Revised 1 Dec. 2010 rm (Vers. 2.1) **RUO**

INTENDED USE

For the Measurement of Human, Monkey, Dog and Bovine Osteocalcin in Serum or Heparinized Plasma.

This kit is intended for Research Use Only. Not for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The assay measures only intact osteocalcin, which is synthesized de novo by the osteoblast, and eliminates any potential confounding interference by circulating fragments. The assay is a sandwich EIA which utilizes monoclonal antibodies directed toward the amino- and carboxy- terminal regions of the protein. It recognizes only intact osteocalcin, requiring the full 49 residue protein for detection. It is rapid, sensitive and reliable.

REFERENCES

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3. Tracy, R.P.; Andrianorivo, A.; Riggs, B.L. and K.G. Mann. Comparison of monoclonal and polyclonal antibody-based immunoassays for osteocalcin: a study of sources of variation in assay results. JBMR 5: 451-61, 1990.
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6. Bodine, P.V.N., Trailsmith, M., and Komm, B.S., Development and Characterization of a Conditionally Transformed Adult Human Osteoblastic Cell Line. JBMR, 11(6): 806-819, 1996.
7. Nuttal, M.E., Paton, A.J., Olivera, D.L., Nadeau, D.P. and Gowen, M. Human Trabecular Bone Cells are able to Express both Osteoblastic and Adipocytic Phenotype: Implications for Osteopenic Disorders. JBMS 13 (3): 371-382 (1998).

REAGENTS: DESCRIPTION AND PREPARATION

Note: *All reagents stable at 4 °C for 6 months.*

1. **Sample Buffer** – One 60 ml bottle. *Store at 4 °C. Stable for 6 months.*

2. **Phosphate-Saline buffer concentrate (Wash buffer)** – One 100ml bottle. Dilute contents to 500 ml with deionized water. *Store at 4 °C. Stable for 6 months.*

3. **Osteocalcin Standards, Five vials, Lyophilized –** Reconstitute each vial with 0.5ml deionized water (use 0.50 ml volumetric pipet), replace stoppers and let stand for 5 minutes. Mix each vial end-over-end several times to obtain a clear solution. **Store these reconstituted standards frozen at -20°C.** *(Stable for 6 months).* Thaw completely and allow reconstituted standards to reach room temperature prior to use. *Stable for 2 freeze thaw cycles.*

4. **Biotinylated Antiserum, One Vial, 0.25ml.** – Biotinylated antibody to human osteocalcin. Dilute with sample

buffer sufficient antiserum for current use and according to dilution ratio printed on the label. *Store at 4°C. Stable for 6 months*

5. **Native Human Osteocalcin Antiserum. One vial, 0.5ml – Dilute contents with 10 ml of sample buffer and use immediately. This is 1:20 dilution. Dilute only enough antibody for current use.** *Store at 4°C. Stable for 6 months.*

6. **Streptavidin Horseradish Peroxidase.** One vial, 11 ml. *Store at 4°C. Stable for 6 months*

7. **Peroxidase Substrate. TMB (3,3',5,5' Tetramethylbenzidine)** – One Vial. *Store at 4°C. Stable for 6 months.*

8. **Stop Solution (1M HCl +1M H3PO4)** – One Vial, 12 ml. *Store at 4°C. Stable for 6 months.*

9. **Hydrogen Peroxide Solution, One Vial** – *Store at 4°C. Stable for 6 months.*

10. **Human Osteocalcin Controls, Two Vials –** Add 200ul deionized water to each, let stand 10 minutes at room temperature, gently mix by inversion (High control 25ng/ml and Low control 5ng/ml).

11. **One 96-Well (8 strip removable well) plate, coated with 1-19 monoclonal antibody.**

OTHER SUPPLIES REQUIRED

1. ELISA Plate Reader which can measure absorbance at 450nm.
2. Pipettes: 100ul and 1-25ul micropipettes. A 0.5ml volumetric pipet.
3. A plate washer is recommended for washing.
4. Deionized water.

PRECAUTIONS

Some components of this kit contain isothiazolones (5ppm) as preservative. Stop solution contains sulfuric acid. Keep these materials away from the skin and eyes!

SAMPLE COLLECTION AND STORAGE

All samples (serum, plasma, cell culture media, etc.) should be aliquoted and stored at -20°C. For long term storage (>1month) store at -70°C. All samples should undergo only one or two freeze-thaw cycles. Serum or Heparinized plasma is ideal for blood samples. Dog serum can be run neat or diluted 1:1. Use the diluent buffer for any sample dilutions. Since bovine oseocalcin (present in bovine serum) is virtually identical with human osteocalcin (and reacts in this assay) it is necessary to wash cells with/and grow in serum free media 24-48 hours prior to taking samples.

ASSAY PROCEDURE

Note: *All reagents should be at room temperature.*

1. Please refer to page 2 for preparation of reagents. **All reagents must be at room temperature.**

2. Remove microtiter plate from resealable bag. Strips not used immediately should be removed from the frame and resealed in the bag for future use.

3. Add 25ul diluent buffer (zero or blank), standards, samples and controls to appropriate wells followed by 50ul **Native** osteocalcin antiserum. The entire plate should be completed in 15 minutes or less. Gently swirl the plate about 1 minute. Cover tightly and incubate at room temperature for 1 hour.

4. Add 50ul of diluted Biotinylated antiserum to all wells. Swirl as above, incubate at room temperature for 1 hour.

5. Aspirate completely and wash the plate 3 times with 0.3ml phosphate-saline wash buffer. Add 100ul Streptavin-Horseradish Peroxidase reagent to all wells. Swirl and then incubate at room temperature for 30 minutes.

6. 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 plate as in step 4. Immediately add 100ul of substrate mix to all wells, incubate at room temperature, in the dark, 10 minutes.

7. Add 100ul stop solution to all wells, swirl, and measure absorbance immediately at 450nm. Collect data.

NOTES:

1. Add stop solution in the same order to the plate as the substrate.
2. Before absorbance measurements are taken, be sure there are no air bubbles floating on top, and the bottom of the wells are clean and dry.
3. Avoid cross-contamination by using new pipet tips for each standard and sample. Dispense samples and standards at bottom of the wells and reagents near the top. Do not agitate or strike the plate so briskly as to cause droplets of liquid to fly up from the wells.

CALCULATION OF RESULTS

Average duplicates for all determinations. Subtract the zero (blank) standard from all averaged 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.

TYPICAL DATA

(Note: Do not use for Determination of Unknowns.)

Standard (ng/ml)	A_{450nm}	Avg-blank
0.00 (Blank)	0.149	
0.00	0.146	0.147
1.00	0.294	
1.00	0.320	0.160
5.00	0.745	
5.00	0.850	0.605
10.00	1.051	
10.00	1.031	0.894
25.00	1.242	
25.00	1.286	1.117
50.00	1.409	
50.00	1.393	1.254

Typical Standard Curve