



Revised 29 Nov. 2010 rm (Vers. 4.1)

For Research Use Only

Please use only the valid version of the package insert provided with the kit.

## FOR RESEARCH USE ONLY

## **Principle of the Assay**

This sandwich ELISA Kit is specific for <u>rat osteocalcin only</u>. Both carboxylated and decarboxylated rat osteocalcin are recognized. Rat osteocalcin can be measured directly from serum, heparinized plasma, bone extracts and serum free cell culture supernates. A monoclonal antibody directed against the N-terminal region of osteocalcin is bound to the polystyrene wells. After an incubation with sample, the plate is washed followed by an incubation with a second antibody (Goat polyclonal) of high specificity for the C-terminus of rat osteocalcin. Detection is achieved by a third incubation using a Horseradish Peroxidase conjugate of a Donkey anti-Goat IgG and subsequent enzyme assay. The osteocalcin concentration is proportional to color development. Standards of highly purified rat osteocalcin are used to generate a standard curve.

# **References / Literature**

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## **Reagents: Description and Preparation**

Store all reagents at 4°C up to 6 months except as noted. \* *See Storage Exception* <u>*CAUTION:*</u> DO NOT USE AZIDE.

- 1. Working sample buffer. One 60 ml bottle and one 125 ml bottle.
- 2. Phosphate-Saline Concentrate (**wash buffer**). One 100 ml bottle. Transfer contents to a graduated cylinder, and bring volume up to 500 ml with deionized water.
- 3. Osteocalcin **Standard**. One vial 200 ng lyophilized. Reconstitute vial with exactly 2.0 ml of sample buffer. Use for making working standards. Store at -20°C.
- 4. Osteocalcin **Antiserum** Concentrate. One vial containing 0.5 ml. Dilute contents to 10 ml with sample buffer and use immediately. This is a 1:20 dilution. <u>Dilute only enough antibody for current use</u>.
- Donkey anti-Goat IgG Peroxidase. 2 vials, lyophilized. Reconstitute 1 vial with 12 ml of sample buffer 30 minutes before use. Discard all leftovers. Store the second vial at 4 °C for later use if needed.
- 6. **Peroxidase Substrate** TMB (3,3',5,5'-tetramethyl benzidine). One 6 ml vial.
- 7. Hydrogen Peroxide Solution. One 6 ml vial.
- 8. Stop Solution. One 12 ml vial.
- Rat Serum Control. 50 μl lyophilized whole serum. Reconstitute with 50 μl deionized water, gently mixing 15-20 minutes. Dilute with 0.45 ml sample buffer, mix thoroughly. Multiply the quantity printed on the vial by 2 to obtain the ng/ml. Store at -20°C.
- 10. One 96 well plate (8 well removable strips) coated with osteocalcin antibody. Extra sealing tape provided.

## **Other Supplies Required**

- 1. ELISA Plate Reader which can measure absorbance at 450 nm.
- 2. Pipettes: micropipettes 5-1000 µL.
- 3. A plate washer is recommended for washing.
- 4. A 37°C Incubator.
- 5. Deionized water.





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## **Precautions**

Some components of this kit contain isothiazolones (5ppm) as preservative. Stop solution contains hydrochloric and phosphoric acids. Keep all materials away from the skin and eyes.

## **Sample Preparation**

Aliquot and freeze immediately all samples for future analysis.

Samples stored frozen are stable for six months. Avoid repeated freeze-thawing of samples, osteocalcin levels will decline considerably. Samples from serum, heparinized plasma and cell culture media can be measured directly using Osteocalcin Rat ELISA Kit. Samples containing azide cannot be assayed.

#### Rat Serum

Rat serum must be diluted at least 10-fold with sample buffer. We recommend a 1/10 to 1/20 dilution with sample buffer. Thus it is possible to quantitate osteocalcin in 5  $\mu$ l of rat serum (10  $\mu$ L in duplicate). Serum samples can conveniently be aliquoted in 25-50  $\mu$ L amounts and stored at -70°C.

