



Rheumera™ Native Type II Collagen Capture ELISA Kit
Catalog Number: ASB-5000-EX

ELISA Assay Kit To Measure Native Type II Collagen.

INTRODUCTION

There are more than 20 known types of collagen and they are classified by a Roman numeral based on their time of discovery. They are found in all connective tissues such as skin, bone, cartilage, vasculature, tendons and ligaments where their function is to provide support. All collagen molecules consist of three polypeptide chains folded into a left-handed helical conformation, and the three helical chains are then wrapped around each other into a right-handed triple helix. Homotrimer collagen types have 3 identical chains, whereas heterotrimer collagen types contain two or three different chains. Every third amino acid is glycine in the repeating Gly-X-Y sequence in each of the chains. Glycine allows for the triple helix confirmation where the three chains come together and X is usually proline and Y, hydroxyproline.

Types I, II, III, V and XI are considered the fibril-forming collagens as they form long fibers consisting of a triple-helical domain and are similar in structure. They are characterized by 67 nm banded fibrils that are quarter -staggered. The length of these collagens is approximately 300 nm and 1.5nm in diameter and consist of 1000 Gly-X-Y repeating residues. Collagens are synthesized as precursor molecules called procollagens that contain amino (N) and carboxy (C) terminal propeptide domains which consist of collagen and non-collagen-like sequences. The N and C terminal propeptides are linked to the main triple-helical domain by short, non-collagenous sequences, called telopeptides. The telopeptides are the primary sites for intermolecular cross-linking, which is important for the stabilization of the collagen fibers.

The procollagen molecules are processed to mature collagen molecules by cleavage of the N and C propeptides by specific N and C proteinases.

Type II collagen is homotrimeric consisting of three identical alpha1(II) chains and is the major collagen of cartilage accounting for approximately 95% of all collagen types. Type II collagen is also in the vitreous body of the eye, the **nucleus pulposus** of intervertebral discs and the inner ear. The COL2A1 gene codes for the production of the pro-alpha1(II) chain of type II collagen. It has been discovered that the gene sequence coding for the N-propeptide has an alternatively spliced exon that codes for an additional 69-amino acid cysteine-rich domain. Type IIA procollagen contains this cysteine-rich globular domain while type IIB lacks it and both have distinct distributions during various stages of chondrogenesis. Type IIA procollagen can be localized to pre-chondrocytes of the perichondrium, while type IIB is localized to differentiated chondrocytes of cartilage.



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Autoimmunity to type II collagen is implicated in the pathogenesis of various diseases including rheumatoid arthritis (RA), eye disease associated with RA, and relapsing polychondritis. Circulating antibodies to type II collagen are found in these diseases and rheumatoid cartilage and synovium contain antibodies to type II collagen more than in serum. Furthermore, immunization of susceptible rodents and non-human primates with native, type II collagen (collagen-induced arthritis; CIA) induces an erosive polyarthritis which closely resembles RA. In CIA, complement fixing antibodies bind to type II collagen in autologous cartilage, and initiate the inflammation cascade. The CIA model has proven to be a valuable model to study the pathogenesis of arthritis and for the development of new therapies.

In cartilage, type II collagen is polymerized to form collagen fibrils. Small-diameter fibrils (10-25 nm) are formed pericellularly, while larger fibrils (up to 300 nm in diameter) are formed in the territorial and interterritorial matrix. The collagen molecules in the fibrils overlap by a quarter-stagger forming a banded pattern. The molecules are covalently cross-linked between the triple-helical domains and the telopeptides.

The Rheumera™ kit is specific for measuring **native** type II collagen, and will not measure denatured collagen. This kit will measure 80 samples, individually or 40 samples in duplicate, and the sensitivity of this assay is in the nanogram range. The Native Type II Collagen Detection Kit is designed to quantify the amount of native type II collagen from most species in cell or tissue culture or from tissue homogenates by ELISA.

Important: Newly synthesized collagen from cells in culture is incorporated into the extracellular matrix and remains in fibril form. Therefore, very little soluble, monomeric collagen exists in culture media or in tissue. Thus, to measure collagen content in cell culture or in tissue using the Native Type II Collagen Detection Kit, the collagen must first be digested with pepsin in acidic conditions and then further digested with pancreatic elastase at neutral pH in order to convert polymeric collagen to monomeric collagen.

