

Rat Osteocalcin ELISA Kit

Enzyme-Linked ImmunoSorbent Assay (ELISA) for the Quantitative Determination of Rat Osteocalcin Levels in Serum, Plasma or Cell Culture Media

Immutopics

Immutopics, Inc.

For RESEARCH Use Only

96 Test Kit
Cat.# 60-1505

Not for use in diagnostic procedures

Store at 2 - 8°C Upon Receipt

INTENDED USE

This kit is intended for research use only in the determination of rat intact osteocalcin levels in serum, plasma or cell culture media.

INTRODUCTION

Rat osteocalcin, a 50 amino acid peptide, is the major noncollagen protein found in rat 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. 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.

TEST PRINCIPLE

The Rat Osteocalcin ELISA Kit is a two-site enzyme-linked immunosorbent assay (ELISA) for the measurement of osteocalcin in rat serum, plasma or cell culture media. Two different goat polyclonal antibodies to rat osteocalcin have been purified by affinity chromatography. The antibody which recognizes epitopes within the midregion/C-terminal portion of the peptide is biotinylated for capture. The other antibody which recognizes epitopes within the N-terminal region is conjugated with the enzyme horseradish peroxidase (HRP) for detection.

In a two-step reaction a sample containing rat osteocalcin is first incubated with the biotinylated antibody in a streptavidin coated microtiter well. After washing the well to remove any unbound antibody and other components, the well is incubated with the HRP conjugated antibody. Osteocalcin contained in the sample is now immunologically bound by the capture antibody and the detection antibody to form a "sandwich" complex:

Well/Biotin Antibody — Rat Osteocalcin — HRP Antibody

Following another wash, the enzyme antibody bound to the well is incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microtiter plate reader. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of rat osteocalcin in the sample. A standard curve is generated by plotting the absorbance versus the respective rat osteocalcin concentration for each standard on linear or logarithmic scales. The concentration of rat osteocalcin in the samples 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. STREPTAVIDIN COATED MICROTITER PLATE (40-0010)**
One plate with 12 eight well strips and frame (96 wells total). This reagent should be stored in the foil pouch with desiccant at 2 - 8°C and is stable until the expiration date on the kit.
- 2. RAT OSTEOCALCIN BIOTINYLATED ANTIBODY (40-1515)**
One vial containing 5.5 mL of biotin labeled anti-rat osteocalcin in TRIS buffered saline with protein stabilizers and a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the kit.
- 3. RAT OSTEOCALCIN HRP CONJUGATED ANTIBODY (40-1525)**
One vial containing 11 mL of horseradish peroxidase conjugated to anti-rat osteocalcin in a stabilized protein solution with a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8°C protected from light and is stable until the expiration date on the kit.
- 4. RAT OSTEOCALCIN STANDARDS (40-1531 to 40-1536)**
Six vials, five of which contain synthetic rat osteocalcin (1-50) lyophilized in a protein matrix with 0.1% sodium azide. **Refer to vial label for exact concentration.** The zero standard is also used as the sample diluent and is supplied as a 20 mL ready-to-use liquid. 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 15 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. The zero standard may be stored at 2 - 8°C. 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.
- 5. RAT OSTEOCALCIN CONTROLS I & II (40-1541 & 40-1542)**
Two vials each containing rat osteocalcin (1-50) 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 15 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.
- 6. ELISA WASH CONCENTRATE (40-0042)**
One bottle containing 30 mL of a 20 fold concentrate. Before use dilute the contents to 600 mL with deionized water and mix well. Upon dilution this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution should be stored at room temperature and is stable until the expiration date on the kit.
- 7. ELISA HRP SUBSTRATE (40-0026)**
One bottle containing 11 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 - 8°C protected from light and is stable until the expiration date on the kit.

8. ELISA STOP SOLUTION (40-0030)

One bottle containing 11 mL of 1 M sulfuric acid. This reagent may be stored at room temperature or at 2 - 8°C and is stable until the expiration date on the kit.

