Chondrex, Inc.

Rat Anti-Type II Collagen IgG Subtype ELISA Kit

Catalog # 20421T, 20422T, 20431T, 20432T, 20441T, 20442T

For Research Use Only - Not Human or Therapeutic Use

INTRODUCTION

Autoantibodies to type II collagen play an important role in the rat collagen-induced arthritis (CIA) model of Rheumatoid arthritis (RA). However, the mere presence of autoantibodies is not sufficient to induce arthritis, since the activation of the complement cascade by immunoglobulin is a more critical step in initiating the inflammatory response in the induction of arthritis. Therefore, instead of IgG1, the production of IgG2a and IgG2b subtype autoantibodies which are capable of activating the complement cascade is essential in the development of arthritis.

In fact, the immunization of type II collagen emulsified with incomplete Freund's adjuvant develops severe arthritis in rats by eliciting IgG2a/2b antibodies dominantly against type II collagen. However, pretreatment with immunization of type II collagen mixed with aluminum adjuvant reduces severity of arthritis by inducing an anti-collagen antibody subclass shift from IgG2a/2b to IgG1 (1).

These results demonstrate the importance of determining the IgG subtype of the antibodies against the heterologous collagen used for immunization as well as the rat type II collagen for the evaluation of the immune response in the rat CIA model (2). In addition, this is especially important for the evaluation of immune modulating adjuvants which can be used for a treatment of autoimmune diseases such as RA (3).

Chondrex provides Rat Anti-Collagen IgG Subtype ELISA Kits (see table below for catalog numbers) for further detailed analysis of antibodies in rat sera from the rat CIA model. These kits can also be used to determine antibodies to type I collagen upon request. Please contact customer service for more information on custom type II collagen and type I collagen-coated plates.

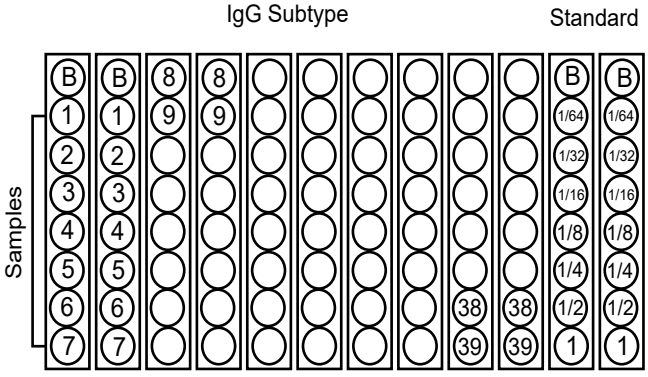
Note: The antibody-antigen affinity may vary significantly among serum samples; additionally the IgG subtypes may recognize different epitopes,. Therefore, the total IgG concentration calculated as the sum of the IgG subtypes may not be the same as the total IgG levels as determined by our Rat Anti-Collagen IgG ELISA Kits.

Species (Type II Collagen Color Coding)	IgG1 Catalog #	IgG2a Catalog #
Rat (Purple)	20441T	20442T
Bovine (Green)	20421T	20422T
Porcine (Pink)	20431T	20432T
Standard (Red)	---	---

ELISA Kit for Single IgG Subtype Assay

Figure 1 shows an example of an ELISA kit for assaying an individual anti-collagen IgG subtype antibody level. Each kit contains one 96-well plate coated with one species of type II collagen for each IgG subtype antibody assay. In addition, two extra 8-well standard strips are included per subtype for two separate assays. "B" represents blank wells to determine background values caused by the secondary antibody. Standards and samples (numbers 1 - 39) are run in duplicate.

Note: Custom coating of plates is available upon request.



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KIT COMPONENTS

Item	Quantity	Amount	Storage
Standard Antibody IgG1or IgG2a	1 vial	20 units, lyophilized	-20°C
Secondary Antibody (Peroxidase-Conjugated Anti-Mouse IgG1or IgG2a)	2 vials	50 µl	-20°C
Solution A - Blocking Buffer (2071)	1 bottle	12 ml	-20°C
Solution B - Sample/Standard Dilution Buffer (2072)	1 bottle	50 ml	-20°C
Solution C - Secondary Antibody Dilution Buffer (2073)	1 bottle	20 ml	-20°C
TMB (90023)	2 vials	0.2 ml	-20°C
Chromogen Dilution Buffer (90022)	1 bottle	20 ml	-20°C
Stop Solution - 2N Sulfuric Acid (9016)	1 bottle	10 ml	-20°C
Wash Buffer, 20X (9005)	1 bottle	50 ml	-20°C
Type II Collagen Coated 8-Well Strips	10 each	8-well strips	-20°C
Reference Standard Strips (two strips per run)	4 each	8-well strips	-20°C

NOTES BEFORE USING ASSAY

Note 1: It is recommended that the standard and samples be run in duplicate.

Note 2: Partially used reagents may be kept at –20°C.

Note 3: Crystals may form in the 20X wash buffer when stored at cold temperatures. If crystals have formed, it is necessary to warm the wash buffer by placing the bottle in warm water until crystals have dissolved completely.

Note 4: Measure exact volume of buffers using a serological pipette prior to diluting. Extra buffer is provided.

ASSAY PROCEDURE

1. **Dilute Wash Buffer**: Dilute 50 ml of 20X wash buffer in 950 ml of distilled water (1X wash buffer). Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

2. **Add Blocking Buffer**: Add 100 µl of Blocking Buffer (Solution A) to all wells. Incubate for 1 hour at room temperature.

