COLLAGEN TYPE I AND II CLEAVAGE ELISA
ITEM #60-1002-001

(C1,2C ELISA)

1. OVERVIEW

1.1 PRINCIPLE OF PROCEDURE

This assay measures the carboxy terminus neoepitope of the three-quarter length piece (C1,2C or Col 2 ¾C Short) generated by the cleavage of types I and II collagens by collagenases.
This assay is for *in vitro* research use only and has been optimized for analyzing human serum. It has also been used to analyze various types of samples and species.this assay 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 plate format using a synthetic peptide representing the neoepitope, which is recognized by rabbit polyclonal C1,2C antibodies (C1,2C antibody formerly called the Col 2 ¾C short antibody) (Billinghurst *et al*., 1997, 2000; Dahlberg *et al*., 2000). The synthetic peptide is conjugated to a protein and pre-coated onto the C1,2C ELISA plate. C1,2C peptide standards and unknown serum samples are added to a polypropylene mixing plate, followed by the C1,2C antibody. This mixture is pre-incubated to allow antibody binding to the free C1,2C peptide. Then, the pre-incubated samples are transferred from the polypropylene mixing plate onto the C1,2C ELISA plate and incubated to allow the antibody to bind either to the immobilized peptide on the plate, to the C1,2C standards or to the endogenous neoepitope in serum samples. After washing the C1,2C ELISA plate, conjugated goat anti-rabbit horseradish peroxidase (GAR-HRP) is added, which binds to any C1,2C antibody on the coated plate. After washing the C1,2C 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 C1,2C epitope present in the samples.

1.3 BACKGROUND

Joint cartilage, like most other connective tissues, is composed of a collagen-based fibrillar network complexed to proteoglycans. Collagen type II is found in cartilages and discs. These collagens consist of 3 α-chains arranged in a triple helix that form fibrils. In cartilage, the large aggregating proteoglycan aggrecan resides within this fibrillar meshwork. In arthritis, type II collagen is extensively cleaved and destroyed by the activity of collagenases namely MMP-1, MMP-8 and MMP-13, which results in loss of type II collagen and increase of cleavage product in serum levels (Billinghurst *et al*, 1997). The neoepitope that is created at the C-terminus of the primary cleavage site is recognized by the C1,2C antibodies (Billinghurst *et al*., 1997).

The C1,2C neoepitope is resistant to degradation by chymotrypsin and can therefore be extracted from cartilage and other tissues, allowing *in situ* collagen degradation to be quantified. This neoepitope is significantly increased in early and advanced human osteoarthritic (OA) cartilage lesions compared to normal adult cartilages (Billinghurst *et al*., 1997; Squires *et al,* 2003; Aurich *et al,* 2005).
Human OA cartilages *in vitro* release significantly more of this neoepitope into the medium compared to normal. This increase could be inhibited in culture in a majority of OA cartilages by an MMP inhibitor (Dahlberg *et al*., 2000). In bovine articular and nasal cartilage explants, interleukin-1 stimulates an increase in cartilage degradation and corresponding increase in C1,2C neoepitope. This increase can be inhibited in a dose-dependant manner by selective inhibitors of type II collagen cleavage (MMP-8 and/or MMP-13 inhibitors) in articular cartilage and partially inhibited in nasal cartilage (Billinghurst *et al*., 2000; Baragi *et al*, 2009).
Type I collagen is similarly cleaved by these collagenases and this cleavage can also be detected with the C1,2C antibodies (Sukhova *et al*., 1999).

