Anti-Citrullinated Human Fibrinogen Assay Kit (mouse)

Item No. 501270

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Customer Service 800.364.9897
Technical Support 888.526.5351
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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
401276	Citrullinated Human Fibrinogen Precoated ELISA Strip Plate	1 plate
401274	Mouse Anti-Citrullinated Fibrinogen Monoclonal Antibody Standard	1 vial
401272	Human Fibrinogen Affinity Sorbent	1 vial/1.2 ml
400071	Anti-Mouse IgG/HRP Conjugate	2 vials/1.5 ml
400054	Immunoassay Buffer B 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	2 vials/12 ml
10011355	HRP Stop Solution	2 vials/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 Anti-Citrullinated Human Fibrinogen Assay Kit (mouse). 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
Fax:	734-971-3641
Email:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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 the ELISA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 9).

INTRODUCTION

Background

Immunization of mice with citrullinated human fibrinogen, especially mice expressing the human HLA-DR4 transgene, induces an arthritic response in 30-40% of the immunized animals.[1,2] By promoting the production of antibodies that recognize citrullinated epitopes, it has been proposed that this model more closely approximates the pathophysiology of the human disease than other mouse models of arthritis, including collagen-induced arthritis. Publications using this model report the generation of polyclonal antibody responses that contain antibodies reactive with both citrullinated human fibrinogen and unmodified, non-citrullinated human fibrinogen. Presumably, it is the response to the citrullinated epitopes that drives the arthritic response.[3,4] This assay kit provides a method that can distinguish the antibody response to citrullinated human fibrinogen from the antibody response to unmodified human fibrinogen in mouse serum or plasma for a more accurate analysis of the anti-citrulline response.

About This Assay

Cayman's Anti-Citrullinated Human Fibrinogen Assay (mouse) is an immunometric (sandwich) assay that can be used to measure citrullinated fibrinogen antibodies in mouse plasma or serum. A human fibrinogen affinity sorbent is provided with the kit so that any antibodies capable of reacting with non-citrullinated (unmodified) fibrinogen can be removed prior to analysis of the remaining anti-citrullinated fibrinogen antibodies.

This kit uses a citrullinated human fibrinogen-coated plate and an anti-citrullinated human fibrinogen monoclonal antibody standard. The standard curve spans the range of 0.15 µg/ml to 10 µg/ml, with an a lower limit of quantification (LLOQ) of 0.15 µg/ml.

Description of Immunometric ELISAs

Each well of the microwell plate supplied in the kit has been coated with citrullinated human fibrinogen. Autoantibodies reactive to citrullinated human fibrinogen, if present in the biological fluid sample, will bind to the immobilized citrullinated human fibrinogen. 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 the 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 anti-citrullinated human fibrinogen antibody.

Absorbance $\propto$ [Anti-mouse IgG/HRP] $\propto$ [Anti-citrullinated human fibrinogen antibody]

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

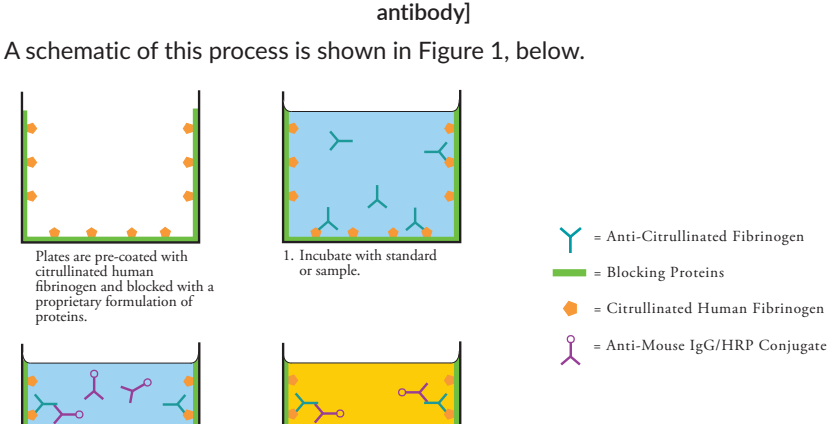


Figure 1. Schematic of the Immunometric ELISA

PRE-ASSAY PREPARATION

Buffer Preparation

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

1. **Immunoassay Buffer B (Assay Buffer) Preparation**

   Dilute the contents of each vial of Immunoassay Buffer B Concentrate (10X) (Item No. 400054) 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

Recommended protocol for testing plasma or serum samples from mice immunized with citrullinated human fibrinogen. This kit provides all of the reagents required for complete analysis of five individual plasma or serum samples.

