PAD4 Autoantibody ELISA Kit

Item No. 500930

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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
400931	Goat Anti-Human Ig (H+L)/HRP Conjugate	2 vials/1.5 ml
400932	PAD4 Precoated 96-Well Strip Plate	1 plate
400934	Anti-PAD4 (human) ELISA Standard*	1 vial
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	1 vial/12 ml
10011355	HRP Stop Solution	1 vial/12 ml
400012	96-Well Cover Sheet	3 covers

*The Anti-PAD4 (human) ELISA Standard was affinity-purified from human plasma. Prior to processing at Cayman Chemical facilities, this standard screened negative for HIV and hepatitis viruses. However, we recommend it still be considered potentially infectious. Wear disposable gloves while handling this reagent and thoroughly wash hands afterwards.

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 PAD4 Autoantibody ELISA Kit. 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: 734-971-3640

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.

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 pure water; glass distilled water or deionized water is acceptable
4. Materials used for **Sample Preparation** (see 8).

INTRODUCTION

Background

Protein arginine deiminase 4 (PAD4) is a guanidino-modifying enzyme belonging to the amidinotransferase superfamily. PAD4 is a homodimer that functions as a transcriptional coregulator to catalyze the conversion of specific arginine residues to citrulline in a calcium-dependent manner. PAD4 activity is increased in rheumatoid arthritis (RA), producing citrulline-containing proteins that induce the formation of arthritogenic autoantibodies. The majority of patients with RA produce autoantibodies against citrullinated proteins that are routinely detected by a cyclic citrullinated peptide (CCP) immunoassay. In addition to anti-CCP autoantibodies, 23-45% of RA patients also produce autoantibodies specific for the PAD4 enzyme itself.

About This Assay

Cayman's PAD4 Autoantibody ELISA Kit is an immunometric assay which can be used to measure anti-PAD4 autoantibodies of any isotype (IgM, IgG, and IgA) in human plasma and serum without prior sample purification. Affinity-purified PAD4 autoantibody isolated from the plasma of a patient with RA is used as the standard. One unit is approximately equal to 1 ng of anti-PAD4 Ig protein. The standard curve spans the range of 15.6-1,000 U/ml, with an LLOQ of 15.6 U/ml.

Definition of Key Terms

Standard Curve: a plot of the absorbance values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Description of Immunometric ELISAs

Each well of the microwell plate supplied in the kit has been coated with recombinant human PAD4. Autoantibodies specific for PAD4, if present in the biological fluid sample, will bind to the immobilized PAD4. A detection antibody recognizing human immunoglobulins (Goat Anti-Human Ig (H+L)) is added to the well. The Goat Anti-Human Ig (H+L) is labeled with HRP, allowing quantitation 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 Goat Anti-Human Ig (H+L)/HRP, which is proportional to the concentration of the anti-PAD4 autoantibody.

Absorbance $\propto$ [Goat Anti-human Ig/HRP] $\propto$ [Anti-PAD4 autoantibody]

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

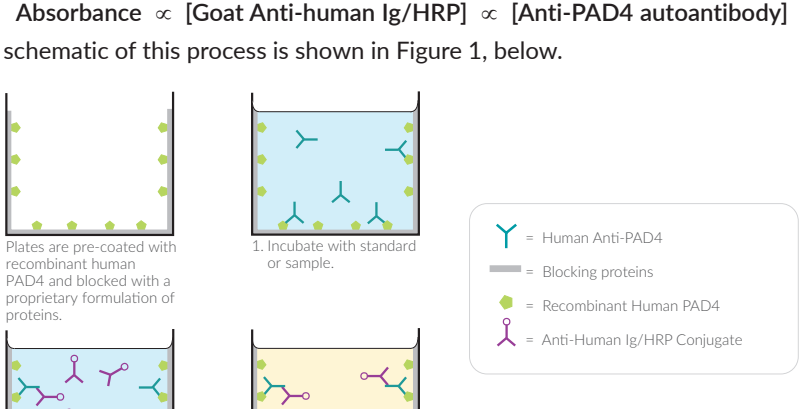


Figure 1. Schematic of the Immunometric ELISA

PRE-ASSAY PREPARATION

Buffer Preparation

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

1. **Assay Buffer Preparation**

   Dilute the contents of one vial of Immunoassay Buffer B Concentrate (Item No. 400054) with 90 ml of deionized 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 deionized 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 human serum or plasma samples be diluted in Assay Buffer at least 1:100 in order to fall within the range of the standard curve. In general, human serum or plasma (prepared using heparin or EDTA as the anticoagulant) can be used directly in the assay following dilution in Assay Buffer.

Parallelism

To assess parallelism, human plasma samples were serially diluted, and evaluated using the PAD4 Autoantibody ELISA Kit. Measured concentrations and concentrations of the standard dilutions were plotted as a function of sample dilution. The results are shown below. Parallelism demonstrates that the anti-PAD4 autoantibody binding characteristics are similar enough to allow accurate determination of native analyte levels in diluted human plasma samples.