The use of C1,2C as a urine biomarker has revealed its value in studies of pre-radiological OA onset in knee OA (Cibere *et al*, 2009). Serum C1,2C is of value in inflammatory arthritis for the study of disease progression (Verstappen *et al*, 2006) and early responses to therapy in rheumatoid arthritis (Mullan *et al*, 2007) and of joint hemophilic arthropathy (Jansen *et al*, 2009).
Increase in serum C2C and C1,2C, but not CPII and CS846, are associated with radiographic knee OA (Kong *et al,* 2006) reflecting the increased cleavage of type II collagen by collagenases viewed *in situ* in diseased joints. It was observed that the ratios of C2C: CPII and C1,2C: CPII both showed an almost significant relationship to radiographic assessment of disease progression not seen with the individual biomarkers (Cahue *et al,* 2007).
C1,2C biomarker was found to be one of the most discriminatory biomarkers for the study of hand OA. (Ramonda *et al,* 2013). Patients at risk for OA following knee ACL injury, with or without abnormal joint space width (JSW) reflective of cartilage loss showed an increased ratio of urine C1,2C : serum CPII compared to controls after 1 and 4 years (Tourville *et al,* 2013). A recent randomized double-blind placebo-controlled clinical trial evaluated the chondroprotective action of salmon nasal cartilage proteoglycan in individuals with knee joint discomfort but without diagnosis of knee osteoarthritis (Tomonoga *et al,* 2017). C1,2C levels dropped significantly in the treatment group compared with the placebo group following a 16 week intervention of subjects with high levels of knee pain and physical dysfunction and subjects with constant knee pain. The C1,2C : PIICP ratio decreased in the treatment group, whereas it increased a little in the placebo group following treatment. Like the C2C assay, the C1,2C assay has also been used to study lung disease (Armstrong *et al,* 1999).

Explant studies demonstrated increased degradation of type II collagen in Osteochondrosis in (Laverty *et al,* 2002).
In foals serum C1,2C was indicative of OCD severity at 5 months of age (Billinghurst *et al,* 2004). In those with lesions at 11 months of age, severity correlated negatively with C1,2C and positively with CPII.

2. PROTOCOL

Bring C1,2C 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.
- Add 50 µL of C1,2C Standard and samples to appropriate wells of polypropylene mixing plate. Serum samples should be diluted 1/2 with Buffer I. This can be done directly on the plate (25 µL serum + 25 µL Buffer I).
- Add 50 µL of C1,2C Antibody diluted in Buffer II to all wells of the polypropylene mixing plate (One Plate: 15 µL of C1,2C Antibody + 6 mL Buffer II).
- Pre-incubate the polypropylene mixing plate on a high speed microplate shaker at 700 ± 10 rpm for 30 minutes (± 2 minutes) at room temperature (20-25°C).
- Remove the C1,2C ELISA plate from the foil bag. Transfer 80 µL 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 ELISA plate on a high speed microplate shaker at 700 ± 10 rpm for 1 hour (± 2 minutes) at room temperature (20-25°C).
- Wash the ELISA Plate 6-times with 1x diluted Wash solution (300 µL/well). Blot the plate dry on absorbent paper after the last wash.
- Add 100 µL/well GAR-HRP conjugate diluted in Buffer III.
  (One Plate: 22µL of GAR-HRP + 11 mL Buffer III).
- Incubate the ELISA plate on a 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 1x diluted Wash solution (300 µL/well). Blot the plate dry on absorbent paper after the last wash.
- Add 100 µL/well of TMB.
- Incubate the 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.)

- C1,2C Standard Stock at 100 µg/mL. Make up 7 levels of Standards from this stock in Buffer I. (Stds.: 0, 0.03, 0.1, 0.3, 1, 3.0, and 10 µg/mL)
- C1,2C ELISA plate. ELISA plate coated with C1,2C peptide conjugate within a stabilizing matrix including BSA. Packaged dry in a foil pouch with desiccant sachet.
- Polypropylene mixing plate.
- Buffer I. Protein based buffer containing BSA and a non-mercury preservative. For dilution of Standards and samples.
- Buffer II. Protein based buffer containing BSA 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 secondary antibody.
- C1,2C antibody. Primary polyclonal rabbit antibody in a protein based buffer containing BSA and a non-mercury preservative.
- 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 antibody and HRP conjugate 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 I is provided to make up Standards for the measurement of serum and plasma samples. Ideally, an equivalent matrix to the sample 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 (600g / 3000rpm, 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.