PRINCIPLE OF THE ASSAY

The Rheumera™ assay measures native type II collagen from cell/tissue culture or cartilage tissue using enzyme-linked immunosorbent assay (ELISA). The kit will measure 80 individual samples or 40 samples in duplicate. Removable strips and aliquoted reagents allow for samples to be tested on partial plates on 2 separate occasions. Samples are incubated in wells that are coated with antibodies to type II collagen. Dilutions of a standard (highly purified type II collagen) is also incubated in wells that are coated with antibodies to type II collagen. The wells are washed to remove unbound type II collagen molecules, and then incubated with antibodies to type II collagen which are conjugated to biotin. The wells are washed to remove unbound antibodies and then incubated with avidin (which has affinity to biotin) conjugated to peroxidase. The wells are washed to remove unbound avidin-peroxidase and then incubated with a chromogen substrate solution, TMB. A blue color develops which then

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turns yellow when the stop solution, mild sulfuric acid, is added. The color intensity is proportion to the amount of type II collagen present in the sample. The sample values, ng/ml of native type II collagen, are determined by the standard curve.

EXAMPLE OF AN ELISA PLATE CONFIGURATION

A Typical Configuration ELISA plate

A typical ELISA plate configuration where samples and standards are run in duplicate. Ten, 8-well strips, are coated with anti-type II collagen.

Two, 8-well strips, depicted in red, are used for the standards (0 to 200 ng/ml). "0" represents blank wells to determine secondary antibody background levels.

	Anti-type II collagen coated										Std(ng/ml)	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	9	9	17	17	25	25	33	33	200	200
B	2	2	10	10	18	18	26	26	34	34	100	100
C	3	3	11	11	19	19	27	27	35	35	50	50
D	4	4	12	12	20	20	28	28	36	36	25	25
E	5	5	13	13	21	21	29	29	37	37	12.5	12.5
F	6	6	14	14	22	22	30	30	38	38	6.25	6.25
G	7	7	15	15	23	23	31	31	39	39	3.12	3.12
H	8	8	16	16	24	24	32	32	40	40	0	0

REAGENTS INCLUDED

- A. Anti-type II collagen-coated strips (10).
- B. Standard coated strips (4).
- C. Diluent Buffer 1X (60ml). Solution
- D. Wash Buffer 10X (50ml). Solution
- E. Anti-type II collagen (2 vials). Lyophilized. Conjugated to biotin.
- F. Standard (1 vial). Purified type II collagen 0.2 ml (1000 ng/ml).
- G. Sample/Standard Dilution Buffer 1X (20ml). Solution
- H. Avidin-Peroxidase (2 vials). Lyophilized
- I. TMB (1X) - 10ml. Chromogen substrate for peroxidase detection.
- J. Stop Solution (1X) – 5ml. Diluted sulfuric acid to stop the color reaction.

REAGENTS NOT INCLUDED

Pepsin
Elastase

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REQUIRED EQUIPMENT/LABWARE/REAGENTS

Microplate reader with 450 nm and 540 nm or 650 nm filters.
Single and multi-channel pipettes and tips.
Deionized or distilled water.
Wash bottle with 8 or 12 spigot manifold dispenser or automated microplate washer.
Graduated cylinder.

PRECAUTIONS

The following agents are hazardous, wear appropriate eye, hand, face, and clothing protection:

Solution I (dilute sulfuric acid).

Solution H (TMB).

SAMPLE PREPARATION

Newly synthesized collagen from cells in culture is incorporated into the extracellular matrix and remains in fibril form. Therefore, very little soluble, monomeric collagen exists in culture media or in tissue. Thus, to measure collagen content in cell culture or in tissue using the Native Type II Collagen Detection Kit, the collagen must first be digested with pepsin in acidic conditions and then further digested with pancreatic elastase at neutral pH in order to convert polymeric collagen to monomeric collagen.

