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GENERAL INFORMATION

Materials Supplied

This kit will arrived packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item	Quantity/Size	Storage
PAD Assay Buffer (Ammonia)	1 vial/30 ml	-20°C
PAD4 (human recombinant) Assay Reagent	2 vials/60 µl	-80°C
PAD Substrate	1 vial/lyophilized	-20°C
PAD Stop Solution	1 vial/2.5 ml	-20°C
PAD Ammonia Detector	2 vials/lyophilized	-20°C
Ethanol Assay Reagent	1 vial/2 ml	RT
DTT (1M) Assay Reagent	1 vial/1 ml	-20°C
Cl-Amidine Inhibitor Assay Reagent	1 vial/lyophilized	-20°C
96-Well Solid Plate (black)	1 plate	RT
96-Well Cover Sheet	1 cover	RT

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.

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence using an excitation wavelength of 405-415 nm and an emission wavelength of 470-480 nm
2. Adjustable pipettes and a multichannel pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

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.[1] It is expressed in neutrophils, as well as a variety of tissues, including the brain, liver, lung, and kidney.[1-3] PAD4 has a key role in NETosis, a lytic form of cell death characterized by the release of neutrophil extracellular traps (NETs).[1] Upon neutrophil activation, PAD4 translocates to the nucleus where it citrullinates histones, initiating chromatin decondensation and the release of NETs.[2,4,5] 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] Production of autoantibodies against PAD4 and various PAD4 targets is associated with disease progression and/or severity in patients with autoimmune diseases, including rheumatoid arthritis, Hashimoto's encephalopathy, and multiple sclerosis.[6-9] PAD4 inhibition reduces tumor growth in various mouse xenograft models and ameliorates inflammatory joint destruction in rodent models of rheumatoid arthritis.[10] Development of novel PAD4 inhibitors has the potential to improve the treatment of diseases progressed by PAD4 overexpression and hypercitrullination of PAD4 targets.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **PAD Assay Buffer (Ammonia)**

   This vial contains 30 ml of 50 mM HEPES, pH 7.7, containing 10 mM $CaCl_2$. Once thawed, add 150 µl of 1 M DTT assay reagent (Item No. 700416). This final buffer should be used in the assay and for diluting reagents. After addition of DTT, the buffer should be used within the same day or stored at -20°C limiting freeze-thaw cycles.

2. **PAD4 (human recombinant) Assay Reagent**

   Each vial contains 60 µl of human recombinant PAD4. Thaw the enzyme on ice, add 540 µl of PAD Assay Buffer (Ammonia) to the vial, and mix thoroughly. The diluted enzyme is stable for four hours on ice. One vial of PAD4 is sufficient enzyme to assay 60 wells. Use the additional vial if assaying the entire plate.

3. **PAD Substrate**

   This vial contains lyophilized N-α-benzoyl-L-arginine ethyl ester (BAEE). Reconstitute the contents of the vial with 1 ml of PAD Assay Buffer (Ammonia). The reconstituted substrate is stable for two weeks at -20°C, limit freeze-thaw cycles. *NOTE: The final concentration of substrate in the assay as described below is 250 µM. The K_m value for the substrate is 355 µM.*

4. **PAD Stop Solution**

   This vial contains a citrate solution (a calcium chelator). It is ready to use as supplied. Store unused reagent at -20°C.

5. **PAD Ammonia Detector**

   Each vial contains lyophilized ammonia detector. Reconstitute the contents of the vial with 600 µl of ethanol (Item No. 700566). One vial of detector is sufficient reagent to assay 60 wells. Reconstitute the additional vial if assaying the entire plate. The reconstituted reagent is stable for three hours at room temperature.

6. **Ethanol Assay Reagent**

   This vial contains 2 ml of ethanol. It is ready to use in the assay.

7. **DTT (1 M) Assay Reagent**

   This vial contains 1 M DTT. Once thawed, the reagent is ready to use and can be stored at -20°C, limiting freeze-thaw cycles.

8. **Cl-Amidine Inhibitor Assay Reagent**

   This vial contains 950 nmol of inhibitor. Reconstitute with 150 µl of PAD Assay Buffer (Ammonia) containing 5 mM DTT. The final concentration of the inhibitor in the reaction is 150 µM.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% Initial Activity and three wells designated as Background. It is suggested that each inhibitor (including the Cl-Amidine Inhibitor Assay Reagent (Item No. 700567)) be assayed in triplicate, and the contents of each well are recorded on the template sheet provided on page 18. A typical layout of samples and inhibitors to be measured in triplicate is shown in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	IA	IA	IA	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	PC	PC	PC

BW - Background Wells
IA - 100% Initial Activity Wells
1-29 - Inhibitor Wells
PC - Cl-Amidine Positive Control Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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.

