Urine CartiLaps® (CTX-II)EIA

For the quantification of degradation products of C-terminal telopeptides of type II collagen (CTX-II) in urine
Immunodiagnostic Systems Limited 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, Immunodiagnostic Systems Limited 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 UrineCartiLaps® (CTX-II)EIA kit nor for any consequences such interpretations may cause.
INTRODUCTION

Intended use (outside the US)
The UrineCartiLaps® (CTX-II)EIA detects degradation products of C-terminal telopeptides of type II collagen. It is intended for in vitro diagnostic use as an indication of degradation of cartilage and may be used as an aid for:
• quantitative assessment of disease activity (structural damage of articular cartilage) in patients with RA and OA
• prognosis of disease activity in patients with RA and OA, and
• early assessment of long-term effect of therapy in patients with RA

Background
Disruption of the structural integrity of cartilage is the major histological finding in osteoarthritis and rheumatoid arthritis. Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (CTX-II) are being released into circulation and subsequently secreted into urine following degradation of cartilage. In urine, the CTX-II fragments can be quantified by UrineCartiLaps® (CTX-II)EIA.
The UrineCartiLaps® (CTX-II)EIA has been reported to be useful in prediction of progression of osteoarthritis (Reijman (2003), Garnero (2003)) and in other clinical and pre-clinical investigations (please refer to REFERENCES).

Principle of the procedure
UrineCartiLaps® (CTX-II)EIA (Christgau (2001) is based on the competitive binding of a monoclonal antibody to urinary fragments of type II collagen or to biotinylated, synthetic peptides bound to the surface of microtitre plates coated with streptavidin.
Initially, biotinylated, synthetic peptides are bound to the surface of streptavidin-coated wells of the microtitre plate. After washing, standards, controls, and urine samples are pipetted into the wells followed by addition of a solution of the monoclonal antibody. The wells are washed, and a solution of peroxidase-conjugated anti-mouse immunoglobulin (rabbit) is added to the wells. Following the second washing step, a chromogenic substrate is added to all wells and the colour reaction is stopped with sulphuric acid and 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.
• Do not use kit components beyond their expiration date and do not mix reagents from different lots of kits.
• Cover working area with disposable absorbent paper.
• Always use clean containers.

Warnings
For in vitro use only.
• All reagents and laboratory equipment should be handled and disposed of as if they were infectious.

Storage
Store the UrineCartiLaps® (CTX-II)EIA kit at 2-8°C upon receipt. 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, but any spot urine sample may be used. Urine samples are stable for 24 hours at 4°C and should be stored frozen (<-18°C) for longer storage. Urine samples are stable for at least 10 freeze-thaw cycles.
Prior to use, urine specimens should be shaken and sedimentation allowed for a minimum of 30 minutes.
Materials supplied
Before using the kit, please read the section on Precautions. The kit contains reagents sufficient for 96 determinations.

**Streptavidin coated microtitre plate**
Microwell strips (12x8 wells) pre-coated with streptavidin. Supplied in a plastic frame.

**CartiLaps Standard**
One vial (min. 3.0 mL) of a ready-for-use TRIS-buffered solution containing protein stabilizer, detergent, and preservative.

**CartiLaps Standards**
Five vials (min. 0.4 mL each) of ready-for-use synthetic peptide in a TRIS-buffered solution containing protein stabilizer, detergent, and preservative. The exact value of each Standard is printed on the QC Report.

**CartiLaps Control**
Two vials (min. 0.4 mL) of ready-for-use synthetic peptide in a TRIS-buffered solution containing protein stabilizer, detergent, and preservative. Please refer to enclosed QC Report for control range.

**Biotinylated CartiLaps Antigen**
One vial (min. 12.0 mL) of ready-for-use biotinylated, synthetic peptide in a PBS-buffered solution containing protein stabilizer, detergent, and preservative.

**Primary Antibody**
One vial (min. 12.0 mL) of ready-for-use monoclonal antibody in a TRIS-buffered solution containing protein stabilizer, detergent, preservative, and a red dye.

**Peroxidase Conjugated Antibody**
One vial (min. 12.0 mL) of ready-for-use peroxidase-conjugated anti-mouse immunoglobulins (rabbit) in a TRIS-buffered solution with protein stabilizer, detergent, preservative, and a blue dye.

**Substrate Solution**
One vial (min. 12.0 mL) of a ready-for-use tetramethylbenzidine (TMB) substrate in an acidic solution. Please note that the chromogenic substrate might appear slightly bluish.

