

**PROCOLLAGEN II C-PROPEPTIDE ELISA
ITEM #60-1003-001**

(CP II ELISA)

1. OVERVIEW

1.1 PRINCIPLE OF PROCEDURE

This assay measures type II collagen carboxy propeptide (C-propeptide, also referred herein as CP II) which is cleaved from type II procollagen following the release of newly synthesized procollagen into the matrix.

This assay is for *in vitro* research use only and has been optimized for analyzing human serum, but has also been used to analyze various types of samples and species. It has been designed to be used for comparative analysis only, and is not to be used for absolute diagnostic purposes. All samples must be treated the same way and in the recommended manner.

1.2 PRINCIPLE OF THE ASSAY

This assay is a competitive immunoassay in a 96-well format utilizing bovine CP II antigen pre-coated onto the ELISA plate. Bovine CP II standards or unknown samples are added to a polypropylene mixing plate, followed by a rabbit polyclonal antiserum specific for CP II. This mixture is pre-incubated to allow the antibodies to bind to the free CP II antigen. The pre-incubated samples are then transferred from the polypropylene mixing plate onto the CP II ELISA plate and incubated to allow the antibodies to bind either to the CP II coated on the bottom of the plate, to the CP II standards or to the endogenous epitope in serum samples. After washing the plate, goat anti-rabbit horseradish peroxidase (GAR-HRP) conjugate is added, which binds to any rabbit antibody on the plate. After washing the plate again, Tetramethylbenzidine (TMB) is added and then HRP degrades H₂O₂ and oxidizes TMB to form a blue product. The reaction is stopped and the signal amplified with an acid, which converts the product from a blue to a yellow colour that can be quantified at 450 nm. The optical density (OD) at 450 nm is inversely proportional to the amount of CP II epitope present in the samples.

1.3 BACKGROUND

Hyaline cartilage is composed of an extracellular matrix containing mainly type II collagen fibrils and a large aggregating proteoglycan called aggrecan. In arthritic joints, the collagen matrix is disrupted and the proteoglycan content decreased. This change in matrix composition involves excessive matrix collagen degradation and changes in collagen synthesis revealed by extraction of CPII (.Squires et al., 2003; Aurich et al., 2005). Type II collagen is synthesized as a procollagen which contains amino and carboxy propeptides. These are removed extracellularly by amino and carboxy proteinases as collagen are incorporated into the collagen fibril. The half-life of the C-propeptide is about 18 hr (Nelson *et al.*, 1998). CP II content directly correlates with type II collagen synthesis (Nelson *et al.*, 1998).

CP II content increases in synovial fluid following knee injury and in primary OA (Lohmander, *et al.*, 1996), and decreases in OA serum (Nelson *et al.*, 1998). When used alone or in conjunction with other collagen degradation biomarkers, it can be used to distinguish preradiographic and established knee OA from no OA (Cibere et al., 2009), OA progression (Cahue et al., 2007) and to identify subsets of hip OA (Conrozier et al., 2006:2008). CPII increases in serum RA (Nelson *et al.*, 1998; Mansson *et al.*, 1995) and can be used with collagen degradation markers to detect early (1 month) responses to therapy predictive of radiologic progression at 1 year (Mullan et al., 2007).

In certain animal models of OA serum, CPII assay can be used in conjunction with C2C assay to identify differences in knee (stifle joint) OA progression in guinea pigs (Huebner et Kraus, 2006). Osteochondral lesions in horses have been studied using CPII assays (Frisbie et al., 1999).

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2. PROTOCOL

Bring CP II ELISA kit to room temperature before use (20-25°C). Wear gloves for all steps of the protocol.

Note 1: Gently centrifuge small tubes before opening to ensure proper recovery of reagents.

Note 2: Reagents and samples must be gently vortexed just before use.

- Make standards according to Table 1.
- Add 50 µL of CP II standards and samples to the polypropylene mixing plate. Serum samples should be diluted 1/2 with Buffer III. This can be done directly on the plate (25 µL serum + 25 µL Buffer III). *Note: CP II binds to glass, make up CP II Std. in polypropylene tubes.*
- Add 50 µL of CP II Antibody diluted in Assay Buffer to all wells of the polypropylene mixing plate. (One Plate: 50 µL of CP II Antibody + 6 mL Assay Buffer).
- Pre-incubate the polypropylene mixing plate on a high speed Titre Plate Shaker at 600-700 rpm, covered, for 60 minutes (\pm 2 minutes) at room temperature (20-25°C).
- Remove the CP II ELISA plate from the foil pouch. Transfer 80 uL of antigen-antibody mixture from each well of the polypropylene mixing plate to the corresponding well of the ELISA plate using a multi-channel pipette.
- Incubate the covered ELISA plate on a high speed Titre Plate Shaker at 600-700 rpm for 2 hours (\pm 8 minutes) at room temperature (20-25°C).
- Wash the ELISA plate 6 times with 350 µL/well diluted Wash buffer (1x) without soak time. Blot the plate dry on towels after the last wash.
- Add 100 µL/well of GAR-HRP conjugate diluted in Buffer III. (One Plate: 50 µL of GAR-HRP / 11 mL Buffer III).
- Incubate the covered ELISA plate on high speed Titre Plate Shaker at 600-700 rpm for 30 minutes (\pm 2 minutes) at room temperature (20-25°C).
- Wash the ELISA plate 6 times with 350 µL/well diluted Wash buffer (1x) without soak time. Blot the plate dry on towels after the last wash.
- Add 100 µL of TMB/well.
- Incubate the covered ELISA plate on a high speed Titre Plate Shaker at 600-700 rpm for approximately 30 minutes at room temperature (20-25°C). Monitor colour development at 630 nm if necessary.
- Stop reaction by adding 100 µL of Stop Solution/well.
- Read plate at 450 nm within 10 minutes.