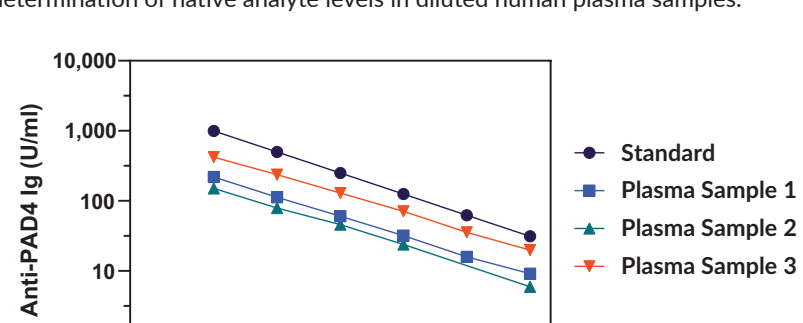


Figure 2. Parallelism of sample matrices in the PAD4 Autoantibody ELISA Kit

Preparation of Assay-Specific Reagents

Anti-PAD4 (human) ELISA Standard

Reconstitute the lyophilized purified Anti-PAD4 (human) ELISA Standard (Item No. 400934) with 2.0 ml of Assay Buffer. Mix gently. The concentration of this solution (the bulk standard) is 1,000 U/ml. The reconstituted standard should be stable for two weeks at 4°C. Enough standard is provided to produce four duplicate-well standard curves for use on different days, if necessary.

To prepare the standard for use in the ELISA: Obtain eight clean test tubes or plastic microfuge tubes and label them, #1 through #8. Aliquot 250 µl of Assay Buffer into tubes #2-8. Transfer 500 µl of freshly prepared stock standard (1,000 U/ml) to tube #1. Serially dilute the standard by removing 250 µl from tube #1 and placing into tube #2. Mix gently. Next, remove 250 µl from tube #2 and place into tube #3; mix gently. Repeat this process for tubes #4-7. Do not add any Anti-PAD4 to tube #8. This tube is the zero-point vial, the lowest point on the standard curve.

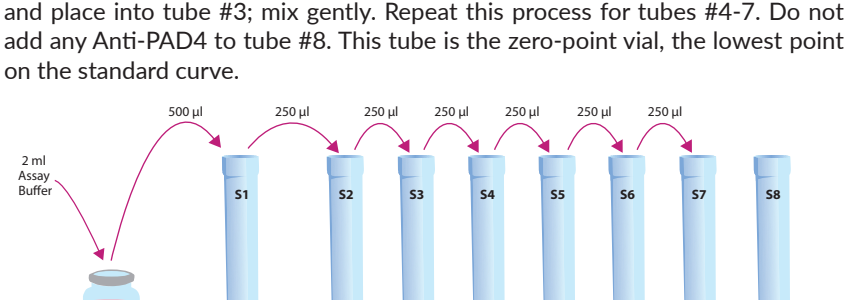


Figure 3. Preparation of the Anti-PAD4 standards

Goat Anti-Human Ig (H+L)/HRP Conjugate

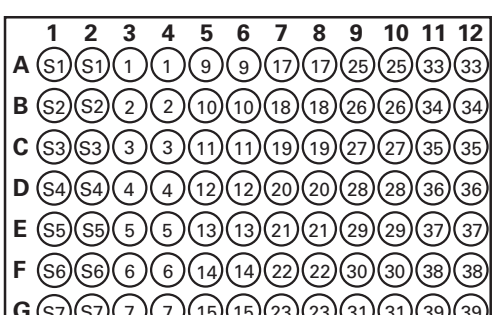
This reagent is supplied as a concentrated (10X) stock solution of Goat anti-Human Ig (H+L) polyclonal antibody conjugated to HRP (Item No. 400931). On the day of the assay, prepare a Working Solution by adding 1.2 ml of the stock solution (Item No. 400931) to 10.8 ml Assay Buffer (12 ml total). This Working Solution should be stable for 48 hours at 4°C. In the event that two or more experiments are performed with this kit more than 48 hours apart, a second vial of stock solution has been provided to produce additional 12 ml of the 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 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.* 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 below in Figure 3. 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 12, for more details). We suggest you record the contents of each well on the template sheet provided (see page 22).



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

Figure 4. 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 Goat Anti-Human Ig (H+L)/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 Goat Anti-Human Ig (H+L)/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 96-Well Cover Sheet and incubate for ten minutes at room temperature in the dark.
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 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 (linear x-axis) for standards (S1-S8) and fit the data with a quadratic or linear equation. Using the equation of the line, calculate the concentration of Ig in each sample.

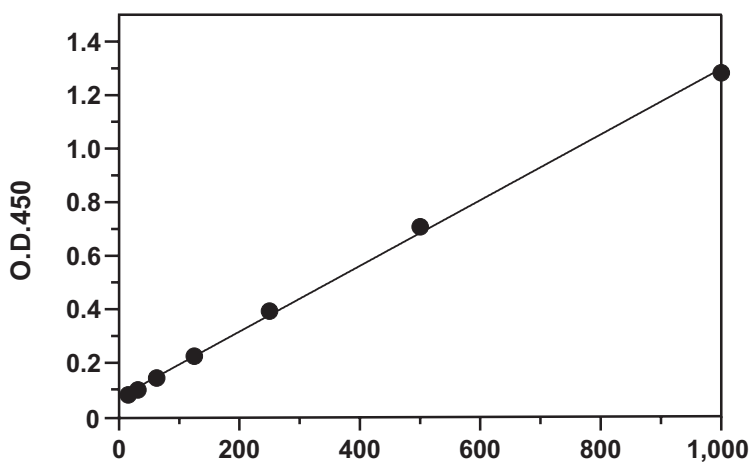
Performance Characteristics

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. Development of the plate for 10 minutes typically results in an absorbance of >1.0 O.D. units for the 1,000 U/ml standard.

