1. Intended Use

Not for use in clinical or diagnostic procedures.

The CrossLaps® for Culture (CTX-I) ELISA 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 bone cell culture supernatant. 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[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 devitalised slices of bone or dentin.

The CrossLaps® for Culture (CTX-I) ELISA is based on the observation that certain C-telopeptide degradation products from type I collagen released during osteoclastic bone resorption occur in the circulation as modified di-peptides[2]. These modified (β-isomerised) and cross-linked di-peptides (Glu-Lys-Ala-His-Asp-β-Gly-Gly-Arg) must be covalently cross-linked through the lysine residue for signal in the CrossLaps® for Culture (CTX-I) ELISA. This epitope is present in type I collagen of many species, including human, bovine, elephant and chicken[3 - 9]; however, not in rat and mouse.

3. Method Description

The CrossLaps® for Culture (CTX-I) ELISA is an enzyme immunoassay which is based on two highly specific monoclonal antibodies against CTX-I. 50 µL of pre-diluted standards, controls or unknown samples are pipetted into microtitre wells coated with streptavidin, followed by application of a mixture of a biotinylated antibody and an enzyme (horseradish peroxidase – HRP) conjugated antibody. Then, a complex between the CTX-I, present in the original standard, control or sample, the biotinylated antibody and HRP conjugated antibody is generated; this complex binds to the streptavidin surface via the biotinylated antibody. Following the one step incubation at room temperature, the wells are emptied and washed. The colour is developed using a chromogenic substrate (TMB). The colour reaction is stopped and the absorbance of the stopped reaction mixture is read in a microtitre plate reader. The colour intensity of the reaction mixture is proportional to the concentration of CTX-I in the original sample.

4. Warnings and Precautions

The CrossLaps® for Culture (CTX-I) ELISA 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.

5. Shelf Life And Storage Of Reagents

Store the kit and components in an **upright** position in the dark at 2 - 8 °C. **Do not freeze** the kit or components.

Reagent	**Storage after opening or preparation**
Streptavidin coated plate MICROPLAT	Store at 2 – 8°C in foil pouch with desiccant sachet. Under these conditions the kit is stable for up to 8 weeks.
Standard diluent CAL 0	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Standard CAL 1	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Control CTRL	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Biotinylated Antibody AB BIOTIN	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Peroxidase Conjugated Antibody ENZYMCONJ	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.

Incubation Buffer BUF	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Substrate Solution SUBS TMB	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Stopping Solution H_2SO_4	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.
Washing Solution WASHBUF 50x	Store at 2 – 8°C after opening. Under these conditions the kit is stable for up to 8 weeks.

6. Sample Collection and Storage

The assay should be performed using culture supernatants harvested form bone cells cultured on the surface of bone or dentine.
NOTE: Ideally the supernatants should be tested on the same day as they are harvested.

Sample Storage	Duration
2-8°C	14 days

7. Materials

Materials Provided

Streptavidin coated plate MICROPLAT	Microwell strips (12 x 8 wells) pre-coated with streptavidin, supplied in a plastic frame
Standard Diluent CAL 0	Ready to use PBS buffered solution containing stabilisers and 0.05% Proclin 300 as preservative; 1 vial, 9.0 mL
Standard CAL 1	PBS buffered solution containing CrossLaps standard (desalted urinary antigens of human origin) with stabilisers and 0.05% Proclin 300 as preservative; 1 vial, 1.0 mL The exact value of each standard is printed on the Quality Control Report Must be serially diluted before performing the ELISA.
Control CTRL	Ready to use PBS buffered solution containing CrossLaps standard (desalted urinary antigens of human origin) with stabilisers and 0.05% Proclin 300 as preservative; 1 vial, 0.4 mL The exact value of each standard is printed on the Quality Control Report
Biotinylated Antibody AB BIOTIN	Concentrated solution containing a biotinylated monoclonal anti- CTX-I antibody with stabilisers; 1 vial, 0.25 mL
Peroxidase Conjugated Antibody ENZYMCONJ	Concentrated solution containing a peroxidise conjugated anti- CTX-I antibody with stabilisers; 1 vial, 0.25 mL
Incubation Buffer BUF	Ready to use buffered solution containing stabilisers, detergent and 0.05% Proclin 300 as preservative; 1 vial, min. 19.0 mL
Substrate Solution SUBS TMB	Ready to use tetramethylbenzidine (TMB) substrate in an acidic buffer, 1 vial; 12.0 mL. Please note that the chromogenic substrate might appear slightly bluish.
Stopping Solution H_2SO_4	Ready to use 0.18 M sulphuric acid; 1 vial; 12.0 mL
Washing Solution WASHBUF 50x	Concentrated washing solution containing detergent and preservative; 1 vial, min. 20.0 mL
Adhesive Plate Sealer	1 pk of 4 per kit
Documentation	Instructions for Use and QC report

Materials Required But Not Provided

- Microtubes (or similar) for preparation of the serial dilutions of the Standard
- Containers for preparing the Antibody Solutions and the Washing Solution
- Precision pipetting devices to deliver 20 – 250 µL
- Distilled water
- Precision 8 or 12 channel multipipette to deliver 100 µL and 150 µL
- Vortex mixer
- Microwell mixing apparatus
- Automatic microplate washer (optional)
- Photometric microplate reader and data analysis equipment

8. Preparation Of Reagents

Allow all reagents to come to room temperature (18 - 22°C) for a minimum of 60 minutes before use.

