

# Rat-MID™ Osteocalcin ELISA

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## **For the quantification of osteocalcin in rat serum and plasma**

The Rat-MID™ Osteocalcin ELISA should be used for the in vitro determination of osteocalcin in rat serum and plasma only. Nordic Bioscience Diagnostics A/S is not responsible for any other use of the kit or consequence hereof than the one specified above. Neither for misuse e.g. uses deviating from the procedure described in this manual.

Furthermore, Nordic Bioscience Diagnostics A/S is not to be made responsible for any diagnoses or conclusions made by the user or third party based on the results obtained with the Rat-MID™ Osteocalcin ELISA nor for any consequences such interpretations may cause.

Nordic Bioscience Diagnostics A/S  
Herlev Hovedgade 207  
2730 Herlev  
Denmark

1104A

## INTRODUCTION

### Intended use and type of procedure

The Nordic Bioscience Diagnostics Rat-MID™ Osteocalcin ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of osteocalcin in rat serum and plasma. The assay is for research-use-only.

### Summary and explanation of the test

Osteocalcin or bone Gla protein (BGP), is a major non-collagenous protein of the bone matrix. It has a molecular weight of approximately 6000 Dalton and consists in most species of 49 amino acids; however, rat osteocalcin consist of 50 amino acids (1-4). Osteocalcin has a unique property for binding to calcium facilitated by the presence of 2-3 gamma-carboxyglutamic acids at position 17, 21 and 24 (5). The mid-molecular part of osteocalcin, especially the amino acids between 20 and 30, exhibits a high degree of inter-species conservatism (4).

The bone forming cells, osteoblasts, synthesizes osteocalcin. After production it is partly incorporated into the bone matrix and partly delivered to the circulatory system. Circulating osteocalcin is associated with changes in the rate of bone turnover and regarded as a specific marker for bone formation (4).

The clinical utility of rat osteocalcin as a marker for bone turnover has been evaluated in several pre-clinical settings. It has been reported that the serum level increases after ovariectomy and this estrogen-deficiency-induced state can be prevented by treatment with either estrogen, selective estrogen receptor modulators (SERMs) or bisphosphonates (6-10).

### Principle of the procedure

The Rat-MID™ Osteocalcin ELISA is based upon the competitive binding of a monoclonal antibody to soluble osteocalcin or to immobilized osteocalcin. Briefly, the monoclonal antibody is raised against human osteocalcin and recognizes the mid-molecular part (amino acids 21-29) of the molecule (11). Synthetic human osteocalcin is used for standardization. Parallelism is observed with purified rat osteocalcin and rat serum.

During the **Pre-incubation** step, biotinylated osteocalcin is immobilized by binding to the streptavidin-coated microtitre wells. The wells are emptied and washed, and standards, control, or unknown serum samples are pipetted into appropriate wells, followed by a solution of monoclonal antibody. Following the **Primary-incubation** step the wells are emptied and washed. In the **Secondary-incubation** step a solution of peroxidase conjugated anti-mouse immunoglobulins is added and binds to the monoclonal antibody. After the third washing step a chromogenic substrate (TMB) is added and the colour reaction is stopped with sulfuric acid. Finally, the absorbance at 450 nm is measured. The absorbance level is inversely related to concentration of osteocalcin in the sample.

## PRECAUTIONS

### Storage

Store the package upon receipt at 2-8°C. Under these conditions the reagents are stable up to the expiry date stated on each vial.

Following reconstitution the **Standards** and the **Control** should be stored below -18°C, and should only be frozen and thawed twice. When **Primary antibody** and the **Primary Incubation**

Buffer have been mixed, the remaining solution should be stored at 2-8°C for maximum 30 days or below -18°C. The remaining reagents and immuno strips should be stored at 2-8°C.

### **Warning**

The Rat-MID™ Osteocalcin ELISA is for **research-use-only**.

The following precautions should be observed in the laboratory:

- Do not eat, drink or smoke where immuno diagnostic materials are being handled.
- Do not pipette by mouth.
- Wear gloves when handling immuno diagnostic materials.
- Do not use reagents beyond their expiration date and do not mix reagents from different lots of kits.

All reagents and laboratory equipment should be handled and disposed of as if they were infectious.

## **MATERIALS**

### **Specimen collection**

When collecting blood take care to avoid haemolysis. Separate the serum from the cells within 3 hours after collection of blood. It is recommended to freeze (<-18°C) samples immediately after use.

### **Materials supplied**

Before opening the kit, please read the section on Precautions. The kit contains reagents sufficient for 96 determinations.

