Collagen Type II Cleavage ELISA
ITEM #60-1001-001

(C2C ELISA)

1. OVERVIEW

1.1 PRINCIPLE OF PROCEDURE

The C2C assay measures a neoepitope created by the cleavage of type II collagen by collagenases. This neoepitope that is created at the carboxy terminus of the three-quarter length piece of the primary cleavage site is recognized by the C2C monoclonal antibody which is specific for type II collagen.
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 mouse monoclonal antibody and a synthetic peptide to represent the neoepitope (Poole *et al*, 2004). This synthetic peptide is conjugated to a protein and pre-coated onto the C2C ELISA plate. C2C peptide standards and unknown serum samples are added to a polypropylene mixing plate, followed by specific mouse IgG antibody (C2C antibody formerly called the col 2 3/4 long antibody). This mixture is pre-incubated to allow antibody binding to the free C2C peptide. The pre-incubated samples are then transferred from the polypropylene mixing plate onto the C2C ELISA plate and incubated to allow the antibody to bind either to the immobilized peptide on the plate, to the C2C standards or to the endogenous neoepitope in serum samples. After washing the C2C ELISA plate, conjugated goat anti-mouse horseradish peroxidase (GAM-HRP) is added, which binds to any mouse antibody on the C2C ELISA plate. After washing the C2C 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

Joint cartilage is composed of a type II collagen-based fibrillar network complexed to proteoglycans. Type II collagen consists of 3 identical α chains arranged in a triple helix that form fibrils. Within this fibrillar meshwork resides the large aggregating proteoglycan aggrecan. 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).

Serum C2C is increased in rheumatoid arthritis (RA) and baseline levels are prognostic of progression (Verstappen *et al,* 2006). In patients with RA, C2C singly or together with C1,2C and CPII assays provide indications of early responses to biologic therapy that are predictive of radiologic changes seen almost a year later. (Mullan *et al*, 2007).

As a ratio to CPII, serum C2C is indicative of progressors from non-progressors of knee OA (Cahue *et al*, 2007). Strong correlations between serum C2C and MRI T2 images have been reported (King *et al,* 2004).
In a study following knee ACL rupture, in which baseline serum pre-injury were available, subsequent serum changes in C2C, C12C, CPII and CS846 were observed compared to age-related changes in uninjured controls (Svoboda *et al,* 2013).

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Patients at increased risk for ACL rupture can be identified prior to injury by differences in serum C2C, C12C, CPII and CS846 levels (Svoboda *et al,* 2016). These findings suggest that fundamental genetic and/or biomechanically related differences exist that influence cartilage metabolism.

Arthroscopic analyses of pre-radiographic knee cartilage degeneration following ACL injury have revealed significant associations of increased synovial fluid C2C with the presence of three or more high Outerbridge graded cartilage lesions (Yoshida *et al,* 2013).

Increase in knee synovial fluid C2C levels occur within a day following injury and persist up to 7 years (Kumahashi *et al,* 2015). C2C concentrations in SF and serum were correlated.

When knee or hip OA patients are haplotyped, the C2C, CPII and C2C:CPII ratio were significantly increased in sera of OA patients carrying the haplogroup H compared to OA carriers of the J haplogroup (Fernandez-Moreno *et al,* 2012). The collagenase MMP-13 is also more elevated in patients of haplogroup H, who are more likely to need total joint replacement, than non-H haplotypes (Soto-Hermida *et al,* 2015).

Although most studies have examined serum, much progress has been made in recent years to indicate the importance of also analyzing urine (Cibere *et al,* 2009). Different results may be obtained which may prove more informative, such as regarding OA onset and progression in OA. Analyses of urine have revealed that the C2C antibody recognizes a 45-mer peptide (Nemirovskiy *et al,* 2007). Urine C2C but not serum C2C is elevated in patients with pre-radiographic knee OA (Cibere *et al,* 2009). The differences would appear related to the concentration of the disease-related 45-mer peptide in urine.

In animal models of arthritis, serum C2C is elevated in a rat polyarthritis model and decreases with effective therapy (Song *et al,* 1999).

Serum C2C in conjunction with CPII assay is reflective of natural OA progression in the guinea pig as in patients (Huebner and Kraus, 2006). Another study with Hartley guinea pigs showed elevation of synovial fluid C2C, as well as MMP-13 than can generate the C2C neoepitope, in joints of post-traumatic OA group compared to the control or primary OA groups (Wei *et al,* 2010).

In a dog model of OA, C2C is elevated in synovial fluid, serum and urine following surgery (Matyas *et al,* 2004; Chu *et al,* 2002). In natural onset of OA in dogs, synovial fluid C2C is also elevated compared to unaffected dogs (Prink *et al*, 2010).

In a rabbit RA model, C2C levels are elevated in SF, but not in serum, following induction of joint inflammation (Kojima *et al,* 2001).

