PAD4 Inhibitor Screening Assay Kit

Item No. 700560

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
700561	PAD Assay Buffer	1 vial/30 ml	-20°C
700562	PAD4 (human recombinant) Assay Reagent	2 vials/60 µl	-80°C
700563	PAD Substrate	2 vials/lyophilized	-20°C
700564	PAD Stop Solution	2 vials/1.2 ml	-20°C
700565	PAD Ammonia Detector	2 vials/lyophilized	-20°C
700566	Ethanol Assay Reagent	1 vial/2 ml	Room temperature
400017	96-Well Solid Plate (black)	1 plate	Room temperature
400012	96-Well Cover Sheet	1 cover	Room temperature

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 laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

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 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 fluorometer with the capacity to measure fluorescence using an excitation wavelength of 405-415 nm and an emission wavelength of 470-480 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Protein Arginine Deiminases (PADs) are guanidino-modifying enzymes belonging to the amidinotransferase superfamily and are designated PAD1-4 and PAD6. All enzymes are cytosolic except for PAD4 which is localized in the nucleus.[1] 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 substrates include histones H2A, H3, and H4, whose post-translational modifications play a large role in gene regulation.[2] PAD4 itself can undergo autocitrullination at several sites which inhibit its enzymatic activity and may play an important role in regulating citrullination in cells.[3] PAD4 activity is increased in rheumatoid arthritis, producing an abundance of citrulline-containing proteins that generate an immune response resulting in production of autoantibodies that ultimately attack the host tissues.[4,5] PAD4 has also been implicated in several other diseases including multiple sclerosis, Alzheimer's disease, glaucoma, and cancer.[2,6]

About This Assay

Cayman's PAD4 Inhibitor Screening Assay provides a convenient method for screening human PAD4 inhibitors. PAD4 deiminates N-α-benzoyl-L-arginine ethyl ester (BAEE), a non-natural substrate with similar kinetic properties to the natural substrates, producing ammonia.[4] Ammonia reacts with a detector resulting in a fluorescent product. Fluorescence is then analyzed with an excitation wavelength of 405-415 nm and an emission wavelength of 470-480 nm.

PRE-ASSAY PREPARATION

Reagent Preparation

1. PAD Assay Buffer - (Item No. 700561)

The vial contains 30 ml of 50 mM borate, pH 8.0, containing 10 mM $CaCl_2$. It is ready to use as supplied. Once thawed, the buffer can be stored at 4°C. It is stable for one month at 4°C.

2. PAD4 (human recombinant) Assay Reagent - (Item No. 700562)

Each vial contains 60 µl of human recombinant PAD4 (Protein Arginine Deiminase 4). Thaw the enzyme on ice, add 540 µl of Assay Buffer to the vial, and vortex. 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 - (Item No. 700563)

Each vial contains lyophilized N-α-benzoyl-L-arginine ethyl ester (BAEE). Reconstitute the contents of the vial with 600 µl of Assay Buffer. One vial of substrate is sufficient reagent to assay 60 wells. Reconstitute the additional vial if assaying the entire plate. The reconstituted substrate is stable for two weeks at -20°C. *NOTE: The final concentration of substrate in the assay as described below is 2 mM. This concentration may be reduced with Assay Buffer at the user's discretion. The K_m value for the substrate is 355 µM.*

4. PAD Stop Solution - (Item No. 700564)

Each vial contains 1.2 ml of a citrate solution (a calcium chelator). It is ready to use as supplied. One vial of Stop Solution is sufficient reagent to assay 60 wells. Thaw the additional vial if assaying the entire plate. Store unused reagent at -20°C.

5. PAD Ammonia Detector - (Item No. 700565)

Each vial contains a clear lyophilized powder of ammonia detector. Reconstitute the contents of the vial with 600 µl of ethanol. 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 - (Item No. 700566)

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

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 wells and three wells designated as background wells. We suggest that each inhibitor be assayed in triplicate and that you record the contents of each well on the template sheet provided on page 19. 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	A	A	A	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	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor 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.
- We recommend assaying 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 150 µl of Assay Buffer, 10 µl of PAD4, and 10 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
2. **Background Wells** - add 160 µl of Assay Buffer and 10 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells** - add 150 µl of Assay Buffer, 10 µl of PAD4, and 10 µl of inhibitor* to three wells.

Well	Assay Buffer (µl)	PAD4 (µl)	Inhibitor (µl)	Solvent (µl)
100% Initial Activity Wells	150	10	-	10
Background Wells	160	-	-	10
Inhibitor Wells	150	10	10	-

*Inhibitors can be dissolved in assay buffer, methanol, dimethylsulfoxide, or ethanol and should be added to the assay in a final volume of 10 µl. In the event that an appropriate concentration of inhibitor is completely unknown, we recommend that several dilutions of the inhibitor be made.

