RatLaps™ ELISA

English

For quantification of fragments of type I collagen in rat/mouse serum or urine and released from rat/mouse bone into cell culture supernatants during bone resorption in vitro

The RatLaps™ ELISA should be used for the in vitro determinations of C-terminal telopeptides in rat/mouse serum, urine or cell culture supernatants. Nordic Bioscience Diagnostics is not responsible for any other use of the kit or consequence hereof than described in this manual. Neither for misuse e.g. uses deviating from the procedure described in this manual.

Furthermore, Nordic Bioscience Diagnostics is not to be made responsible neither for any diagnosis or conclusions made by the user or third party based on the results obtained with the RatLaps™ ELISA nor for any consequences such interpretations may cause.

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INTRODUCTION

Intended use and type of procedure
The Nordic Bioscience Diagnostics A/S RatLaps™ ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of bone-related degradation products from C-terminal telopeptides of type I collagen in rat/mouse serum or urine and from rat/mouse bone released into cell culture supernatants by osteoclasts. The assay is for research-use-only.

Summary and explanation of the test
Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesized primary in bone (1). During renewal of the skeleton bone matrix is degraded and consequently fragments of type I collagen is released into circulation. The resorption process can be studied in vitro by culturing bone cells on slices of bone or dentin.

The RatLaps™ ELISA are based on the observation that certain C-telopeptide degradation products from type I collagen released during osteoclastic bone resorption. With RatLaps™ ELISA it is possible to measure this degradation products in rat/mouse serum and urine and bone cell culture supernatants (2-8).

Principle of the procedure
The RatLaps™ ELISA is based upon the competitive binding of a polyclonal antibody to soluble RatLaps antigens EKSQDGGR or to immobilized RatLaps antigens. Briefly, the polyclonal antibody is raised against a synthetic peptide having a sequence (EKSQDGGR) specific for a part of the C-terminal telopeptide α1 chain of rat type I collagen. For standardization of the RatLaps™ ELISA a synthetic peptide (EKSQDGGR), which is specific for the C-terminal telopeptide α1 chain of type I collagen in rats has been used.

During the pre-incubation step, biotinylated EKSQDGGR is immobilized by binding to the streptavidin-coated microtitre wells. The wells are emptied and washed. Standards, control, or unknown samples (culture supernatant or rat/mouse serum or urine) are pipette into appropriate wells, followed by a solution of a primary antibody (polyclonal rabbit). Following the primary-incubation step the wells are emptied and washed. In the secondary-incubation step a solution of a Goat anti-Rabbit antibody conjugated with peroxidase (secondary antibody) is added and binds to the polyclonal rabbit antibody. After the third washing step a chromogenic substrate (TMB) is added and the color reaction is stopped with sulfuric acid. Finally, the absorbence at 450 nm is measured with 650 nm as reference if possible. The absorbence level is inversely related to concentration of RatLaps antigens in the sample.

PRECAUTIONS

Storage
Store the RatLaps™ ELISA upon receipt at 2-8°C.
Under these conditions the reagents are stable until the expiry date stated on each vial.

Warning
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
- The RatLaps™ ELISA is for research-use-only

MATERIALS

Specimen collection
Please note that we strongly recommend using serum samples for investigation of in vivo models of bone disease, since this will give the best results due to reduced variability. However, the procedure can also be utilized with urinary samples.

SERUM: Rat/mouse serum samples should be collected as fasting samples, either in the morning after an over night fasting or in the afternoon after a period of minimum 6 hours of fasting. It is recommended to store rat serum samples at or below -20˚C.

URINE: Urinary samples can be collected as spot samples. In order to avoid influence from diurnal variation, urinary samples can also be collected as 24-hour urine samples by using metabolic cages or similar devices. It is recommended to store collected rat urine at 2-8˚C for no more than one week, but in general at or below -20˚C for prolonged storage. Results obtained by using urinary samples should be corrected for creatinine prior to evaluation.

