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Protein Arginine Deiminases(PADs) Activity Detection Kit

General Instruction Manual

For the sensitive and accurate measurement of total PAD enzyme activity/inhibition in complex biological samples or purified PAD proteins.

This assay kit is for research use only – NOT intended for use in diagnostic or therapeutic procedures.


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ASSOCIATED KIT PRODUCTS

Detection Kit	Catalog No.
PAD Activity Kit	P312-863

Inhibitor Screening Kits	Catalog No.
PAD1 Inhibitor Screening Kit	P310G-863
PAD2 (mouse) Inhibitor Screening Kit	P310BG-863
PAD2 (human) Inhibitor Screening Kit	P318BG-863
PAD3 Inhibitor Screening Kit	P310CG-863
PAD4 Inhibitor Screening Kit	P310DG-863
PAD Cocktail Inhibitor Screening Kit	P37C-863

CONTENTS

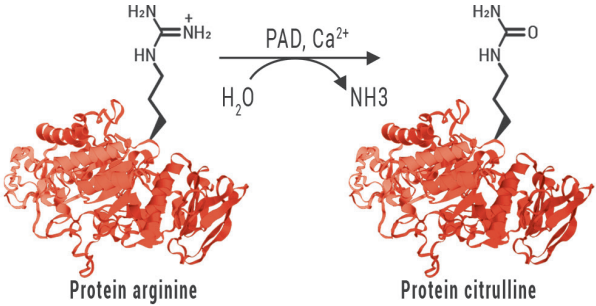
INTRODUCTION

Background

Protein citrullination is a post-translational modification of proteins in which arginine residues are deiminated and converted into citrullines. The deimination of arginine side chains results in the loss of positive charge and an increase in local hydrophobicity of the modified protein. Consequently, protein unfolding, altered protein-protein or protein-DNA interactions and novel antigenic epitopes that elicit aberrant immune responses can occur.

Although many citrullinated proteins play essential roles in physiological processes such as differentiation, organelle maintenance, embryonic development, autoimmunity and gene regulation, dysregulated citrullination has been linked to the pathogenesis of a number of diseases, including rheumatoid arthritis (RA), atherosclerosis, Alzheimer's disease, lupus, and cancers. As such, citrullinated proteins have been proposed to serve as powerful biomarkers for these diseases. Assays that reliably monitor the activity of protein citrullination in complex biological samples therefore become crucial.

The protein arginine deiminases (PAD) family of enzymes is responsible for the catalysis of arginine-to-citrulline conversion in target proteins. There are 5 mammalian PAD isoforms (PAD1-4, PAD6) that share ~50% sequence homology. In vitro experiments have demonstrated only PAD1-4 are active, while PAD6 is inactive due to critical mutations. Each PAD enzyme has its own typical subcellular localization, tissue distribution and substrate specificity, although no substrate protein has been found so far for PAD6.



The catalytic activities of PADs have an absolute requirement for calcium (1-10 mM). Under normal conditions PADs are inactive because of the relatively low calcium concentrations in the cytosol and nucleoplasm. PAD activities are highly elevated in tissues from disease models or patients of various inflammatory conditions and cancers. Concomitantly, PAD inhibitors are showing prominent efficacy in many of these disease models. Overall, regulation of PAD activity is becoming a promising strategy to treat and prevent inflammatory and autoimmune diseases linked to abnormal protein citrullination.

About this assay

The PAD Activity Kits are direct Enzyme-Linked ImmunoSorbent Assay (ELISA) kits for the quantitative determination of total PAD activity in cell or tissue lysates (P312-863). In addition, when combined with recombinant PAD enzymes, the PAD Inhibitor Screening Kits enable users to perform PAD-targeted inhibitor profiling studies -

Product Name	Catalog Number
PAD1, Active	P310G-863
PAD2 (mouse), Active	P310BG-863
PAD2 (human), Active	P318BG-863
PAD3, Active	P310CG-863
PAD4, Active	P310DG-863
PAD Cocktail (0.5 mg/ml)	P37C-863

The assay was designed based on the fact that the citrullination of arginine interferes with the ability of trypsin to hydrolyze the carboxyl side of the amide bond. A universal PAD substrate is first fixed onto NeutrAvidin-coated strips of ELISA plate. Incubation of the plate with lysate sample followed by trypsin removes the peptide fragment on the carboxyl-side of the arginine, but not that of the PAD generated citrulline. The epitope remaining on these peptidyl citrulline is then detected by an antibody in the presence of a chromogenic substrate. The signal intensity would be quantitatively correlated with PAD activity from the sample.

