N-MID® Osteocalcin ELISA

English

For the Quantitative Determination of Osteocalcin in human serum and plasma

The Nordic Bioscience Diagnostics A/S N-MID® Osteocalcin ELISA kit should be used only for *in vitro* diagnostic determination of osteocalcin in human serum or plasma. Nordic Bioscience Diagnostics A/S is not responsible for any misuse of the kit in a way deviating from what is described in this manual. Likwise, Nordic Bioscience Diagnostics is not responsible for any conclusions or diagnosis made by the doctor and/or user of the kit or for any consequences such interpretations may cause.

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INTRODUCTION

Intended use
The N-MID® Osteocalcin ELISA is an enzyme immunological test for the quantitative measurement of osteocalcin, an indicator of osteoblastic activity in human serum and plasma and is **intended to be used as an aid in the prevention of osteoporosis.**

Limitations
Osteocalcin values may vary depending upon the person's age (years post menopause), "circadian rhythm", rate of glomerular filtration and duration of treatment
Results should be used in conjunction with information available from the clinical evaluation of the patient and other diagnostic procedures. Therefore, osteocalcin values are not recommended for use as a screening procedure to detect the presence of osteoporosis in the general population. Also, medication dosage should not be changed or stopped based solely on the osteocalcin values.
When evaluating subsequent samples, collect at the same time of day as baseline and use the same specimen type, serum or anticoagulated plasma.

Summary and explanation of the test
Osteocalcin, or bone Gla protein (BGP), is the major non-collagenous protein of bone matrix. It has a molecular weight of approximately 5800 Dalton and consists of 49 amino acids, including three residues of gamma-carboxyglutamic acid.
Osteocalcin is synthesized in bone by osteoblasts. After production, it is partly incorporated into the bone matrix and partly delivered to the circulatory system. The precise physiological function of osteocalcin is still unclear. A large number of studies have shown that the circulating level of osteocalcin reflects the rate of bone formation (1-14).
Determination of serum osteocalcin has proved to be valuable as an aid in identifying women at risk of developing osteoporosis, for monitoring bone metabolism during the perimenopause and postmenopause and during antiresorptive therapy.

Principle of the procedure
The N-MID® Osteocalcin ELISA is based upon the application of two highly specific monoclonal antibodies (Mabs) against human osteocalcin. An antibody recognizing the midregion (amino acids 20-29) is used as the capture antibody and for detection a peroxidase conjugated antibody recognizing the N-terminal region (amino acids 10-16) is used. In addition to intact osteocalcin (amino acid 1-49) the N-terminal-Mid fragment (amino acids 1-43) is also detected.
Standards, control and unknown samples are pipetted into the appropriate microtitre wells coated with streptavidin. Then a mixture of a biotinylated antibody and a peroxidase conjugated antibody is added. Following incubation for 2 hours at room temperature the wells are washed and a chromogenic substrate is added and the colour reaction is stopped with sulfuric acid. Finally, the absorbance is measured.

PRECAUTIONS

The following precautions should be observed in the laboratory:
- Do not eat, drink, smoke or apply cosmetics where immunodiagnostic materials are being handled
- Do not pipette by mouth
- Wear gloves when handling immunodiagnostic materials and wash hands thoroughly afterwards

- Cover working area with disposable absorbent paper

Storage
Store the N-MID® Osteocalcin ELISA kit upon receipt at 2-8°C. Under these conditions the kit is stable up to the expiry date stated on the box.
Following reconstitution the **Standards** and the **Control** should be stored below -18°C for up to 3 months, and should only be frozen and thawed twice. When the components of the **Antibody Solution** are mixed, the remaining solution should be stored at 2-8°C for no longer than 1 month or frozen below -18°C. The remaining reagents and immunostrips should be stored at 2-8°C.

Warnings
For *in vitro* use only.
- All reagents and laboratory equipment should be handled and disposed of as if they were infectious.
- Do not use kit components beyond the expiry date and do not mix reagents from different lots.

MATERIALS

Specimen collection
Collect blood by venipuncture taking 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.
When analysing plasma, both heparin and EDTA plasma may be used.

Materials supplied
Before opening the kit, read the section on **Precautions.** The kit contains reagents sufficient for 96 determinations. For reconstitution of lyophilized material add appropriate volume of distilled water and leave for 10 minutes before mixing. Make sure to avoid foam.

Streptavidin coated microtitre plate (MTP)
Microwell strips (12 x 8 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 below -18°C after use.