General Information

- The final volume of the assay is 210 µl in all the wells.
- All reagents, except the enzyme, must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 405-415 nm and an emission wavelength of 470-480 nm.

Performing the Assay

1. **100% Initial Activity Wells -** add 155 µl of PAD Assay Buffer (Ammonia), 10 µl of PAD4, and 5 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
2. **Background Wells -** add 165 µl of PAD Assay Buffer (Ammonia) and 5 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells -** add 155 µl of PAD Assay Buffer (Ammonia), 10 µl of PAD4, and 5 µl of test inhibitors or the positive control, Cl-Amidine Inhibitor Assay Reagent, to at least three wells. *NOTE: Inhibitors may be prepared in organic solvents such as DMSO or DMF, as long as the final concentration of organic solvent in the assay is <2% or <1% respectively. Methanol and ethanol are not compatible in this assay. In the event that an appropriate concentration of inhibitor is unknown, it is recommended that several dilutions of the inhibitor are assayed.*

Well	PAD Assay Buffer (Ammonia) (µl)	PAD4 (µl)	Inhibitor (µl)	Solvent (µl)
100% Initial activity wells	155	10	-	5
Background wells	165	-	-	5
Inhibitor wells	155	10	5	-

Table 1. Pipetting summary

4. Incubate for five minutes at room temperature.
5. Initiate the reactions by adding 10 µl of PAD Substrate to all of the wells being used.
6. Cover the plate with the plate cover and incubate for thirty minutes at 37°C.
7. Remove the plate cover, add 20 µl of PAD Stop Solution, and 10 µl of PAD Ammonia Detector to all of the wells being used.
8. Cover the plate with the plate cover and incubate for fifteen minutes at 37°C.
9. Remove the plate cover and read the fluorescence in a plate reader at an excitation wavelength of 405-415 nm and an emission wavelength of 470-480 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of each sample.
2. Subtract the average fluorescence of the Background wells from the average fluorescence of the 100% Initial Activity and Inhibitor wells.
3. Determine the percent Inhibition or percent Initial Activity for each inhibitor using one of the following equations:

$$\% \text{ Inhibition} = \left[\frac{\text{Initial Activity - Inhibitor Activity}}{\text{Initial Activity}} \right] \times 100$$

$$\% \text{ Initial Activity} = \left[\frac{\text{Inhibitor Activity}}{\text{Initial Activity}} \right] \times 100$$

4. Graph the percent inhibition or percent initial activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). Inhibition of human recombinant PAD4 by Cl-Amidine is shown in Figure 2 (see page 14).

Performance Characteristics

Z´ Factor:

Z´ Factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[8]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{\left| \mu_{c+} - \mu_{c-} \right|}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control or Inhibitor Sample
c-: Negative control or 100% Initial Activity

The theoretical upper limit for the Z´ factor is 1.0. A robust assay has a Z´ factor >0.5. The Z´ factor for Cayman's PAD4 Inhibitor Screening Assay Kit (Ammonia) was determined to be 0.84.

Precision:

When a series of twelve PAD4 100% Initial Activity measurements were performed on the same day, the intra-assay coefficient of variation was 2.9%. When a series of four PAD4 100% Initial Activity measurements were performed on multiple days under the same experimental conditions, the inter-assay coefficient of variation was 4.8%.

Sample Data:

The data shown here is an example of inhibition data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

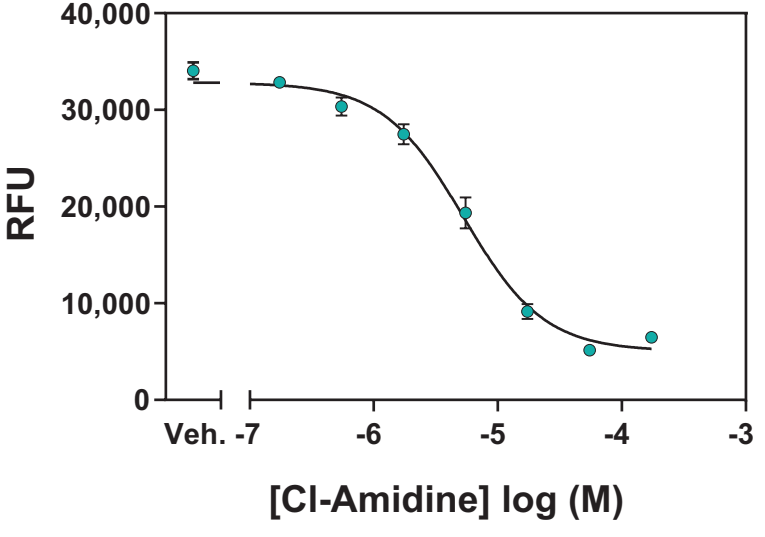

Figure 2. Inhibition of human recombinant PAD4 by Cl-Amidine. "Veh." represents 100% initial activity.

