Urine BETA CrossLaps® ELISA

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

The Urine BETA CrossLaps® ELISA kit is for in vitro use only.

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 Urine BETA CrossLaps® ELISA kit nor for any consequences such interpretations may cause.

For US:
The Urine BETA CrossLaps® ELISA is For Research Use Only. Not for use in diagnostic procedures.
INTRODUCTION

Intended use
The Urine BETA CrossLaps® ELISA is an enzyme immunological test for the quantification of degradation products of C-terminal telopeptides of Type-I collagen in human urine. The test is intended for Research Use Only. Not for use in diagnostic procedures. The Urine BETA CrossLaps® ELISA assay may be used as an indication of human bone resorption as an aid in

A. Monitoring bone resorption changes of
   1) Anti-resorptive therapies in postmenopausal women:
      a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
      b) Bisphosphonate therapies

B. Predicting skeletal response (Bone Mineral Density) in postmenopausal women undergoing anti-resorptive therapies
   a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
   b) Bisphosphonate therapies

Limitations
The use of the test has not been established to predict the development of osteoporosis or future fracture risk.

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 (1). During renewal of the skeleton, type I collagen is degraded, and small peptide fragments are excreted into the urine. These fragments can be measured by Urine BETA CrossLaps® ELISA. Urine BETA CrossLaps® ELISA demonstrate a high correlation to corresponding measurement of serum samples in Serum CrossLaps® ELISA. Serum CrossLaps® ELISA has been reported to be useful for follow-up of anti-resorptive treatment of patients with metabolic bone diseases (3 17).

Principle of the procedure
The Urine BETA CrossLaps® ELISA is based on two highly specific monoclonal antibodies against the amino acid sequence of EKAHD-β-GGR, where the aspartic acid residue (D) is β-isomerized. In order to obtain a specific signal in the Urine BETA CrossLaps® ELISA, two chains of EKAHD-β-GGR must be cross-linked.

Standards, controls, or unknown urine samples are pipetted into the appropriate microtitre wells coated with streptavidin, followed by application of a mixture of a biotinylated antibody and a peroxidase-conjugated antibody. Then, a complex between CrossLaps 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 room temperature, 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, or smoke 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 Urine BETA 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
As bone resorption has a marked circadian variation primarily regulated by food intake (18), it is recommended to use second morning void urines obtained after an overnight fast.

Keep the urine sample refrigerated (2-8°C) for storage less than one week, or freeze the sample (<-18°C) for longer storage. Please note that for optimal results it is recommended to mix the samples and then centrifuge the samples (e.g. 2000g; 10 min) before testing.

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.

CrossLaps Standard (Vial A)
One vial (min. 10.0 mL/vial) of ready-for-use PBS buffered solution with protein stabiliser and preservative.

CrossLaps Standards (Vial B F)
Five vials (min. 0.3 mL/vial) of ready-for-use, CrossLaps standard in a PBS-buffered solution with protein stabiliser and preservative. The exact concentration is stated on each vial.

Controls (Vial CO 1-2)
Two vials (min. 0.5 mL/vial) CrossLaps peptide in a PBS-buffered solution with protein stabiliser and preservative. Please refer to enclosed certificate of analysis for control range.

Biotinylated Antibody (Vial no. 1)
One vial (min. 0.25 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 and preservative.

Peroxidase Conjugated Antibody (Vial no. 2)
One vial (min. 0.25 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 and preservative.

Incubation Buffer (Vial no. 3)
One vial (min. 19 mL) of a ready-for-use buffered solution with protein stabiliser, detergent, and preservative.

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 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 10-200 μL
- Distilled water
- Precision 8- or 12-channel multipipette to deliver 100 μL and 150 μL
- Microwell mixing apparatus
- Microtiter plate reader

**ASSAY PROCEDURE**

**Assay Procedure**

For optimal performance of the assay, it is important to comply with the instructions given below. Equilibrate all reagents to room temperature (18-22°C) prior to use. 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 16 wells are needed for standards and controls. Place the appropriate number of strips in the plastic frame. Store unused immunostrips in the tightly closed foil bag with desiccant capsules. Mix all reagents and samples before use (avoid foam).

1. **Preparation of the Antibody Solution:**
   ATTENTION: Prepare the Antibody Solution maximum 30 minutes before starting the assay. Mix 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.

2. **Pre-dilution of test specimens**
   All specimens (unknown samples and CO 1-2), except the standards delivered with the kit must be pre-diluted 1+3 in standard A prior to testing (e.g. 20 μL+60 μL).