Additional materials required

1. Samples to be tested for PAD activity, or purified PAD enzyme and test compounds if needed
2. Milli-Q water or other source of pure water
3. Pipettes, or multichannel pipettes
4. Incubator for 37°C incubation
5. Microplate reader capable of reading absorbance at 450 nm and 540 nm

GENERAL INFORMATION

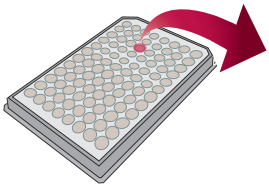
Materials supplied

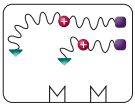
	Part #	Part	Size/Quantity	Storage
Kit Box	AB99-61DM	Detection Antibody, 500X	25 µL x 1 vial	4°C for up to 1 month
	CP01-506	NeutrAvidin™ Coated Plate	1 plate	
	CS01-506	TMB Substrate	6 mL x 1 vial	
	P312-09	PAD Buffer	8 mL x 1 vial	
	SS01-09	Stop Solution	3 mL x 1 vial	
	T01-09	Trypsin Digestion Buffer	15 mL x 1 vial	
	WB20-09	Wash Buffer, 20X	15 mL x 2 vials	
Dry Ice Pack	P312-37C	PAD Cocktail, 0.5 mg/ml	5 µg x 1 vial	-70°C for up to 1 year*
	P312-58	PAD Substrate, lyophilized	0.5 mg x 1 vial	Reconstituted substrate -70°C for up to 1 year*
	T575-31N	Trypsin, 10 mg/ml	200 µg x 1 vial	-20°C for up to 1 year*
	D86-09	DTT, 1M	10 µL x 1 vial	
	Optional**	Active PAD Enzyme		-70°C for up to 1 year*
	Inhibitor Screening Kit Optional Components			Kit Cat #
Optional	P312-310G	PAD1, Active	50 µg x 1 vial	P310G-863
	P312-310BG	PAD2 (mouse), Active	100 µg x 1 vial	P310BG-863
	P312-318BG	PAD2 (human), Active	100 µg x 1 vial	P318BG-863
	P312-310CG	PAD3, Active	100 µg x 1 vial	P310CG-863
	P312-310DG	PAD4, Active	50 µg x 1 vial	P310DG-863
	P312-37C	PAD Cocktail, Active	50 µg x 1 vial	P37C-863

* Avoid repeat handling and multiple freeze-thaw cycles.

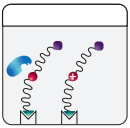
** Optional component for Inhibitor Screening kits only – not included in P312-863 PAD Activity Kit

ASSAY WORKFLOW

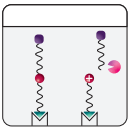




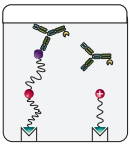
Substrate binds to well



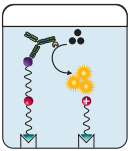
Add sample



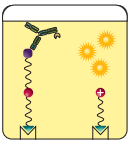
Wash plate & add trypsin



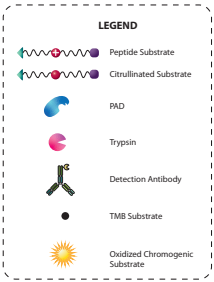
Wash plate & add detection antibody



Wash plate & add TMB



Add stop solution & read plate



PROTOCOLS

Reagent preparation

The materials supplied in this kit are sufficient for 100 PAD activity assays using the provided protocol. Users may adjust the amount of reagents to be prepared according to number of assays needed and store unused reagents at appropriate conditions as indicated.