1. Mix 2 µl of mouse serum or plasma sample with 2 ml of Immunoassay Buffer B (1:1,000) in a clean test tube. Store at 4°C until needed.
2. Shake or vortex the Human Fibrinogen Affinity Sorbent (Item No. 401272) to resuspend the slurry.
3. Transfer 100 µl of slurry to individual 1.5 ml microcentrifuge tubes (one tube per sample to be tested).
4. Centrifuge for 30 seconds at 400 x g.
5. Aspirate supernatant from atop the packed sorbent, taking care not to disturb the packed sorbent.
6. Add 1 ml of the diluted plasma or serum from step #1 to the tube containing the packed sorbent. Keep the remaining 1 ml “non-adsorbed” sample at 4°C until needed for the ELISA.
7. Incubate the sorbent-containing sample for two hours at room temperature with rotation or frequent vortexing.
8. Centrifuge for 30 seconds at 400 x g.
9. Transfer the supernatant to a new test tube (adsorbed sample).
10. Perform a serial two-fold dilution of each non-adsorbed and adsorbed sample as follows:
11. Obtain 8 clean test tubes per sample and label them #1 through #8.
12. Transfer the 1 ml of pre-adsorbed (step 6) or adsorbed sample (step 9) into tube #1.
13. Serially dilute the sample by removing 500 µl from tube #1 to tube #2. Mix gently. Next, remove 500 µl from tube #2 and place into tube #3. Mix gently. Repeat this process for tubes #4-8.

Preparation of Assay-Specific Reagents

Mouse Anti-Citrullinated Fibrinogen Monoclonal Antibody Standard

The Mouse Anti-Citrullinated Fibrinogen Monoclonal Antibody Standard (Item No. 401274) is supplied as a liquid solution at a concentration of 10 µg/ml. Label this solution #1.

To prepare the standard for use in the ELISA: Obtain seven clean test tubes and label them #2 through #8. Aliquot 500 µl of Immunoassay Buffer B into tubes #2-8. Serially dilute the standard by removing 500 µl from tube #1 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. 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.

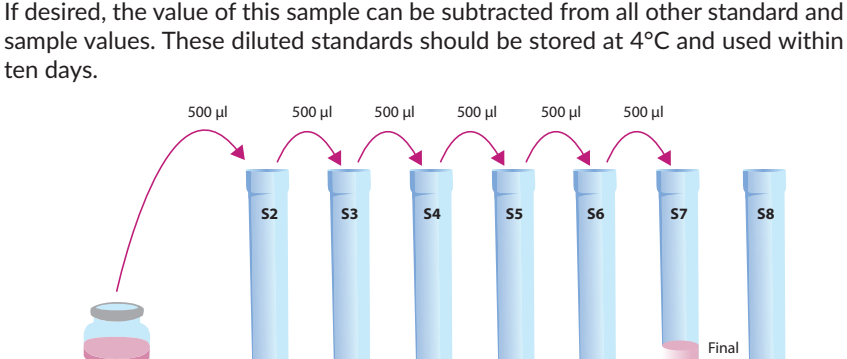


Figure 2. Preparation of the Anti-Citrullinated Fibrinogen Monoclonal Antibody standards

Anti-Mouse IgG/HRP Conjugate

This reagent is supplied as a concentrated stock solution of donkey anti-mouse IgG polyclonal antibody conjugated to HRP. On the day of the assay, prepare a working solution by adding 1.2 ml of the Anti-Mouse IgG/HRP Conjugate (Item No. 400071) to 10.8 ml Immunoassay Buffer B (12 ml total). This working solution should be stable for 24 hours at 4°C, protected from light. In the event that two or more experiments are performed with this kit more than 24 hours apart, sufficient stock solution has been provided to produce an additional 12 ml of the working solution.

Plate Set Up

The 96-well plate included with this kit is supplied ready to use. It is not necessary to rinse the plate 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 according to the plate insert 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*.

A suggested plate format is shown in Figure 3, on page 13. 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 25).