9. PLATE SEALER (10-2016)

Two included in kit.

SAFETY PRECAUTIONS

Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid (i.e. ELISA HRP Substrate and ELISA Stop Solution). In case of contact with any of these reagents, wash thoroughly with water. TMB is a suspected carcinogen. Use Good Laboratory Practices. Wash hands before eating. Do not eat, drink or smoke in the work area.

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 buildup (Manual Guide-Safety Management No. CDC-22, Center for Disease Control, Atlanta, Georgia, April 30, 1976).

MATERIALS REQUIRED BUT NOT PROVIDED

1. 1.0 mL and 2.0 mL volumetric pipettes for reconstituting standards and controls.
2. Precision pipets capable of delivering 20 µL, 25 µL, 50 µL, 100 µL and 400 µL.
3. Aluminum foil.
4. Automated microtiter plate washer OR
5. Repeating dispenser for delivering 350 µL and suitable aspiration device.
6. Container for storage of wash solution.
7. Spectrophotometric microtiter plate reader capable of reading absorbance at 450 nm.
8. Deionized water.
9. Horizontal rotator capable of maintaining 180 - 220 RPM.
10. Timer.

SPECIMEN COLLECTION

Measurement of the rat osteocalcin concentration may be made on serum, plasma or cell culture media. Since serum and plasma samples are diluted 1:21 prior to assay only twenty microliters are required to assay the sample in duplicate. If obtaining serum, collect blood and allow it to clot at room temperature. Centrifuge the sample and separate the serum, plasma, or media from the cells. Samples should be assayed immediately or stored frozen at -20°C or below. Avoid repeated freezing and thawing of specimens.

ASSAY PROCEDURE

1. **Dilute both controls and each serum or plasma sample 1:21 prior to assay.** (Standards do not require dilution and are ready-to-use after reconstitution.) For sample dilution pipette 20 µL of sample and 400 µL of zero standard into appropriately labeled tubes and vortex. Cell culture media samples may have to be diluted differently to obtain optimum results.
2. Place a sufficient number of Streptavidin Coated Strips in a holder to run osteocalcin standards, controls and unknown samples.
3. Pipet 25 µL of standard, diluted control, or diluted sample into the designated or mapped well. Freeze the remaining standards and controls except standard zero, as soon as possible after use.
4. Pipet 50 µL of Rat Osteocalcin Biotinylated Antibody into each well and cover the plate with one plate sealer.
5. Incubate plate at room temperature for 1 hour on a horizontal rotator set at 180 - 220 RPM.

6. Remove the plate sealer. **Using an automated microtiter plate washer aspirate the contents of each well. Wash each well five times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents.** A suitable aspiration device may also be used.
7. Pipet 100 µL of Rat Osteocalcin HRP conjugated antibody into each well.
8. Re-cover the plate with the plate sealer and aluminum foil to protect from exposure to light. Incubate at room temperature for 1 hour on a horizontal rotator set at 180-220 RPM.
9. Remove the aluminum foil and plate sealer. **Using an automated microtiter plate washer aspirate the contents of each well. Wash each well five times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents.** A suitable aspiration device may also be used.
10. Pipet 100 µL of ELISA HRP Substrate into each of the wells.
11. Re-cover the plate with the Plate Sealer and aluminum foil. Incubate at room temperature for 30 minutes on a horizontal rotator set at 180 - 220 RPM.
12. Remove the aluminum foil and plate sealer. Immediately pipet 50 µL of ELISA Stop Solution into each of the wells. Mix on a horizontal rotator for 1 minute.
13. Read the absorbance at 450 nm within 10 minutes in the microtiter plate reader against a reagent blank of 100 µL of Substrate and 50 µL of Stop Solution.

If dual wavelength correction is available set the Measurement wavelength to 450 nm and Reference wavelength to 595 nm to 650 nm.