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3. EQUIPMENT & MATERIALS

Required Equipment not Supplied

- ELISA plate reader that can perform dual wavelength (OD at 450 nm and reference at 630 nm) would be preferred.
- Precision pipettes to deliver 25-1000 µL
- 8 channel adjustable pipette
- Vortexer
- Titre plate shaker capable of 600-700 revolutions per minute (rpm)
- Automatic ELISA plate washer.

Supplied Materials (Store all reagents at 2-8°C unless indicated otherwise until the expiration date on the label.)

- CP II ELISA plate. ELISA plate coated with bovine CP II within a stabilizing matrix including BSA. Packaged dry in foil pouch with desiccant sachet.
- Polypropylene mixing plate.
- Bovine CP II Standard Stock (10 µg/mL). Make up 7 levels of Standards from this stock in Buffer III (Stds. 0, 50, 100, 250, 500, 1000, and 2000 ng/mL).
- CP II Antibodies. Primary rabbit polyclonal antibodies to CP II diluted in a protein based buffer containing BSA and a non-mercury preservative.
- Assay Buffer. Protein based buffer containing BSA, goat normal serum and a non-mercury preservative. For dilution of primary antibody.
- Buffer III. Protein based buffer containing BSA and a non-mercury preservative. For dilution of standards, samples and secondary antibody.
- GAR-HRP Conjugate. Secondary antibody in a protein based buffer containing BSA and a non-mercury preservative.
- Wash buffer (25X). Contains buffered saline with a non-ionic detergent. Prepare wash buffer as needed by dilution in deionized water. e.g. For 1000 ml of wash buffer solution, take 40 ml of the concentrated Wash buffer (25X) and complete to 1000 ml with deionized water. **Wash buffer contains no preservative. Store at 4°C after dilution for no more than one week.**
- TMB. A solution of tetramethylbenzidine (TMB) in a proprietary buffer.
- Stop Solution. Contains 0.2 M sulfuric acid.

CHEMICAL HAZARD

Avoid contact with reagents containing sulfuric acid. In case of contact with any of these reagents, wash thoroughly with water.

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4. PROCEDURAL NOTES

- The following recommendations must be followed in order to achieve the most reliable results:
 - Collect your samples, freeze them at -70°C and, if applicable, run all timepoints at the same time (refer to section 5 for details).
 - Analyze all timepoints from the same individual on one plate and group your samples as much as possible.
 - Use the same kit lot number for the entire study.
 - The results from the timepoints of one individual should be compared in proportion to each other in order to evaluate the trends over time.
 - The best results will be obtained by analyzing test samples in triplicate.
 - If a sample must be re-tested, perform the re-test with associated timepoints, i.e. if one timepoint is unexpected, retest this timepoint along with at least one or two additional timepoints from the same individual.

- We recommend centrifuging small tubes to maximize recovery of reagents. Reagents are overfilled to ensure required volume can be recovered.

- All reagents and samples must be gently vortexed just before use.

- Stock reagents should be diluted shortly before use. Final dilutions of antibodies and conjugates are not stable for more than one day. Final dilutions of standards are stable for up to 1 week at 2-8°C.

- A set of standards (in duplicate) must be included on each plate.

- Buffer III is provided to make up Standards for the measurement of serum and plasma samples. For other samples an equivalent matrix should be used to make up standards. For example, for tissue culture, supernatant medium may be used.

- Samples should ideally be analyzed as triplicates. All samples to be compared must be subject to the same treatment.

- Avoid microbial contamination of reagents, especially the stock antibodies and conjugates.

- GAR-HRP is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic hydrocarbons often found in laboratory water supplies.

- TMB is highly sensitive to light and should not be exposed to silica based materials such as glass or metal.

5. SPECIMEN COLLECTION & STORAGE

Blood should be collected without anticoagulants and in such a way as to avoid haemolysis. Ideally, serum samples should be aliquoted, immediately frozen and stored at -70 °C following centrifugation (600 g/3000 rpm, 10 min) to remove particulate material and any clots. Aliquots of serum can be stored at -70 °C for more than a year. Repeated freeze thaw cycles should be avoided.