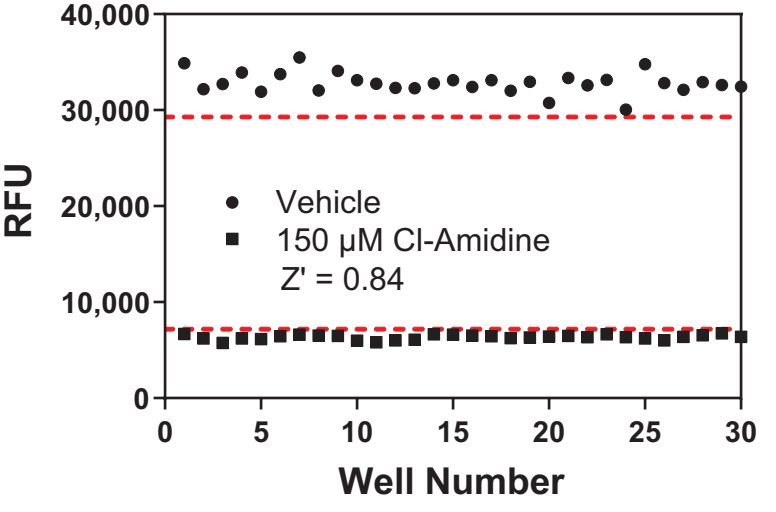

Figure 3. Typical Z´ data for the PAD4 Inhibitor Screening Assay Kit (Ammonia). Data are shown from wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z´ factor from this experiment was 0.84. The red lines correspond to three standard deviations from the mean for each control value.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence was detected above background in the inhibitor wells	A. Enzyme or substrate was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all of the components to the well(s) B. Reduce the concentration of the inhibitor and re-assay
The plate reader exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition was seen with inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

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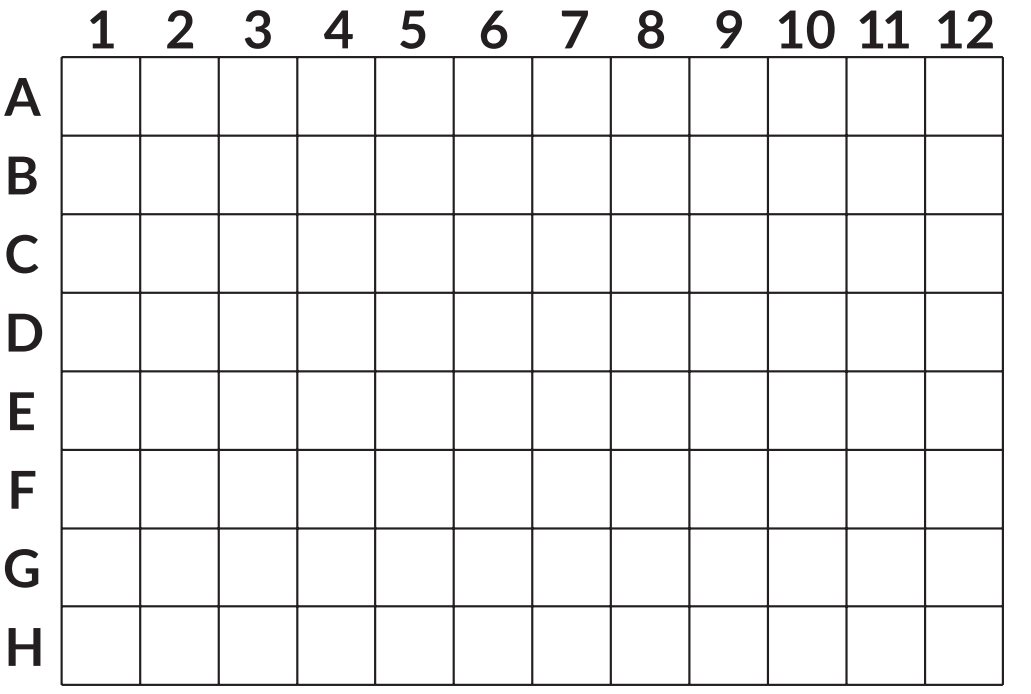
1 2 3 4 5 6 7 8 9 10 11 12
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