CrossLaps® for Culture ELISA

For quantification of fragments of type I collagen released into bone cell culture supernatants during bone resorption *in vitro*

This ELISA kit should only be used for *in vitro* determination of the antigenic type I collagen specific peptide sequence in bone cell culture supernatants.

The CrossLaps® for Culture kit should only be used for *in vitro* determinations. Nordic Bioscience Diagnostics is not responsible for any other use of the kit or consequence hereof than the one specified above. Neither for misuse e.g. uses deviating from the procedure described in this manual.

Furthermore, Nordic Bioscience Diagnostics is not to be made responsible for any diagnoses or conclusions made by the user or third party based on the results obtained with the CrossLaps® for Culture kit nor for any consequences such interpretations may cause.

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INTRODUCTION

Intended use
The test is an enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of degradation products of C-terminal telopeptides of type I collagen in bone cell culture supernatants. It represents a technically improvement of the assay originally described in 1996 (6).

Summary and explanation of the test
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 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 (9). 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 ELISA. This epitope is present in type I collagen of many species, including human, bovine, elephant and chicken (2-5, 7, 8, 10). However, not in rat and mouse.

Principle of the procedure
The assay is based on the simultaneous binding of the collagen fragments by two monoclonal antibodies, one of which will subsequently mediate the binding to the solid surface of the microtitre plate.

Standards, control, or unknown samples are pipetted into the appropriate well of a microtitre plate coated with streptavidin, and subsequently a mixture of a biotinylated antibody and a peroxidase-conjugated antibody is added. Then a complex between the collagen fragments and the two antibodies is generated, and the complex will bind to the streptavidin surface via the biotinylated antibody. Following this one step incubation at room temperature the wells are washed and a chromogenic substrate added. The colour reation is stopped with sulphuric acid and the absorbance is measured.

PRECAUTIONS

Storage
Store the CrossLaps® for Culture kit upon receipt at 2-8˚C. Under these conditions the kit is stable up to the expiry date stated on the box.

Warnings
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 CrossLaps® for Culture ELISA is for research-use-only

MATERIALS

Specimen collection
The determination should be carried out using culture supernatants harvested from bone cells cultured on surfaces of bone or dentin. The culture supernatants preferably should be tested on the same day as they are harvested, but current date indicates that culture supernatants can be stored for 2 weeks at 4˚C. Fetal and newborn bovine serum in a concentration up to 10% (v/v) in the culture supernatant does not interfere with the test.

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.

All specimens, except those delivered with the kit (i.e. standards and control) should be pre-diluted 1+4 in Standard Diluent prior to testing.

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) precoated with streptavidin. Supplied in a plastic frame.

Standard Diluent (Vial A)
One vial (9 mL) of ready-for-use PBS-buffered solution with protein stabiliser and preservative.

Standard (Vial B)
One vial (1 mL) containing CrossLaps® standard (desalted urinary antigens) in a PBS-buffered solution with protein stabiliser and preservative. Please refer to the enclosed Technical Data Sheet for the concentration.
Serial dilutions of the Standard (vial B) in the Standard Diluent (vial A) must be made prior to performing the ELISA.

Control (Vial CO)
One vial (0.5 vial) of ready-for-use control reagent, containing desalted urinary antigens of human origin in a PBS-buffered solution with protein stabiliser and preservative. Please refer to the enclosed Technical Data Sheet for concentration range.

Biotinylated Antibody (Vial no. 1)
One vial (0.25 mL) of a concentrated solution containing a biotinylated monoclonal antibody specific for degradation products of C-terminal telopeptides of Type I collagen in a buffered solution with protein stabiliser and preservative. Mix vials 1, 2 and 3: 1+1+100 before use.

Peroxidase Conjugated Antibody (Vial no. 2)
One vial (0.25 mL) of a concentrated solution containing a peroxidase conjugated monoclonal antibody specific for degradation products of C-terminal telopeptides of Type I collagen in a buffered solution with protein stabiliser and preservative. Mix vials 1, 2 and 3: 1+1+100 before use.

Incubation Buffer (Vial no. 3)
One vial (min. 19 mL) of a ready-for-use buffered solution with protein stabiliser, detergent and preservative. Mix vials 1, 2 and 3: 1+1+100 before use.

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. 12 mL) of ready-for-use 0.18 M sulphuric acid.

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

Sealing tape
Adhesive film for covering wells during incubation.

Materials required – not supplied
- Microtubes (or similar) for preparation of serial dilutions of the Standard
- Containers for preparing the Antibody Solution and the Washing Solution
- Precision micropipettes to deliver 20-250 µL
- Distilled water
- Precision 8-or 12-channel multipipette to deliver 100 µL and 150 µL
- Microwell mixing apparatus (300 rpm)
- ELISA plate reader with both 450 nm and 650 nm filters

ASSAY PROCEDURE

Prior to use, equilibrate all solution to room temperature. **The assay should be performed at room temperature (18-22ºC).**

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 standards and 2 wells are recommended for the Control. Furthermore, for each experiment (but independently of the number of ELISA plates used), a total of 16 wells are recommended for the 2 Medium control specimens (4 wells), the 2 Cell control specimens (4 wells) and the 4 Slice control specimens (8 wells).

