Rat-Laps® (CTX-I) EIA

For Research Use Only Instructions For Use

Product Name	RatLaps® (CTX-I) EIA	REF	AC-06F1
Abbreviated Product Name	RatLaps EIA		

1. Intended Use

Not for use in clinical or diagnostic procedures.

The RatLaps™ (CTX-I) EIA assay is an enzyme-linked immunosorbent assay intended for the quantitative determination of bone related degradation products from C-terminal telopeptides of type I collagen in rat/mouse serum and rat urine. The assay is for research use only.

2. Summary and Explanation

Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesised 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™ (CTX-I) EIA assay is based on the observation that certain C-telopeptide degradation products from type I collagen released during osteoclastic bone resorption. With the RatLaps™ (CTX-I) EIA it is possible to measure this degradation products in rat/mouse serum and rat urine[2-8].

3. Method Description

The RatLaps™ (CTX-I) EIA is an enzyme-linked immunosorbent assay which requires the pre-incubation of the streptavidinated microtitre plate MICROPLAT with a biotinylated RatLaps Antigen AG BIOTIN. The microtitre plate wells are then washed and 20 µL of each calibrator CAL 0-5, control CTRL or unknown sample are incubated together with the Primary Antibody Ab (polyclonal antibody against the peptide sequence EKSQDGGR) at 2 - 8°C. The wells are then washed and enzyme conjugate ENZYMCONJ (peroxidase conjugated anti- IgG) added to bind to the primary antibody. A further wash step is conducted, following which a chromogenic substrate SUBS TMB is added to allow colour to be developed. The colour reaction is stopped upon the addition of Stopping Solution H_2SO_4 and the absorbance read in a microtitre plate reader, with the colour intensity developed being inversely proportional to the concentration of RatLaps antigen in the original sample.

4. Warnings and Precautions

The RatLaps™ (CTX-I) 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 these Instructions For Use (IFU). Immunodiagnostic Systems Limited (IDS) 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 practice 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.

Reagents containing Sodium Azide

Some reagents in this kit contain sodium azide (NaN_3) <0.1 % (w/w) which may react with lead, copper or brass plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent azide build up.

5. Shelf Life And Storage Of Reagents

Store the kit and components in an **upright** position at 2 - 8 °C. **Do not freeze** the kit or components.
Do not use any kit component beyond their expiry date.

Reagent	After opening or preparation
Kit & components	9 weeks Store at 2 – 8°C* after opening

* The Streptavidin coated plate MICROPLAT must be stored in foil pouch with desiccant sachet after opening.

6. Sample Collection and Storage

The assay should be performed using rat or mouse 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 be utilised with rat urinary samples.
IMPORTANT:

- Samples should be collected as **fasting** samples. It is advised that the samples are taken after a minimum of 6 hours fasting.
- Urine samples can be collected as spot samples. Alternatively, the samples can also be collected as 24-hour urine samples by using metabolic cages or similar devices. Results obtained by using urine samples should be corrected for creatinine prior to evaluation.

Sample Type	Storage Temperature	Duration
Serum, Urine	18 - 22°C	Up to 2 hours
	2 - 8°C	Up to 4 hours
	-20°C	Up to 18 months
	Freeze / thaw cycles	Up to 3 freeze/thaw cycles

7. Materials

Materials Provided

MICROPLAT **Antibody coated plate**
Microwell strips (12x8 wells) pre-coated with streptavidin, supplied in a plastic frame

AG BIOTIN **Biotinylated RatLaps Antigen**
Ready to use PBS buffer containing biotinylated peptide (EKSQDGGR) stabilisers and 0.05% sodium azide as preservative (0.05%); 1 vial, 12.0 mL

Ab **Primary Antibody**
Ready to use buffered solution containing a rabbit polyclonal antibody specific for a part of the C-telopeptide α chain of rat type I collagen with stabilisers and sodium azide as preservative (0.05%); 1 vial, 12.0 mL

ENZYMECONJ **Peroxidase Conjugated Antibody**
Ready to use buffered solution containing a peroxidise conjugated anti- rabbit IgG antibody with stabilisers and ProClin® 300 as preservative; 1 vial, 12.0 mL

WASHBUF 50x **Washing Solution**
Concentrated washing buffer with detergent and preservative; 1 vial, 20.0 mL

SUBS TMB **Substrate Solution**
Ready to use tetramethylbenzidine (TMB) substrate in an acidic buffer; 1 vial, 12.0 mL
Please note that the chromogenic substrate might appear slightly blueish.

H_2SO_4 **Stopping Solution**
Ready to use solution of 0.18 mol/L sulfuric acid; 1 vial, 12.0 mL

CAL 0 **RatLaps Calibrator**
Ready for use TRIS buffered solution containing stabilisers and 0.05% sodium azide as preservative; 1 vial, 5.0 mL

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CAL 1 - 5 **RatLaps Calibrators**
Ready for use TRIS buffered solution a synthetic peptide (EKSQDGGR) with stabilisers and <0.05 % sodium azide as preservative; 1 each of 5 concentration levels, 0.4 mL per vial
The exact value of each calibrator is printed on the QC report.