Osteocalcin Standards (Vials B-F)
Five vials (lyophilized) containing synthetic human osteocalcin in a PBS-buffered solution with protein stabilizer and preservative. Reconstitute with 0.5 mL of distilled water. The exact concentration is stated on each vial. The standards must be stored below -18°C after use, and should only be frozen and thawed twice.

Control (Vial CO 1-2)
Two vials (lyophilized) containing synthetic human osteocalcin in a PBS-buffered solution with protein stabilizer and preservative. Reconstitute with 0.5 mL of distilled water. Control must be stored below -18°C, and should only be frozen and thawed twice. Please refer to enclosed technical datasheet for control range.

Peroxidase Conjugated Antibody (Vial no. 1)
One vial (0.25 mL) of a concentrated solution of a peroxidase conjugated murine monoclonal antibody specific against the N-terminal region of osteocalcin in a TRIS-buffered solution with protein stabilizer, detergent and preservative. Prior to use, add 10 mL **Conjugate Diluent Solution** (vial no.3).

Biotinylated Antibody (Vial no. 2)
One vial (0.25 mL) of a concentrated conjugate solution of biotinylated murine monoclonal antibody against the mid-region of osteocalcin in a TRIS-buffered solution with protein stabilizer, detergent and preservative. Prior to use, add 10 mL **Conjugate Diluent Solution** (vial no.3).

Conjugate Diluent Solution (Vial no. 3)
One vial (min. 22 mL) of a PBS-buffered solution with protein stabilizer, detergent and preservative.

Substrate Solution (Vial TMB)
One vial (min. 12 mL) of a ready-for-use tetramethylbenzidine (TMB) substrate in an acidic buffer.
Please note that the chromogenic substrate might appear slightly bluish.

Stopping Solution (Vial ST)
One vial (min. 15 mL) of ready-for-use 0.18 mol/L sulfuric acid.

Washing Solution (Vial W)
One vial (min. 20 mL) of a concentrated washing buffer with detergent and preservative. Dilute 1+ 50 times in distilled water before use.

Sealing tape
Adhesive film for covering wells during incubation.

Materials required - not supplied
- Containers for preparing the Antibody Solution and the Washing Solution.
- Precision micropipettes to deliver 20 µL
- Distilled water
- Precision 8- or 12-channel multipipette to deliver 100 µL and 150 µL.
- Microtiter plate reader with both 450 nm and 650 nm filters

ASSAY PROCEDURE

For optimal perfomance of the assay it is important to comply with the instructions given below

Assay procedure
Prior to use, prepare and equilibrate all solutions to room temperature. **Perform the assay at 18-22°C.**

Determine the number of strips needed for the assay. It is recommended to test all samples in duplicate. In addition, for each run a total of 14 wells are needed for the standards and control. Place the appropriate number of strips in the plastic frame. Store unused immuno strips in the tightly closed foil bag with desiccant capsules.

1 Preparation of the Antibody Solution:
The **Antibody Solution** is prepared by 1) adding 10 mL of **Conjugate Diluent Solution** (vial no. 3) to both the **Peroxidase Conjugated Antibody Solution** (vial no. 1) and the **Biotinylated Antibody Solution** (vial no. 2), and 2) mixing the two conjugate solutions in equal volumes.

2 Incubation in Immuno Strips
Pipette 20 µL of either **Standards** (*vial A-F*), **Control** (*vial CO 1-2*), or unknown samples into appropriate wells followed by 150 µL of the **Antibody Solution.** Cover the immunostrips with sealing tape and incubate for 120±5 minutes at 18-22°C (without any mixing)

3 Washing
Wash the immunostrips 5 times manually with **Washing Buffer** (*vial W*) diluted 1+50 in distilled water. Using an automated plate washer, follow the instructions of the manufacturer or the guidelines of the laboratory. Usually 5 washing cycles are adequate. Make sure that the wells are **completely emptied** after each manual or automatic washing cycle.

4 Incubation with chromogenic substrate solution
Pipette 100 µL of the **Substrate Solution** *(vial TMB*) into each well and incubate for 15±2 minutes at 18-22°C in the dark (without any mixing). Use sealing tape. Do not pipette directly from the vial containing TMB substrate but transfer the needed volume to a clean reservoir. Remaining substrate in the reservoir should be discarded and not returned to vial TMB.

5 Stopping of colour reaction
Pipette 100 µL of the **Stopping Solution** *(vial ST*) into each well.

6 Measurement of absorbance
Measure the absorbance at 450 nm with 650 nm as reference within two hours.

Limitations of the procedure
If the absorbance of a sample exceeds that of **Standard F,** the sample should be diluted in **Standard A** and re-analysed.

