Mouse Anti-Type II Collagen IgG Assay Kit (bovine)

Item No. 500410

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
400405	Bovine Type II Collagen Precoated ELISA Strip Plate	1 plate
400407	Mouse Anti-Type II Collagen Polyclonal IgG Standard	1 vial
400071	Anti-Mouse IgG/HRP Conjugate	1 vial/1.5 ml
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml
400035	Polysorbate 20	1 vial/3 ml
400074	TMB Substrate Solution	1 vial/12 ml
10011355	HRP Stop Solution	1 vial/12 ml
400012	96-Well Cover Sheet	3 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman's Mouse Anti-Type II Collagen IgG Assay Kit (bovine). This kit may not perform as described if any reagent or procedure is replaced or modified. The Stop Solution provided with this kit is an acid solution. Please wear appropriate personal protection equipment (*e.g.*, safety glasses, gloves, and lab-coat) when using this material.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at 4°C and used before the expiration date indicated on the outside of the box. *Do not freeze this kit!*

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 10).

INTRODUCTION

Background

Collagen-induced arthritis (CIA) is an experimental autoimmune disease that is widely used as a model of human rheumatoid arthritis (RA) in the study of the pathogenic mechanisms of this disease and for testing potential therapeutics. CIA is elicited in susceptible strains of rodents (mouse and rat) and nonhuman primates after immunization with heterologous type II collagen (CII), the major constituent protein of articular cartilage. Following immunization, these animals develop an autoimmune-mediated polyarthritis that shares several clinical, histological, immunological, and genetic features with human RA. The immune response to CII is characterized by both the stimulation of collagen-specific T cells and the production of high titers of antibody reactive to both heterologous CII and autologous CII, with the latter being most critical for the development of CIA. Antibody generated against type II collagen localizes in the joint of the host by binding to the intact autologous collagen. This event is followed by the activation of the complement and the initiation of the inflammation cascade, consisting of pro-inflammatory cytokines, chemokines, and cartilage degrading enzymes. Because circulating levels of type II collagen antibody are strongly associated with the development of arthritis, this antibody can be used as a biomarker of CIA.

About This Assay

Cayman's Mouse Anti-Type II Collagen IgG Assay is an immunometric (*i.e.*, sandwich) assay that can be used to measure type II collagen antibody in plasma and serum without prior sample purification. Cayman is offering this assay on three kinds of collagen coated plates: bovine, chick, and mouse. The standard is an affinity-purified polyclonal antibody isolated from mice with CIA.

This kit uses a bovine collagen-coated plate. One unit of standard in this assay is approximately equal to 1 ng of IgG protein. The standard curve spans the range of 15.6-1,000 U/ml, with an LLOQ of 15.6 U/ml.

Description of Immunometric ELISAs

Each well of the microwell plate supplied in the kit has been coated with bovine type II collagen. Autoantibodies reactive to type II collagen, if present in the biological fluid sample, will bind to the immobilized collagen. A detection antibody recognizing mouse IgG is added to the well. This antibody is labeled with HRP, allowing quantification of the autoantibody. Addition of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB), followed by Stop Solution produces a yellow colored product which can be measured spectrophotometrically. The intensity of the color is directly proportional to the amount of bound Anti-mouse IgG/HRP, which is proportional to the concentration of the type II collagen autoantibody.

Absorbance $\propto$ [Anti-mouse IgG/HRP] $\propto$ [Collagen autoantibody]

A schematic of this process is shown in Figure 1, below.

