AGGRECAN CHONDROITIN SULFATE 846 EPITOPE ELISA
ITEM #60-1004

(CS846 ELISA)

1. OVERVIEW

1.1 PRINCIPLE OF PROCEDURE

This assay uses a mouse IgM monoclonal antibody to measure an epitope on chondroitin sulfate chains of the cartilage proteoglycan aggrecan which is increased in content in arthritis. In arthritis, articular cartilage is progressively destroyed. This is characterized by degradation of key molecules such as type II collagen and the proteoglycan aggrecan. It is accompanied by attempts at repair with an increase in synthesis of these molecules.
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 enzyme immunoassay in a 96-well format. Unlabelled CS846 standards and unknown diluted serum samples are added to the appropriate wells of the goat anti-mouse antibody (GAM) pre-coated plate. CS846-Biotin is added to all the wells and mouse CS846 antibody is added to start the competition. This mixture is incubated to allow binding between biotin-labeled aggrecan (CS846-Biotin) and unlabeled aggrecan to the CS846 antibody as well as binding of the CS846 antibody to the GAM on the plate. After washing the plate, Streptavidin conjugated to horseradish peroxidase (Streptavidin-HRP) is added. Streptavidin-HRP binds to any bound CS846-Biotin. After washing the plate again, Tetramethylbenzidine (TMB) substrate is added, then HRP degrades H_20_2 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 epitope present in the sample.

1.3 BACKGROUND

Hyaline cartilage is composed of an extracellular matrix containing mainly type II collagen and a large aggregating proteoglycan called aggrecan. In arthritic joints, the collagen matrix is disrupted and the proteoglycan content is decreased. This change in matrix composition appears to be due to abnormal matrix degradation and synthesis, although the exact sequence of events during the development of arthritis remains unknown. The CS846 epitope is an indicator of large or fetal-like aggrecan synthesis. This epitope is only present on the largest aggrecan molecules and was originally identified in human fetal cartilage; its content decreases considerably with cessation of growth (Glant *et al*, 1986). In osteoarthritis (OA), where synthesis is increased, content is elevated, both in articular cartilage (Rizkalla *et al*., 1992) and in synovial fluid and serum (Lohmander *et al.,* 1999; Poole *et al*, 1994; Otterness *et al*, 2001). In rheumatoid arthritis (RA) it is elevated in serum in chronic disease although depressed in rapid progressive disease (Mansson *et al*., 1999). Similar increases are seen in hemophilic arthropathy (Jansen *et al*, 2009). Synovial fluid concentrations are much higher in OA and RA than in serum (Poole *et al,* 1984). Increased serum and synovial fluid concentrations of epitope CS846 are associated with osteochondral fragmentation (OC) in (Frisbie *et al*., 1999). Concentrations are decreased in the joint fluids of young horses with osteochondrosis (Laverty *et al*., 2000). In a dog preclinical model of OA, serum CS846 is elevated within 3 weeks following anterior cruciate section and remains elevated at 3 months (Matyas *et al*, 2004).

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2. PROTOCOL

Bring CS846 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.
- Remove the CS846 ELISA plate from the foil pouch.
- Add 50 µL/well of CS846 Standards and samples to appropriate wells of the ELISA plate. Serum samples should be diluted 1/5 with Buffer III. This can be done directly on the plate (10 µL serum + 40 µL Buffer III).
- Add 50 µL/well of CS846-Biotin diluted in Assay Buffer to all wells of the ELISA plate (One Plate: 50 µL of CS846-Biotin + 6 ml of Assay Buffer).
- Add 50 µL/well of CS846 Antibody diluted in Assay Buffer to all wells of the ELISA plate (One Plate: 50 µL of CS846 Antibody + 6 ml of Assay buffer).
- Incubate the ELISA plate on high speed Titre Plate Shaker at 600-700 rpm for 2 hours (± 8 minutes) at room temperature (20-25°C).
- Wash the ELISA plate 6 times. Vigorously blot the plate dry on towels after the last wash.
- Add 100 µL/well of Streptavidin-HRP conjugate diluted in Buffer III. (One Plate: 50 µL of Strep-HRP + 11 mL Buffer III).
- Incubate the ELISA plate on 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. Vigorously 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. 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 min.

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 dispense 6-1000 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).

