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 and 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 antibody binding 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 of the immobilized CPII on the plate, to the CP II standards or to the endogenous epitope in serum samples. After washing the CPII ELISA plate, conjugated goat anti-rabbit horseradish peroxidase (GAR-HRP) is added, which binds to any rabbit antibody on the plate. After washing the CPII ELISA plate again, Tetramethylbenzidine substrate (TMB) is added to each well which react with HRP 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 neoepitope present in the sample.

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*. 2007;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 J.L and Kraus V.B, 2006). Osteochondral lesions in horses have been studied using CPII assays (Frisbie *et al.* 1999; Laverty *et al.* 2000). CPII levels increases in SFs above baseline after 14 days in a CCL preclinical model in dog OA

(Trumble *et al.* 2003). Study of chronic lameness in Asian elephant showed decreased CPII level in lame animals (Kilgallon *et al,* 2015).

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2. PROTOCOL

Bring CP II ELISA kit to room temperature (20-25°C) for at least 30 minutes before use.
Wear gloves for all steps of the protocol.

Note 1: Gently centrifuge micro 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. ***Note****: CP II binds to glass*; *make up CP II Standards in polypropylene tubes*.
- 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).
- 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 covered polypropylene mixing plate on a high speed microplate shaker at 700 ± 10 rpm , for 60 minutes (± 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 wells of the ELISA plate using a multi-channel pipette.
- Incubate the covered ELISA plate on a high speed microplate shaker 700 ± 10 rpm for 2 hours (± 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 absorbent paper 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 microplate shaker at 700 ± 10 rpm for 30 minutes (± 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 absorbent paper after the last wash.
- Add 100 µL/well of TMB.
- Incubate the covered ELISA plate on a high speed microplate shaker at 700 ± 10 rpm for 30 minutes at room temperature (20-25°C). Stop the colour development by adding 100 µL/well of Stop Solution.
- Read plate absorbance at 450 nm, preferably with the reference wavelength set to 630 nm within 10 minutes.

3. EQUIPMENT & MATERIALS

Material required - not supplied

- Deionized water
- Graduated cylinder for preparation of wash solution
- Precision pipette to deliver 20 – 1000 µL with disposable tips
- Precision multichannel pipette to deliver 100 µL with disposable tips
- Vortex mixer
- Orbital microplate shaker capable of 700 revolutions per minute (rpm)
- Automated microplate washer
- Microtiter plate reader with dual wavelength reading 450 nm and 630 nm (Reference filter: 590 – 650 nm)
- Software capable of calculating a 4 or 5-parameter curve fit for data analysis.

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 purified 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 2-8°C after dilution for no more than one week***.
- TMB. Ready-for-use tetramethylbenzidine (TMB) solution in a proprietary buffer..
- Stop Solution. Ready-for-use 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.

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 micro 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.