CTRL **Control**
Ready for use TRIS buffered solution a synthetic peptide (EKSQDGGR) with stabilisers and <0.05 % sodium azide as preservative; 1 vial, 0.4 mL
The established range for the control is printed on the QC report.

Adhesive Plate Sealer 8 per kit.

Documentation Instructions for Use and QC report.

Materials Required But Not Provided

- Containers for preparing the Wash Solution
- Precision pipetting devices to deliver 20 µL
- Distilled or deionised water
- Precision 8 or 12 channel multipipette to deliver 100 µL
- ELISA plate reader with 450 nm and 650 nm reference wavelength
- 2 – 8°C incubator
- Vortex mixer (optional)
- Automatic microplate washer (optional)

8. Preparation Of Reagents

Allow all reagents to come to room temperature for a minimum of 60 minutes before use. Do not interchange kit components from different lots.

Wash Solution preparation

Prepare by adding 1-part Wash Concentrate WASHBUF 50x to 50-parts distilled or deionised water. Mix carefully and avoid formation of foam.

Prepare a fresh solution before each run of the assay.

All other reagents are supplied ready for use and should be mixed by repeated inversion before use.

N.B. To avoid potential microbial and / or chemical contamination, unused reagents should never be returned into the original vials.

9. Assay Procedure

Prepare reagents as described in § 8. Preparation of Reagents. Mix all reagents and samples before use (avoid formation of foam).

NOTE: To ensure consistent results between runs, between operators, and to minimise any drift effect, strictly adhere to the following procedure:

a. Bring all reagents to room temperature (18 - 22 ºC) prior to use – this will take approximately 60 minutes.
b. Seal the plate during incubations using the plate sealers which are supplied with the assay kit.
c. Do not stack plates during incubation in order to ensure a consistent temperature for all plates.
d. Do not under or over-fill the assay wells during the washing steps.
e. Calibrators and control **must** be run in columns 1 and 2 only.
f. Add calibrators, controls and samples within 30 minutes of washing.
g. It is recommended to add conjugate within 5 minutes of washing.
h. It is recommended to add substrate within 5 minutes of washing; stagger stop times as needed.
i. Add reagents in the same sequence each time to reduce time deviation between reactions

Do not pipette directly from the vial containing TMB substrate. The required volume should first be transferred to a clean container. Solution remaining in the container should be discarded following use and NOT returned to the stock vial SUBS TMB

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

1. Pipette 100 µL of Biotinylated RatLaps antigen AG BIOTIN to the appropriate wells on the Antibody Coated Plate MICROPLAT
2. Cover with a plate seal and incubate at room temperature (18 - 22 °C) for 30 ±5 minutes
3. Wash all wells 5 times with wash solution WASHBUF SOLN
    a. Automatic plate wash — Set plate washer to dispense 300 µL of wash solution per well
        a. Fill and aspirate for 5 cycles
    b. Manual wash — Decant the contents of the wells by inverting sharply
        a. Pipette 300 µL of wash solution into each well, decant and repeat 5 times
        b. Remove excess wash buffer by tapping firmly on absorbent tissue before proceeding
4. Pipette 20 µL of each standard CAL 0 – 5, control CRTL or unknown sample to the appropriate wells on the Antibody coated plate MICROPLAT in duplicate followed by 100 µL of Primary Antibody Ab
5. Cover with a plate seal and incubate at 2 – 8°C overnight 18 ±3 hours
6. Wash all wells 5 times with wash solution WASHBUF SOLN as step 3
7. Pipette 100 µL of Peroxidase conjugated anti- IgG ENZYMCONJ into each well
8. Cover with a plate seal and incubate at room temperature (18 - 22 °C) for 60 ±5 minutes
9. Wash all wells 5 times with wash solution WASHBUF SOLN as step 3
10. Pipette 100 µL of Substrate Solution SUBS TMB into each well
11. Cover with a plate seal and incubate at room temperature (18 - 22 °C) for 30 ±5 minutes in the dark
    NOTE: do not pipette directly from the vial containing TMB substrate. The required volume should first be transferred to a clean container. Solution remaining in the container should be discarded following use and NOT returned to the stock vial SUBS TMB
12. Pipette 100 µL of Stopping Solution H_2SO_4 into each well
13. Measure absorbance at 450 nm with reference at 650 nm using a microplate reader within 2 hours of stopping the reaction

N.B. Microplate readers measure vertically; when pipetting, do not touch the bottom of the wells

Automated Platforms

The RatLaps™ (CTX-I) EIA kit was designed and developed to be performed manually using the protocol described above. The protocol is not necessarily applicable to automated platforms.