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, 16 wells are recommended for the standards and control. Place the appropriate number of strips in the plastic frame. Store any unused immunostrips in the tightly closed foil bag with desiccant capsules.

Antibody Solution

Prepare the following antibody solution a maximum of 30 minutes before starting the assay.

Mix the Biotinylated Antibody AB BIOT, Peroxidase Conjugated Antibody ENZYMCONJ and Incubation Buffer BUF in the volumetric ratio 1 + 1 + 100 in an empty container. Mix carefully and avoid formation of foam.

Prepare a fresh solution before each run of the assay.

Standards

Prepare a two-fold dilution row of the Standard CAL1 in Standard Diluent CAL0. For each dilution 2 x 50 µL will be needed in order to run the ELISA.

a. Add 300 µL of CAL1 to the first microtube (I)
b. 150 µL of CAL0 into 7 other tubes labelled 0 - 6
c. From the first tube (I), transfer 150 µL into another tube containing 150 µL of CAL0 (6)
d. Mix well
e. From tube 6, transfer 150 µL into another tube containing 150 µL of CAL0 (5)
f. Repeat steps d & e until tube 1 leaving tube 0 with only Standard Diluent CAL0

A summary of the dilution scheme can be found in Appendix 1.

Washing Solution

Prepare the Washing Solution WASHBUF 50x by diluting in distilled or deionised water adding 1 part WASHBUF 50x to 50 parts water. Mix carefully and avoid formation of foam.

Prepare a fresh solution before each run of the assay.

Pre-dilution of test samples

All test samples (i.e. not reagents supplied with the assay kit) must be pre-diluted 1 + 4 in Standard Diluent CAL0 prior to testing e.g. 30 µL test sample + 120 µL CAL0.

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

In order to measure the background release of collagen fragments, three types of control specimens are recommended for each experiment:

Medium control specimens: medium on plastic surface (culture dishes/microwells) under culture conditions. At least 2 specimens are recommended for each experiment.

Cell control specimens: bone cells in medium on plastic surface (culture dishes/microwells) under culture conditions. At least 2 specimens are recommended for each experiment.

Slice control specimens: slices of bone or dentin without cells in medium under culture conditions. At least 4 specimens are recommended for each experiment.

9. Assay Procedure

Prepare reagents as described in section 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. Set up the assay within 30 minutes of preparing the antibody solution.
c. Seal the plate during incubations using the plate sealers which are supplied with the assay kit.
d. Do not stack plates during incubation in order to ensure a consistent temperature for all plates.
e. Do not under or over-fill the assay wells during the washing steps.
f. It is recommended to add substrate within 5 minutes of washing; stagger stop times as needed.

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. For each run a total of 14 wells are needed for the standards and 2 for the controls. For each experiment, but independent of the number of ELISA plates used, a further total of 16 wells are recommended for the control specimens to be run in duplicate – 2 Medium control specimens (4 wells), the 2 Cell control specimens (4 wells) and the 4 Slice control specimens (8 wells).

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

- Pipette 50 µL of each prepared standard, control CTRL or pre-diluted sample to the appropriate wells on the Streptavidin Coated Plate MICROPLAT
- Pipette 150 µL of prepared antibody solution into each well
- Cover the plate with an adhesive plate seal
- Incubate at room temperature (18 – 22°C) 120 ±5 minutes on a microtitre plate mixer (300 rpm)
- Wash all wells 5 times with prepared wash solution WASHBUF SOLN

  Automatic plate wash — Set plate washer to dispense 300 µL of wash solution per well
  Fill and aspirate for 5 cycles

  Manual wash — Decant the contents of the wells by inverting sharply
  Pipette 300 µL of wash solution into each well, decant and repeat 5 times
  Remove excess wash buffer by tapping firmly on absorbent tissue before proceeding
- Pipette 100 µL of Substrate Solution SUBS TMB into each well
- Cover the plate with an adhesive plate seal
- Incubate at room temperature (18 – 22°C) 15 ±2 minutes in the dark on a microtitre plate mixer (300 rpm)
- Pipette 100 µL of Stopping Solution H_2SO_4 into each well
- Measure absorbance at 450 nm with reference at 650 nm using a microplate reader within 2 hours of stopping the reaction

10. Calculation of Results

A calibration curve should be prepared by plotting mean absorbance of all prepared standards on the Y-axis against concentration of CTX-I on the X-axis and a best fit or quadratic curve assigned.

