COSMO BIO CO., LTD.
Inspiration for Life Science

Astarte Biologics, LLC

# Rheumera™ Human Anti-Type II Collagen ELISA Kit
# Catalog Number: 4000

# ELISA Assay Kit to measure *human* antibodies to type II collagen

## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joints, which ultimately results in the destruction of cartilage and bone. Components of the inflammatory process include cells, cytokines and matrix metalloproteinases. Although the etiology of the disease is unknown, one potential autoantigen, type II collagen, has been implicated in RA. Type II collagen is the major protein of articular cartilage and consists of three pro-alpha1(II) chains twisted together to form a triple-stranded helical molecule.

Autoantibodies to type II collagen have been widely reported in adult and juvenile rheumatoid arthritis and relapsing polychondritis (see references). In these patients, antibodies have been found in serum, synovial fluid and eluted from cartilage explants. It is hypothesized that antibodies to type II collagen are involved in the pathogenesis of RA because 1) Type II collagen is highly immunogenic and immunization of susceptible mice, rats and non-human primates results in inflammatory arthritis, known as collagen-induced arthritis (CIA). 2) Antibodies to type II collagen are found in the serum of CIA animals and can be passively transferred to naïve recipients to induce disease and 3) antibodies to type II collagen found in RA are complement-fixing. Antibodies to type II collagen have also been found in other diseases such as relapsing polychondritis (RP), SLE, scleroderma, diseases of the heart and vasculature and otosclerosis.

## PRINCIPLE OF THE ASSAY

Research has determined that antibodies to type II collagen from patients with RA or RP react to various species of type II collagen such as chicken, bovine and porcine (see references). It has been hypothesized that antibodies are generated to these various species of dietary collagen which *crossreact* to human collagen, possibly inducing disease if they bind to cartilage, activate complement and subsequently the inflammation cascade. Added proof for this hypothesis comes from studies showing antibody reactivity to various species of type II collagen in healthy persons. Therefore, Rheumera™ ELISA kits can be ordered with various species of type II collagen (see table below).

One of the challenges of measuring antibodies from serum of patients with autoimmune disease, is that it contains high levels of protein components which bind to ELISA wells whether they are coated or uncoated. This results in false positive reactions yielding

high background levels. The Rheumera™ ELISA kits use 2 methods to deal with false positive reactions. First, a unique blocking buffer is applied to the ELISA wells which inhibit non-specific binding. Secondly, samples are incubated in uncoated wells to determine background levels. These background levels are then subtracted from their collagen-coated counterparts, to determine specific antibody binding.

This assay measures antibodies to native human type II collagen from serum or synovial fluid using enzyme-linked immunosorbent assay (ELISA). The kit will measure up to 39 samples in duplicate. Removable strips and aliquoted reagents allow for samples to be tested on 2 partial plates on 2 separate occasions.

Samples are incubated in wells that are uncoated or coated with type II collagen which have been first pre-treated with blocking buffer. Standards are incubated in wells that are coated with type II collagen. The wells are washed to remove unbound antibodies, and then incubated with an anti-human IgG antibody, conjugated with a biotin label. The wells are washed to remove unbound antibodies and then incubated with avidin (which binds to biotin), conjugated to peroxidase. The wells are washed to remove unbound avidin-peroxidase, then incubated with a chromogen substrate solution, TMB. A blue color develops which then turns yellow when the stop solution, mild sulfuric acid, is added. The color intensity is proportion to the amount of anti-collagen antibody bound to type II collagen The sample values, measured as Units/ml, are determined by the standard curve.

The kit can be ordered with various species of type II collagen (see chart below).