- CS846 Standard Stock 10µg/mL. Make up 7 level of Standards from this stock in Buffer III (0, 20, 50, 100, 200, 500 and 1000 ng/mL).
- ELISA plate coated with stabilized Goat anti-mouse antibody (GAM).
- CS846-Biotin. Prepared in a protein based buffer containing BSA and a non-mercury preservative.
- CS846 Antibody. Prepared 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 CS846-Biotin conjugate and CS846 Antibody.
- Buffer III. Protein based buffer containing BSA and a non-mercury preservative. For dilution of standards and Streptavidin-HRP Conjugate.
- Streptavidin-HRP Conjugate. Prepared 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.

- The use of a high speed plate shaker is critical to the success of this assay.

- Stock reagents should be diluted shortly before use. Final dilutions of antibody 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.

- Serum samples should be diluted to reduce matrix interference. We recommend a dilution of 1/5 in Buffer III.

- 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.

- Streptavidin-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 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

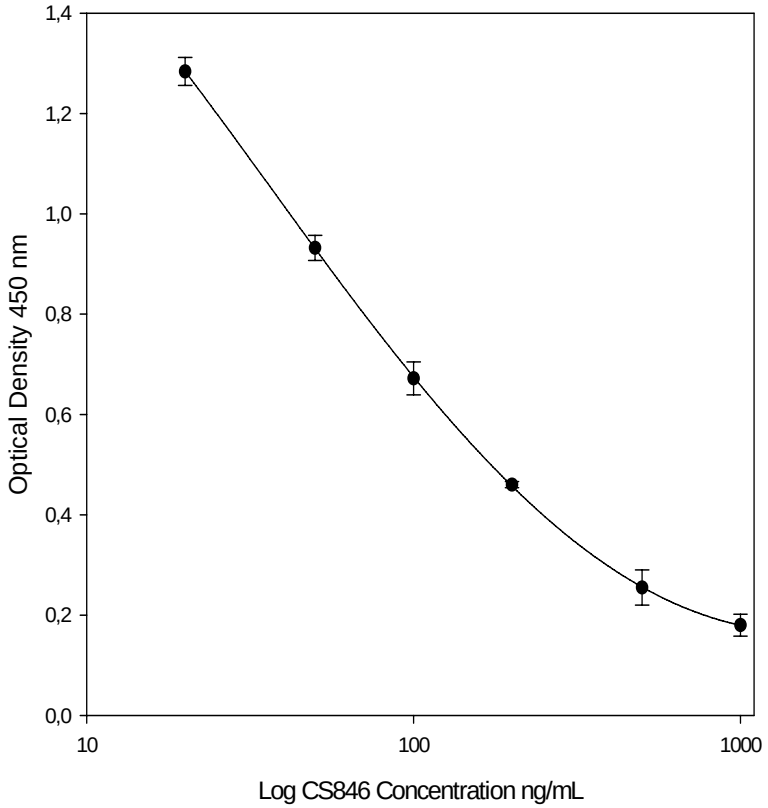
- Plot mean standard absorbance readings on the y-axis versus log concentration on the x-axis using a logistic equation (4 or 5 parameter). Note that the standard curve is sigmoidal and not linear. Ideally, appropriate curve fitting software should be used.

- Any sample reading higher than the highest standard should be diluted with Buffer III and reassayed. Any sample reading lower than the lowest standard should be re-assayed or discarded.

Table 1

CS846 Assay PG Standard Dilution Table				
Std #	**Concentration**	**Volume Stock**	**Vol. Buffer III**	**Dilution**
1)	1000 ng/mL	50 µL of 10 µg/mL Stock	450 µL	1:10
2)	500 ng/mL	25 µL of 10 µg/mL Stock	475 µL	1:20
3)	200 ng/mL	10 µL of 10 µg/mL Stock	490 µL	1:50
4)	100 ng/mL	50 µL of Std 1	450 µL	1:10
5)	50 ng/mL	50 µL of Std 2	450 µL	1:10
6)	20 ng/mL	50 µL of Std 3	450 µL	1:10
7)	0 ng/mL	-----	500 µL	

Representative CS846 Standard Curve



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7. SPECIFIC PERFORMANCE CHARACTERISTICS

7.1 CROSS REACTIVITY AND SPECIFICITY

The CS846 antibody reacts strongly with an epitope on the chondroitin sulphate (CS) chains of the proteoglycan aggrecan. CS, dermatan sulphate and keratan sulfate alone, either in their native or degraded state, show no reactivity. The epitope recognized by the CS846 antibody is chondroitinase ABC labile.

This antibody has broad cross reactivity and recognizes human, as well as monkey, horse, bovine, dog, rat, rabbit and guinea pig. The antibody has not been tested in other species.

This assay is suitable for serum. It has also been used by Ibex customers for synovial fluid and tissue culture media.

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.

8. REFERENCES

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