ALPHA CrossLaps® ELISA

For the quantification of degradation products of C-terminal telopeptides of Type-I collagen in human urine

The ALPHA CrossLaps® ELISA kit is For Research Use Only. Not for use in diagnostic procedures.

Nordic Bioscience Diagnostics A/S is not responsible for any other use of the kit or consequence hereof than the one specified above. Neither for misuse, e.g. use deviating from the procedure described in this manual.

Furthermore, Nordic Bioscience Diagnostics A/S 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 ALPHA CrossLaps® ELISA kit nor for any consequences such interpretations may cause.

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INTRODUCTION

Intended use
The ALPHA CrossLaps® ELISA is an enzyme immunological test for the quantification of degradation products of C-terminal telopeptides of Type-I collagen (ALPHA CTX) in human urine. The test is intended For Research Use Only. Not for use in diagnostic procedures**.**

Limitations
The use of the test has not been established for determination of the level of bone resorption.

Summary and explanation of the test
Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesized primarily in bone. During renewal of the skeleton, Type I collagen is degraded, and small peptide fragments are excreted into the bloodstream. These fragments can be measured by ALPHA CrossLaps® ELISA. The measurements of the specific degradation products of Type I collagen (ALPHA CTX) in human urine have been reported as useful assessment of bone resorption in Paget's disease (1) and for detection of bone metastases in prostate (2) and breast cancer (3, 4).

Principle of the procedure
The ALPHA CrossLaps® ELISA is based on one highly specific monoclonal antibodies against the amino acid sequence of EKAHDGGR. In order to obtain a specific signal in the ALPHA CrossLaps® ELISA, two chains of EKAHDGGR must be cross-linked.
Standards, control, or unknown urine samples are pipetted into the appropriate microtitre wells coated with streptavidin, followed by application of a mixture of biotinylated antibody and peroxidase-conjugated antibody. Then, a complex between ALPHA CTX antigens, biotinylated antibody and peroxidase-conjugated antibody is generated, and this complex binds to the streptavidin surface via the biotinylated antibody. Following the one-step incubation at 2-8°C, the wells are emptied and washed. A chromogenic substrate is added and the colour reaction is stopped with sulfuric acid. Finally, the absorbance is measured.

PRECAUTIONS

The following precautions should be observed in the laboratory:
- Do not eat, drink, smoke or apply cosmetics where immunodiagnostic materials are being handled
- Do not pipette by mouth.
- Wear gloves when handling immunodiagnostic materials and wash hands thoroughly afterwards
- Cover working area with disposable absorbent paper

Warnings
For *in vitro* use only.
- All reagents and laboratory equipment should be handled and disposed of as if they were infectious.
- Do not use kit components beyond the expiry date and do not mix reagents from different lots.

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

MATERIALS

Specimen collection
It is recommended to use second morning void urine specimens from fasting individuals.
Urine samples are stable for 7 days at 4 and 20°C. For longer storage, the urine samples should be stored frozen (<-18°C).
Prior to use, urine specimens should be shaken and sedimentation allowed for a minimum of 30 minutes.

Materials supplied
Before opening the kit, read the section on **Precautions.** The kit contains reagents sufficient for 96 determinations.

Streptavidin coated microtitre plate (MTP)
Microwell strips (12x8 wells) pre-coated with streptavidin. Supplied in a plastic frame.
Standard A (vial A)
One vial (min. **12.0 mL**) of ready-for-use solution with protein stabiliser, detergent and reservative.
Standards B-F (vial B-F)
Five vials (min. 0.4 mL/vial) of ready-for-use, standard in a buffered solution with protein stabiliser, detergent and preservative. The exact concentration of ALPHA CTX is stated on each vial.
Control (vial CO 1-2)
Two vials (min. 0.4 mL) of ready-for-use, di-peptide in a buffered solution with protein stabiliser, detergent and preservative. The exact concentration of ALPHA CTX is stated on the vial.
Biotinylated Antibody (vial no. 1)
One vial (min. 0.2 mL) of a concentrated solution of a biotinylated monoclonal murine antibody specific for degradation products of C-terminal telopeptides of Type I collagen. Prepared in a buffered solution with protein stabiliser, detergent and preservative.
Peroxidase Conjugated Antibody (vial no. 2)
One vial (min. 0.2 mL) of a concentrated solution of a peroxidase conjugated murine monoclonal antibody specific for degradation products of C-terminal telopeptides of Type I collagen. Prepared in a buffered solution with protein stabiliser, detergent and preservative.
Incubation Buffer (vial no. 3)
One vial (min. 13.0 mL) of a ready-for-use buffered solution with protein stabiliser, detergent and preservative.
Substrate Solution (vial TMB)
One vial (min. 12.0 mL) of a ready-for-use tetramethylbenzidine (TMB) substrate in an acidic buffer.
Please note that the chromogenic substrate might appear sligthy blueish.

