
7. Count each tube in a gamma counter for one minute and record the counts.

PROCEDURAL NOTES

1. It is recommended that all standards, controls, and patient samples be assayed in duplicate. The average counts per minute of each duplicate should then be used for data reduction and calculation of results.
2. The use of ¹²⁵I Labeled Human Osteocalcin Antibody should be pipetted carefully into the bottom one-fourth of the tube.
3. The washing step is an important part of the total assay procedure. Accurate dispensing of the wash solution and thorough and complete aspiration of the tube contents is essential. The length of time the wash solution sits in each tube is also an important factor. The washing procedure should be performed such that this timing is as consistent as possible.
4. If data reduction requires total count tubes, label duplicate tubes appropriately and pipet 200 µL of the ¹²⁵I Labeled Human Osteocalcin Antibody into each tube and cap.
5. Patient samples (non-uremic) with values greater and 40 ng/mL should be diluted 1:10 with the 0 ng/mL Standard or the optional Sample Diluent reagent and reassayed. Multiply the result by 10. (See Limitations, #1 and #2).

- Plasma samples may contain fibrin clots or cellular debris. Freeze/thaw of plasma samples may accelerate clot formation. These samples must be centrifuged and decanted prior to assay to remove all particulate material which can cause random high non-specific binding on tube or bead surface.

CALCULATION OF RESULTS

The standard curve is generated using the human osteocalcin standards contained in the kit. **Refer to individual vial labels for exact concentrations.** Generate the curve as follows:

- Calculate the average CPM for each pair of duplicate assay tubes.
- Subtract the average CPR of the 0 ng/mL Standard from all other average CPMs to obtain corrected CPM.
- The standard curve is generated by plotting the CPM of each standard level on the ordinate against the standard concentration on the abscissa using log-log paper. Appropriate computer assisted data reduction programs may also be used for calculation of results.

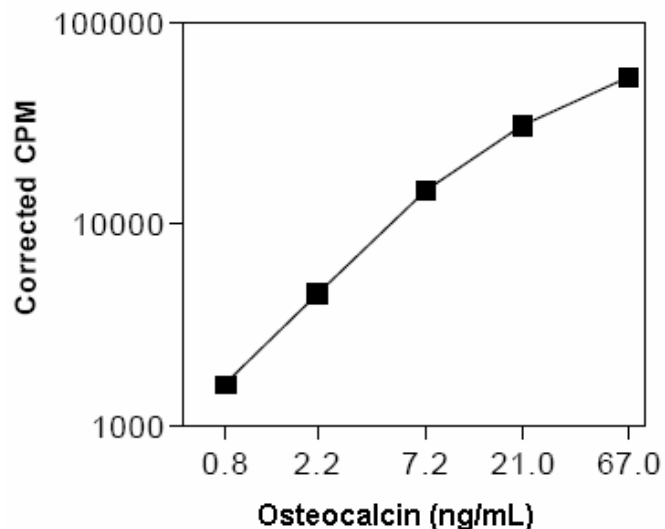
The osteocalcin concentrations of the controls and patient samples are read directly from the standard curve using their respective corrected CPM. Samples having a corrected CPM between the 0 ng/mL Standard and the next highest standard should be calculated by the formula:

$$\text{Value of unknown} = \frac{\text{Corrected RLU (unknown)}}{\text{Corrected RLU (2}^{\text{nd}} \text{ Std.)}} \times \text{Value of the 2}^{\text{nd}} \text{ Std.}$$

EXAMPLE DATA AND STANDARD CURVE

The following are representative examples of data and the resulting standard curve. **This curve should not be used in lieu of a standard curve run with each assay.**

Tube #	Tube I.D.	CPM	Average CPM	Corrected CPM	Results ng/mL
1	0 ng/mL	587			
2		677	632		
3	0.8 ng/mL	2286			
4		2196	2241	1609	
5	2.2 ng/mL	5115			
6		5173	5144	512	
7	7.2ng/mL	15174			
8		15314	15244	14512	
9	21.0 ng/mL	29839			
10		32161	31000	30368	
11	67.0 ng/mL	50954			
12		55788	53371	52739	
13	Control 1	3757			
14		3749	3753	3121	1.5
15	Control II	25347			
16		24347	24847	24215	14.3
17	Sample 1	18216			
18		19146	18681	18049	9.4
19	Sample 2	6283			
20		6456	6369	5737	2.8