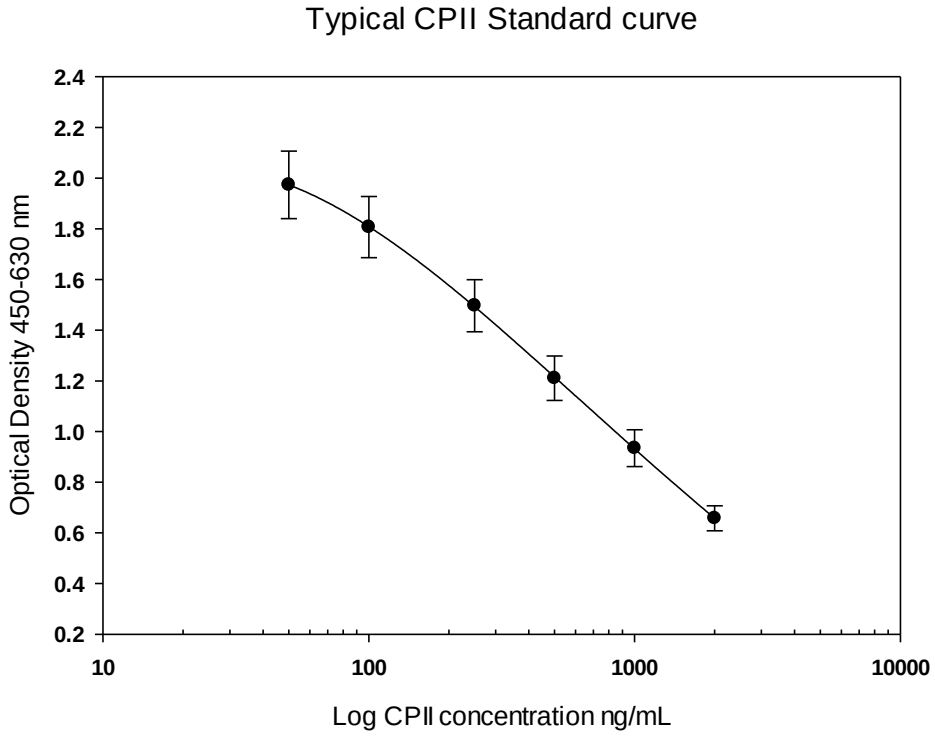
6. RESULTS

- Plot mean standard absorbance readings on the y-axis versus log concentration of Standards 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				
Standard	**Concentration**	**Volume Stock**	**Volume Buffer III**	**Dilution**
Std-1	2000 ng/mL	100 µL of 10 µg/mL Stock	400 µL	1:5
Std-2	1000 ng/mL	50 µL of 10 µg/mL Stock	450 µL	1:10
Std-3	500 ng/mL	25 µL of 10 µg/mL Stock	475 µL	1:20
Std-4	250 ng/mL	60 µL of Std-1	420 µL	1:8
Std-5	100 ng/mL	50 µL of Std-2	450 µL	1:10
Std-6	50 ng/mL	50 µL of Std-3	450 µL	1:10
Std-7	0 ng/mL	-----	500 µL	



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 pro-collagen 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 S^{35} 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 IBEX 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.

REFERENCES

1. Aurich M.,M., Squires G.,Reiner A., Mollenhauer JA., Kuettner KE., Poole AR. and Cole AA. (2005). Differential matrix degradation and turnover in early cartilage lesions of human knee and ankle joints. Arthritis Rheum. 52: 112-119.
2. Cahue S., Sharma l., Dunlop D., Ionescu M., Song J., Lobanok T., King L. and Poole AR. (2007). The ratio of type II collagen breakdown to synthesis and its relationship with the progression of knee osteoarthritis. Osteoarthritis Cartilage 15: 819-823.
3. Cibere J, Zhang H, Garnero P, Poole AR, Lobanok T. et al. (2009). Association of biomarkers with with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based cohort. Arthritis Rheum 60: 1372-1380.
4. Conrozier T.,Ferrand F., Poole AR., Verret C., Mathieu P., Ionescu M. et al. (2007) Differences in biomarkers of type II collagen in atrophic and hypertrophic osteoarthritis of the hip: implications for the differing pathologies.Osteoarthritis Cartilage 25: 462-467.
5. Conrozier T., Poole AR., Ferrand F., Mathieu P., Vincent F., Piperno M et al. (2008). Serum concentrations of type II collagen biomarkers (C2C, C1,2C and CPII) suggest different pathophysiologies in patients with hip osteoarthritis. Clin. Exp. Rheumatol. 26: 430-435.
6. Frisbie D.D., Ray C.S., Ionescu M., Poole R.A., Chapman P.L. and McIlwraith W. (1999). Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy propeptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. AJVR 60:305-309.
7. Huebner JL, Kraus VB. (2006). Assessment of the utility of biomarkers of osteoarthritis in the guinea pig. Osteoarthritis Cartilage. 14: 923-930.
8. Kilgallon CP, Larsen S, Wong A, Yellowley C. (2015). Analysis of a collagen II degradation protein C2C and a collagen II formation protein CPII in serum of Asian elephants (Elephas maximus). J Zoo Wild Led 46: 146-149.
9. Laverty S, Ionescu M, Marcoux M, Boure L, Doize B, Poole AR. (2000). Alterations in cartilage type II procollagen and aggrecan contents in synovial fluid in equine osteochondrosis. J Orthop Res 18: 399-405.

10. Lohmander S.L., Yoshihara Y., Roos H., Kobayashi T., Yamada H. and M. Shinmei. (1996) Procollagen II C-propeptide in joint fluid: Changes in concentration with age, time after knee injury and osteoarthritis. J. Rheumatol. 23: 1765-1769.

11. Månsson B., Carey D., Alini M., Ionescu M., Rosenberg L.C., Poole A.R., Heinegård D. and Saxne T. (1995). Cartilage and bone metabolism in rheumatoid arthritis: difference between rapid and slow progression of disease identified by serum markers of cartilage metabolism. J.Clin.Invest. 95:1071-1078.

12. Mullan RH., Matthews C., Bresnihan B., FitzGerald O., King L., Poole AR., Fearon U. and Veale DJ. (2007). Early changes in serum type II collagen biomarkers predict radiographic progression at one year in inflammatory arthritis patients after biologic therapy. Arthritis Rheum. 56: 2919-2928.

13. Nelson F., Dalhberg L., Laverty S., Reiner A., Pidoux I., Ionescu M., Fraser G.L., Brooks E., Tanzer M., Rosenberg L.C., Dieppe P. and Poole A.R. (1998). Evidence for altered synthesis of type II collagen in patients with osteoarthritis. J. Clin. Invest. 102: 2115-2125.

14. Squires G.,Okouneff S., Ionescu M.and Poole AR. (2003). Pathobiology of focal lesion development in aging human articular cartilage reveals molecular matrix changes characteristic of osteoarthritis. Arthritis Rheum. 48: 1261-1270.

15. Trumble TN, Billinghurst RC and McIlwraith CW. (2004). Correlation of prostaglandin E2 concentrations in synovial fluid with ground reaction forces and clinical variables for pain or inflammation in dogs with osteoarthritis induced by transection of the cranial cruciate ligament. Am J Vet Res. 65(9):1269-75.

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