Determine the CrossLaps® for Culture (CTX-I) ELISA concentration of the Control CTRL, Medium, Cell and Slice control samples and each of the test samples by interpolation on the curve. CrossLaps® for Culture (CTX-I) ELISA concentration determined for the Control CTRL should be within the range giving on the QC Report contained within the assay kit.

Due to the pre-dilution, the CrossLaps® for Culture (CTX-I) ELISA concentration of the Medium, Cell and Slice control samples as well as the test samples should be multiplied by 5 to obtain the true concentration.

NOTE: For all bone cell culture supernatants, the results obtained must be corrected for the total background effect, which is calculated as the sum of the background effects from the medium, cell and slice controls.
For example:
Medium control: 0.15 nM
Cell control: 0.48 nM
Slice control: 2.26 nM
Total Background effect: 0.15 + 0.48 + 2.26 = 2.89 nM

Test sample concentration following dilution correction: 5.05 – total background effect (2.89 nM)
Actual sample concentration: 2.16 nM

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. Controls should be treated as unknown samples, and the results analysed with appropriate statistical methods.

The kit control provided in the kit should be tested as unknown and is 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 duplicate in six locations across 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.

12. Measurement Range
The measuring range of the assay is 0.75 to 112.7 nM.

13. Limitations of the Procedure
The content of antigenic collagen fragments in foetal calf serum (FCS) varies from product to product and from lot to lot. However, when used as additive to the bone cell culture medium in final concentrations up to 10% (v/v), all of the more than 20 commercially available foetal and new-born bovine serum products tested until now have shown a CrossLaps® for Culture (CTX-I) ELISA concentration below 1nM and therefore do not cause a problem for the analysis. If a serum additive is used for the bone cell culture it is recommended to:

a) Check its concentration of CrossLaps® for Culture (CTX-I) ELISA and if necessary select a different product that gives a lower CrossLaps® for Culture (CTX-I) ELISA concentration.

b) Reduce the concentration of serum in the culture medium. Most osteoclast preparations grow well at a 5% (v/v) or even lower concentration of serum additive

14. 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.

14.1 Detection limit
Detection limit: 0.75 nM CrossLaps
This is the concentration corresponding to three standard deviations above the mean of 11 determinations of the Standard 0.

14.2 Precision
The precision of the CrossLaps® for Culture (CTX-I) ELISA was evaluated for three serum samples. The results are summarised in the table below:

Sample	n	Mean (nM)	Inter-assay Variation		Intra-assay Variation	
			SD	CV%	SD	CV%
1	10	1.5	0.2	11.1%	0.1	7.7%
2	10	20.0	0.9	3.8%	0.4	2.0%
3	10	60.4	2.0	4.1%	0.8	1.2%

15. Symbols used

REF	Catalogue Number
	Manufacturer

16. Bibliography

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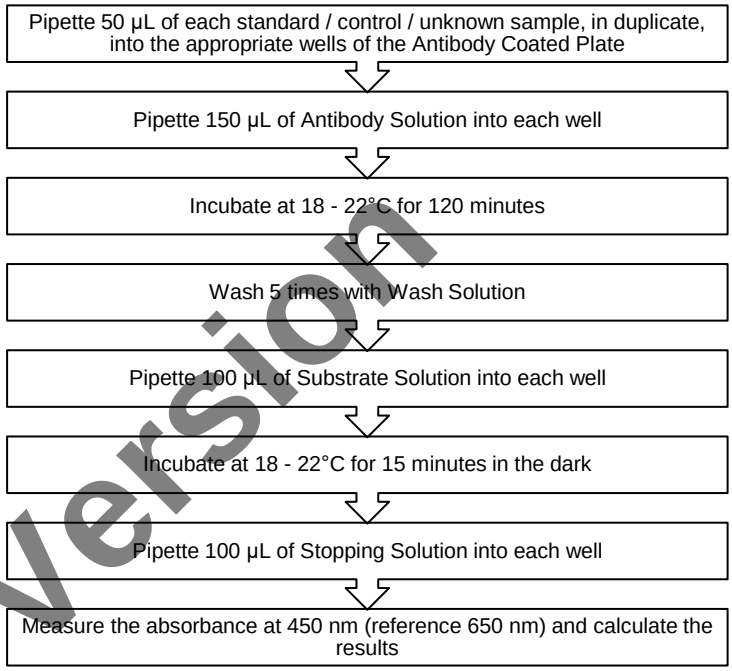
17. Appendix 1

Assay Procedure



Recommended dilution scheme