The following is a brief protocol for digesting sample specimens:

Cells grown in 24-well plates

1. Most collagen produced by cells will be incorporated immediately into the matrix. Intact collagen is not expected to be in the supernatant. **Important: perform a count of the cells so that the cell density can be correlated to the amount of collagen measured.** Remove the culture media and add 0.5 mL (starting volume) of 0.05M acetic acid (pH 2.8 - 3.0 with formic acid) to the cell layer. The starting volume may be adjusted according to the quantity of cells processed. Transfer the cells to a microcentrifuge tube using a cell scraper.
2. Add 1/10 starting volume of pepsin solution (1 - 10 mg/mL dissolved in 0.05M acetic acid) and digest the collagen at 4°C overnight with gentle mixing on a rotator or rocker. If

If additional digestion is necessary, homogenize the sample and repeat incubation at 4°C overnight or 48 to 72 hours. Do not overheat the sample when homogenizing, as this will denature the collagen.

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3. Add 1/10 starting volume of 10X TBS (1.0M Tris-2.0M NaCl-50mM CaCl₂, pH 7.8 - 8.0) and adjust the pH to 8.0 with 1N NaOH.

4. Add 1/10 the starting volume of pancreatic elastase (1 mg/mL dissolved in 1X TBS, pH 7.8 - 8.0) and incubate at 4°C overnight with gentle mixing on a rotator or rocker. If additional digestion is necessary, homogenize the sample and incubate at 4°C overnight with gentle mixing. This will cleave the polymeric collagen molecules into monomers by digesting the intra and inter-crosslinkages within the polymeric collagen molecule.

Note: Elastase digests the collagen molecule at the N-terminal region, which contains the intra- and inter-crosslinkages. As a result of N-terminal cleavage by elastase, the dimeric and trimeric collagen molecules are converted into monomeric collagen. Elastase digests the degraded collagen into small fragments, so avoid increasing the sample temperature to more than 20°C.

5. Centrifuge the sample at 10,000 rpm for 5 minutes at room temperature. Dilute the supernatant with Sample/Standard Dilution Buffer (Solution G) and assay for type II collagen content with the Rheumera™ Native Type II Collagen Detection Kit.

Tissue Specimens

1. Obtain a small piece of sample tissue (i.e. 0.5 cm³ or 1-5 mg), blot it dry and **measure the weight so that the collagen content can be correlated to the weight of the tissue sample**. In addition, a dry weight can be obtained by freeze drying a second sample of equal size or wet weight.

2. Homogenize the tissue using a small electric homogenizer and centrifuge for 3 minutes at 10,000 rpm. Alternatively, the sample can be pulverized by freezing in liquid nitrogen or on dry ice and then crushing using a metal grinder.

3. Resuspend the tissue in 0.8 mL of 0.05M acetic acid containing 0.5M NaCl, pH 2.9 - 3.0 (adjust pH with formic acid).

4. Add 0.1 mL of pepsin (10 mg/mL) dissolved in 0.05M acetic acid and mix at 4°C for 48 hours. Pepsin digests telopeptides located on both N- and C-terminals of the collagen molecule and solubilizes the collagen from collagen fibrils.

Note: Pepsin cannot digest the intra- and inter-molecule crosslinkages.

5. Add 0.1 mL of 10X TBS (1.0M Tris-2.0M NaCl-50mM CaCl₂, pH 7.8-8.0) and adjust the pH to 8.0 with 1N NaOH.

6. Add 0.1 mL of pancreatic elastase (1 mg/mL dissolved in 1X TBS, pH 7.8 - 8.0) and mix at 4°C overnight on a rotator rocker.

Note: Elastase digests the collagen molecule at the N-terminal region, which contains the intra- and intercrosslinkages. As a result of N-terminal cleavage by elastase, the dimeric and trimeric collagen molecules are converted into monomeric collagen.



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Elastase digests the degraded collagen into small fragments, so avoid increasing the sample temperature to more than 20°C.

7. Centrifuge the sample at 10,000 rpm for 5 minutes and collect the supernatant.

Note: The tissue should be completely solubilized. However, trace amounts of insoluble material might remain.

8. Dilute the supernatant at 1:100 - 1:10,000 with the Sample/Standard Dilution Buffer (Solution G) provided in the Rheumera™ Native Type II Collagen Detection Kit.