QUALITY CONTROL

Good Laboratory Practice (GLP) 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 analysed with appropriate statistical methods.

RESULTS

Calculation of results
A four-parametric logistic curve fit can be used.

Alternatively, calculate the mean of the duplicate absorbance determinations. Construct a standard curve on graph paper by plotting the mean absorbances 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.

Example of results obtained:

Standards/ Controls/ Samples	Osteocalcin (ng/mL)	$A_{450-650}$ (nm) Obs 1/ Obs 2	Mean $A_{450-650}$ (nm)	Interpolated Osteocalcin (ng/mL)
Standard A	0.0	0.086 / 0.076	0.081	
Standard B	6.3	0.176 / 0.171	0.174	
Standard C	12.5	0.251 / 0.267	0.259	
Standard D	25.0	0.532 / 0.517	0.525	
Standard E	50.0	1.085 / 1.088	1.087	
Standard F	100.0	2.480 / 2.422	2.451	
Control CO 1		0.550 / 0.500	0.525	25.0

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

Performance characteristics

Detection limit: 0.5 ng/mL Osteocalcin

This is the concentration corresponding to three standard deviations above the mean of 21 determinations of the blank ("Osteocalcin Standard A").

Precision

The precision of the N-MID® Osteocalcin ELISA was evaluated for three serum samples. Inter-assay variation was evaluated by analyzing 3 samples in 13 consecutive runs. Intra-assay was evaluated by analyzing 3 samples in 3 consecutive runs using 21 determinations in each run. The results are summarised in the table below:

InterAssay Variation (n=13)

Sample	Mean (ng/mL)	Inter-assay CV (%)
A	6,8	3.6
B	25,0	4.1
C	50,5	6.4

IntraAssay Variation (n=21)

Sample	Mean (ng/mL)	Intra-assay CV (%)
D	7,0	3.4
E	21,8	2.0
F	43,2	2.4

Dilution/Linearity

It was investigated if the N-MID® Osteocalcin ELISA assay was sensitiv to any effect of the serum matrix. Four serum samples were diluted in Standard A, and the concentrations were determined in the N-MID® Osteocalcin ELISA.

Sample	Dilution	Observed	Expected	Recovery

		[ng/mL)	(ng/mL)	[% of expected value]
1	1 / 1	12.0	12.0	100
	1 / 2	5.1	6.0	84
	1 / 4	2.7	3.0	88
	1 / 8			
2	1 / 1	36.1	36.1	100
	1 / 2	18.1	18.1	106
	1 / 4	9.0	9.0	105
	1 / 8	4.7	4.5	104
3	1 / 1	38.2	38.2	100
	1 / 2	20.5	19.1	107
	1 / 4	9.9	9.6	104
	1 / 8	4.3	4.8	90
4	1 / 1	48.1	48.1	100
	1 / 2	23.8	24.1	99
	1 / 4	12.2	12.0	101
	1 / 8	6.3	6.0	105

Recovery

The accuracy of the N-MID® Osteocalcin ELISA was determined by spiking human serum with different amounts of synthetic osteocalcin. Three serum samples were mixed in equal volumes with 5 standard solutions and assayed in the N-MID® Osteocalcin ELISA.

Sample	**Standards synthetic osteocalcin (ng/mL)**	**Observed [ng/mL)**	**Expected (ng/mL)**	**Recovery [% of expected value]**
1	100	50.7	55.5	91
	50	26.9	30.5	88
	25	16.4	18.0	91
	12.5	10.7	11.7	92
	6.3	8.4	8.6	98
2	100	54.1	58.1	93
	50	30.8	35.6	87
	25	19.9	20.6	97
	12.5	14.6	14.3	102
	6.3	11.0	11.2	98
3	100	61.7	65.5	94
	50	37.5	40.5	93
	25	26.6	28.0	95
	12.5	21.2	21.7	98
	6.3	18.6	18.6	100

Measuring Range

The measuring range for N-MID® Osteoclacin ELISA is between 0.5 ng/mL and 100 ng/mL osteocalcin.

Interference:
The interference of Lipid on the measurement of osteocalcin in serum samples by N-MID® Osteocalcin ELISA was investigated.
In the concentration listed below no interference was detected:

Lipid (IntraLipid): 15 g/L
Bilirubin: 200 mg/L
Hemoglobin: 5 g/L

Expected values
It is advisable for a laboratory to establish its own range of normal and pathological values. As an example, the mean values and standard deviations for various populations are given below. For further reading, please refer to the reference list.