In equine studies of osteochondral injuries, synovial fluid analyses with serum C2C revealed increases in injured joints that correlated with the severity of injury (Trumble *et al*, 2009)

2. PROTOCOL

Bring C2C 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 C2C Standards and samples to appropriate wells of 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 C2C Antibody diluted in Assay Buffer to all wells of the polypropylene mixing plate (One Plate: 50 µL of Ab + 6 mL Assay Buffer).
- 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 C2C 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 700 ± 10 rpm for 1 hour (± 2 minutes) at room temperature (20-25°C).
- Wash the ELISA plate 3-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 GAM-HRP conjugate diluted in Buffer III.
  (One Plate: 22 µL of GAM-HRP conjugate + 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).

- C2C Standard Stock at 10 µg/mL. Make up 7 levels of Standards from this stock in Buffer III. (Stds.: 0, 10, 50, 100, 200, 500 and 1000 ng/mL).
- C2C ELISA plate. ELISA plate coated with C2C peptide conjugate within a stabilizing matrix including BSA. Packaged dry in a foil pouch with desiccant sachet.
- Polypropylene mixing plate.
- Assay Buffer. Protein based buffer containing BSA, normal goat 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.
- C2C antibody. Primary mouse antibody in a protein based buffer containing BSA and a non-mercury preservative.
- GAM-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 reagent. 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 III is provided to make up Standards for the measurement of serum and plasma samples. Ideally, a matrix equivalent 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.

- GAM-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.

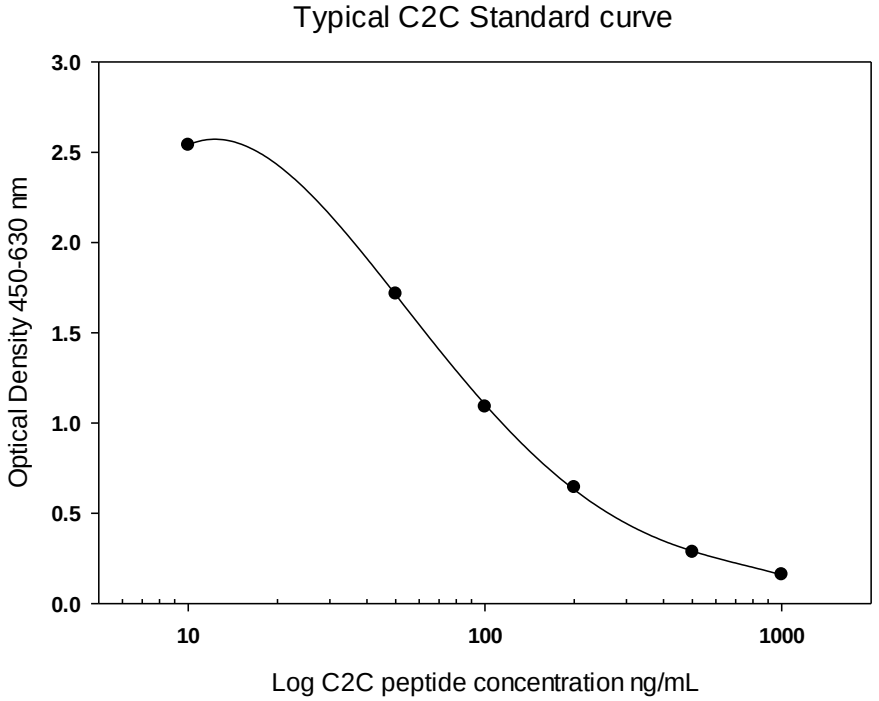
6. RESULTS

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

Table 1

C2C Working Standards Dilution Table				
Standard	**Concentration**	**Volume Stock**	**Volume Buffer III**	**Dilution**
Std-1	1000 ng/mL	50 µL of 10 µg/mL Stock	450 µL	1:10
Std-2	500 ng/mL	25 µL of 10 µg/mL Stock	475 µL	1:20
Std-3	200 ng/mL	100 µL of 1000 ng/mL (Std-1)	400 µL	1:5
Std-4	100 ng/mL	50 µL of 1000 ng/mL (Std-1)	450 µL	1:10
Std-5	50 ng/mL	50 µL of 500 ng/mL (Std-2)	450 µL	1:10
Std-6	10 ng/mL	50 µL of 100 ng/mL (Std-4)	450 µL	1:10
Std-7	0 ng/mL	---	500 µL	



7. SPECIFIC PERFORMANCE CHARACTERISTICS

7.1 CROSS REACTIVITY AND SPECIFICITY

The C2C antibody has selective high affinity for collagenase-cleaved human type II collagen. It recognizes the α-chain fragments containing an approximate 9 amino acid sequence from the carboxy terminus of the three-quarter length piece produced by collagenase (MMP-1, MMP-8 and MMP-13) cleavage of type II collagen. This antibody demonstrates no cross reactivity with uncleaved human type I or II collagen α-chains or uncleaved triple-helical and heat denatured human types I and II collagen. It also shows no reactivity to similarly cleaved type I collagen α-chains.

This antibody has broad cross reactivity and recognizes human, as well as monkey, horse, bovine, dog, rabbit, rat, mouse and guinea pig C2C. The antibody has 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 plasma, synovial fluid, urine and culture medium.

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

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

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