The design of the animal experiment is most important. Many variables effect serum osteocalcin levels: age, growth rate, hormonal status, vitamin D intake, stress, circadian rhythm, etc. It is desirable to take blood samples under the same conditions: time of day, without stress, etc. and process the serum (or plasma) the same way for each sampling.

#### **Rat Serum Osteocalcin Levels**

The following results were obtained with male Spraque Dawley rats of given ages. (Blood was allowed to clot at room temperature for 15 minutes and then 15 minutes at 4°C. Serum was drawn off and frozen immediately.)

Age	ng/ml Serum Osteocalcin
1 month	78
3 months	45
5 months	17

A serum sample went through 2 freeze-thaw cycles and was incubated at room temperature for 4 hours without any loss of osteocalcin concentration.

## Rat Bone

Typical levels are 1.5-2.0ng osteocalcin/ $\mu$ g dry bone powder. EDTA extracts of bone powder can be assayed after appropriate dilution.

## **Rat Osteoblast Culture Medium**

Concentrations of osteocalcin in conditioned media range from <1ng/ml to 400ng/ml depending on the cell type and culture conditions.

It is best to wash cells and grow in serum free media (24-48 hours) prior to measuring osteocalcin levels. Bovine serum products (Bovine IgG and osteocalcin) can interfere in the assay. Many researchers find that osteocalcin synthesis can be stimulated in primary bone cell cultures with  $10^{-9}$ M 1,25 dihydroxy Vitamin D<sub>3</sub>. Full dilution series should be done to





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establish linearity of dilution. Medium and serum interference should be assessed by appropriate blanks and internal standards of medium with added rat osteocalcin.

## Assay Procedure

**CAUTION:** KEEP AZIDES AWAY FROM ALL SOLUTIONS AND SAMPLES <u>All Reagents must be at room temperature prior to use.</u>

## **Procedure**

- 1. Please refer to "preparation of reagents" and " sample preparation".
- 2. Remove microtiter plate from resealable bag. Strips not used should be removed from the frame and resealed in the bag for future use.
- Dilute the stock standard (100 ng/ml) in polypropylene tubes with sample buffer to give six or seven standards in the range of 0.25 to 20 ng/ml.
  0.33, 1.0, 2.5, 5.0, 10 and 20 ng/ml results in a good curve.
- Pipet 25 μl of sample buffer (Blank), Standards, Controls and Unknowns into designated duplicate wells followed by 100 μl of osteocalcin antiserum in each well. Cover tightly with plastic seal provided, incubate at 37 °C, 2.5 hours.
- Aspirate wells completely and wash the plate 3 times (plate washer) or 5 times (by hand) with 0.3 ml/well Phosphate-Saline wash buffer.
  (Complete removal of wash buffer after each wash is important for good reproducibility).
- 6. Add 100  $\mu$ L of the diluted Donkey anti-Goat IgG Peroxidase to each well. Incubate at **room temperature for 1 hour**.
- Mix one volume of TMB solution with one volume of Hydrogen Peroxide solution and put aside. (Only mix an amount sufficient for the number of wells in use) Wash the plate as in step 5. Immediately add 100 μL of substrate mix to all wells and incubate at room temperature, in the dark for 30 minutes.
- 8. Add 100 µL of Stop Solution to all wells, swirl and measure absorbance at 450nm within 15 minutes.

## **Calculation of Results**

Average duplicates for all determinations. Subtract the (Blank) from all average readings. Plot net optical density of the standards vs. log of the concentration of each, draw the best curve. Obtain concentration of each unknown from this standard curve. Always generate a standard curve for each new assay.







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# **Typical Data**

(Do Not Use for determination of Unknowns)

ID	A450	Average – Blank
Standards:		
(Blank) 0ng/ml	.201	
(Blank)	.196	(.199)
0.33 ng/ml	.308	
0.33 ng/ml	.272	.091
1 ng/ml	.510	
1 ng/ml	.486	.300
2.5 ng/ml	.771	
2.5 ng/ml	.856	.615
5 ng/ml	1.120	
5 ng/ml	1.157	.940
10 ng/ml	1.384	
10 ng/ml	1.375	1.181
20 ng/ml	1.539	
20 ng/ml	1.604	1.373







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**Typical Standard Curve** 