Prepare Standards (recommended dilutions)

Prepare a two-fold dilution row of the Standard (vial B) in the Standard Diluent (vial A). For each dilution 2x50 µL will be needed for the ELISA. E.g., pipette 300 µL of the Standard (vial B) into the first microtube (I) and 150 µL of Standard Diluent (vial A) into each 7 other microtubes (II-VIII), transfer 150 µL from I to II and mix, transfer 150 µL from II to III and mix, continue until VII. Leave VIII with only the Standard Diluent (vial A). Of the 150 µL in each microtube, 2x50 µL are used in the ELISA. The rest (50 µL) is discarded.

1. Pre-dilution of test specimens

All specimens, except those delivered with the kit (i.e. standard and control) must be pre-diluted 1+4 in **Standard Diluent** prior to testing (e.g. 30 µL (specimen) +120 µL (vial A)).

2. Preparation of the Antibody Solution

ATTENTION: prepare the following Antibody Solution maximum 30 minutes before starting the test;

Mix the solutions of vial no. 1 (**Biotinylated Antibody**), vial no. 2 (**Peroxidase Conjugated Antibody**) and vial no. 3 (**Incubation Buffer**) in the volumetric ratio 1+1+100 in an empty container. Mix carefully and avoid formation of foam. Prepare a fresh solution before each test.

3. One Step incubation

Pipette 50 µL of each **Standards**, **Control** (vial CO), 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 room temperature (18-22ºC) on a microtitre plate mixing apparatus (300 rpm).

4. Washing

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

5. 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 temperature (18-22ºC) in the dark on the mixing apparatus (300 rpm). Use sealing tape.

Do not pipette directly form 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.

6. Stopping of colour reaction

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

7. 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.

RESULTS

Calculation of results

- Calculate the mean of the duplicate absorbance determinations. Construct a standard curve by plotting the mean absorbances of the eight Standards I-VIII (ordinate) against the corresponding concentrations (abscissa). Draw the best fitting curve.
  Alternatively, a quadratic curve fit can be used.
- Determine the CrossLaps® for Culture concentration of the Control (CO), Medium, Cell and Slice control Specimens and each of the Test Specimens by interpolation on the curve.
- CrossLaps® for Culture concentration determined for the Control (CO) should be within the range giving at the enclosed Technical Data Sheet.
- As a consequence of the pre-dilution the CrossLaps® for Culture concentration of the Medium, Cell and Slice control Specimens as well as the Test Specimens should be multiplied by 5 to obtain the true concentration.

Example:

Standards/ Controls/ Specimen	CrossLaps® conc. (nM)	$A_{450-650}$ (nM)	Mean $A_{450-650}$ (nM)	CrossLaps® conc. in prediluted specimen (nM)	CrossLaps® conc. in undiluted specimen (nM)
Zero Std (VIII)	0.00	0.041/0.042	0.042		
Std VII	1.32	0.067/0.071	0.069		
Std VI	2.65	0.096/0.100	0.098		
Std V	5.29	0.147/0.155	0.151		
Std IV	10.59	0.249/0.266	0.258		
Std III	21.18	0.447/0.507	0.477		
Std II	42.35	0.897/0.973	0.935		
Std I	84.70	1.844/1.915	1.880	11.88	
Co		0.291/0.281	0.286		
Medium control		0.041/0.045	0.043	0.05	0.25
Medium control		0.041/0.042	0.042	0.01	0.05
Cell control		0.046/0.044	0.045	0.15	0.75
Cell control		0.041/0.047	0.044	0.10	0.50
Slice control		0.049/0.051	0.050	0.40	2.00
Slice control		0.053/0.055	0.054	0.59	2.95
Slice control		0.048/0.052	0.050	0.40	2.00
Slice Control		0.053/0.052	0.053	0.54	2.70
Sample I		0.064/0.061	0.063	1.01	5.05
Sample II		0.181/0.181	0.184	6.80	34.00
Sample III		0.521/0.512	0.571	22.91	114.55

Please note: *The data above were calculated from a quadratic curve fit of the standard curve and are for illustration only. They should not be used to calculate the results of tests.*

For all bone cell culture supernatants the results obtained by interpolations must be corrected for the medium, cell and slice background effects. Using the value from the table below:

Medium control	:	0.15 nM (Mean of 0.25 & 0.05)
Cells control	:	0.48 nM (Mean of 0.75 & 0.50) *minus* 0.15
Slice control	:	2.26 nM (Mean of 2.00, 2.95, 2.00 & 2.70) *minus* 0.15
Total Background effect:		2.89 nM (0.15 nM + 0.48 nM +2.26 nM)

Corrected values for :

Sample I	:	2.16 nM (5.05 - 2.89 nM)
Sample II	:	31.11 nM (34.00 - 2.89 nM)
Sample III	:	111.66 nM (114.55 - 2.89 nM)

SPECIAL INSTRUCTIONS

Limitations of the procedure

1. The content of antigenic collagen fragments in fetal 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 fetal and newborn bovine serum products tested until now have shown a CrossLaps® for Culture 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, however still recommended to:

a) Check its concentration of CrossLaps® for Culture and if necessary choose another product with a lower CrossLaps® for Culture concentration.

b) Reduce the concentration of serum in the culture medium. Most osteoclast preparations are growing well at 5% (v/v) or even lower concentration of serum additive and in one study (1) their ability to resorb bone was found to be invariable at serum concentrations form 0.04 to 5%.

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