1. Wash Buffer, 1X

Add 570 mL of Milli-Q water to 30 mL of the 20X Wash Buffer to make the 1X wash buffer. The wash buffer can be stored at 4°C for up to one week. For future use, store at -20°C.

2. Substrate Coating Solution

The PAD Substrate vial contains 0.5 mg of lyophilized peptide (Mw = 3687 Da). Reconstitute the contents with 1 mL water. Take desired volume and dilute with 1X wash buffer at a ratio of 1:1000. Unused substrate solution can be stored at -80°C for up to one year.
For standard curve, perform 2-fold serial dilutions in wash buffer starting from 0.5 µg/mL. A typical standard curve has 8 points including zero.

3. PAD Buffer

The PAD Buffer bottle contains a solution of 0.1 M Tris-HCl pH7.4, 10 mM $CaCl_2$. Upon receival, the solution can be stored at 4°C for up to two weeks. Prior to use, add DTT to obtain a final concentration of 0.5 mM. Mix thoroughly and keep on ice until use.

4. PAD Cocktail

The PAD Cocktail contains a mixture of all five PAD isoforms (PAD1,2,3,4,6) expressed as recombinant proteins, with each isoform at 0.1 mg/ml. The storage buffer contains 50 mM Tris-HCl pH7.5, 150 mM NaCl, 10 mM glutathione, 0.1 mM EDTA, 0.25 mM DTT, 0.1 mM PMSF and 25% glycerol.
Thaw the vial on ice. Briefly spin down to bring all content to bottom of vial, gently tap to mix. Take desired enzyme amount and dilute with PAD buffer. The enzymes can be kept on ice for four hours without significant loss of activity. For each assay well, 100 ng of the enzyme mixture is a good starting point.
Note: Avoid repeated freeze/thaw cycles.

5. Trypsin Digestion Reagent

The Trypsin Buffer bottle contains a solution of 50 mM Tris-HCl pH8.0, 2.5 mM EDTA. Upon receival, the solution can be stored at 4°C for up to one month. For future use, store at -20°C. Prepare trypsin digestion reagent by diluting the Trypsin stock solution with Trypsin Buffer at a ratio of 1:1000. Unused trypsin stock solution can be stored at -20°C for up to 12 months.

6. Detection Antibody

The vial contains 25µL of 500X Detection Antibody. Dilute the antibody 500-fold in wash buffer. Keep on ice until use.

Assay procedure

General considerations:

a) The PAD Cocktail is included as a positive control but also can be used for generating a standard curve.
b) It is recommended to perform several dilutions of your sample with the PAD buffer.
c) It is recommended to assay all samples and controls in duplicate or triplicate.
d) For inhibitor screening assays, an optimal concentration of the target PAD enzyme must be used. Pre-incubate with the test compound 15-30min.
e) All assay reagents, except the PAD enzymes or PAD samples, should be equilibrated to room temperature prior to use.

1. Place the NeutrAvidin coated strips onto an ELISA plate frame. Wash the wells with wash buffer for 3 times. Invert the plate and blot it against clean paper towels.

2. Add 100 µL of the substrate coating solution in each well. Incubate at room temperature for 1 hour.

3. Decant solution from the plate. Wash the wells with wash buffer for 4 times. After the last wash, invert the plate and blot it against clean paper towels.

4. Add 50 µL of the test sample, enzyme standard or PAD buffer to each well. Cover all wells with a plate sealer and incubate at 37°C for the desired time period. This is usually 20-60 minutes depending on the activity present in the sample.

5. Repeat **Step 3** as described.

6. Add 100 µL trypsin buffer to each well and aspirate by pipetting.

7. Add 100 µL trypsin digestion reagent in all wells except for the standard curve, in which trypsin buffer is used instead. Cover all wells with a plate sealer and incubate at 37°C for 1 hour.
Note: The duration of trypsin digestion can be shortened if using a higher concentration of trypsin. It is advisable to obtain a trypsin dose response to determine the optimal concentraion of trypsin if a different digestion time is used.