**Stopping Solution**
One vial (min. 12.0 mL) of ready-for-use 0.18 M sulfuric acid.

**Washing Solution**
One vial (min. 20.0 mL) of a concentrated washing buffer with detergent and preservative.

**Sealing tape**
Adhesive film for covering wells during incubation.

**Materials required — not supplied**
- Container for preparing the Washing Solution.
- Precision micropipette to deliver 40 µL.
- Precision 8 or 12-channel multipipette to deliver 100 µL.
- Distilled water.
- Refrigerator (2-8°C).
- Microtiter plate reader for reading at both 450 nm and 650 nm.
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.

Assay Procedure

1 Pre-incubation
   Add 100 µL of **Biotinylated Urine CartiLaps Antigen** to each well, cover with sealing tape, and incubate for 30±5 minutes at room temperature (18-22°C) without shaking.

2 Washing
   Wash the immuno strips 5 times manually with 300 µL **Washing Solution** (WASHBUF 50x 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 automated washing cycle.

3 Primary incubation
   Pipette 40 µL of either **Urine CartiLaps Standards** or **Controls** or unknown urine samples into appropriate wells followed by 100 µL **Primary Antibody**. Cover the immunostrips with sealing tape and incubate for 21±3 hrs in a refrigerator (2-8°C) without shaking.

4 Washing
   See step 2.

5 Secondary incubation
   Add 100 µL of the **Peroxidase-Conjugated Antibody** solution to each well. Cover the immunostrips with sealing tape and incubate for 60±5 minutes at room temperature (18-22°C) without shaking.

6 Washing
   See step 2.

7 Incubation with chromogenic substrate solution
   Pipette 100 µL of the Substrate Solution into each well, cover the immunostrips with sealing tape and incubate for 15±2 minutes in the darkness at room temperature (18-22°C) without shaking.

8 Stopping of color reaction
   Pipette 100 µL of the Stopping Solution into each well.

9 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 is lower than **Standard 5**, the sample should be diluted in **Standard 0** 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
Construct a standard curve using a four-parametric logistic curve fit, and determine the Urine CartiLaps concentration of the Controls and each of the patient specimens by interpolation on the curve.

Example of results obtained:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Urine CartiLaps concentration (µg/L)</th>
<th>Abs\text{\textsubscript{450-650nm}} Obs 1 / Obs 2 (Abs.)</th>
<th>Mean absorbance (Abs.)</th>
<th>Interpolated Urine CartiLaps concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 0</td>
<td>0.00</td>
<td>1.914/1.879</td>
<td>1.897</td>
<td></td>
</tr>
<tr>
<td>Standard 1</td>
<td>0.73</td>
<td>1.069/1.046</td>
<td>1.058</td>
<td></td>
</tr>
<tr>
<td>Standard 2</td>
<td>1.32</td>
<td>0.822/0.806</td>
<td>0.814</td>
<td></td>
</tr>
<tr>
<td>Standard 3</td>
<td>2.50</td>
<td>0.538/0.529</td>
<td>0.534</td>
<td></td>
</tr>
<tr>
<td>Standard 4</td>
<td>5.00</td>
<td>0.317/0.300</td>
<td>0.309</td>
<td></td>
</tr>
<tr>
<td>Standard 5</td>
<td>10.06</td>
<td>0.186/0.171</td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.792/0.754</td>
<td>0.773</td>
<td>1.40</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.369/0.348</td>
<td>0.359</td>
<td>4.31</td>
</tr>
<tr>
<td>Sample 1</td>
<td></td>
<td>0.998/0.967</td>
<td>0.983</td>
<td>0.88</td>
</tr>
<tr>
<td>Sample 2</td>
<td></td>
<td>0.407/0.433</td>
<td>0.420</td>
<td>3.52</td>
</tr>
<tr>
<td>Sample 3</td>
<td></td>
<td>0.238/0.227</td>
<td>0.233</td>
<td>7.23</td>
</tr>
</tbody>
</table>

Note: The data above are for illustration only and should not be used for calculation of results.

Correction with creatinine
The CTX-II value determined as described above should be corrected with creatinine concentration.

Determine the concentration of creatinine (mmol/L) in the sample using an enzymatic colorimetric routine method for clinical chemistry analysers and perform the correction using the equation:

\[
\text{Corrected CTX-II Value (ng/mmol)} = \frac{1000 \times \text{Urine CartiLaps (µg/L)}}{\text{Creatinine (mmol/L)}}
\]

Performance characteristics

\text{Lot-to-Lot variability} < 7.0%  

The lot-to-lot variability was determined by testing three urine samples in three different lots of Urine CartiLaps\textsuperscript{®} (CTX-II)EIA

<table>
<thead>
<tr>
<th>Mean (µg/L)</th>
<th>SD (µg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW</td>
<td>0.47</td>
<td>0.033</td>
</tr>
<tr>
<td>MEDIUM</td>
<td>1.87</td>
<td>0.064</td>
</tr>
<tr>
<td>HIGH</td>
<td>5.44</td>
<td>0.136</td>
</tr>
</tbody>
</table>

\text{Detection limit} 0.20 µg/L  

The detection limit was determined to 0.20 ng/mL, which is the concentration corresponding to three standard deviations below the mean of 21 determinations of the absorbance of the Urine CartiLaps Standard 0.