3. **Prepare Standard Dilutions**: Prepare Standard Dilutions: Dissolve one vial of standard (20 units/vial) in 1.25 ml of Sample/Standard Dilution Buffer (Solution B) to make 16 units/ml solution. Prepare serial dilutions of the standard by mixing 250 ml of 16 units/ml standard with 250 ml of Solution B - 8 units/ml . Then repeat this procedure to make five more serial dilutions of standard - 4, 2, 1, 0.5, and 0.25 units/ml solutions. The 16 units/ml standard may be stored at -20°C for use in a second assay. We recommend making fresh serial dilutions for each assay.

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4. **Prepare Sample Dilutions**: If necessary, centrifuge serum samples at 10,000 rpm at room temperature for 3 minutes to remove insoluble materials and lipids. Dilute 10 µl of sample with 0.99 ml of Solution B (1:100). Keep this as a stock solution for future assays. Then, further dilute the stock solution with Solution B; for example.

   Note: The serum antibody levels differ significantly among individual strains of rats and timing of serum collection after immunization. In addition, a cross-reactivity of anti-immunized collagen (bovine or porcine) antisera to rat type II collagen is about 50 -70%. We recommend confirming sample dilutions using a couple of your selected samples before running a large number of samples.

   Examples of sample dilutions for arthritic rat sera:
   IgG1: x100, x1000, and x10,000
   IgG2a: x1000, x10,000, and x100,000

   Examples of sample dilutions for non-arthritic rat sera:
   IgG1 and IgG2a: x100, x1000, and x10,000

5. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

6. **Add Standards and Samples**: Add 100 µl of standards, Solution B (blank) and samples to wells in duplicate. Incubate at room temperature for 2 hours.

7. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

8. **Add Secondary Antibody**: Dissolve one vial of Secondary Antibody specific to the individual IgG subtype in 10 ml of Secondary Antibody Dilution Buffer (Solution C). Add 100 µl of secondary antibody solution to each well and incubate at room temperature for 1 hour.

9. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

10. **TMB**: Use new tubes when preparing TMB. Dilute one vial (200 µl) of TMB in 10 ml Chromogen Dilution Buffer just prior to use. Add 100 µl of TMB solution to each well immediately after washing the plate. Incubate for 15 minutes at room temperature. If you plan to use less, the remaining stock solution can be stored in its original vial at –20°C.

11. **Stop**: Add 50 µl of 2N sulfuric acid (Stop Solution) to each well.

12. **Read Plate**: Read the OD values at 450 nm. If the OD values of samples are greater than the OD values of the highest standard, re-assay the samples at a higher dilution. A 630 nm filter can be used as a reference.

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CALCULATION OF ANTIBODY TITERS

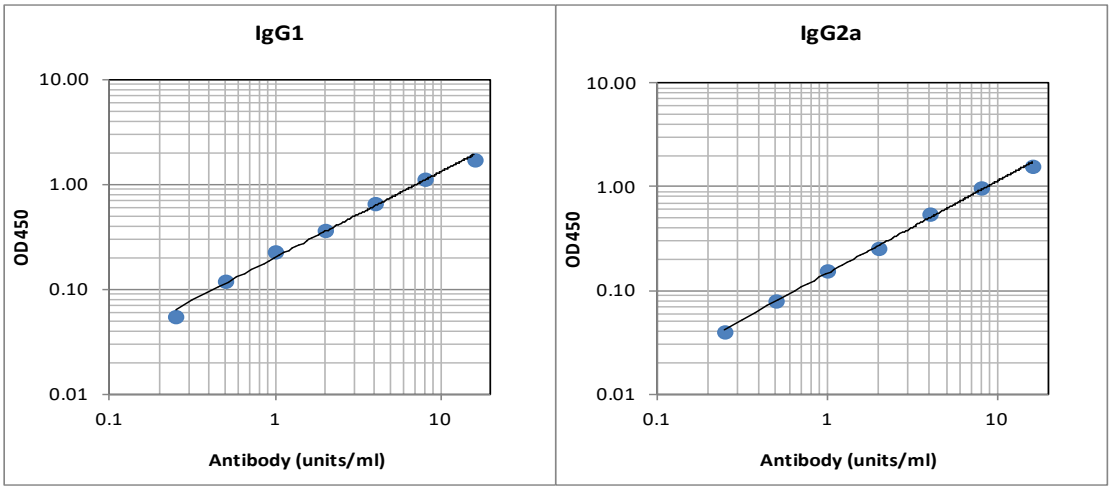
1. Average the duplicate OD values for the standards, blanks (B) and test samples.

2. Subtract the blank (B) values from the averaged OD values of the standards and test samples.

3. Plot the OD values of standards against the units/ml of antibody standard. Using a log/log plot will linearize the data. Figure 2 shows an example of standard curves of IgG1 and IgG2a subtype antibodies.

4. The units/ml of antibody in test samples can be calculated using regression analysis. Multiply it by the sample dilution ratio to obtain the antibody concentration (units/ml) in original sample specimens.

Figure 2 - IgG1 and IgG2a antibody standard curves



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2. IgG subclasses in collagen-induced arthritis in the rat. Firth SA, Morgan K, Evans HB, Holt PJ. Immunol Lett. 1984;7(5):243-7.

3. TGF-beta-treated antigen presenting cells suppress collagen- induced arthritis through the promotion of Th2 responses. Jung S, Park YK, Lee H, Shin JH, Lee GR, Park SH. Exp Mol Med. 2010 Mar 31;42(3):187-94.

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