

**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 neopeptide of the $\frac{3}{4}$ piece (C1,2C or Col 2 $\frac{3}{4}$ C 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, 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 plate format using a synthetic peptide representing the neopeptide, which is recognized by rabbit polyclonal C1,2C antibodies (C1,2C Antibody formerly called the Col 2 $\frac{3}{4}$ C Short antibody) (Billingham *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 or unknown serum samples are added to a polypropylene mixing plate, followed by the C1,2C antibody. This mixture is pre-incubated to allow the antibody to bind 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 C1,2C coating conjugate on the plate, to the C1,2C standards or to the endogenous neopeptide in serum samples. After washing the plate, goat anti-rabbit horseradish peroxidase (GAR-HRP) conjugate is added, which binds to any C1,2C antibody on the plate. After washing the plate again, Tetra-methylbenzidine (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 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, which results in loss of type II collagen (Poole *et al.*, 1995; Billingham *et al.*, 1997; Hollander *et al.*, 1994). Different collagenases, namely MMP-1, MMP-8 and MMP-13, can cleave type II collagen. The neopeptide that is created at the C terminus of the primary cleavage site is recognized by the C1,2C antibodies (Billingham *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). Like the C2C assay, the C1,2C assay has also been used to study lung disease (Armstrong *et al.*, 1999).

2. PROTOCOL

Bring C1,2C ELISA kit to room temperature (20-25°C) before use. 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 C1,2C Standard and samples to appropriate wells of polypropylene mixing plate. Serum samples should be diluted ½ 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 Titre Plate Shaker at 600-700 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 Titre Plate Shaker at 600-700 rpm for 1 hour (± 5 minutes) at room temperature (20-25°C).
- Wash the ELISA Plate 6 times. Blot the plate dry on towels 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 Titre Plate Shaker at 600-700 rpm for 30 minutes (± 2 minutes) at room temperature (20-25°C).
- Wash the ELISA Plate 6 times. Blot the plate dry on towels after the last wash.
- Add 100 µL/well of TMB.
- Incubate the 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.

3. EQUIPMENT & MATERIALS

Required Equipment not Supplied

- ELISA plate reader that can perform dual wavelengths (OD at 450 nm and reference at 630 nm) would be preferred.
- Precision pipettes to deliver 15-1000 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.)

- C1,2C Standard Stock. 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.
- 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 4°C after dilution for no more than one week.**
- TMB. A solution of tetramethylbenzidine (TMB) in 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.

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 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.

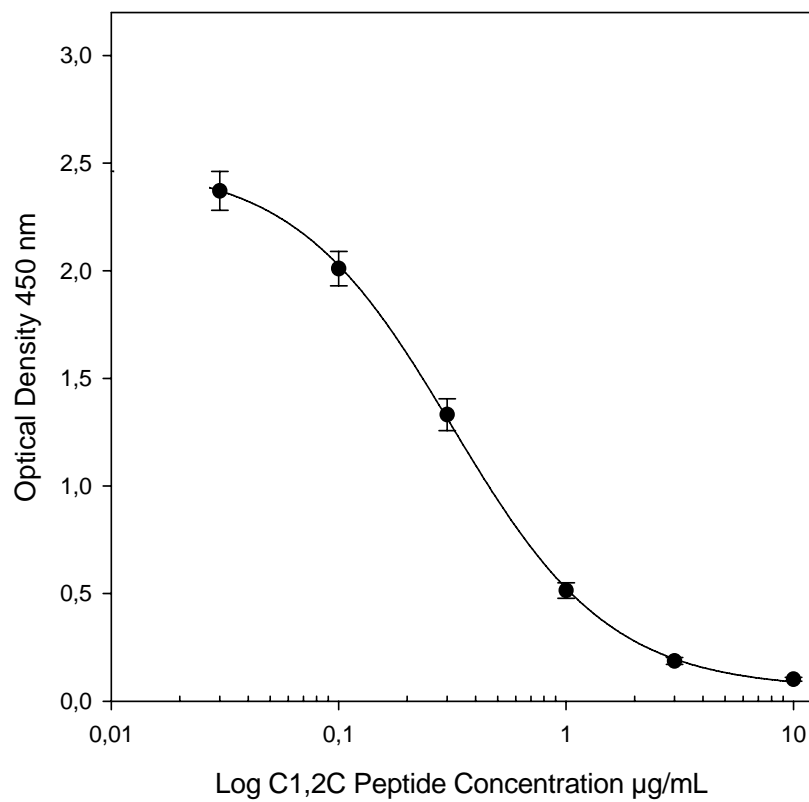
6. RESULTS

- Plot mean standard absorbance readings on the y-axis versus log concentration 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				
Std #	Concentration	Volume Stock	Vol. Buffer I	Dilution
1)	10 µg/mL	100 µL of 100 µg/mL Stock	900 µL	1:10
2)	3.0 µg/mL	30 µL of 100 µg/mL Stock	970 µL	1:33
3)	1 µg/mL	100 µL of Std 1	900 µL	1:10
4)	0.30 µg/mL	100 µL of Std 2	900 µL	1:10
5)	0.1 µg/mL	100 µL of Std 3	900 µL	1:10
6)	0.03 µg/mL	100 µL of Std 4	900 µL	1:10
7)	0 µg/mL	-----	1000 µL	

Representative 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, cardiovascular 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.

7. REFERENCES

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