CULTURE: Culture supernatants harvested from bone cells cultured on surfaces of bone or dentin from rat or mouse. Preferably culture supernatants should be tested on the same day they are harvested. The culture supernatants can be stored at or below -20˚C.

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.

RatLaps Standard (Vial A)
One vial (5.0 mL) of a ready-for-use TRIS-buffered solution with protein stabilizer and preservative.

RatLaps Standards (Vial B-F)
Five vials (0.5 mL/vial) ready-for-use, containing a synthetic peptide, EKSQDGGR, in a TRIS-buffered solution with protein stabilizer and preservative. Please refer to the enclosed Technical Data Sheet for the exact concentrations.

Control (Vial CO)
One vial (0.5 mL) ready-for-use, containing a synthetic peptide, EKSQDGGR, in a TRIS-buffered solution with protein stabilizer and preservative. Please refer to the enclosed Technical Data Sheet for the exact concentrations.

Biotinylated RatLaps Antigen (Vial no. 1)
One vial (12.0 mL) of a ready-for-use solution containing a biotinylated peptide, EKSQDGGR, in a PBS-buffered solution with protein stabilizer and preservative.

Primary Antibody (Vial no. 2)
One vial (12.0 mL) of ready-for-use solution containing polyclonal antibody specific for a part of the C-telopeptide α1 chain of rat type I collagen, in a buffered solution with protein stabilizer and preservative.

Peroxidase Conjugated Goat anti-Rabbit IgG (Vial no.3)
One vial (12.0 mL) of ready-for-us solution of peroxidase conjugated Goat anti-Rabbit IgG antibody in a buffered solution with protein stabilizer and preservative.

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

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

Washing Solution. (Vial W)
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
- Precision micropipettes to deliver 20 µL
- Distilled water
- Precision 8 or 12-channel multipipette to deliver 100 µL
- ELISA plate reader with both 450 nm and 650 nm filters
- 2 – 8˚C incubator

ASSAY PROTOCOL

Prior to use, equilibrate all solutions to room temperature. 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 12 wells are recommended for standards and 2 wells are recommended for the Control.

Place the appropriate number of strips in the plastic frame. Store unused immunostrips in the tightly closed foil bag with desiccant capsules.

1. **Pre-incubation**
   Add 100 µL of **Biotinylated RatLaps Antigen** (vial no. 1) to each well, cover with sealing tape, and incubate for 30 ± 5 minutes at room temperature (18-22°C).

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**
   Add 20 µL of **Standards** (vial A-F), **Control** (vial CO) or unknown samples into the appropriate wells followed by 100 µL of **Primary Antibody** (vial no. 2). Cover the immuno strips with sealing tape and incubate over night (18 ± 3 hours) at 2-8°C.

4. **Washing**
   See step 2

5. **Secondary incubation**
   Add 100 µL of the **Peroxidase conjugated Goat anti-Rabbit IgG Antibody** (vial no. 3) to each well, cover with sealing tape, and incubate for 60 ± 5 minutes at room temperature (18-22°C).

6. **Washing**
   See step 2

7. **Incubation with chromogenic substrate solution**
   Pipette 100 µL of the **Substrate Solution** (vial TMB) into each well and incubate for 15 ± 2 minutes at room temperatur in darkness. Use sealing tape

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

9. **Measurement of absorbance**
   The absorbance is measured within two hours 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 that the sample is diluted 1+1 with Standard A (Vial A). It is not recommended to dilute a high sample more than 1+3.

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 using 4-parametric logistic curve fit with mean absorbances of the six standards A-F (ordinate) against the corresponding RatLaps concentrations (abscissa). The RatLaps analyte concentration of the controls and each patient sample is determined by interpolation.