PROCEDURAL NOTES

1. It is recommended that all standards, controls and samples be assayed in duplicate. The average absorbance reading of each duplicate should then be used for data reduction and the calculation of results.
2. Store light sensitive reagents (i.e. HRP Conjugated Antibody and ELISA HRP Substrate) in the original amber bottles or other suitable container which is well protected from light.
3. Store any unused Streptavidin Coated Strips in the resealable aluminum pouch with desiccant to protect them from moisture.
4. The sample and all reagents should be pipetted carefully to minimize air bubbles in the wells.
5. The sequence and timing of each reagent addition is important as both the immunological and enzymatic reactions are in kinetic modes. The washing step is also an important part of the total assay procedure. **The use of an automated microtiter plate washer is strongly recommended.** All pipeting and washing steps should be performed such that the timing is as consistent as possible.
6. Samples with values greater than the highest standard should be further diluted 1:10 with the 0 ng/mL Standard and reassayed. Multiply the result by 10. (See Limitations, # 1 and # 2)
7. Plasma or cell culture media 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 well surface.
8. Rarely, upon opening the streptavidin plate, small white crystals may be observed in some of the wells. This is entirely cosmetic and will not affect the assay. This condition is reported by other kit manufacturers and results from the final stabilizing buffer used in the coating process.

CALCULATION OF RESULTS

The absorbance readings taken after the addition of the ELISA Stop Solution allow for the construction of a standard curve using the rat osteocalcin standards contained in the kit. **Refer to the individual vial label for exact concentration.** The curve should be generated as follows:

1. Calculate the average absorbance for each pair of duplicate assay wells.
2. Subtract the average absorbance of the 0 ng/mL Standard from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by plotting the corrected absorbance of each standard level on the ordinate against the standard concentration on the abscissa using linear-linear or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of rat osteocalcin results.

The rat osteocalcin concentration of the controls and samples are read directly from the standard curve using their respective corrected absorbance. If log-log graph paper or computer assisted data reduction programs utilizing logarithmic transformation are used, samples having corrected absorbance between the 0 ng/mL Standard and the next highest standard should be calculated by the formula:

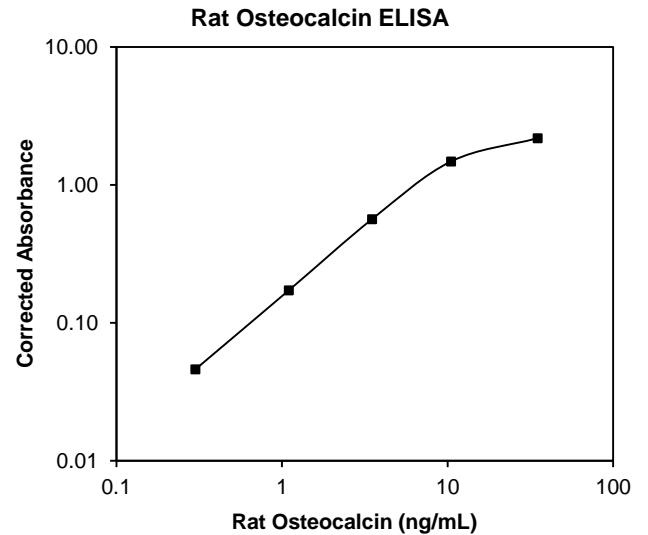
$$\text{Value of unknown} = \frac{\text{Corrected Absorbance (unknown)}}{\text{Corrected Absorbance (2}^{\text{nd}} \text{ Std.)}} \times \text{Value of the 2}^{\text{nd}} \text{ Std.}$$

To obtain the final rat osteocalcin concentrations for controls and samples multiply the observed values by the dilution factor.

