immunodiagnosticsystems

Rat-MID™ Osteocalcin EIA

Enzymeimmunoassay for the quantitative determination of osteocalcin in rat serum and plasma

For Research Use Only. Not for use in diagnostic procedures.

REF AC-12F1 Σ 96

INTRODUCTION

Intended use
The Rat-MID™ Osteocalcin EIA 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 EIA 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 absorbence at 450 nm is measured. The absorbence 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.

Warnings
The Rat-MID™ Osteocalcin EIA is for research-use-only and is not for internal use in humans or animals. This product must be used strictly in accordance with the instructions set out in the Package Insert. IDS Limited will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of non-compliance with the instructions provided.
CAUTION: this kit contains material of animal origin. Handle kit reagents as if capable of transmitting an infectious agent.
Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. Disposal of kit reagents should be in accordance with local regulations..
Do not use reagents beyond their expiration date and do not mix reagents from different lots of kits.

MATERIAL

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

Osteocalcin Standard **CAL 0**
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 **CAL 1 - 5**
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 vial labels for the exact concentrations.

Control **CTRL**
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 QC Report for control range.

Biotinylated Osteocalcin **Ag BIOTIN**
One vial (min. 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 **Ab**
One vial (min. 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 Primary Antibody and Primary Incubation Buffer: 1+100 before use.

Primary Incubation Buffer **BUF**
One vial (min. 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 **ENZYMCONJ**
One vial (min. 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 **SUBS TMB**
One vial (min. 12.0 mL) of a ready-for-use tetramethylbenzidine (TMB) substrate in an acidic solution.

Stopping Solution **H2SO4**
One vial (min. 12.0 mL) of ready-for-use 0.18 M sulfuric acid.

Washing Solution **WASHBUF 50x**
One vial (min. 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 µL
- Distilled water
- Precision 8 or 12-channel multipipette to deliver 100 µL and 150 µL
- Microtitre plate mixing apparatus (300 rpm)
- ELISA plate reader with both 450 nm and 650 nm filters

ASSAY PROCEDURE

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 µL of Biotinylated Osteocalcin **Ag BIOTIN** to each well, cover with sealing tape, and incubate for 30±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 300 µL Washing Solution. 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 Primary Antibody **Ab** and Primary Incubation Buffer **BUF** in the volumetric ratio 1+100 in an empty container. Mix carefully and avoid formation of foam.
   B: 20 µL of either Standards **CAL** **0** - **5**, Control **CTRL** or unknown samples are pipetted into appropriate wells followed by 150 µL of the mixture of Primary Antibody in Primary Incubation Buffer. Cover the immuno strips with sealing tape and incubate for 60±5 minutes at room temperature (18-22°C) on a microtitre plate mixing apparatus (300 rpm).

4. **Washing**
   See step 2

5. **Secondary incubation**
   Add 100 µL of the Secondary Antibody **ENZYMCONJ** to each well, cover with sealing tape, and incubate for 60±5 minutes at room temperature (18-22°C) on a microtitre plate mixing apparatus (300 rpm).

6. **Washing**
   See step 2

7. **Incubation with chromogenic substrate solution**
   Pipette 100 µL of the Substrate Solution **SUBS** **TMB** into each well and incubate for 15±2 minutes at room temperature (18-22°C) in the dark on the microtitre plate mixing apparatus (300 rpm). Use sealing tape.

8. **Stopping of colour reaction**
   Pipette 100 µL of the Stopping Solution **H2SO4** 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 5, 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 0-5 (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/ Specimen	Osteocalcin conc. (ng/mL)	$A_{450-650}$ (Abs)	Mean $A_{450-650}$ (Abs)	Interpolated Osteocalcin conc. (ng/mL)
Standard 0	0.0	1.997/1.991	1.994	
Standard 1	45.9	1.725/1.756	1.741	
Standard 2	169.9	1.063/1.030	1.147	
Standard 3	401.0	0.528/0.502	0.515	
Standard 4	801.0	0.329/0.320	0.325	
Standard 5	1510.0	0.191/0.178	0.185	
Control		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 EIA. 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 0.**

Imprecision
The imprecision of the Rat-MID™ Osteocalcin EIA 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 (%)
948 215 152	7.7 5.5 6.6	3.4 5.0 3.6