Example:

Standards/ Controls/ Samples	RatLaps Conc. (ng/mL)	$A_{450-650}$ (nm)	Mean $A_{450-650}$ (nm)	Interpolated RatLaps Conc. (ng/mL)
Standard A	0	2.144/2.100	2.122	
Standard B	7.7	1.566/1.622	1.594	
Standard C	16.0	1.346/1.351	1.349	
Standard D	44.3	0.914/0.922	0.918	
Standard E	86.9	0.631/0.575	0.603	
Standard F	188.1	0.361/0.355	0.358	
Control CO		0.748/0.703	0.726	67.4
Sample I		1.641/1.606	1.624	10.2
Sample II		0.845/0.962	0.904	55.2
Sample III		0.545/0.535	0.504	134.5

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

Performance characteristics

Detection limit: **2.0 ng/mL**

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

Imprecision

The imprecision of RatLaps™ ELISA was evaluated for three samples (low, medium, high)

Intra-assay variation

The number of determination of each sample was 21.

Sample	Mean level (ng/mL)	SD (ng/mL)	CV (%)
Low	13.0	1.2	9.2
Medium	49.8	2.8	5.6
High	143.0	8.3	5.8

Inter-assay variation

The inter-assay variation is based on 10 consecutive runs according to NCCLS EP5-A (2)

Sample	Mean level (ng/mL)	SD (ng/mL)	CV (%)
Low	13.5	2.0	14.8
Medium	65.0	6.8	10.5
High	140.1	15.0	10.7

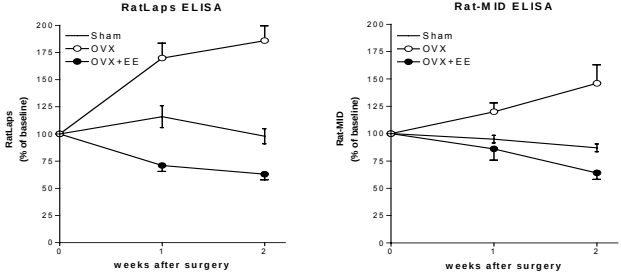
Linearity

A rat serum sample was diluted with Standard A determined in the RatLaps™ ELISA.
The result is summarized in the table below.

Dilution procedure Serum 60.7 ng/mL (parts)	Standard A 0.0 ng/mL (parts)	*Expected* (ng/mL)	*Observed* (ng/mL)	*Recovery* (%)
1	1	30.4	34.8	114.5
1	3	15.2	17.5	115.1
1	7	7.6	8.7	114.5
Serum 49.6 ng/mL (parts)	Standard A 0.0 ng/mL (parts)			
1	1	24.8	20.1	81.0
1	3	12.4	12.8	103.2
1	7	6.2	5.1	82.3

EXPERIMENT EXAMPLE

Evaluation Protocol for RatLaps™ ELISA and Rat-MID™ Osteocalcin ELISA with Serum Samples from THE RAT OVX MODEL of Metabolic Bone Disease:



Three-month-old female rats (Sprague-Dawley) were randomly allocated into three groups:

(1) Sham operation (n=10), (2) ovariectomy (OVX) (n=10), and (3) ovariectomy and subsequent subcutaneous placement of slow release 17b-estradiol pellets (0.5 mg, corresponding to 8 mg/day) (OVE+EE) (n=10).

All serum samples were collected after 6 hours of fasting (no food and water). Start of fasting was at 7.00 in the morning, and samples were collected after 13.00.

The first blood samples were collected on day 0 before the operation. Serum samples were tested in RatLaps™ ELISA for fragments of the type I collagen (CTX) and in Rat-MID™ Osteocalcin ELISA for the mid-molecular part (amino acid 21-29) of osteocalcin. All

measurements were expressed in % of baseline measurement, for each individual rat. The error bars on the figure above are SEM.

The RatLaps™ ELISA rapidly detects the increase in bone resorption following ovariectomy. Within two weeks after surgery RatLaps increases to 186 % of pre-operation levels. This increase in bone resorption could be completely inhibited with estradiol. Similarly, Rat-MID™ Osteocalcin ELISA detects the increase in bone formation induced by ovariectomy. Within two weeks after surgery Rat-MID™ increases to 146 % of pre-operation levels. Also this increase could be completely inhibited with estradiol.

Conclusion: Serum measurement of RatLaps™ and Rat-MID™ Osteocalcin detects the change in bone resorption and bone formation that is induced by ovariectomy of the rat.

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