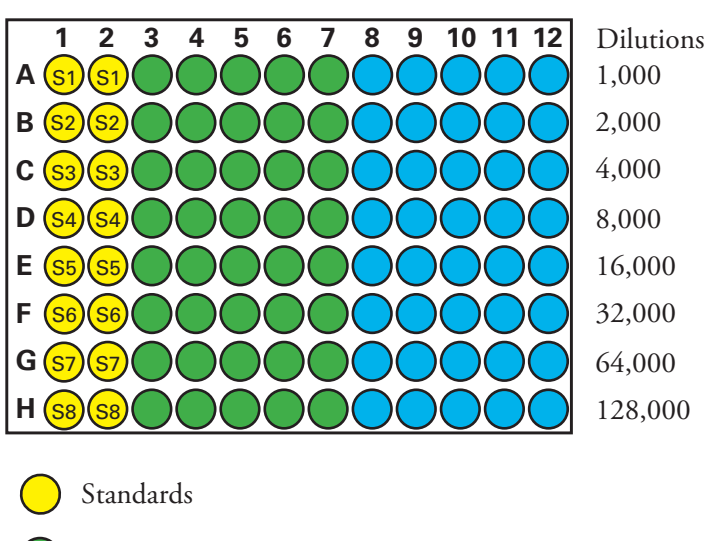


Figure 3. Sample plate format

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.
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 ten minutes at room temperature.
4. DO NOT WASH THE PLATE. Add 100 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should 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 absorbance 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 quadratic equation. Using the equation of the line, calculate the concentration of IgG in each sample.

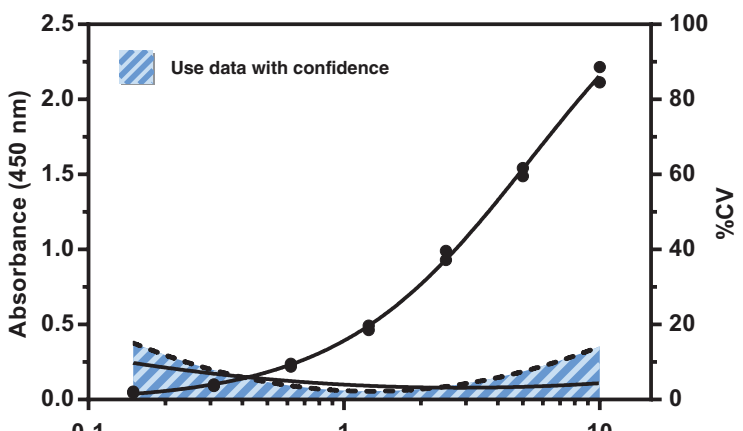
Performance Characteristics

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.

Anti-Citrullinated Fibrinogen IgG (µg/ml)	Absorbance (450 nm)
10	2.16
5	1.54
2.5	0.99
1.25	0.49
0.62	0.24
0.31	0.10
0.15	0.04
0	0.00

Table 1. Typical results



Assay Range = 0.15-10 µg/ml
LLOQ = 0.15 µg/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 Immunoassay Buffer B.

Anti-Citrullinated Fibrinogen Standard curve
Anti-Citrullinated Fibrinogen Intra-assay variation
Anti-Citrullinated Fibrinogen 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-Citrullinated Fibrinogen IgG (µg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
10	4.88	15.02
5	2.68	5.28
2.5	3.27	3.95
1.25	3.07	4.37
0.62	5.83	3.12
0.31	7.12	6.30
0.15	9.38	15.84

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

Anti-Citrullinated Fibrinogen IgG (µg/ml)	Mean of O.D.	Standard Deviation (S.D.)	O.D. - (1.64 x S.D.)
10	2.164	0.037	2.103
5	1.533	0.025	1.493
2.5	0.941	0.022	0.906
1.25	0.485	0.013	0.463
0.62	0.233	0.016	0.207
0.31	0.095	0.007	0.083
0.15	0.043	0.005	0.036
0	0.002	0.013	0.023*

*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 0.15 µg/ml.

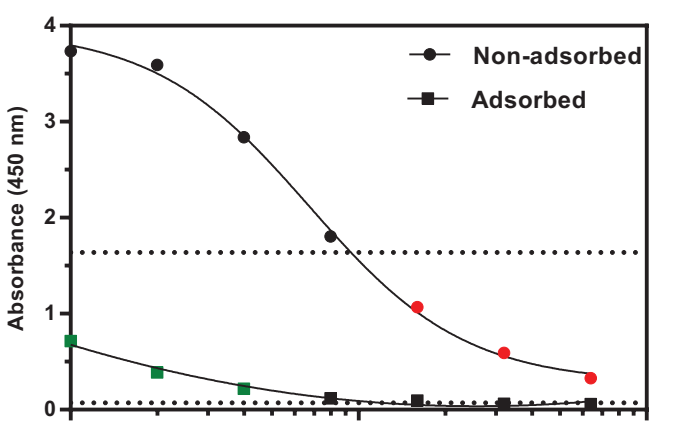


Figure 5. Analysis of pre-sorbent and post-sorbent anti-citrullinated fibrinogen response.