#### *Streptavidin coated microtitre plate (MTP)*

Microwell strips (12 pcs. of 1x8 wells) pre-coated with streptavidin. Supplied in a plastic frame.

#### *Osteocalcin Standard (Vial A)*

One vial (lyophilized) containing a PBS-buffered solution with protein stabilizer and preservative. Reconstitute with 5.0 mL of distilled water. The standard must be stored at or below -18°C after use.

#### *Osteocalcin Standards (Vial B-F)*

Five vials (lyophilized) containing synthetic human osteocalcin in a PBS-buffered solution with protein stabilizer and preservative. Reconstitute each of the vials with 0.5 mL of distilled water. The standard must be stored below -18°C after use, and must only be frozen and thawed twice. Please refer to the enclosed Technical Data Sheet for the exact concentrations.

#### *Control (Vial CO)*

One vial (lyophilized) containing synthetic human osteocalcin in a PBS-buffered solution with protein stabilizer and preservative. Reconstitute with 0.5 mL of distilled water. The control must be stored below -18°C after use, and must only be frozen and thawed twice. Please refer to the enclosed Technical Data Sheet for control range.

#### *Biotinylated Osteocalcin (Vial no. 1)*

One vial (12.0 mL) of a ready for use solution containing biotinylated synthetic human osteocalcin in a PBS-buffered solution with protein stabilizer and preservative.

#### *Primary Antibody (Vial no. 2)*

One vial (0.5 mL) of a concentrated solution of a monoclonal antibody specific the mid-molecular part (amino acids 21-29) of osteocalcin, in a buffered solution with protein stabilizer and preservative. Mix vials 2 and 3: 1+100 before use.

#### *Primary Incubation Buffer (Vial no. 3)*

One vial (20.0 mL) of a ready-for-use PBS-buffered solution with protein stabilizer, detergent and preservative for dilution of Primary Antibody. Mix vials 2 and 3: 1+100 before use.

#### *Secondary Antibody (Vial no. 4)*

One vial (12.0 mL) of a ready-for-use solution of a peroxidase conjugated antibody specific for mouse IgG in a buffered solution with protein stabilizer and preservative.

#### *Substrate Solution (Vial TMB)*

One vial (12.0 mL) of a ready-for-use tetramethylbenzidine (TMB) substrate in an acidic solution.

#### *Stopping Solution (Vial ST)*

One vial (12.0 mL) of ready-for-use 0.18 M sulfuric acid.

#### *Washing Solution. (Vial W)*

One vial (20.0 mL) of a concentrated washing solution containing detergent and preservative. Dilute 1+50 in distilled water before use.

#### *Sealing tape*

Adhesive film for covering wells during incubation.

### **Materials required - not supplied**

- Containers for preparing the dilutions of the Primary Antibody and the Washing Solution
- Precision micropipettes to deliver 20  $\mu$ L
- Distilled water
- Precision 8 or 12-channel multipipette to deliver 100  $\mu$ L and 150  $\mu$ L
- Microtitre plate mixing apparatus (300 rpm)
- ELISA plate reader with both 450 nm and 650 nm filters

## **ASSAY PROTOCOL**

Prior to use, equilibrate all solutions to room temperature. Perform the assay at room temperature (18-22°C). Determine the number of strips needed for the entire experiment. It is recommended to test all samples in duplicate. In addition, for each ELISA plate 14 wells are recommended for standards and 2 wells are recommended for the Control.

### **1. Pre-incubation**

Add 100  $\mu$ L of **Biotinylated Osteocalcin** (vial no. 1) to each well, cover with sealing tape, and incubate for 30 $\pm$ 5 minutes at room temperature (18-22°C) on a microtitre plate mixing apparatus (300 rpm).

## 2. Washing

Wash the immuno strips 5 times manually with **Washing Solution** (vial W). Make sure that the wells are completely emptied after each washing cycle. Using an automated plate washer, follow the instructions of the manufacturer or the guidelines of the laboratory. Usually 5 washing cycles are adequate.

## 3. Primary incubation

**A:** Prior to use mix the solutions in vial no. 2 (**Primary Antibody**) and vial no. 3 (**Primary Incubation Buffer**) in the volumetric ratio 1+100 in an empty container. Mix carefully and avoid formation of foam.

**B:** 20  $\mu$ L of either **Standards** (vial A-F), **Control** (vial CO) or unknown samples are pipetted into appropriate wells followed by 150  $\mu$ L of the mixture of **Primary Antibody** in **Primary Incubation Buffer**. Cover the immuno strips with sealing tape and incubate for  $60 \pm 5$  minutes at room temperature ( $18-22^{\circ}\text{C}$ ) on a microtitre plate mixing apparatus (300 rpm).