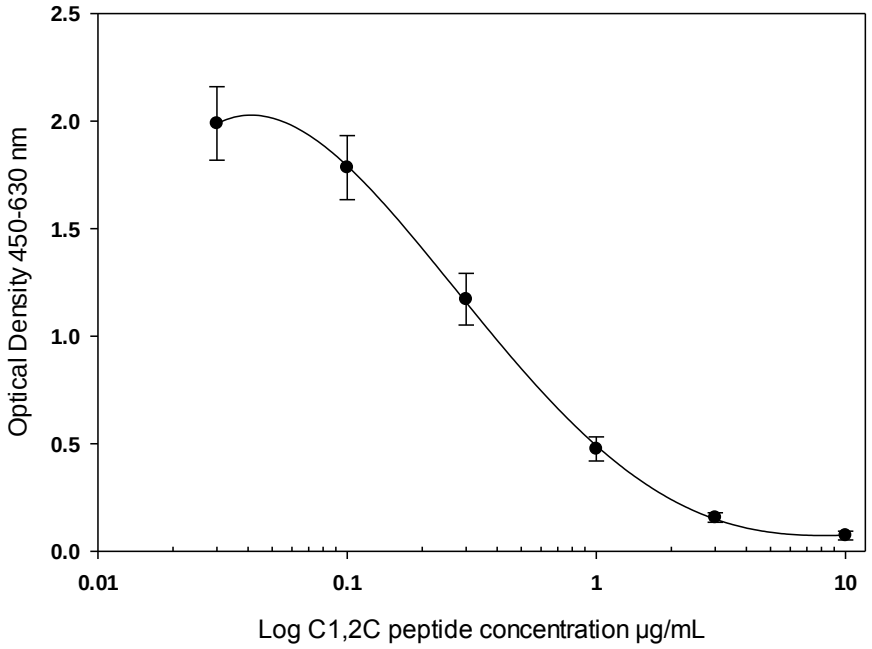
6. RESULTS

- 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 I and re-assayed. Any sample reading lower than the lowest standard should be re-assayed or discarded.

Table 1

C1,2C Working Standards Dilution Table				
Standard	**Concentration**	**Volume Stock**	**Volume Buffer I**	**Dilution**
Std 1	10 µg/mL	100 µL of 100 µg/mL Stock	900 µL	1:10
Std 2	3.0 µg/mL	30 µL of 100 µg/mL Stock	970 µL	1:33
Std 3	1 µg/mL	100 µL of Std 1	900 µL	1:10
Std 4	0.30 µg/mL	100 µL of Std 2	900 µL	1:10
Std 5	0.1 µg/mL	100 µL of Std 3	900 µL	1:10
Std 6	0.03 µg/mL	100 µL of Std 4	900 µL	1:10
Std 7	0 µg/mL	-------	1000 µL	

Typical C1,2C Standard curve



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7. SPECIFIC PERFORMANCE CHARACTERISTICS

7.1 CROSS REACTIVITY AND SPECIFICITY

The C1,2C antibodies have high affinity for collagenase-cleaved human type II collagen. More specifically, they recognize an α-chain fragment containing an approximate 8 amino acid sequence on the carboxy terminus of the three-quarter length piece produced by collagenase (MMP-1, MMP-8 and MMP-13) cleavage of type II collagen. These antibodies demonstrate affinity for similarly cleaved human type I collagen α-chains. There is negligible reactivity with uncleaved triple-helical and heat denatured human types I and II collagen and intact or cleaved type II collagen α-chains.

These antibodies have broad cross reactivity and recognize human, as well as monkey, horse, bovine, dog, rat and mouse C1,2C. The antibodies have not been tested in 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 in tissues, urine, synovial fluid, cells and culture medium and bronchoalveolar lavage. It may be used for tissues that contain type I or II collagen degradation products including cartilage, skin, ligaments, tendons, bone, cardiovacular tissues, lung and intervertebral disc to name a few. Tissues must first be digested with α-chymotrypsin to solubilize the cleavage epitope (Billinghurst *et al*., 1997).

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

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

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