Note: Store samples at 4°C to prevent the collagen from denaturing.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Sample Preparation (see above).

Solution D. Wash Buffer (10X) – 50ml. Add the contents (50 ml) of Wash Buffer to 450 ml of dH₂O to make 1X.

Solution E. Anti-Type II Collagen, conjugated to biotin. Lyophilized. Dilute the contents of one vial in 5.0 ml **Diluent Buffer (C)**. **IMPORTANT NOTE** * Make sure to rinse the vial several times with Diluent Buffer (C) to ensure that all lyophilized material is in solution.

Solution H. Avidin-Peroxidase. Lyophilized. Dilute the contents of one vial in 5.0 ml **Diluent Buffer (C)**. **IMPORTANT NOTE** * Make sure to rinse the vial several times with Diluent Buffer (C) to ensure that all lyophilized material is in solution.

Solution F. Standard 0.2 ml (1000 ng/ml). Add 0.8 ml of **Sample/Standard Dilution Buffer (G)**, and vortex well. This solution is now 200 ng/ml. Prepare serial dilutions of the standard by mixing 0.2 ml of the 200 ng/ml standard with 0.2 ml of **Sample/Standard Dilution Buffer (G)** in a separate tube to make a 100 ng/ml solution. Repeat this procedure to make 50, 25, 12.5, 6.25, 3.125 ng/ml and using **Sample/Standard Dilution Buffer (G)**. 0 or blank is **Sample/Standard Dilution Buffer (G)** only, with no standard. The unused 200 ng/ml standard may be stored at 4° for up to one month for use in a second assay.

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ASSAY PROCEDURE

It is recommended that test samples and positive controls be run in duplicate.

1. Prepare Samples and Reagents as described above.

2. Sample and Standards (see Example ELISA Plate above). Add 100 ml of diluted samples to anti-type II collagen-coated wells. Add 100 ml of diluted standards to Standard (Red) anti-type II collagen-coated wells. Incubate for 2 hours at room temperature. **Best results (i.e. higher OD values) are achieved by incubating overnight at 4°C.**

3. Wash 3 times with **1 X Wash Buffer (D)** using a wash bottle with manifold or automated plate washer. Blot the inverted plate on a paper towel to remove excess liquid.

4. Anti-type II collagen, conjugated to biotin. Add 100 ml of diluted **Solution E**, antitype II collagen, conjugated to biotin to all wells and incubate for 1 hour at room temperature.

5. Wash 3 times with **1 X Wash Buffer (D)** using a wash bottle with manifold or automated plate washer. Blot the inverted plate on a paper towel to remove excess liquid.

6. Avidin-Peroxidase. Add 100 ml of diluted **Solution H**, Avidin-Peroxidase, to all wells and incubate for 1 hour at room temperature.

7. Wash 3 times with **1 X Wash Buffer (D)** using a wash bottle with manifold or automated plate washer. Blot the inverted plate on a paper towel to remove excess liquid.

8. Chromogen. Add 100 ml of **Solution I**, TMB, to all wells and incubate 30 minutes at room temperature.

9. Stop reaction. Add 50 ml of **Solution J**, Stop Solution, to each well.

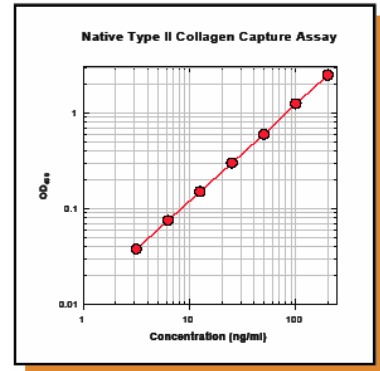
10. Read OD value at 450 nm (A 540 or 650 nm filter can be used as a reference).

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CALCULATION OF RESULTS

1. Average the duplicate OD values for the standards and test samples.
2. Subtract the 0 or blank values from the averaged OD values in step 1.
3. Plot the OD values of the type II collagen standards against their known concentrations (3.125—200 ng/ml). Using a log/log plot will make the data linear. The graph shows a representative experiment where the standard range is from 3.125 to 200 ng/ml.



4. The ng/ml of type II collagen in test samples can be calculated using regression analysis.

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