\text{Precision} \leq 12.2%  

The precision was determined using ten analytical runs, each with duplicate determinations of urine samples.
Dilution/Linearity  96%
The dilution recovery of the Urine CartiLaps® (CTX-II)EIA was determined to 96%. Four urine samples were diluted in Urine CartiLaps Standard 0, the concentration of CTX-II was determined in the Urine CartiLaps® (CTX-II)EIA and the recovery calculated after correction with the dilution factor.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (µg/L)</th>
<th>Intraassay ≤7.8%</th>
<th>Interassay ≤12.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW</td>
<td>0.52</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>MEDIUM</td>
<td>1.84</td>
<td>0.08</td>
<td>0.20</td>
</tr>
<tr>
<td>HIGH</td>
<td>5.50</td>
<td>0.28</td>
<td>0.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>SD (µg/L)</th>
<th>CV (%)</th>
<th>SD (µg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW</td>
<td>0.04</td>
<td>7.8</td>
<td>0.06</td>
<td>12.2</td>
</tr>
<tr>
<td>MEDIUM</td>
<td>0.08</td>
<td>4.6</td>
<td>0.20</td>
<td>10.8</td>
</tr>
<tr>
<td>HIGH</td>
<td>0.28</td>
<td>5.2</td>
<td>0.38</td>
<td>6.9</td>
</tr>
</tbody>
</table>

DF: Dilution Factor; RC: Recovery

Interference
No interference could be detected in Urine CartiLaps® (CTX-II)EIA with addition of the following compounds into human urine samples:
- Urea up to 30 g/L
- Ibuprofen up to 50 g/L
- Creatinine up to 10 mg/L
- Acetylsalicylic acid up to 50 g/L
- Glucose up to 5 mg/L
- Paracetamol up to 50 g/L
- Ascorbic acid up to 5 mg/L
- Albumin up to 50 mg/L

Specificity
The epitope being detected in the Urine CartiLaps® (CTX-II)EIA is highly conserved and therefore the test can be applied to urine samples from most other species, including non-human primates, bovines, horses, pigs, rabbits, rats, and mice.

Expected values
It is advisable for each laboratory to establish its own range of healthy and pathological CTX-II values. As an example, the geometric mean values and 95% confidence interval (CI) for various populations are given below. For further information, please refer to the reference list at the end of these instructions.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Number of subjects</th>
<th>Age (years)</th>
<th>Mean CTX-II (ng/mmol)</th>
<th>95% CI (ng/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All women</td>
<td>459</td>
<td>20-85</td>
<td>299</td>
<td>79-1137</td>
</tr>
<tr>
<td>Pre 20-30 yrs</td>
<td>38</td>
<td>20-30</td>
<td>464</td>
<td>103-2086</td>
</tr>
<tr>
<td>Pre 30-60 yrs</td>
<td>165</td>
<td>30-59</td>
<td>200</td>
<td>65-618</td>
</tr>
<tr>
<td>Post</td>
<td>256</td>
<td>46-85</td>
<td>363</td>
<td>112-1172</td>
</tr>
<tr>
<td>Pre 48-53 yrs</td>
<td>28</td>
<td>48-53</td>
<td>164</td>
<td>66-410</td>
</tr>
<tr>
<td>Post 48-53 yrs</td>
<td>38</td>
<td>48-53</td>
<td>318</td>
<td>89-1132</td>
</tr>
<tr>
<td>All male</td>
<td>247</td>
<td>22-87</td>
<td>278</td>
<td>87-895</td>
</tr>
<tr>
<td>Male 20-30 yrs</td>
<td>27</td>
<td>20-30</td>
<td>501</td>
<td>214-1171</td>
</tr>
<tr>
<td>Male 30-60 yrs</td>
<td>141</td>
<td>30-60</td>
<td>236</td>
<td>89-628</td>
</tr>
<tr>
<td>Male &gt; 60 yrs</td>
<td>79</td>
<td>60-87</td>
<td>305</td>
<td>85-1096</td>
</tr>
</tbody>
</table>
REFERENCES

1. Ceunick F De. et al., Urinary collagen type II C-telopeptide fragments are sensitive markers of matrix metallo-proteinase dependent cartilage degradation in rat adjuvant induced arthritis. *J Rheumatol* (2003); 30: 1561-1564.


15. Mazières B. et al., Molecular markers of cartilage breakdown and synovitis are strong independent predictors of structural progression of hip osteoarthritis (OA). the ECHODIAH cohort. *ACR 2003*.