A BALB/c female mouse was immunized with citrullinated human fibrinogen, and boosted with citrullinated human fibrinogen 24 and 48 days later. One week after the second boost, the mouse was bled. The plasma was treated as described in the **Sample Preparation** section on page 9 and analyzed in the ELISA using the standard curve described on page 18, with the assay performed as described on page 14. The data are reported as OD450 versus dilution. The range of the standard curve was from 1.64 (S1) to 0.07 (S7), and is indicated by the dashed horizontal lines. For the non-adsorbed samples, only plasma dilutions greater than 8,000 fell within the range of the standard curve (red symbols). For the adsorbed samples, only dilutions less than 8,000 fell within the range of the standard curve (green symbols). The calculated concentration of anti-citrullinated fibrinogen antibodies is 76.77 mg/ml for the non-sorbent sample, and 3.00 mg/ml for the adsorbed sample. Therefore, in this example, 96.1% of the antibody response was directed against non-citrullinated fibrinogen epitopes and 3.9% of the antibody response was directed against citrullinated epitopes.

Performance of the Fibrinogen Sorbent

Volume of affinity sorbent used (µl)	% depletion
400	98.33
200	98.35
100	98.93
50	97.26
25	95.54
12.5	93.09

Table 4.
Mouse plasma containing 19.4 mg/ml anti-fibrinogen antibody was diluted 1:1,000 (19.4 µg/ml), and 1 ml fractions were adsorbed with various amounts of the human fibrinogen affinity sorbent slurry. The amount of anti-fibrinogen antibody remaining after adsorption at two hours was determined by ELISA, and the percent anti-fibrinogen depletion was calculated.

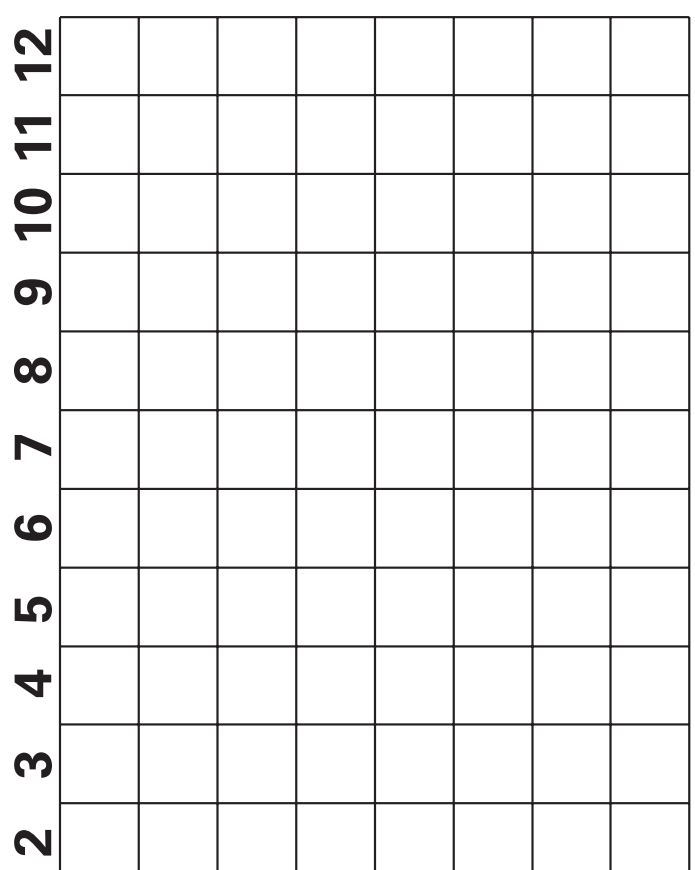
RESOURCES

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. Hill, J.A., Bell, D.A., Brintnell, W., *et al*. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. *J. Exp. Med*. **205(4)**, 967-979 (2008).
2. Yue, D., Brintnell, W., Mannik, L.A., *et al*. CTLA-4Ig blocks the development and progression of citrullinated fibrinogen-induced arthritis in DR4-transgenic mice. *Arthritis Rheum*. **62(10)**, 2941-2952 (2010).
3. Hill, J.A., Al-Bishri, J., Gladman, D.D., *et al*. Serum autoantibodies that bind citrullinated fibrinogen are frequently found in patients with rheumatoid arthritis. *J. Rheumatol*. **33(11)**, 2115-2119 (2006).
4. Zhao, X., Okeke, N.L., Sharpe, O., *et al*. Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. *Arthritis Res. Ther*. **10(4)**, (2008).



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.