Anti-PAD4 (U/ml)	Absorbance (10 min. development)
1,000	1.284
500	0.708
250	0.393
125	0.225
62.5	0.143
31.3	0.099
15.6	0.080
0	0.055

Table 1. Typical results



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

The lower limit of quantification (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 Assay Buffer.

Figure 5. Typical standard curve

Precision:

The intra- and inter-assay CVs have been determined for each point of the standard curve from the multiple measurements. This data is summarized in the table below.

Dose (U/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
1,000	0.2	2.7
500	1.9	3.0
250	4.4	4.7
125	4.3	3.0
62.5	4.4	4.5
31.3	8.8	16.1
15.6	26.1	16.8
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.

Sample Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (human plasma) in a single assay.

Plasma Control (U/ml)	%CV
192,318.4	16.1
51,148.4	14.4
22,137.2	8.1

Table 3. Sample Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (human plasma) in eight separate assays on different days.

Plasma Control (U/ml)	%CV
183,245.4	6.9
45,085.7	10.3
18,873.3	21.4

Table 4. Sample Inter-assay precision

Anti-PAD4 (U/ml)	Mean of O.D.	Standard Deviation (S.D.)	O.D. - (1.64 x S.D.)
1,000	1.142	0.014	1.190
500	0.643	0.008	0.630
250	0.350	0.025	0.309
125	0.210	0.009	0.195
62.5	0.133	0.005	0.125
31.3	0.092	0.001	0.009
15.6	0.072	0.002	0.069
0	0.052	0.001	0.054*

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

Table 6. 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.

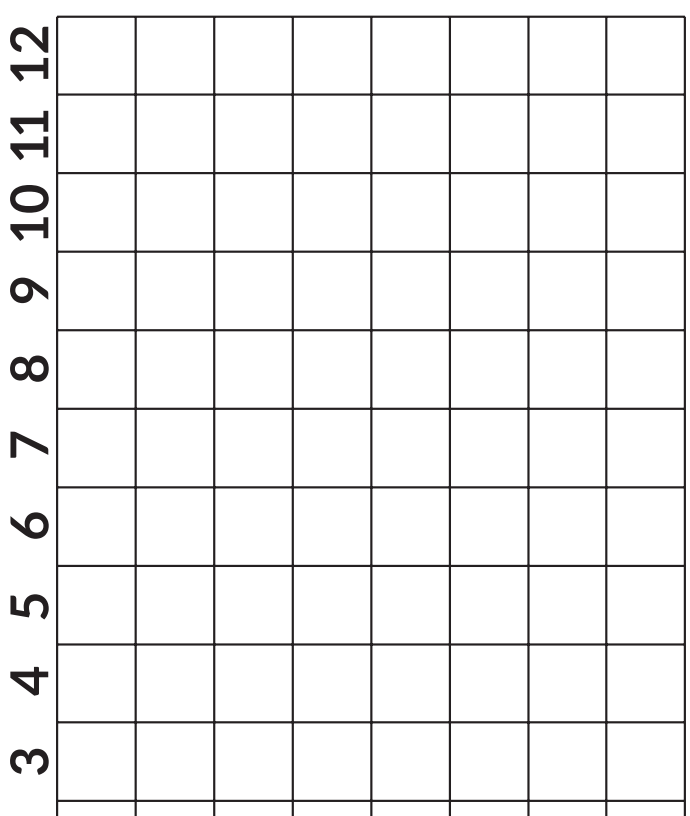
RESOURCES

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 deionized 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. Shirai, H., Blundell, T.L., and Mizuguchi, K. A novel superfamily of enzymes that catalyze the modification of guanidino groups. *Trends Biochem. Sci.* **26(8)**, 465-468 (2001).
2. Takizawa, Y., Sawada, T., Suzuki, A., *et al.* Peptidylarginine deiminase 4 (PADI4) identified as a conformation-dependent autoantigen in rheumatoid arthritis. *Scand. J. Rheumatol.* **34(3)**, 212-5 (2005)
3. Zhao, J., Zhao, Y., He, J., *et al.* Prevalence and significance of anti-peptidylarginine deiminase 4 antibodies in rheumatoid arthritis. *J. Rheumatol.* **35(6)**, 969-974 (2008).
4. Halvorsen, E.H., Pollmann, S., Gilboe, I.-M., *et al.* Serum IgG antibodies to peptidylarginine deiminase 4 in rheumatoid arthritis and associations with disease severity. *Ann. Rheum. Dis.* **67**, 414-17 (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.