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

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)	(ng/mL)
1 1 1	1 3 7	569 430 305	470 376 323	82.6 87.4 105.9
High Serum 593 ng/mL (parts)	Low Serum 292 ng/mL (parts)			
1 1 1	1 3 7	443 367 330	410 341 291	92.6 92.9 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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EXP	GB	*Use By*	LOT	GB	*Batch code*
	DE	*Verwendbar bis*		DE	*Chargenbezeichnung*
	ES	*Fecha de caducidad*		ES	*Código de lote*
	IT	*Utilizzare entro*		IT	*Codice del lotto*
	FR	*Utiliser jusque*		FR	*Code du lot*
	NL	*Houdbaar tot*		NL	*Lot nummer*
	DK	*Holdbar til*		DK	*Lotnummer*
	CZ	*Použitelné do*		CZ	*Číslo šarže*
	SK	*Použiteľné do*		SK	*Číslo šarže*
	GR	*Ημερομηνία λήξης*		GR	*Αριθμός Παρτίδας*
	PT	*Prazo de validade*		PT	*Código do lote*
	HU	*Felhasználható*		HU	*Sarzsszám*
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	IT	*Numero di catalogo*		IT	*Fabbricante*
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	PT	*Referência de catálogo*		PT	*Fabricante*
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	PL	*Numer katalogowy*		PL	*Producent*
Σ	GB	*Contains sufficient for <n> tests*	IVD	GB	*In Vitro Diagnostic Medical Device*
	DE	*Inhalt ausreichend für <n> Prüfungen*		DE	*In-Vitro-Diagnostikum*
	ES	*Contenido suficiente para <n> ensayos*		ES	*Producto sanitario para diagnóstico in vitro*
	IT	*Contenuto sufficiente per "n" saggi*		IT	*Dispositivo medico-diagnostico in vitro*
	FR	*Contenu suffisant pour "n"tests*		FR	*Dispositif médical de diagnostic in vitro*
	NL	*Inhoud voldoende voor "n" testen*		NL	*Medisch hulpmiddel voor in-vitro diagnostiek*
	DK	*Indeholder tilstrækkeligt til "n" test*		DK	*Medicinsk udstyr til in vitro-diagnostik*
	CZ	*Lze použít pro <n> testů*		CZ	*In Vitro diagnostický zdravotnický prostředek*
	SK	*Obsah postačuje na <n> stanovení*		SK	*Zdravotnícka pomocka in vitro*
	GR	*Περιεχόμενο επαρκές για «ν» εξετάσεις*		GR	*In Vitro Διαγνωστικό Ιατροτεχνολογικό προϊόν*
	PT	*Conteúdo suficiente para "n" ensaios*		PT	*Dispositivo médico para diagnóstico in vitro*
	HU	*A doboz tartalma <n> vizsgálat elvégzéséhez elegendő*		HU	*In vitro diagnosztikum*
	SE	*Räcker till "n" antal tester*		SE	*Medicintekniska produkter för in vitro diagnostik*
	PL	*Wystarczy na wykonanie <n> testów*		PL	*Wyrób do diagnistyki In Vitro*
	GB	*Temperature limitation*		GB	*Consult Instructions for Use*
	DE	*Temperaturbegrenzung*		DE	*Gebrauchsanweisung beachten*
	ES	*Límite de temperatura*		ES	*Consulte las instrucciones de uso*
	IT	*Limiti di temperatura*		IT	*Consultare le istruzioni per l'uso*
	FR	*Limites de température*		FR	*Consulter les instructions d'utilisation*
	NL	*Temperatuurlimiet*		NL	*Raadpleeg de gebruiksaanwijzing*
	DK	*Temperaturbegrænsning*		DK	*Se brugsanvisning*
	CZ	*Teplotní rozmezí od do*		CZ	*Viz návod k použití*
	SK	*Teplotné rozmedzie od do*		SK	*Vid' návod na pužitie*
	GR	*Περιορισμοί θερμοκρασίας*		GR	*Συμβουλευτείτε τις οδηγίες χρήσης*
	PT	*Limites de temperatura*		PT	*Consulte as instruções de utilização*
	HU	*Hőmérséklettartomány*		HU	*Nézze meg a Használati utasítást*
	SE	*Temperaturbegränsning*		SE	*Se handhavandebeskrivningen*
	PL	*Przestrzegać zakresu temperatury*		PL	*Sprawdź w instrukcji obsługi*

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