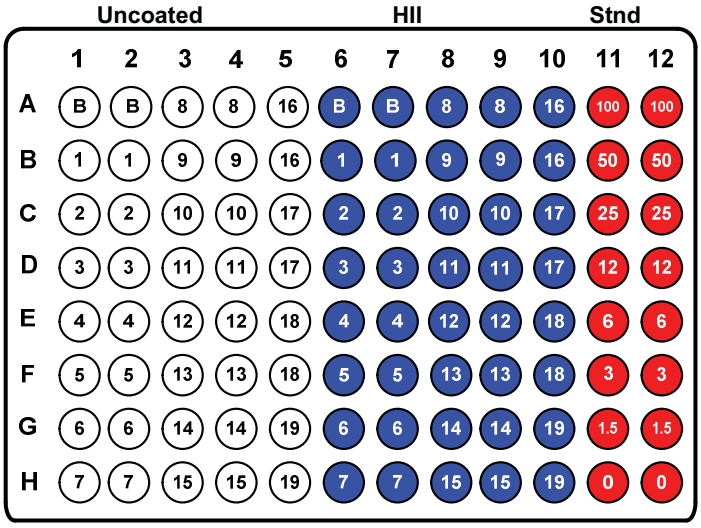
| Species of Type II Collagen | Color Code |
|---|---|
| Standard | Red |
| Chicken | Yellow |
| Bovine | Green |
| Porcine | Purple |
| Human | Blue |
| Rat | White |
| Mouse | Black |
| uncoated | Clear (no color) |

## EXAMPLES OF DIFFERENT ELISA PLATE CONFIGURATIONS

### Typical ELISA plate

A typical ELISA plate where samples and standards are run in duplicate. Five, 8-well strips are uncoated, depicted in white circles, and serve as controls for sample background levels.

Five additional 8-well strips are coated with one species of type II collagen (such as human; HII depicted in blue) to determine specific antibody levels. Two, 8-well strips are used for the standards (0 to 100 units/ml), depicted in red. "B" represents blank wells to determine secondary antibody background levels.

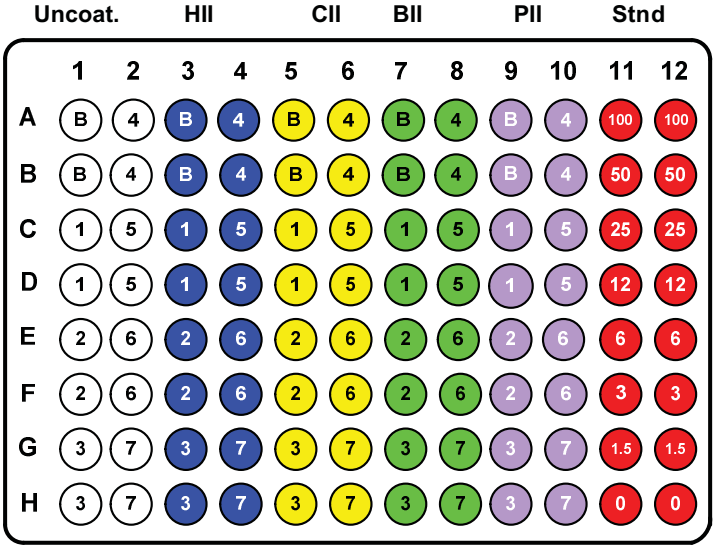



## EXAMPLES OF DIFFERENT ELISA PLATE CONFIGURATIONS

### A Custom ELISA plate

A custom ELISA plate where samples and standards are run in duplicate. Two, 8-well strips are uncoated and serve as controls for sample background levels.

Two each, 8-well strips are coated with multiple species of type II collagen, such as human (HII), in blue, chick (CII) in yellow, bovine (BII) in green and porcine (PII) in purple to determine specific antibody levels. Two, 8-well strips are used for the standards (0 to 100 units/ml) depicted in red. "B" represents blank wells to determine secondary antibody background levels



## REAGENTS INCLUDED

**A. Type II collagen-coated strips (10).** Includes 2 Standard strips (see above chart for color coding).
**B. Un-coated strips (10).** Includes 2 Standard strips.
**C. Blocking Buffer 1X (20ml).**
**D. Diluent Buffer 1X (60ml)**
**E. Wash Buffer - 1 tablet.** Dissolve tablet in 1000 ml $dH_2O$
**F. Standard (2 vials).** Lyophilized. Dilute one vial with 1.0 ml 1X Diluent Buffer (D)
**G. Secondary Ab (2 vials).** Lyophylized. Anti-human IgG, conjugated to biotin. Dilute one vial in 10.0 ml 1X Diluent Buffer (D).
**H. Avidin-peroxidase (2 vials).** Lyophylized. Steptavidin-Avidin. Dilute one vial in 10.0 ml 1X Diluent Buffer (D).
**I. TMB (1X) - 20ml.** Chromogen substrate for peroxidase detection.
**J. Stop Solution (1X) – 10 ml.** Dilute sulfuric acid to stop the color reaction.