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.**

Well I.D.	ABSORBANCE - 450 nm			Results ng/mL	Corrected Results ng/mL
	ABS	Average ABS	Corrected ABS		
Reagent Blank	0.000				
	0.000	0.000			
0 ng/mL	0.018				
	0.021	0.019	0.000		
0.3 ng/mL	0.065				
	0.065	0.065	0.046		
1.1 ng/mL	0.194				
	0.188	0.191	0.172		
3.5 ng/mL	0.582				
	0.584	0.583	0.564		
10.5 ng/mL	1.518				
	1.476	1.497	1.478		
35 ng/mL	2.221				
	2.174	2.198	2.179		
Control I	0.205			1.34	28
	0.199	0.202	0.183		
Control II	0.677			4.11	86
	0.646	0.662	0.643		
Sample 1	0.471			2.89	61
	0.478	0.475	0.456		
Sample 2	0.662			4.14	87
	0.670	0.666	0.647		
Sample 3	0.976			6.59	138
	0.998	0.987	0.968		



LIMITATIONS OF THE PROCEDURE

1. The lowest concentration of rat osteocalcin measurable is 0.05 ng/mL (assay sensitivity) and the highest concentration of rat osteocalcin measurable is the value of the highest standard.
2. The reagents in this Rat Osteocalcin ELISA kit have been optimized so that the high dose "hook effect" is not a problem for samples with elevated rat osteocalcin values. Samples with rat osteocalcin levels between the highest standard and 6,000 ng/mL will read greater than the highest standard and should be further diluted 1:10 with the 0 ng/mL Standard and reassayed for correct values.
3. Grossly lipemic serum or plasma samples may affect the immunological response and it is recommended that results obtained with such samples be scrutinized accordingly.
4. Differences in protein concentration and protein type between samples and standards in an immunoassay contribute to "protein effects" and dose biases. When measuring low protein concentration culture media samples against high protein concentration standards, it is recommended that like samples be assayed together in the same assay to minimize this bias.

QUALITY CONTROL

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

PERFORMANCE CHARACTERISTICS: SENSITIVITY

The sensitivity of the rat osteocalcin 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 diluted samples each performed in a single assay.

Observed Mean Value (ng/mL)	Coefficient of Variation
1.3	1.6 %
3.9	1.8 %

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

Observed Mean Value (ng/mL)	Coefficient of Variation
1.3	3.0 %
4.2	6.9 %

PARALLELISM

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

SAMPLE DILUTION		CORR. OBSERVED VALUE	CORR. EXPECTED VALUE	% O/E
1	1:21	118	118	100
	1:42	122	118	103
	1:84	126	118	107
	1:168	126	118	107
2	1:21	116	116	100
	1:42	113	116	97
	1:84	118	116	102
	1:168	111	116	96
3	1:21	187	187	100
	1:42	210	187	112
	1:84	185	187	99
	1:168	201	187	107

RECOVERY

Various amounts of rat osteocalcin were added to different rat serum and/or plasma samples and assayed. Results, corrected for dilution, in ng/mL are as follows:

SAMPLE	ORIG. VALUE	AMOUNT ADDED	OBSERVED VALUE	EXPECTED VALUE	% O/E
1	35.0	11.8	45.2	46.8	97
		23.6	55.5	58.6	95
		35.4	67.7	70.4	96
2	10.9	17.8	30.2	28.7	105
		35.6	47.2	46.5	102
		53.4	65.5	64.3	102
3	22.6	14.9	35.3	37.5	94
		29.8	49.5	52.4	95
		44.7	64.4	67.3	96

CROSS-REACTIVITY

Human osteocalcin and mouse osteocalcin were each diluted in an osteocalcin-free protein matrix and measured in the Rat Osteocalcin ELISA Kit. The results show zero cross-reactivity from either of these species.

CORRELATION

A correlation study was performed with rat serum and plasma samples comparing results obtained from the Rat Osteocalcin ELISA Kit vs. results obtained from the Rat Osteocalcin IRMA Kit (Immutopics, Inc.). A linear regression analysis gave the following results: $n = 52$, $ELISA = 0.92 \times IRMA + 0.2$; correlation coefficient: $(r) = 0.99$.

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.

REFERENCES

1. Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA and Wozney JM, "Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones", *EMBO J.* 5(8), p.1885-1890 (1986).
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4. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, and Karsenty G, "Increased bone formation in osteocalcin-deficient mice", *Nature*, Vol 382, 1 Aug 1996, 448-452.

CLIENT SERVICES

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

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