4. Initiate the reactions by adding 10 µl of PAD Substrate to the 100% Initial Activity, Background, and Inhibitor wells.
5. Cover the plate with the plate cover and incubate for thirty minutes at 37°C.
6. Remove the plate cover, add 20 µl of PAD Stop Solution, and 10 µl of PAD Ammonia Detector to all of the wells that are being used.
7. Cover the plate with the plate cover and incubate for fifteen minutes at 37°C.
8. Remove the plate cover and read fluorescence at an excitation wavelength of 405-415 nm and an emission wavelength of 470-480 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of the background, 100% initial activity (IA), and inhibitor wells.
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 Streptomycin and Chlortetracycline is shown in Figures 2 and 3 (see pages 12 and 13, respectively).[7]

Performance Characteristics

Precision:

When a series of sixteen PAD4 measurements were performed on the same day, the intra-assay coefficient of variation was 2.3%. When a series of sixteen PAD4 measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 2.8%.

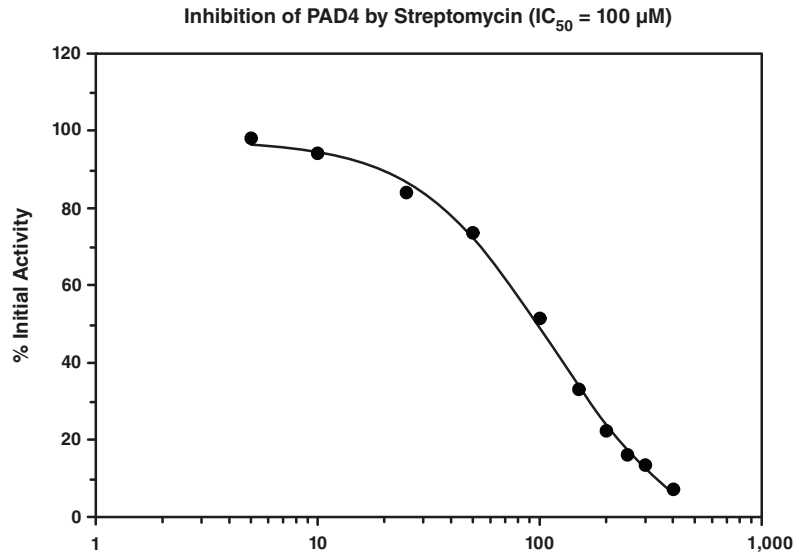

Figure 2. Inhibition of human recombinant PAD4 by Streptomycin (IC_{50} = 100 µM)

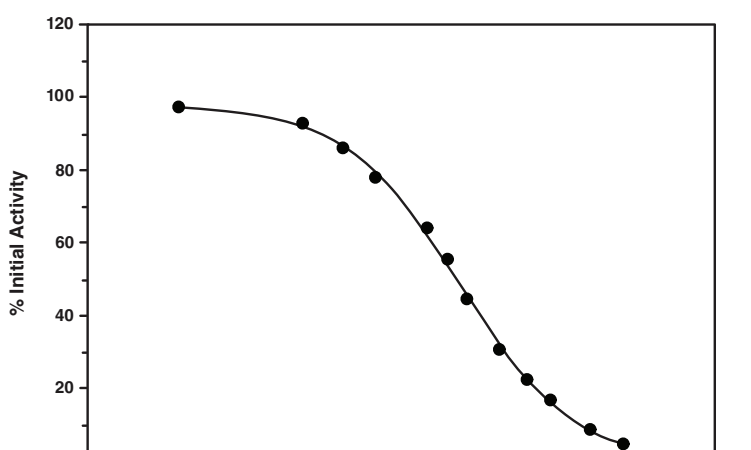

Figure 3. Inhibition of human recombinant PAD4 by Chlortetracycline (IC_{50} = 240 µM)

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 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 wells B. Reduce the concentration of the inhibitor and re-assay
The fluorometer 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