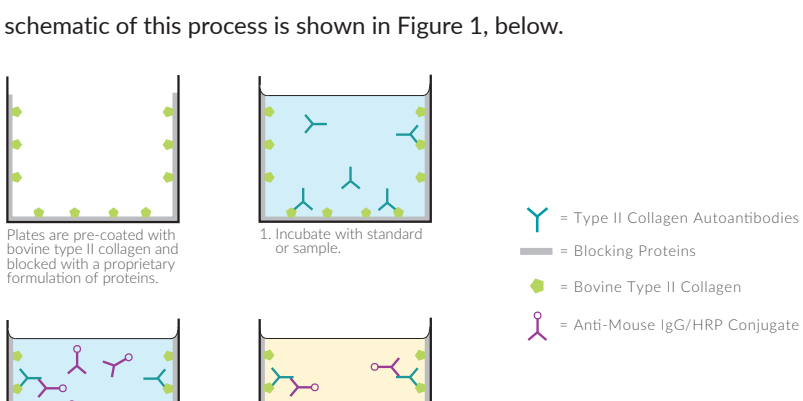


Figure 1. Schematic of the Immunometric ELISA

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **ELISA Buffer Preparation**

   Dilute the contents of each vial of ELISA Buffer Concentrate (10X) (Item No.400060) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (Item No. 400062): Dilute to a total volume of 2 L with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

Sample Preparation

Prior to use, it is recommended that mouse serum or plasma samples be diluted in ELISA Buffer at least 1:5,000 in order to fall within the range of the standard curve. In general, mouse serum or plasma (prepared using heparin or EDTA as the anticoagulant) can be used directly in the assay following dilution in ELISA Buffer.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Mouse Anti-Type II Collagen Polyclonal IgG Standard

Reconstitute the contents of the Mouse Anti-Type II Collagen Polyclonal IgG Standard (Item No. 400407) with 1 ml of ELISA Buffer. Mix gently. The concentration of this solution (the bulk standard) will be 1,000 U/ml. Label this solution #1.

To prepare the standard for use in the ELISA: Obtain seven clean test tubes or microcentrifuge tubes and label them #2 through #8. Aliquot 500 µl of ELISA Buffer into tubes #2-8. Serially dilute the standard by transferring 500 µl from the bulk standard to tube #2. Mix gently. Next, remove 500 µl from tube #2 and place into tube #3. Mix gently. Repeat this process for tubes #4-7. Do not add any antibody to tube #8. This tube contains no antibody, and serves as an indicator of non-specific binding (NSB). If desired, the value of this sample can be subtracted from all other standard and sample values. These diluted standards should be stored at 4°C and used within ten days.

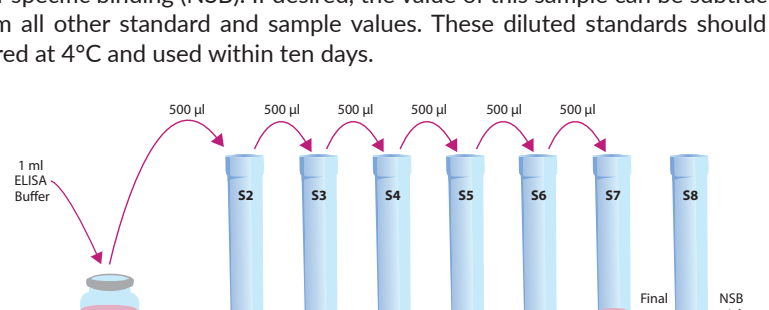


Figure 2. Preparation of the Anti-Collagen standards

Anti-Mouse IgG/HRP Conjugate

This reagent is supplied as a concentrated (20X) stock solution of donkey anti-mouse IgG polyclonal antibody conjugated to HRP. Just before use, prepare a Working Solution by adding 0.6 ml of the Anti-Mouse IgG/HRP Conjugate (Item No. 400071) to 11.4 ml Assay Buffer (12 ml total). In the event that two or more experiments are performed with this kit, sufficient stock solution has been provided to produce additional 12 ml of the Working Solution. Discard any leftover Working Solution.

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all of the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 16, for more details). We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	9	9	17	17	25	25	33	33
B	S2	S2	2	2	10	10	18	18	26	26	34	34
C	S3	S3	3	3	11	11	19	19	27	27	35	35
D	S4	S4	4	4	12	12	20	20	28	28	36	36
E	S5	S5	5	5	13	13	21	21	29	29	37	37
F	S6	S6	6	6	14	14	22	22	30	30	38	38
G	S7	S7	7	7	15	15	23	23	31	31	39	39
H	S8	S8	8	8	16	16	24	24	32	32	40	40

S1-S8 - Standards 1-8
1-40 - Samples

Figure 3. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of Standards and Samples and First Incubation

1. Add 100 µl of the standards or diluted sample to the appropriate wells on the plate. Each sample should be assayed in duplicate, triplicate recommended.
2. Cover the plate with a 96-Well Cover Sheet (Item No. 400012). Incubate for two hours at room temperature on an orbital shaker.