8. Repeat **Step 3** as described.

9. Add 100 µL detection antibody to each well. Incubate at room temperature for 1 hour.

10. Repeat **Step 3** as described.

11. Add 50 uL of TMB Substrate to each well. Incubate at room temperature for 20 minutes and protected from light.

12. Add 25 µL stop solution to each well. The color in the wells should change from blue to yellow. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

13. Within 30 minutes, read the optical density (OD) of all assay wells at 450nm and 540nm in a microplate reader.

DATA ANALYSIS

Calculation of Specific Activity

In order to calculate the specific activity of a PAD sample, a standard curve that correlates the OD with the amount of citrullinated substrate must be generated in the same experiment the sample is assayed (Figure 1).

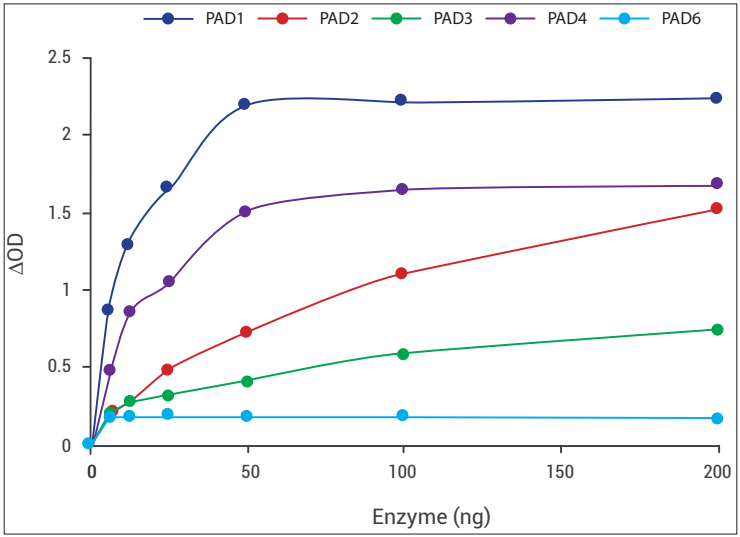


Figure 1. Demonstration of high sensitivity and specificity of the assay achieved by using human recombinant PAD enzymes with PAD Activity Kit.

Calculation of Specific Activity (cont'd)

1. Subtract OD value measured at 540 nm from that measured at 450 nm for each assay well. This subtraction will correct for optical imperfections in the plates. Readings made directly at 450 nm without correction may be higher and less accurate.

2. Subtract the average OD value of the blank wells from the values of those wells that have PAD reactions.

3. Using the standard curve and OD value for a PAD sample, determine concentration or quantity of citrullinated substrate.

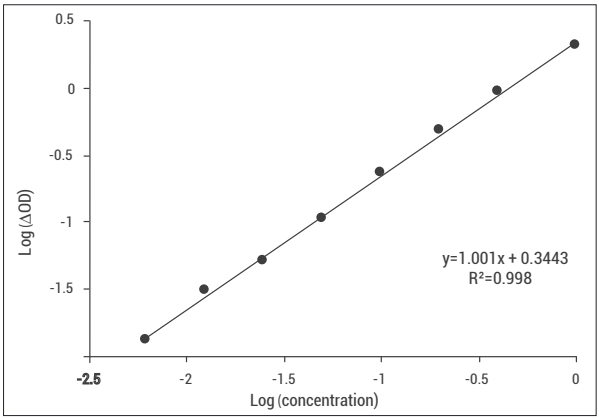


Figure 2. Typical dose response curve of the supplied PAD substrate. Data was analyzed using MS Excel® software.

4. Calculate activity using the equation below:

$$\text{Specific Activity}((\text{nmol}/\text{min})/\text{mg}) = \frac{\text{Substrate Conc } (\mu g/mL) \times \text{Coating Solution Vol } (\mu L)}{\text{Substrate Mw (Da)} \times \text{Reaction Time (min)} \times \text{Protein Amount (mg)}}$$

where the Substrate Mw is 3687 Da, Protein Amount is the mass of total protein in a sample containing PAD activity.

Inhibition Assay

When a several dilutions of a test compound are co-incubated with a purified PAD enzyme with the substrate-coated plate, an inhibition profile of the compound can be plotted on completion of the previously defined assay protocol (Figure 3). Note that the maximum volume of the compound should be less than 5% of the total reaction volume to avoid any inhibitory effect of the solvent.

