

## Measurement of Total and Uncarboxylated osteocalcin

Summary: Osteocalcin is measured in serum by an equilibrium radioimmunoassay utilizing an antibody made against osteocalcin purified from human bone. The assay recognizes intact osteocalcin and the 1-43 major fragment with equimolar reactivity and both carboxylated and uncarboxylated osteocalcin equivalently. Antibody, non-immune serum, and tracer is stored in small aliquots to avoid repeated freeze and thaw cycles. Each standard is stored in individual aliquots such that they are used only for 3 assays. Quality control is routinely monitored in each assay with a pool of three known samples with low (<5ng/ml), mid (12-18 ng/ml), and high (>35 ng/ml) osteocalcin concentrations. For uncarboxylated osteocalcin, controls containing 0%, 25%, 50%, 75% and 100% carboxylated osteocalcin (total osteocalcin concentration 10 ng/ml) are prepared with native and thermally decarboxylated osteocalcin to ensure hydroxyapatite binding fidelity.

Carboxylated osteocalcin is separated from uncarboxylated osteocalcin by differential hydroxyapatite binding. After adsorption of serum onto a standard suspension of HA, uncarboxylated OC is measured in the supernatant. Total osteocalcin is measured in the original serum. The binding assay depends upon both the concentration of osteocalcin in the sample and the concentration of HA. Samples with total osteocalcin > 15 ng/ml are diluted with osteocalcin free serum to the 10 ng/ml and hydroxyapatite binding repeated. Results are expressed as total osteocalcin and %uncarboxylated osteocalcin.

Reference: Gundberg CM, Nieman SD, Abrams S, Rosen H. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin. *J Clin Endocrinol Metab* 1998;83:3258-66.

### Detailed Method:

1. Make a slurry of 4 mg/ml Calbiochem Hydroxylapatite, Fast Flow catalog # 391947 with dH<sub>2</sub>O in a small beaker (10 ml) containing a magnetic flea. Swirl vigorously on a magnetic shaker. While mixing, pipet 100 µl aliquots of the suspension into microtubes.
2. Pipet 100 µl of sample into microtubes and cap. Shake microtubes vigorously for 1 hour on vortex mixer (Fisher Vortex Genie 2 cat# 12-812 fitted with 60 micro tube holder cat# 12-812B).
3. Spin in a Micro Centrifuge for 1 minute. The supernatant contains uncarboxylated osteocalcin.
4. Assay original sample and hydroxyapatite supernatant.
5. Carboxylated osteocalcin can be determined from Total OC-ucOC=cOC.
6. Uncarboxylated osteocalcin is generally expressed as %ucOC because total osteocalcin is a function of osteoblastic activity and the carboxylation status is dependent upon vitamin K availability.

7. The binding assay depends upon the relative amount of osteocalcin in the sample with respect to the amount hydroxyapatite used for binding. Therefore, any sample containing >15 ng/ml of total osteocalcin is diluted in osteocalcin free serum to a final value of 10 ng/ml and re-assayed. %ucOC is calculated.

**Quality Control.** Quality control is routinely monitored in each assay with a pool of three known samples with low (<5ng/ml), mid (12-18 ng/ml) , and high (>35 ng/ml) osteocalcin concentrations. For uncarboxylated osteocalcin, controls containing 0%, 25%, 50%, 75% and 100% carboxylated osteocalcin (total osteocalcin concentration 10 ng/ml) are prepared with native and thermally decarboxylated osteocalcin to ensure hydroxyapatite binding fidelity.

To avoid freeze-thaw, individual aliquots are stored at -80°C. Acceptable limits of error (mean  $\pm$  2 SD) have been established for each control. This is accomplished by assaying each control by replicate analyses in 8 separate assays. Once this is completed, the controls are employed. If any values are outside the acceptable range, the data are reevaluated before any results are reported. If at any time, results for 2 of the 3 controls exceed three SDs of the calculated mean, the sample batch will be reanalyzed. Once a source of error is found and the assay is re-established, samples are repeated. If no apparent error can be found, new control serum is assayed to determine if the error resides with the control sera. If an upward or downward drift in control values are detected, new standards and quality control samples are evaluated. If degradation of a control occurs over the course of the study, it is replaced and the new concentration again established. The intra-day precision of the assay is assessed periodically by analyzing multiple sets of control serum.

### Commercial Assays

**PINP:** Human P1NP a RIA kit from Orion Diagnostica UniQ (Espoo, Finland, distributed by Immunodiagnostic systems, Inc., Scottsdale, AZ). This is a competitive equilibrium assay that is completed in a single day. 100 ul of serum is required for duplicate analysis. Quality control is monitored by provided samples included in the kits.

**CTX:** Human serum CTX is quantitated by an ELISA kit (Serum CrossLaps) purchased from Nordic Biosciences (Herlev, Denmark) and distributed by Immunodiagnostic Systems, Inc. (Scottsdale AZ) This kit is a sandwich ELISA's based on 2 monoclonal antibodies to the same C-telopeptide region of type 1 collagen. In this case the aspartic acid (D) is isomerized EKAHD- $\beta$ -GGR. Detection requires the crosslinked peptide. 100 ul of serum is required for duplicate analysis. Quality control is monitored by provided samples included in the kit.