Stopping Solution (vial ST)
One vial (min. 12.0 mL) of ready-for-use 0.18 mol/L sulfuric acid.
Washing Buffer (vial W)
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
- Containers for preparing the Antibody Solution and the Washing Solution
- Precision micropipettes to deliver 25-200 µL
- Distilled water
- Precision 8- or 12-channel multipipette to deliver 100 µL
- Microtiter plate reader

ASSAY PROCEDURE

Assay Procedure
Prior to use, prepare and equilibrate all solutions to room temperature (18-22°C). Mix all reagents and samples before use (avoid foam).

1 Pre-dilution of urine samples
Dilute urine samples 1+3 in **Standard A** (vial A).

2 Preparation of the Antibody Solution:
ATTENTION: Prepare the **Antibody Solution *immediately before*** use. The **Antibody Solution** is prepared by mixing the solutions in 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 run of the assay.**

3 One Step incubation
Pipette 25 µL of either **Standards** (vial A-F), **Control** (vial CO 1-2), or unknown samples into appropriate wells followed by 100 µL, of the **Antibody Solution.** Cover the immunostrips with sealing tape and incubate for **60±5 minutes at 2-8°C** without shaking.

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

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 (18-22°C) in the dark without shaking. Use sealing tape. Do not pipette directly from 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
Measure the absorbance at 450 nm with 650 nm as reference within two hours.

Limitations of the procedure
- If the absorbance of a (pre-diluted) sample is above that of **Standard F,** the sample should be additionally diluted in **Standard A** and re-analysed.

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.

RESULTS

Calculation of results
It is recommended to use a **4-parameter** curve fit.

Example of results obtained:

Standards Controls Samples	ALPHA CTX conc. (ng/mL)	$A_{450-650}$ (Abs) Obs 1/ Obs 2	Mean $A_{450-650}$ (Abs)	ALPHA CTX Interpolated conc. (ng/mL)	ALPHA CTX Conc. corrected for dilution (ng/mL)
Standard A	0.00	0.070 / 0.072	0.071		
Standard B	1.77	0.598 / 0.569	0.583		
Standard C	4.03	1.192 / 1.145	1.168		
Standard D	6.01	1.639 / 1.554	1.597		
Standard E	8.06	1.948 / 1.927	1.938		
Standard F	10.09	2.232 / 2.198	2.215		
Control CO 1		1.730 / 1.683	1.707	6.64	6.64
Sample I		1.558 / 1.471	1.515	5.63	22.52
Sample II		1.071 / 1.092	1.082	3.67	14.68
Sample III		0.377 / 0.373	0.375	1.02	4.08

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

Calculation of corrected ALPHA CTX value
For each urine sample the ALPHA CTX concentration (ng/mL) and the creatinine concentration (mM= mmol/L) should be determined using an enzymatic colometric method for clinical chemistry analysers (e.g. CREA plus for Roche/Hitachi analysers) or equivalent.

The following equation corrects the ALPHA CTX concentration for variation in urine concentration:

$$\text{Corr. ALPHA CTX Value } (\mu g/mmol) = \frac{\text{ALPHA CTX (ng/mL)}}{\text{Creatinine (mmol/L)}}$$

Performance characteristics
Detection limit: 0.10 ng/mL ALPHA CTX
This is the concentration corresponding to three standard deviations above the mean of 21 determinations of the blank (Standard A).

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

InterAssay Variation (n=10)

Mean (ng/mL)	SD (ng/mL)	CV (%)
6.37	0.34	5.3
3.85	0.17	4.3
1.04	0.06	6.0

IntraAssay Variation (n=10)

Mean (ng/mL)	SD (ng/mL)	CV (%)
6.37	0.15	2.3
3.85	0.13	3.4
1.04	0.04	2.3

Dilution/Linearity
The ALPHA CrossLaps® ELISA is linear in the range 0.20 ng/mL to 11.00 ng/mL of ALPHA CTX.

Urine samples were diluted in **Standard A** and the concentration of ALPHA CTX were determined with ALPHA CrossLaps® ELISA. The urine neat sample is set to 100%.

Urine [%]	Standard A [%]	Recovery [% of expected value]
100.0	0.0	100
87.5	12.5	106
75.0	25.0	106
62.5	37.5	98
50.0	50.0	113
37.5	62.5	109
25.0	75.0	102
12.5	87.5	99
Mean		103

Interference:
To be determined.

Expected values
It is advisable for a laboratory to establish its own range of normal and pathological values. As an example, the mean values and 95% CI for various populations are given below. For further reading, please refer to the reference list. All samples were morning fasting samples from healthy individuals.

Populations	Number of subjects	Mean Values (µg/mmoL)	95% CI
Post-menopausal women	220	0.636	0.17-2.38
Pre-menopausal women	76	0.302	0.10-0.94
Males	209	0.382	0.13-1.13

REFERENCES

1. Alexandersen et al. Submitted.
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3. Cloos et al. Breast cancer patients with bone metastases are characterised by increasing levels of nonisomerised type I collagen fragments. Breast Cancer Res (2003);5:R103-9.
4. Cloos et al. An immunoassay for measuring fragments of newly synthesized collagen type I produced during metastatic invasion of bone. Cli. Lab. (2004); 50:279-289.