3. **One Step incubation**
   Pipette 10 μL of un-diluted Standards (vial A-F), pre-diluted Controls (vial CO 1-2), and pre-diluted 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 (18-22°C) on a microtitre plate mixing apparatus (300 rpm).

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 on the mixing apparatus (300 rpm). 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 sample exceeds that of Standard F, the sample should be further 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
A quadratic curve fit should be used.

Alternatively, calculate the mean of the duplicate absorbance determinations. Construct a standard curve on graph paper by plotting the mean absorbances of the six standards A-F (ordinate) against the corresponding CrossLaps concentrations (abscissa). Determine the CrossLaps concentration of the controls and each patient sample by interpolation.

Example of results obtained:

<table>
<thead>
<tr>
<th>Standards/ Controls/ Samples</th>
<th>CrossLaps conc. (µg/L)</th>
<th>A450-650 (nm) Obs 1/ Obs 2</th>
<th>Mean A450-650 (nm)</th>
<th>Interpolated CrossLaps conc. (µg/L)</th>
<th>Conc. Corrected for 4X dilution (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard A</td>
<td>0.00</td>
<td>0.046 / 0.045</td>
<td>0.046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard B</td>
<td>1.43</td>
<td>0.164 / 0.160</td>
<td>0.162</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard C</td>
<td>4.24</td>
<td>0.402 / 0.383</td>
<td>0.393</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard D</td>
<td>8.27</td>
<td>0.742 / 0.700</td>
<td>0.721</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard E</td>
<td>16.6</td>
<td>1.461 / 1.366</td>
<td>1.414</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard F</td>
<td>23.7</td>
<td>2.027 / 1.923</td>
<td>1.975</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control CO 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control CO 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample II</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sample III</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Calculation of corrected CrossLaps value
For each sample the CrossLaps concentration (ng/mL) and the creatinine concentration should be determined. Determine the concentration of creatinine (mmol/L) in the sample using an routine enzymatic colorimetric method for clinical chemistry analysers, and perform the correction using the equation:

\[
\text{Corr. CrossLaps Value (µg/mmol)} = \frac{\text{CrossLaps (ng/mL)}}{\text{Creatinine (mM)}}
\]

Performance characteristics
Detection limit: 0.80 µg/L CrossLaps

This is the concentration corresponding to three standard deviations above the mean of 21 determinations of the blank (“CrossLaps Standard A”) multiplied by the dilution factor 4.
The precision of the Urine BETA CrossLaps® ELISA was evaluated for three urine samples. The results are summarised in the table below (the results have not been corrected by dilution factor).

<table>
<thead>
<tr>
<th>Intraassay ≤ 3.9% (n=10)</th>
<th>Interassay ≤ 6.9% (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (µg/L)</strong></td>
<td><strong>SD (µg/L)</strong></td>
</tr>
<tr>
<td>5.38</td>
<td>0.05</td>
</tr>
<tr>
<td>21.6</td>
<td>0.15</td>
</tr>
<tr>
<td>56.0</td>
<td>0.42</td>
</tr>
</tbody>
</table>

**Dilution/Linearity**

The Urine BETA CrossLaps® ELISA is linear in the range 0.20 ng/mL to 40.7 ng/mL of CrossLaps.

Urine samples with the concentration of 16.7 – 52.8 ng/mL CrossLaps were diluted with standard A and the concentration of CrossLaps were determined with Urine BETA CrossLaps® ELISA.

The data below is calculated from 3 different urine samples:

<table>
<thead>
<tr>
<th>Dilution Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine [%]</td>
</tr>
<tr>
<td>Exp. (µg/L)</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>90</td>
</tr>
<tr>
<td>80</td>
</tr>
<tr>
<td>70</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>10</td>
</tr>
</tbody>
</table>

**Expected values**

It is advisable for a laboratory to establish its own range of normal and pathological values.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Number of subjects (n)</th>
<th>Age (Years)</th>
<th>Geometric Mean Values (µg/mmol)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-menopausal women</td>
<td>25</td>
<td>40-49</td>
<td>1.66</td>
<td>0.83-3.32</td>
</tr>
<tr>
<td>Post-menopausal women</td>
<td>272</td>
<td>52-75</td>
<td>2.27</td>
<td>0.73-7.07</td>
</tr>
<tr>
<td>Males</td>
<td>25</td>
<td>50-78</td>
<td>1.46</td>
<td>0.53-4.04</td>
</tr>
</tbody>
</table>
**Day to Day Individual Variation**

The Day to Day Intra-individual Variation was assessed by analyzing urine samples (morning fasting) from 11 healthy post menopausal women at five time points over 2 weeks.

