Version 22-04/24

User's Manual

PAD4 Autoantibody EIA Kit

REF **DEIA5084**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The 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.

General Description

Protein arginine deiminase 4 (PAD4) catalyzes the conversion of arginine residues to citrulline within cellular protein substrates, resulting in the loss of a positive charge, which can alter protein structure and/or function. It is expressed in neutrophils, as well as a variety of tissues, including the brain, liver, lung, and kidney. PAD4 has a key role in NETosis, a lytic form of cell death characterized by the release of neutrophil extracellular traps (NETs). Upon neutrophil activation, PAD4 translocates to the nucleus where it citrullinates histones, initiating chromatin decondensation and the release of NETs. Neutrophils isolated from Pad4-/- mice exhibit decreased citrullination of histone H3 under both basal and LPS-stimulated conditions and are defective for NET formation in response to stimulation with LPS, phorbol 12-myristate 13-acetate (PMA), or hydrogen peroxide.4 Citrullination of PAD4 targets contributes to disease progression and the generation of autoantibodies in patients with autoimmune diseases, including rheumatoid arthritis, Hashimoto's encephalopathy, and multiple sclerosis. Additionally, PAD4 autoantibodies isolated from the serum of patients with rheumatoid arthritis are associated with disease severity.

Principles of Testing

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.

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

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

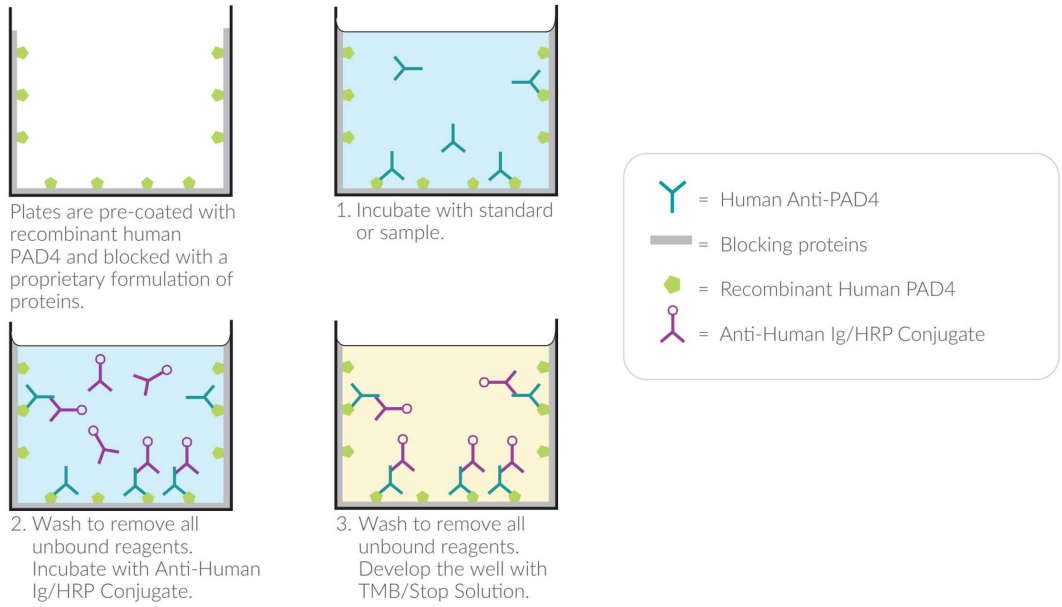


Figure 1. Schematic of the Immunometric ELISA

Reagents And Materials Provided

1. Goat Anti-Human Ig(H+L)/HRP Conjugate, 2 vials/1.5 ml
2. PAD4 Precoated 96-Well Strip Plate, 1 plate
3. Anti-PAD4 (human) ELISA Standard*, 1 vial
4. Immunoassay Buffer B (10×), 2 vials/10 ml
5. Wash Buffer Concentrate (400×), 1 vial/5 ml
6. Polysorbate 20, 1 vial/3 ml
7. TMB Substrate Solution, 1 vial/12 ml
8. HRP Stop Solution, 1 vial/12 ml
9. 96-Well Cover Sheet, 3 covers

*The Anti-PAD4 (human) ELISA Standard was affinity-purified from human plasma.

Materials Required 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.

Storage

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.

Plate Preparation

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 points 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 2. The user may vary the location and type of wells present as necessary for efach particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet (see below, for more details).

	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 2. Sample plate format

Reagent 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 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 (400×): Dilute to a total volume of 2 L with deionized water and add 1 ml of Polysorbate 20.

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.