## REQUIRED EQUIPMENT/LABWARE/REAGENTS (not included)

Microplate reader with 450 nm and 540 nm or 650 nm reference 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.

## SAMPLE/REAGENT PREPARATION

Bring all reagents to room temperature before use.

**Sample Preparation**. The dilution range for samples will vary greatly depending upon the antibody concentration and specificity. A recommended range is 1:100 to 1:1,000 in **Diluent Buffer D**.

**Solution F. Standard**. Add 1.0ml of **Diluent Buffer (D)** and vortex well. This solution is 100 Units/ml. Prepare serial dilutions of the standard by mixing 250ul of the 100 Units/ml standard with 250ul of **Diluent Buffer (D)** in a separate tube to make 50 Units/ml solution. Repeat this procedure to make 25, 12.5, 6.25, 3.125 and 1.6 Units/ml. 0 Units/ml is **Diluent Buffer (D)** only. The unused 100 Units/ml standard may be stored at 4°C for up to one month for use in a second assay.

**Solution E. Wash Buffer – one tablet.** Dilute 1 tablet in 1000 ml $dH_2O$ to make 1X**.**

**Solution G. Anti-Human IgG Ab, conjugated to biotin.** Lyophylized. Dilute the contents of one vial in 10.0 ml **Diluent Buffer (D). IMPORTANT NOTE *** Make sure to rinse the vial several times with **Diluent Buffer (D)** to ensure that all lyophilized material is in solution.

**Solution H. Avidin-peroxidase.** Lyophylized. Dilute the contents of one vial in 10.0 ml **Diluent Buffer (D). IMPORTANT NOTE** * Make sure to rinse the vial several times with **Diluent Buffer (D)** to ensure that all lyophilized material is in solution

## PRECAUTIONS

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

**Solution J** (dilute sulfuric acid).
**Solution I** (TMB).

## ASSAY PROCEDURE

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

**1. Prepare Samples and Reagents as described above.**

**2. Blocking**. Add 100 µl of **Blocking Buffer (C)** to all wells**.** Incubate for 60 minutes at room temperature. Empty the plate by inverting it and blot on a paper towel.

**3. Sample and Standards (see Examples 1 and 2 above).** Add 100 µl of diluted samples to uncoated and type II collagen-coated wells. Add 100 µl of diluted standards to Standard-coated wells (Red). Incubate for 1-2 hours at room temperature or overnight at $4^0$C, for better results (i.e. higher OD values).

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

**5. Secondary Ab (G).** Add 100 µl of diluted anti-human IgG, conjugated to biotin to all wells and incubate for 1 hour at room temperature.

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

**7. Avidin-peroxidase (H).** Add 100 µl of diluted avidin-peroxidase to all wells and incubate for 1 hour at room temperature.

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

**9. Chromogen.** Add 100 µl of **TMB (I)** to all wells and incubate 30 minutes at room temperature.

**10. Stop reaction.** Add 50 µl of **Stop Solution (J)** to each well.

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

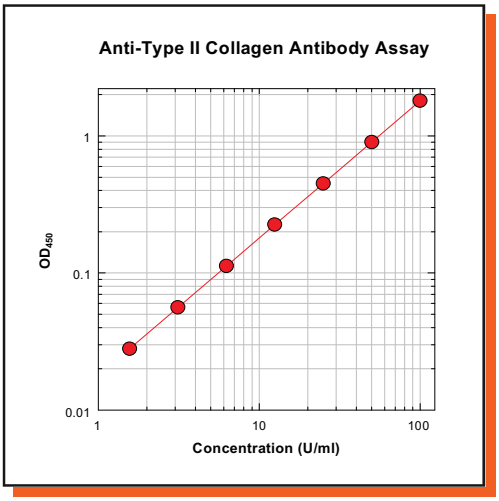
## CALCULATION OF RESULTS

1. Average the duplicate OD values for the standards and test samples in uncoated wells and collagen-coated wells.

2. Subtract the blank (B) values from the averaged OD values in step 1. The blank (B) wells for each different antigen should be used.

3. Subtract the OD values of samples tested in uncoated wells (background values) from their counterpart OD values in type II collagen-coated wells from step 2.

4. Plot the OD values of standards against the Units/ml of antibody standard. Using a log/log plot will make the data linear. The figure to the right shows a representative experiment where the standard range is from 0 to 100 units/ml.




5. The Units/ml of antibody in test samples can be calculated using regression analysis.

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