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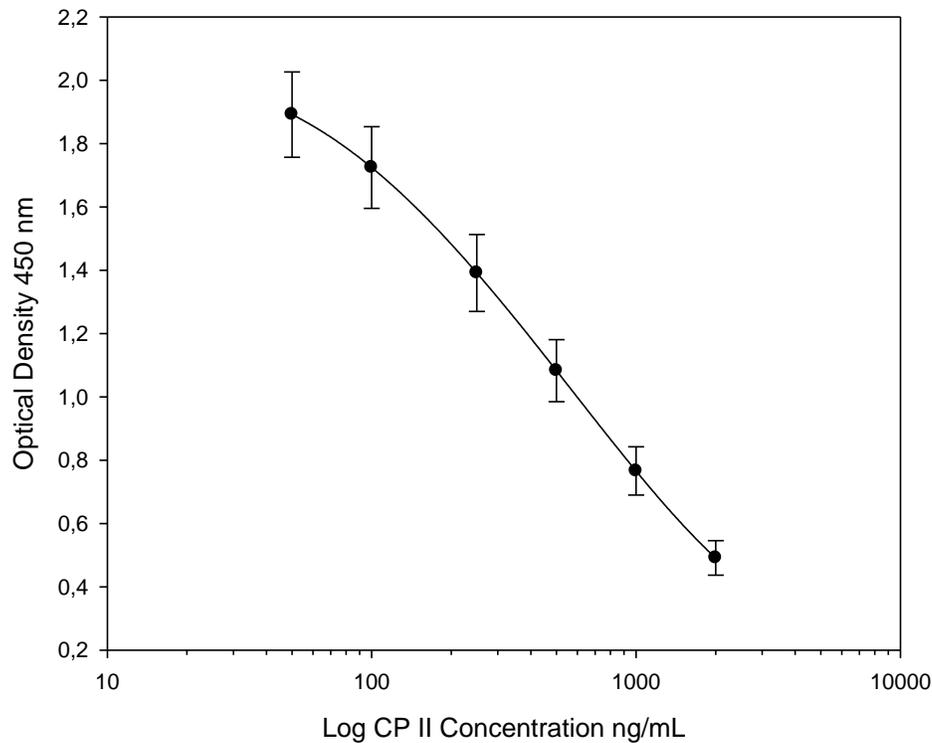
6. RESULTS

- Plot mean standard absorbance readings on the y-axis versus log concentration on the x-axis using a logistic equation (4 or 5 parameter). Note that the standard curve is sigmoidal and not linear. Ideally, appropriate curve fitting software should be used.
- Any sample reading higher than the highest standard should be diluted with Buffer III and re-assayed. Any sample reading lower than the lowest standard should be re-assayed or discarded.

Table 1

CP II Working Standards Dilution Table				
Std #	Concentration	Volume Stock/ Std	Vol Buffer III	Dilution
1)	2000 ng/mL	100 µL of 10 µg/mL Stock	400 µL	1:5
2)	1000 ng/mL	50 µL of 10 µg/mL Stock	450 µL	1:10
3)	500 ng/mL	25 µL of 10 µg/mL Stock	475 µL	1:20
4)	250 ng/mL	60 µL of Std 1	420 µL	1:8
5)	100 ng/mL	50 µL of Std 2	450 µL	1:10
6)	50 ng/mL	50 µL of Std 3	450 µL	1:10
7)	0 ng/mL	-----	500 µL	

Representative CP II Standard Curve



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7. SPECIFIC PERFORMANCE CHARACTERISTICS

7.1 CROSS REACTIVITY AND SPECIFICITY

The C-propeptide of type II collagen (CP II) is composed of three identical 35 KDa chains covalently bonded by inter-chain disulfide bonds. The CP II non-helical domain is cleaved from the type II procollagen molecule as it forms fibrils in the cartilage matrix. The specificity of the polyclonal antibodies to CP II was confirmed by western blotting using purified CP II, and recombinant type II collagen digested with bacterial collagenase. In western blots/autoradiography of S³⁵ labeled CP II, the antibodies recognize CP II and at least one natural degradation product, which lack 10 aa at the amino terminus.

Historically, this assay has broad cross reactivity and recognizes human, as well as monkey, horse, bovine, dog, rat, mouse and guinea pig CP II. However, the new antibodies have not been tested with these or any other species. We strongly recommend testing the performance of the kit in your conditions.

This assay is suitable for serum. It has also been used by Ibox customers for synovial fluid, plasma, culture medium and guanidine HCL extracted cartilage.

This ELISA utilizes rabbit antibodies. Testing of samples containing high concentrations of rabbit antibodies (such as rabbit serum) can result in high background interference.

End-users should conduct their own validation for these and any other samples.

8. REFERENCES

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