<table>
<thead>
<tr>
<th>Subject No</th>
<th>Urine BETA CrossLaps® ELISA (µg/mmol)</th>
<th>Mean (µg/mmol)</th>
<th>SD (µg/mmol)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Visit 1</td>
<td>Visit 2</td>
<td>Visit 3</td>
<td>Visit 4</td>
</tr>
<tr>
<td>1</td>
<td>3.04</td>
<td>3.17</td>
<td>3.88</td>
<td>3.50</td>
</tr>
<tr>
<td>2</td>
<td>0.96</td>
<td>1.21</td>
<td>0.99</td>
<td>1.17</td>
</tr>
<tr>
<td>3</td>
<td>1.15</td>
<td>1.04</td>
<td>1.56</td>
<td>1.78</td>
</tr>
<tr>
<td>4</td>
<td>1.33</td>
<td>1.51</td>
<td>1.65</td>
<td>0.99</td>
</tr>
<tr>
<td>5</td>
<td>1.35</td>
<td>1.60</td>
<td>1.54</td>
<td>1.37</td>
</tr>
<tr>
<td>6</td>
<td>2.40</td>
<td>1.91</td>
<td>1.88</td>
<td>1.90</td>
</tr>
<tr>
<td>7</td>
<td>1.29</td>
<td>1.04</td>
<td>1.18</td>
<td>1.32</td>
</tr>
<tr>
<td>8</td>
<td>1.18</td>
<td>1.21</td>
<td>1.20</td>
<td>1.07</td>
</tr>
<tr>
<td>9</td>
<td>5.77</td>
<td>5.67</td>
<td>4.92</td>
<td>4.78</td>
</tr>
<tr>
<td>10</td>
<td>1.42</td>
<td>1.40</td>
<td>1.28</td>
<td>1.22</td>
</tr>
<tr>
<td>11</td>
<td>4.33</td>
<td>4.47</td>
<td>5.61</td>
<td>5.14</td>
</tr>
</tbody>
</table>

**CLINICAL DATA**

Correlation between serum samples measured in Serum CrossLaps® ELISA and corresponding urine samples measured in Urine BETA CrossLaps® ELISA (n=159). The 159 samples are from pre- and postmenopausal women before and after anti-resorptive therapy, as well as males. The women were treated with Ibandronate, Raloxifene, Drospirone, or Aerodin.

**Anti-resorptive therapies**

Below is the Urine BETA CrossLaps® ELISA data [µg/mmol creatinine] from two anti-resorptive studies. The geometric mean value and the 95% confidence interval of mean are shown.

**Bisphosphonate**

- Postmenopausal women between age 55 and 75 years, more than 5 years since menopause, and a BMD of spine or femoral neck at osteoporotic level
- 20 participants received placebo (500 calcium and 400 mg vitamin D daily)
- 20 participants received active treatment (2.5 mg Ibandronate, 500 mg calcium, and 400 mg vitamin D daily)
- Treatment period 1 year
HRT

- Postmenopausal women between age 45 and 65 years, more than 2 years since menopause, and a BMD of hip within the reference range of premenopausal women
- 57 participants received placebo (500 mg calcium)
- 175 participants received active treatment (1 mg of estradiol continuously combined with 1 mg of drospirenone (n=59), 2 mg of drospirenone (n=58), or 3 mg of drospirenone (n=58))
- Treatment period 2 years

<table>
<thead>
<tr>
<th></th>
<th>Placebo group Mean [95% CI] (µg/mmol)</th>
<th>Bisphosphonate group Mean [95% CI] (µg/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>2.69 [2.11;3.42]</td>
<td>2.17 [1.49;3.14]</td>
</tr>
<tr>
<td>After 6 months of treatment</td>
<td>1.97 [1.36;2.85]</td>
<td>0.67 [0.43;1.04]</td>
</tr>
<tr>
<td>After 12 months of treatment</td>
<td>1.92 [1.39;2.65]</td>
<td>0.53 [0.29;0.95]</td>
</tr>
</tbody>
</table>

REFERENCES

7. Rosen HN. et al., Serum CTX: A new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. Calcif Tissue Int (2000); 66: 100-103.