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. Jones, J.E., Causey, C.P., Knuckley, B., *et al.* Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. *Curr. Opin. Drug Discov. Devel.* **12(5)**, 616-627 (2009).
3. Andrade, F., Darrah, E., Gucek, M., *et al.* Autocitrullination of human peptidylarginine deiminase 4 regulates protein citrullination during cell activation. *Arthritis Rheum.* (2010).
4. Kearney, P.L., Bhatia, M., Jones, N.G., *et al.* Kinetic characterization of protein arginine deiminase 4: A transcriptional corepressor implicated in the onset and progression of rheumatoid arthritis. *Biochemistry* **44**, 10570-10582 (2005).
5. Hill, J.A., Southwood, S., Sette, A., *et al.* Cutting edge: The conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J. Immunol.* **171**, 538-541 (2003).
6. Slack, J.L., Causey, C.P., and Thompson, P.R. Protein arginine deiminase 4: A target for an epigenetic cancer therapy. *Cell. Mol. Life Sci.* (2010).
7. Knuckley, B., Luo, Y., and Thompson, P.R. Profiling protein arginine deiminase 4 (PAD4): A novel screen to identify PAD4 inhibitors. *Bioorg. Med. Chem.* **16(2)**, 739-45 (2008).

Related Products

Demethylase (Jumonji-type) Activity Assay Kit - Item No. 700390
Demethylase (LSD-type) Activity Assay Kit - Item No. 700400
HAT Inhibitor Screening Assay Kit - Item No. 10006515
HDAC Fluorometric Activity Assay Kit - Item No. 10011563
HDAC1 Inhibitor Screening Assay Kit - Item No. 10011564
HDAC8 Inhibitor Screening Assay Kit - Item No. 700230
JMJD2A Inhibitor Screening Assay Kit - Item No. 700360
JMJD2D Inhibitor Screening Assay Kit - Item No. 700370
LSD1 Inhibitor Screening Assay Kit - Item No. 700120
Methyltransferase Colorimetric Assay Kit - Item No. 700140
Methyltransferase Fluorometric Assay Kit - Item No. 700150
PAD4 Autoantibody EIA Kit - Item No. 500930
PAD4 (human recombinant) - Item No. 10500
SET7/9 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700270
SET8 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700350
SIRT1 Direct Fluorescent Screening Assay Kit - Item No. 10010401
SIRT1 FRET-Based Screening Assay Kit - Item No. 10010991
SIRT2 Direct Fluorescent Screening Assay Kit - Item No. 700280
SIRT3 Direct Fluorescent Screening Assay Kit - Item No. 10011566
SIRT6 Direct Fluorescent Screening Assay Kit - Item No. 700290

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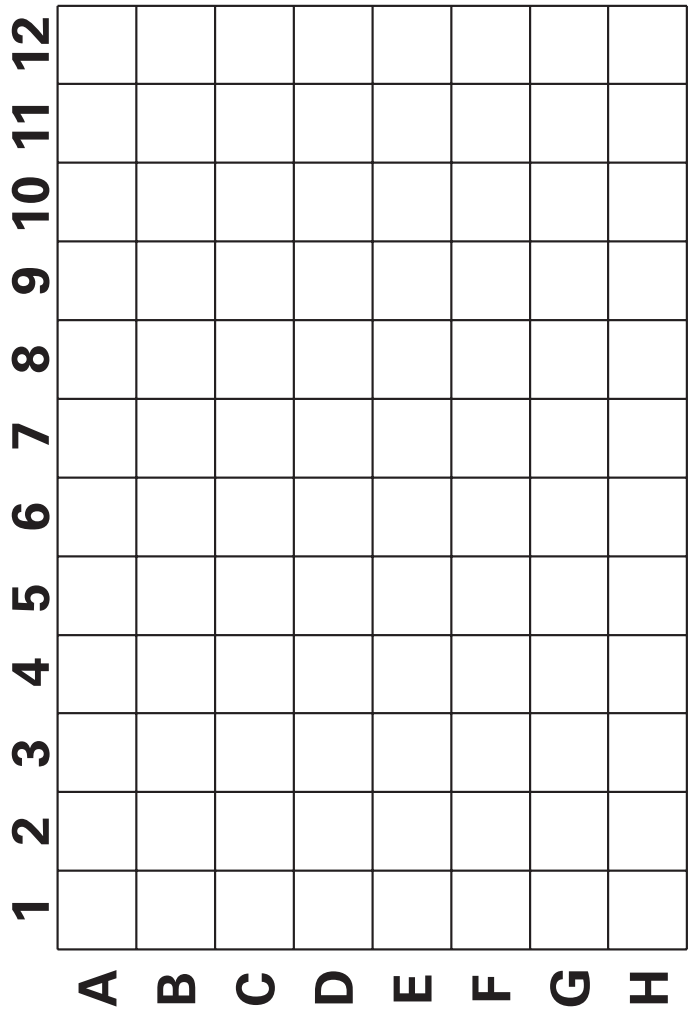
Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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