immunodiagnosticsystems

CrossLaps® for Culture ELISA

Enzymeimmunoassay for the quantitative determination of fragments of type I collagen released into bone cell culture supernatants during bone resorption in vitro

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

REF AC-07F1 Σ 96

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 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 (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 ELISA 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 CrossLaps® for Culture 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 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
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 **MICROPLAT**
Microwell strips (12x8 wells) precoated with streptavidin. Supplied in a plastic frame.

Standard Diluent **CAL** | **0**
One vial (min. 9 mL) of ready-for-use PBS-buffered solution with protein stabiliser and preservative.

Standard **CAL** | **1**
One vial (min. 1 mL) containing CrossLaps standard (desalted urinary antigens) in a PBS-buffered solution with protein stabiliser and preservative. The exact concentration is stated on the bottle label. Serial dilutions of the Standard in the Standard Diluent must be made prior to performing the ELISA.

Control **CTRL**
One vial (min. 0.5 mL) 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 QC Report for concentration range.

Biotinylated Antibody **Ab BIOTIN**
One vial (min. 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.

Peroxidase Conjugated Antibody **ENZYMCONJ**
One vial (min. 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.

Incubation Buffer **BUF**
One vial (min. 19 mL) of a ready-for-use buffered solution with protein stabiliser, detergent and preservative.

Substrate Solution **SUBS** | **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 **H2SO4**
One vial (min. 12 mL) of ready-for-use 0.18 M sulphuric acid.

Washing Buffer **WASHBUF** | **50x**
One vial (min. 20 mL) of a concentrated washing buffer with detergent and preservative.

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 solutions 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 **CAL 1** in the Standard Diluent **CAL 0**. For each dilution 2x50 µL will be needed for the ELISA. E.g., pipette 300 µL of the Standard **CAL 1** into the first microtube (I) and 150 µL of Standard Diluent **CAL 0** 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 **CAL 0**. 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 **CAL 0**).

2. Preparation of the Antibody Solution
ATTENTION: prepare the following Antibody Solution maximum 30 minutes before starting the test;
Mix the solutions of Biotinylated Antibody **Ab BIOTIN**, 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 test.

3. One Step incubation
Pipette 50 µL of each Standards, Control **CTRL**, 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 300 µL diluted Washing Buffer (**WASHBUF 50x** 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 **SUBS 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 **H2SO4** 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 ELISA concentration of the Control, Medium, Cell and Slice control Specimens and each of the Test Specimens by interpolation on the curve.
- CrossLaps® for Culture ELISA concentration determined for the Control should be within the range giving at the enclosed QC Report.
- As a consequence of the pre-dilution the CrossLaps® for Culture ELISA 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		
Control		0.291/0.281	0.286	11.88	
Medium co		0.041/0.045	0.043	0.05	0.25
Medium co		0.041/0.042	0.042	0.01	0.05
Cell co		0.046/0.044	0.045	0.15	0.75
Cell co		0.041/0.047	0.044	0.10	0.50
Slice co		0.049/0.051	0.050	0.40	2.00
Slice co		0.053/0.055	0.054	0.59	2.95
Slice co		0.048/0.052	0.050	0.40	2.00
Slice co		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)

Performance characteristics

Detection limit: 0.44 nM CrossLaps

This is the concentration corresponding to three standard deviations above the mean of 21 determinations of the Standard 0.

Precision

The precision of the CrossLaps® for Culture ELISA was evaluated for three serum samples. The results are summarised in the table below:

InterAssay Variation (n=10)			IntraAssay Variation (n=10)		
Mean (nM)	**SD (nM)**	**CV (%)**	**Mean (nM)**	**SD (nM)**	**CV (%)**
1.5	0.2	11.1	1.5	0.1	7.7
20.0	0.9	4.8	20.0	0.4	2.0
60.4	2.0	3.1	60.4	0.8	1.2

Measuring Range

The measuring range for the CrossLaps® for Culture ELISA is between 0.44 nM and 112.7 nM CrossLaps.

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 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, however still recommended to:

a) Check its concentration of CrossLaps® for Culture ELISA 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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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*
	SE	*Använd före*		SE	*Lot nummer*
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REF	GB	*Catalogue number*		GB	*Manufacturer*
	DE	*Bestellnummer*		DE	*Hersteller*
	ES	*Número de catálogo*		ES	*Fabricante*
	IT	*Numero di catalogo*		IT	*Fabbricante*
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	NL	*Catalogus nummer*		NL	*Fabrikant*
	DK	*Katalognummer*		DK	*Producent*
	CZ	*Katalogové číslo*		CZ	*Výrobce*
	SK	*Katalógové číslo*		SK	*Výrobca*
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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	*Viď 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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