LIMITATIONS OF THE PROCEDURE

- The lowest concentration of osteocalcin measurable is 0.05 ng/mL (assay sensitivity) and the highest concentration of osteocalcin measurable without dilution is 40 ng/mL. Although the highest standard provided in this kit is greater than 40 ng/mL, inherent statistical variation at the highest standard as observed in any IRMA cannot preclude that a sample with a concentration exceeding the highest standard will not, on occasion, read less than the highest standard.
- Levels of intact osteocalcin, large N-terminal midregion fragments, and other smaller osteocalcin fragments are often elevated in patients with renal impairment. High levels of small immunoreactive fragments will suppress the measurement of the larger peptides in undiluted samples. **Therefore, it is necessary that samples from dialysis patients and uremic patients be pre-diluted 1:10 before immunoassay using the 0 ng/mL Standard or the optional Sample Diluent reagent. Values read from the curve must be multiplied by the dilution factor to obtain final results. If this calculated result is >40 ng/mL the sample should be repeated at a 1:50 or greater dilution that is appropriate for the laboratory patient population.**
- The reagents in this Human Osteocalcin IRMA Kit have been optimized so that the high dose "hook effect" is not a problem for samples with elevated osteocalcin values. Samples with osteocalcin levels between 40 ng/mL and 1,000 ng/mL will read greater than 40 ng/mL and should be diluted 1:10 with the 0 ng/mL Standard or the optional Sample Diluent reagent and reassayed for correct values.
- Grossly lipemic serum or plasma samples may affect the immunological response and it is recommended that results obtained with such samples be scrutinized accordingly.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls with known levels of osteocalcin. Immutopics recommends that all assays include the laboratory's own osteocalcin controls in addition to those provided with this kit.

EXPECTED VALUES

Each laboratory should establish its own normal range. The geometric mean and range of serum samples from apparently healthy individuals assayed at Immutopics and based on 95% confidence limits was:

Sample	n	Age Years	Mean ng/mL	Range ng/mL
All	101	19 - 60	5.3	2.4 - 11.7
Male	49	20 - 60	6.1	3.4 - 11.7
Female	52	19 - 58	4.6	2.4 - 10.0

**PERFORMANCE CHARACTERISTICS:
SENSITIVITY**

The sensitivity of the Human Osteocalcin IRMA assay as determined by the 95% confidence limit on 20 duplicate determinations of the 0 ng/mL Standard is 0.05 ng/mL.

PRECISION

To assess intra-assay precision the mean and coefficient of variation were calculated from 20 duplicate determinations of two samples each performed in a single assay.

Mean Value (ng/mL)	Coefficient of Variation
1.6	5.2 %
14.2	3.9 %

To assess inter-assay precision the mean and coefficient of variation were calculated from duplicate determinations of two samples performed in 20 assays.

Mean Value (ng/mL)	Coefficient of Variation
1.5	6.7 %
14.2	5.5 %

PARALLELISM

Non-uremic serum samples were diluted with the 0 ng/mL Standard and assayed. Results in ng/mL are as follows:

SAMPLE	DILUTION	OBSERVED VALUE	EXPECTED VALUE	%O/E
1	undiluted	9.4		
	1:2	5.0	4.7	106
	1:4	2.5	2.4	104
	1:8	1.3	1.2	108
2	undiluted	9.5		
	1:2	4.6	4.7	98
	1:4	2.3	2.4	96
	1:8	1.2	1.2	100
3	undiluted	7.4		
	1:2	3.6	3.7	97
	1:4	2.1	1.9	111
	1:8	0.9	0.9	100

RECOVERY

Various amounts of human osteocalcin were added to different non-uremic serum samples and assayed. Results in ng/mL are as follows:

SAMPLE	ORIG. VALUE	AMOUNT ADDED	OBSERVED VALUE	EXPECTED VALUE	%O/E
1	4.0	4.3	8.9	8.3	107
		8.4	13.5	12.4	109
		12.6	15.11	16.6	91
2	5.7	3.7	9.4	9.4	100
		7.4	13.1	13.1	100
		11.0	16.3	16.7	98
3	3.4	4.1	8.1	7.5	108
		8.2	11.4	11.6	98
		12.2	15.6	15.6	100

SPECIFICITY AND CROSS-REACTIVITY

The antibodies used in the Immutopics Human Osteocalcin IRMA Kit are purified by affinity chromatography to be specific for well defined regions of the osteocalcin molecule. The ¹²⁵I labeled antibody only recognizes epitopes within the N-terminal 1-19 amino acid sequence of human osteocalcin and has zero cross-reactivity with the 20-49 segment. The antibody immobilized on the bead only recognizes epitopes within the midregion 20-36 amino acid sequence of the molecule and has zero cross-reactivity with the 1-19 segment.

Various human osteocalcin peptide fragments representing highly elevated physiological conditions were studied to further validate the cross-reactivity performance characteristics of this kit. Human osteocalcin fragments 1-19, 37-49, 43-49, and 45-49, each at concentrations of 1000 ng/mL, were spiked into the 0 ng/mL Standard and assayed. The results show zero cross-reactivity from any of these fragments assayed in this manner.

WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Immutopics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Immutopics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights which vary from state to state.

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CLIENT SERVICES

To place an order or for technical assistance, contact Immutopics International's at (800) 681-6665 or (949) 369-9207 or FAX to (949) 369-9405 or e-mail: info@immutopicsintl.com..

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