If automated platforms are used it is the responsibility of the user to ensure the kit has been appropriately tested. To improve the performance of the kit on automated platforms, it is recommended to increase the number of wash cycles at each wash step.

10. Calculation of Results

A variety of data reduction software packages are available, which may be employed to generate the mean calibration curve and to calculate the mean concentrations of unknown samples and controls. A 4 parameter logistic (4PL) curve fit, **including Calibrator 0 is required.**

Alternatively, a calibration curve may be prepared on semi-log graph paper by plotting mean absorbance on the Y-axis against concentration of RatLaps on the X-axis. Calibrator 0 should be included in the calibration curve. Read the mean absorbance value of each unknown sample off the curve.

NOTE: If the absorbance of a sample is lower than that of **Calibrator 5,** the sample should be diluted 1 + 1 in in **Calibrator 0** and re-analysed. Urine samples may be diluted up to 1 + 3 in **Calibrator 0**.

11. 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. Control should be treated as an unknown sample, and the results analysed with appropriate statistical methods.

The kit control provided in the kit should be tested as unknowns and are intended to assist in assessing the validity of results obtained with each assay plate.

The mean concentration of the control level is documented in the QC report included with each kit. This mean concentration level is determined over a number of assays which are run in quadruplicate on each plate. The established control range is equivalent to ± 2 standard deviations based on a nominal % coefficient variation of the kit control.

IDS recommends the users to maintain graphic records of the control values generated with each assay run, including the running means, SDs and %CVs. This information will facilitate the controls trending analysis relating to the performance of current and historical control lots relative to the supplied Quality Control data. The trending will assist in the identification of assays which give control values significantly different from their average range.

When interpreting control data, users should note that this product was designed and developed as a manual product. The range stated on the QC certificate should be appropriate for assays that are performed manually and with strict adherence to the Assay Procedure described above. It is recognised by Quality Control professionals, that as a result of differences in conditions and practices, there will always be variability in the mean values and precision of control measurements between different laboratories[9].

12. Representative Performance Data

Representative performance data are shown which are calculated based on a limited level of testing and are provided **for guidance only**. Results obtained at individual laboratories may vary.

12.1 Sensitivity

The limit of blank (LoB), limit of detection (LoD) and limit of quantitation (LoQ) were determined using 30 blanks and 15 low level samples.

Sensitivity	Concentration (ng/mL)
Limit of Blank (LoB)	2.2
Limit of Detection (LoD)	4.5
Limit of Quantitation (LoQ)	8.7

12.2 Precision

Intra-assay precision was evaluated by running a total of 5 samples in 21 replicates using 1 lot of reagent.

Sample	Sample type	n	Mean conc. (ng/mL)	Intra-assay	
				SD	CV%
1	Mouse serum*	21	23.9	3.0	13%
2	Rat urine*	21	141.3	15.6	11%
3	Rat serum	21	71.4	7.4	10%
4	Mouse serum	21	21.2	2.2	10%
5	Rat serum	21	44.4	4.6	10%

* Diluted in Cal 0

Inter-assay precision was evaluated by running a total of 2 serum and 1 urine sample, and kit control using 1 lot of reagent in duplicate, across 38 experiments.

Sample	Sample type	n	Mean conc. (ng/mL)	Inter-assay	
				SD	CV%
1	Kit control	38	60.5	6.7	11%
2	Mouse serum*	38	20.8	3.0	14%
3	Rat serum	38	47.2	5.3	11%
4	Rat urine*	38	110.5	11.8	11%

* Diluted in Calibrator 0

12.3 Linearity
Linearity was evaluated by diluting a high sample with RatLaps Calibrator 0 prior to assay, with each dilution being assayed in duplicate. The resulting mean concentrations were compared with the predicted concentration. The mean value for recovery for each sample type (2 dilutions of 3 samples per sample type) was determined as:

Rat		Mouse
Serum	Urine	Serum
106%	117%	105%

12.4 Method Comparison
The RatLaps™ (CTX-I) EIA, using the new formulation of Primary Antibody Ab was compared against the previous formulation of the assay. A total of 58 neat samples selected to include the various sample types was assayed by each method. Passing-Bablok regression analysis was performed on the comparative data:

n	Slope	95% CI	Intercept (ng/mL)	95% CI	Correlation Coefficient (r)
58	1.01	0.92 to 1.15	3.80	-1.89 to 7.79	0.93

Additional analysis of a total of 85 samples including diluted serum and urine samples was assessed by each method. Passing-Bablok regression analysis was performed on the data:

n	Slope	95% CI	Intercept (ng/mL)	95% CI	Correlation Coefficient (r)
85	1.02	0.94 to 1.10	3.89	0.92 to 6.76	0.94

13. Symbols used

REF	Catalogue Number
	Manufacturer

14. Bibliography

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Assay Procedure