The assay for PAD enzyme inhibition uses the same general assay procedure defined on page 10, with added step of pre-incubation of the PAD enzyme with the test compound for 15-30min prior to step 4.

Calculation of Inhibition

After correcting the OD values for optical imperfections in the plates, the following equation can then be used to calculate PAD inhibition from a test compound.

$$\%Inhibition = \left(1 - \frac{Inhibitor\ Sample\ OD - Blank\ OD}{No\ Inhibitor\ Sample\ OD - Blank\ OD} \right) \times 100$$

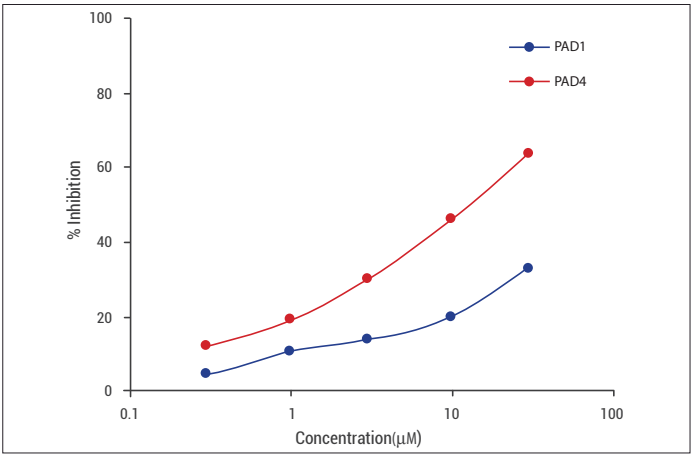


Figure 3. Inhibition of human recombinant PAD1 and PAD4 by Cl-amidine.

Related products

Active PAD Enzymes

Name	Catalog Number	Species	Tag	Expression System	Sequence	Genbank Number
PAD1, Active	P312-310G	Human	GST	Sf9	Full Length	NM_013358
PAD2, Active	P312-310BG	Mouse	GST	Sf9	Full Length	BC049947
PAD2, Active	P312-318BG	Human	GST	Sf9	Full Length	NM_007365
PAD3, Active	P312-310CG	Human	GST	Sf9	Full Length	NM_016233
PAD4, Active	P312-310DG	Human	GST	Sf9	Full Length	BC025718
PAD Cocktail, Active	P312-37C		GST	Sf9	Full Length	

Inhibitor Screening Kits

Catalog #	Product Name	Included Optional Component	
P310G-863	PAD1 Inhibitor Screening Kit	P312-310G	PAD1, Active
P310BG-863	PAD2 (mouse) Inhibitor Screening Kit	P312-310BG	PAD2, Active
P318BG-863	PAD2 (human) Inhibitor Screening Kit	P312-318BG	PAD2, Active
P310CG-863	PAD3 Inhibitor Screening Kit	P312-310CG	PAD3, Active
P310DG-863	PAD4 Inhibitor Screening Kit	P312-310DG	PAD4, Active
P37C-863	PAD Cocktail Inhibitor Screening Kit	P312-37C	PAD Cocktail

Related products (cont'd)

siRNA Sets

Name	Catalog Number
PAD1 siRNA Set I	P312-911
PAD2 siRNA Set I	P312-911B
PAD3 siRNA Set I	P312-911C
PAD4 siRNA Set I	P312-911D
PAD6 siRNA Set I	P312-911E

RESOURCES

References

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2. Raijmakers, R. et al. (2007). Methylation of Arginine Residues Interferes with Citrullination by Peptidylarginine Deiminases in vitro. *J. Mol. Biol.* **367**, 1118–29.
3. Tilvawala, R., & Thompson, P.R. (2019). Peptidyl arginine deiminases: detection and functional analysis of protein citrullination. *Curr. Opin.* Struct. Biol. **20**, 1-11.
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5. Clancy K.W. et al. (2016). Detection and identification of protein citrullination in complex biological systems. Curr. Opin. *Chem. Biol.* **30**, 1-6.

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