Populations	Number of subjects	Mean Values (ng/mL)	SD (ng/mL)
Pre-menopausal women	77	17.9	6.5
Post-menopausal women [1)]	125	28.4	9.5
Males	81	21.4	9.1

1) The average years after menopause is 10.3 years.

The menopause induces a dramatic increase in bone turnover which peaks 1-3 years after cessation of ovarian function and slows down there after for the next 8-10 years. *(Delmas P.D. Clin obstes & Gynaecol 5;4: 817-830, 1991.)*

Day to Day Intra-individual Variation
The Day to Day Intra-individual Variation was assessed by analyzing serum samples (morning fasting) from 11 healthy post menopausal women at five time points over 2 weeks.

Subject	Mean (ng/mL)	SD (ng/mL)	CV (%)
1	22.0	3.5	16
2	13.4	1.0	7
3	19.6	1.3	7
4	18.0	3.1	17
5	12.9	1.1	9
6	9.9	0.9	9
7	14.4	2.2	15
8	7.5	0.4	5
9	15.3	2.2	15
10	14.5	1.6	11
11	14.8	0.6	4

CLINICAL DATA

The N-MID® Osteocalcin ELISA has been used to monitor treatment in several clinical studies and the osteocalcin values have been compared to Bone Mineral Density (BMD_{spine}) measurements.

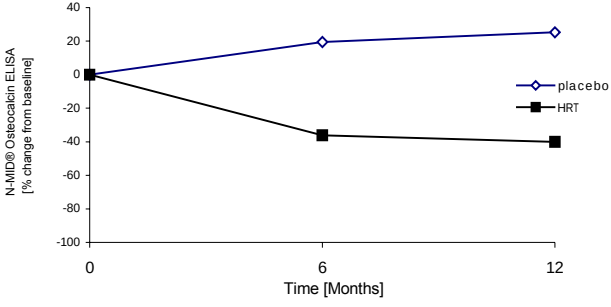
All the clinical studies presented below were performed according to the European Standard for good clinical practice (GCP and GLP).
The clinical studies presented here were conducted on white Danish women. However, several studies have been published showing that other demographic groups display similar osteocalcin decrease in response to anti-resorptive therapies (7, 8, 11, 12, 14).
The Bone Mineral Density (BMD) was measured at the Lumbar spine (L 1 - L4).
The change in the bone mineral density is presented below α-BMD.
α -BMD is defined as the slope of the linear regression line for BMD_{spine} versus time (years) for the period of treatment. In most cases the calculation of α -BMD involves a minimum of 5 BMD_{spine} measurements. The α -BMD thus represents the % change in BMD_{spine} per year.
To this date there is no universal agreement as to what constitutes a BMD response above the average bone loss (most BMD measurements have an impression error of about 1%).

Determination of cut-off for a-BMD:
Since the bisphosphonate participants (average 1.7 years since menopause) are less menopausal than the HRT participants (average 3.2 years since menopause) one may expect that they would have a larger annual decrease in bone mass than the HRT participants.
We believe that in order to determine the most "correct" cut-off one should consider both the required significant annual change of 2.1% (based on 5 measurements over 2 years) and the average annual change of the placebo group.
In other words; for the alendronate study to be sure to determine an increase in BMD compared to what would be expected for an untreated women the α -BMD must be above (2.1-2.8) = -0.7%
For the HRT study the cut-off point is calculated to (2.1-1.2) = 0.9%
For the combined studies the cut-off point is calculated to (2.1-1.6) = 0.5%:
For comparison reasons we have used a cut-off for a-BMD of 0.5% for both studies.

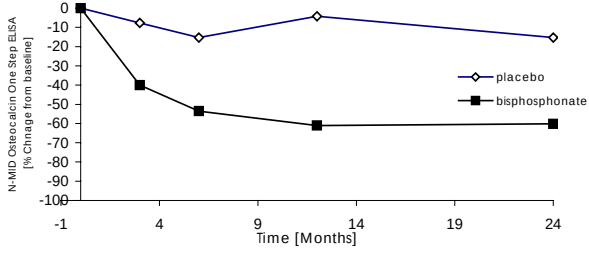
HRT study:
- Women more than 45 year and 1-6 years since menopause
- 35 participants on placebo
- 26 participants on active treatment

Bisphosphonate study:
- Women between age 40 and 59 years, and 6 months to 3 years since menopause.
- 12 participants on placebo
- 31 participants on active treatment