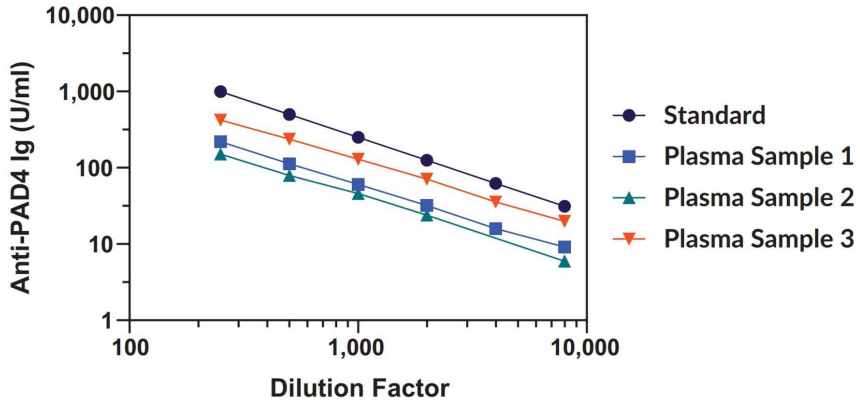


Figure 3. Parallelism of sample matrices in the PAD4 Autoantibody ELISA Kit

Preparation of Assay-Specific Reagents

Anti-PAD4 (human) ELISA Standard

Reconstitute the lyophilized purified Anti-PAD4 (human) ELISA Standard 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.

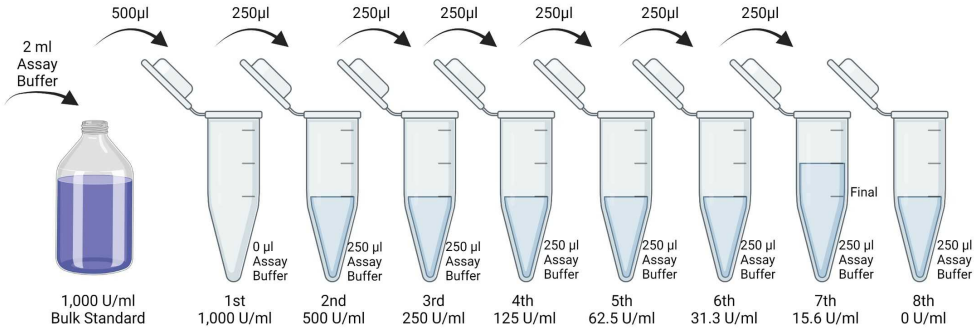


Figure 4. Preparation of the Anti-PAD4 standards

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

This reagent is supplied as a concentrated (10×) stock solution of Goat anti-Human Ig(H+L) polyclonal antibody conjugated to HRP. On the day of the assay, prepare a Working Solution by adding 1.2 ml of the stock solution 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.

Assay Procedure

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. 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 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 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.

Calculation

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.

Typical 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.

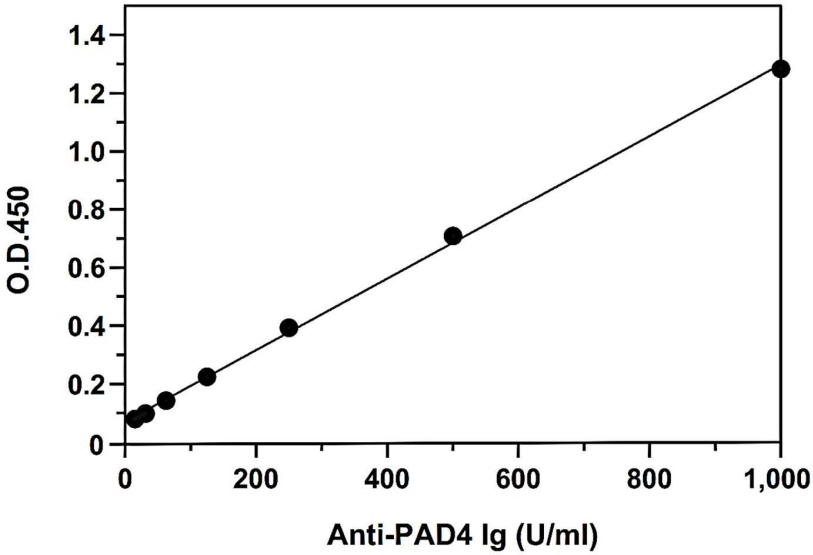
Performance Characteristics

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 5. 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.

Detection Range

15.6-1,000 U/ml

Detection Limit

15.6 U/ml

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with 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.

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	