## 4. Washing

See step 2

## 5. Secondary incubation

Add 100  $\mu$ L of the **Secondary Antibody** (Vial No. 4) to each well, cover with sealing tape, and incubate for  $60 \pm 5$  minutes at room temperature ( $18-22^{\circ}\text{C}$ ) on a microtitre plate mixing apparatus (300 rpm).

## 6. Washing

See step 2

## 7. Incubation with chromogenic substrate solution

Pipette 100  $\mu$ L of the **Substrate Solution** (vial TMB) into each well and incubate for  $15 \pm 2$  minutes in the darkness on the microtitre plate mixing apparatus (300 rpm). Use sealing tape.

## 8. Stopping of colour reaction

Pipette 100  $\mu$ L of the **Stopping Solution** (vial ST) into each well.

## 9. Measurement of absorbance

The absorbance is measured within two hour at 450 nm. It is recommended to use the reading at 650 nm as reference.

## Limitations of the procedure

If the absorbance of a sample is lower than **Standard F**, it is recommended to dilute the sample to be diluted in rat serum with a low osteocalcin concentration.

## Quality control

Good Laboratory Practice requires the use of quality control specimens in each series of assays in order to check the performance of the assay. Controls should be treated as unknown samples, and the results analyzed with appropriate statistical methods.

## RESULTS

### Calculation of results

Calculate the mean of the duplicate absorbance determinations. Construct a standard curve on log-linear graph paper by plotting the mean absorbencies of the six standards A-F (ordinate) against the corresponding osteocalcin concentrations (abscissa). Determine the osteocalcin concentration of the controls and each patient sample by interpolation.

Alternatively, a four-parametric logistic curve fit can be used.

Example:

Standards/ Controls/ Samples	Osteocalcin conc. (ng/mL)	A <sub>450-650</sub> (Abs)	Mean A <sub>450-650</sub> (Abs)	Interpolated Osteocalcin conc. (ng/mL)
Standard A	0.0	1.997/1.991	1.994	
Standard B	45.9	1.725/1.756	1.741	
Standard C	169.9	1.063/1.030	1.147	
Standard D	401.0	0.528/0.502	0.515	
Standard E	801.0	0.329/0.320	0.325	
Standard F	1510.0	0.191/0.178	0.185	
Control CO		1.322/1.243	1.262	118.5
Sample I		0.263/0.267	0.265	940.2
Sample II		0.869/0.922	0.896	211.6
Sample III		1.676/1.654	1.665	56.9

**Please note:** The data above are for illustration only and should not be used to calculate the results of any run.

### Performance characteristics

*Cross reactivity towards rat osteocalcin:* **99±4% (mean±SD)**

Purified rat osteocalcin was diluted two-fold in Standard Buffer and measured in the Rat-MID™ Osteocalcin ELISA. The observed concentrations spanned from 48.1 to 1660 ng/mL. The recoveries were calculated as (Observed conc./Expected conc.)\*100%.

*Detection limit:*

**50.0 ng/mL**

This is the concentration corresponding to two standard deviations below the mean of 21 determinations of **Osteocalcin Standard A** (vial A).

*Imprecision*

The imprecision of the Rat-MID™ Osteocalcin ELISA was evaluated for three serum samples. The imprecision result is based on 10 consecutive runs according to NCCLS EP5-A (11).

The results are summarized in the table below.

	Interassay variation	Intraassay variation
Mean Osteocalcin (ng/mL)	CV (%)	CV (%)
948	7.7	3.4
215	5.5	5.0
152	6.6	3.6

### Linearity

A rat serum sample was diluted with another rat serum (low concentration) and determined in the Rat-MID™ Osteocalcin ELISA.

The result is summarized in the table below.

Dilution procedure		Expected	Observed	Recovery
High Serum 845 ng/mL (parts)	Low Serum 292 ng/mL (parts)	(ng/mL)	(ng/mL)	(%)
1	1	569	470	82.6
1	3	430	376	87.4
1	7	305	323	105.9
High Serum 593 ng/mL (parts)	Low Serum 292 ng/mL (parts)			
1	1	443	410	92.6
1	3	367	341	92.9
1	7	330	291	88.2

### Expected values

It is advisable for a laboratory to establish its own range of expected values. As an example, the mean value, standard deviation and standard error of the mean for populations of 3 months-old SPRD female rats are given below.

Rat strain	Age (Months)	Number of subjects	Mean values (ng/mL)	Standard Deviation (SD) (ng/mL)	Standard Error of the Mean (SEM) (ng/mL)
SPRD	3	30	417.8	83.5	15.3

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