Addition of Anti-Mouse IgG/HRP Conjugate and Second Incubation

1. Empty the wells and rinse four times with Wash Buffer. Each well should be completely filled with Wash Buffer during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.
2. Add 100 µl of the diluted Anti-Mouse IgG/HRP Conjugate to each well of the plate.
3. Cover the plate with a 96-Well Cover Sheet and incubate for one hour at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse four times with Wash Buffer.
2. Add 100 µl of TMB Substrate Solution (Item No. 400074) to each well of the plate.
3. Cover the plate with a 96-Well Cover Sheet and incubate for 15 minutes at room temperature in the dark. Development of the blue color can be monitored at 650 nm. When the maximum standard (S1) O.D. value reaches 0.5-0.6, Stop Solution should be added to the entire plate.
4. DO NOT WASH THE PLATE. Add 100 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells will turn yellow and colorless wells will remain colorless. *NOTE: The Stop Solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Read the plate at a wavelength of 450 nm.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/immuno) to obtain a free copy of this convenient data analysis tool.*

Calculations

Plotting the Standard Curve and Determining the Sample Concentration

Using computer reduction software, plot absorbance (linear y-axis) *versus* concentration (logarithmic x-axis) for standards (S1-S7) and fit the data with a 4-parameter logistic equation. Using the equation of the line, calculate the concentration of IgG in each sample.

NOTE: The choice of x and y-axis (linear or logarithmic) is arbitrary and can be altered at the discretion of the user without changing the results of the assay.

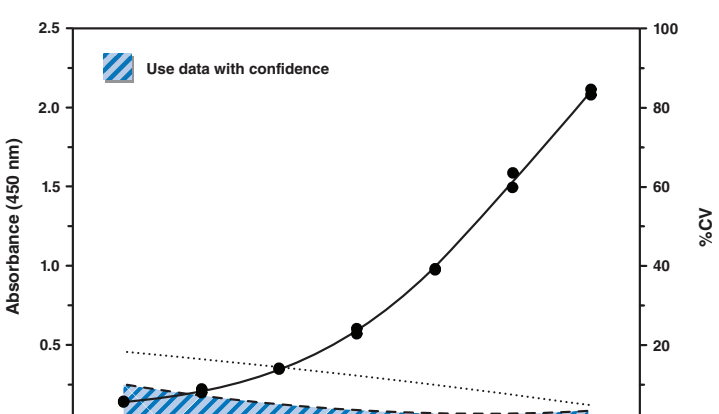
Performance Characteristics

Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially. Development of the plate for 15 minutes typically results in an absorbance of >1.0 O.D. units for the 1,000 U/ml standard.

Anti-Type II Collagen IgG (U/ml)	Absorbance (450 nm)
1,000	2.094
500	1.540
250	0.978
125	0.586
62.5	0.350
31.3	0.211
15.6	0.144
0	0.068

Table 1. Typical results



Assay Range = 15.6-1,000 U/ml
LLOQ = 15.6 U/ml

The lower limit of quantitation (LLOQ) is defined as the lowest standard concentration in which O.D. – (1.64 x S.D.) is higher than the blank value of O.D. + (1.64 x S.D.).The standard was diluted with ELISA Buffer.

Type II Collagen Antibody Standard curve
Type II Collagen Antibody Intra-assay variation
Type II Collagen Antibody Inter-assay variation

Figure 4. Typical standard curve

Precision:

The intra- and inter-assay CVs have been determined at multiple points on the standard curve. These data are summarized in the graph on page 18 and in the table below.