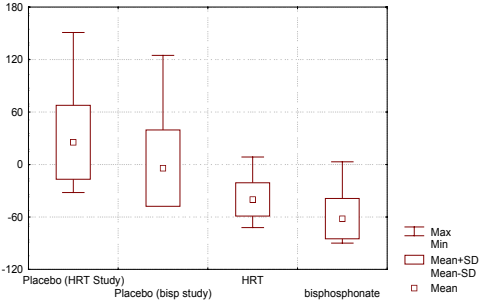
N-MID® Osteocalcin ELISA
(% Change from Baseline)
Response to HRT



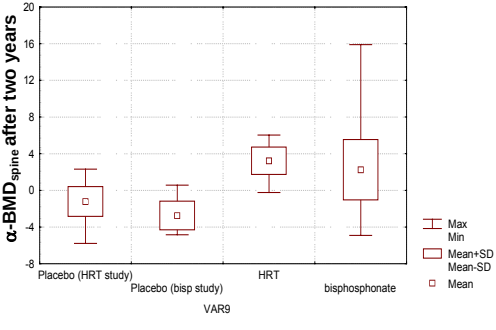
N-MID® Osteocalcin ELISA
(% Change from Baseline)
Response to Bisphosponate therapy



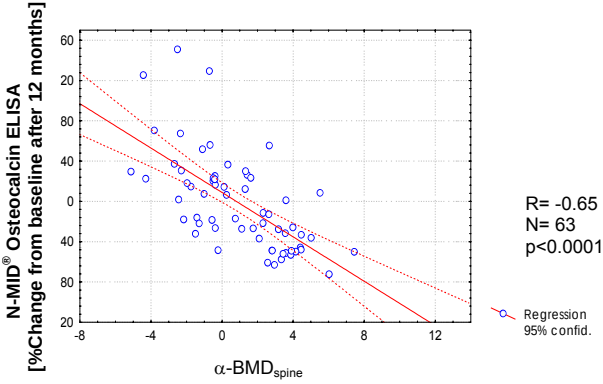
Response of N-MID® Osteocalcin ELISA to HRT or Bisphosphonate Treatment



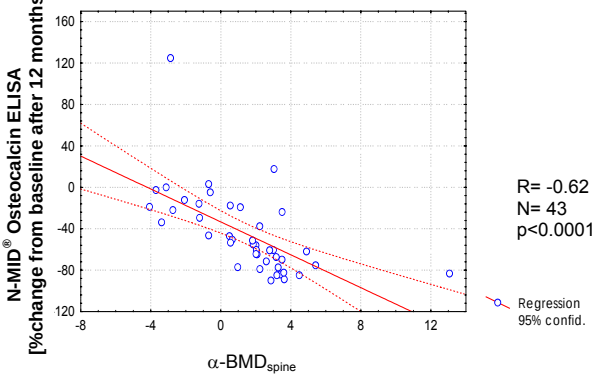
Response of BMD_{spine} to HRT or Bisphosphonate Treatment



Correlation between α-BMD$_{spine}$ and N-MID®
Osteocalcin ELISA - HRT study



Correlation between α-BMD$_{spine}$ and
N-MID® Osteocalcin ELISA - Bisphosphonate study



Below is summarized the specificity and sensitivity using different cut-off values of N-MID® Osteocalcin ELISA, change from baseline after 12 months HRT or Bisphosphonate treatment for α-BMD cut-off of 0.5%.
95% confidence intervals are indicated.

HRT study

Cut-off of change in osteocalcin	Specificity	Sensitivity
-10 [% change from baseline]	*83 (64 – 94)*	*74 (55 – 87)*
-20 [% change from baseline]	*76 (56 – 90)*	*85 (69 – 95)*
-30 [% change from baseline]	*62 (42 – 79)*	*94 (80 – 99)*
-40 [% change from baseline]	*48 (29 – 67)*	*97 (85 – 100)*

Bisphosphonate study

Cut-off of change in osteocalcin	Specificity	Sensitivity
-10 [% change from baseline]	*100 (89 – 100)*	*35 (14 – 62)*
-20 [% change from baseline]	*96 (80 – 100)*	*59 (33 – 82)*
-30 [% change from baseline]	*96 (80 – 100)*	*76 (50 – 93)*
-40 [% change from baseline]	*92 (75 –99)*	*82 (57 – 96)*

REFERENCES

The information contained herein is provided solely for medical education purposes and may discuss research regarding use of test developed and marketed by Nordic Bioscience Diagnostics that exceeds the labelled intended use statement in the product package inserts. Please see the current Nordic Bioscience package inserts for the Intended Use and Limitations of the Procedure.

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