Anti-Type II Collagen IgG (U/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
1,000	2.7	6.7
500	3.2	6.0
250	3.5	7.2
125	4.4	12.2
62.5	4.1	16.6
31.3	5.4	18.8
15.6	11.6	16.0
0	†	†

Table 2. Intra- and inter-assay variation
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.
†Outside of the recommended usable range of the assay.

Anti-Type II Collagen IgG (U/ml)	Mean O.D.	Standard Deviation (S.D.)	O.D. - (1.64 x S.D.)
1,000	2.10	0.08	2.09
500	1.51	0.10	1.34
250	0.98	0.05	0.90
125	0.57	0.04	0.51
62.5	0.37	0.07	0.25
31.3	0.21	0.02	0.17
15.6	0.15	0.02	0.12
0	0.07	0.01	0.09*

*O.D. + (1.64 x S.D.)

Table 3. Determination of LLOQ

The lower limit of quantitation (LLOQ) is defined as the lowest standard concentration in which O.D. - (1.64 x S.D.) is higher than the blank value of O.D. + (1.64 x S.D.). The LLOQ is 15.6 U/ml.

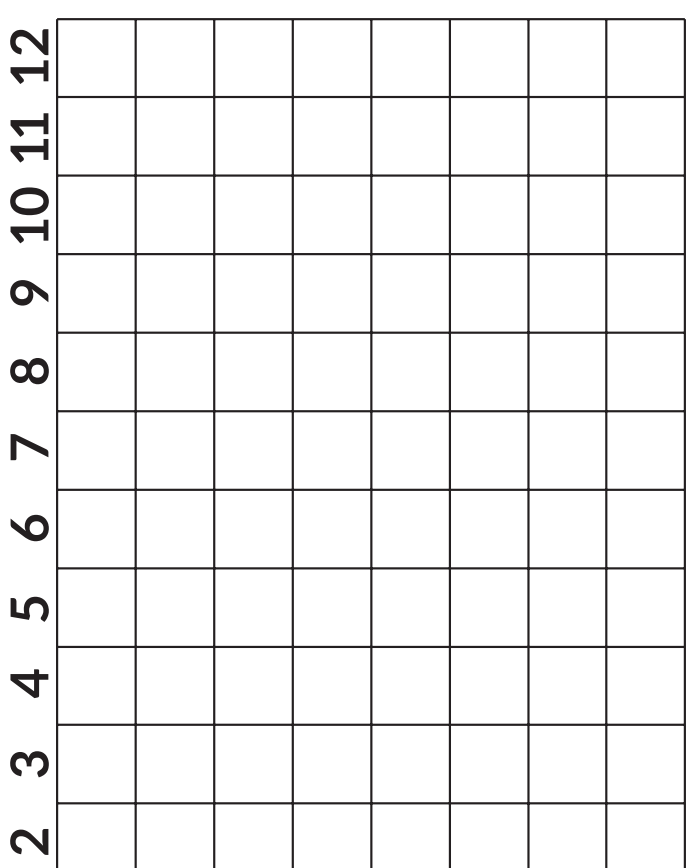
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
Poor development (low signal) of standard curve	A. Plate required more development time B. Standard was diluted incorrectly C. Standard is degraded	

References

1. Trentam, D.E. Collagen arthritis as a relevant model for rheumatoid arthritis. *Arthritis Rheum.* **25(8)**, 911-6 (1982).
2. Terato, K., Hasty, K.A., Cremer, M.A., *et al.* Collagen-induced arthritis in mice: Localization of an arthritogenic determinant to a fragment of the type II collagen molecule. *J. Exp. Med.* **162(2)**, 637-646 (1985).
3. Stuart, J.M., Townes, A.S., and Kang, A.H. Nature and specificity of the immune response to collagen in type II collagen-induced arthritis in mice. *J. Clin. Invest.* **63(3)**, 673-683 (1982).
4. Myers, L.K., Rosloniec, E.F., Cremer, MA., *et al.* Collagen-induced arthritis, an animal model of autoimmunity. *Life Sci.* **61(19)**, 1861-78 (1997).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.