PAD1 Inhibitor Screening Assay Kit (Ammonia)

Item No. 701450

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GENERAL INFORMATION

Materials Supplied

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

Item Number	Item	Quantity/Size	Storage
701451	PAD Assay Buffer (Ammonia)	1 vial/30 ml	-20°C
701452	PAD1 (human recombinant) Assay Reagent	2 vials/60 µl	-80°C
700563	PAD Substrate	1 vial/lyophilized	-20°C
700564	PAD Stop Solution	1 vial/2.5 ml	-20°C
700565	PAD Ammonia Detector	2 vials/lyophilized	-20°C
700566	Ethanol Assay Reagent	1 vial/2 ml	RT
700416	DTT (1M) Assay Reagent	1 vial/1 ml	-20°C
700567	Cl-Amidine Inhibitor Assay Reagent	1 vial/lyophilized	-20°C
400017	96-Well Solid Plate (black)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

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.

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 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 deiminases (PADs) are guanidino-modifying enzymes belonging to the amidinotransferase superfamily and are designated PAD1-4 and PAD6. PADs are calcium-dependent enzymes that catalyze the post-translational modification of target proteins by converting arginine to citrulline.[1] The various PADs exhibit tissue specific expression and different subcellular localization.[2] PAD1 is a homodimer that is expressed in the uterus, epidermis, and hair follicles. Substrates of PAD1 include keratin, filaggrin, and other proteins.[3] Both PAD1 and PAD3 are speculated to maintain skin hydration.[4] PAD1 activity is decreased in Psoriasis, a type of dermatitis that is characterized by excessive proliferation and leads to flaky, dry patches of skin. The lack of citrullinated keratin causes excessive cornification and an inflammatory response.[3]

About This Assay

Cayman's PAD1 Inhibitor Screening Assay Kit (Ammonia) provides a convenient method for screening human PAD1 inhibitors. PAD1 deiminates N-α-benzoyl-L-arginine ethyl ester (BAEE), a non-natural substrate with similar kinetic properties to the natural substrates, producing ammonia.[5] 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. **DTT (1 M) Assay Reagent - (Item No. 700416)**

   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.

2. **PAD Assay Buffer (Ammonia) - (Item No. 701451)**

   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). The final concentration of DTT in the buffer is 5 mM. 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.

3. **PAD1 (human recombinant) Assay Reagent - (Item No. 701452)**

   Each vial contains 60 µl of human recombinant PAD1. Thaw the enzyme on ice, add 540 µl of PAD Assay Buffer (Ammonia) containing 5 mM DTT to the vial, and mix thoroughly. The diluted enzyme should be stable for four hours on ice. One vial of PAD1 is sufficient enzyme to assay 60 wells. Use the additional vial if assaying the entire plate.

4. **PAD Substrate - (Item No. 700563)**

   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) containing 5 mM DTT. The reconstituted substrate should be stable stable for two weeks at -20°C, limiting freeze-thaw cycles. *NOTE: The final concentration of substrate in the assay as described below is 250 µM.*

5. **PAD Stop Solution - (Item No. 700564)**

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

6. **PAD Ammonia Detector - (Item No. 700565)**

   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. If sealed, the reconstituted reagent should be stable for three hours at room temperature.

7. **Ethanol Assay Reagent - (Item No. 700566)**

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

8. **Cl-Amidine Inhibitor Assay Reagent - (Item No. 700567)**

   This vial contains 950 nmol of inhibitor. Reconstitute with 150 µl of PAD Assay Buffer (Ammonia) containing 5 mM DTT. The concentration in the reaction of the inhibitor is 176 µM. The reconstituted substrate should be stable for two weeks at -20°C, limiting freeze-thaw cycles.

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 wells.

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 (negative) Wells -** add 155 µl of PAD Assay Buffer (Ammonia) containing 5 mM DTT, 10 µl of PAD1, 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) containing 5 mM DTT and 5 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor/Positive Control Wells -** add 155 µl of PAD Assay Buffer (Ammonia) containing 5 mM DTT, 10 µl of PAD1, and 5 µl of test inhibitor or the positive control, Cl-Amidine Inhibitor Assay Reagent (Item No. 700567), to at least three wells. *NOTE: Inhibitors may be prepared in solvents such as DMSO, ethanol, or methanol and should be added to the assay in a final volume of 5 µl. 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 (µl)	PAD1 (µl)	Inhibitor/ Positive Control (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 ten 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 30 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 cover sheet and incubate for 15 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. These are the corrected values.
3. Determine the percent Inhibition or percent Initial Activity for each inhibitor using one of the following equations:

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

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

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 PAD1 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.[6]

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

Where σ: Standard deviation

μ: Mean

c+: Positive control or Inhibitor Sample

c-: Negative control or 100% Inital 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 PAD1 Inhibitor Screening Assay Kit (Ammonia) was determined to be 0.83.

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.

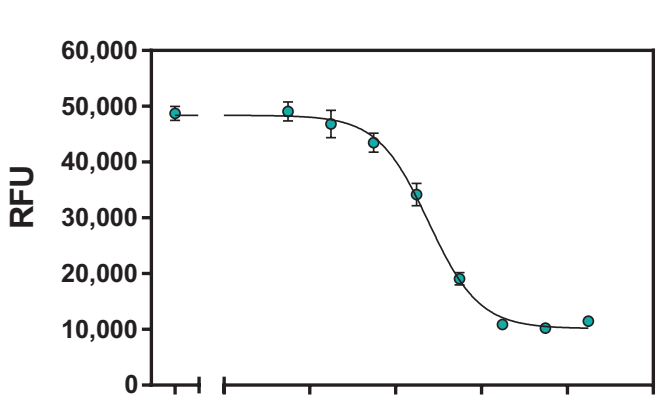


Figure 2. Inhibition of human recombinant PAD1 by Cl-Amidine. "Veh." represents 100% initial activity vehicle control.

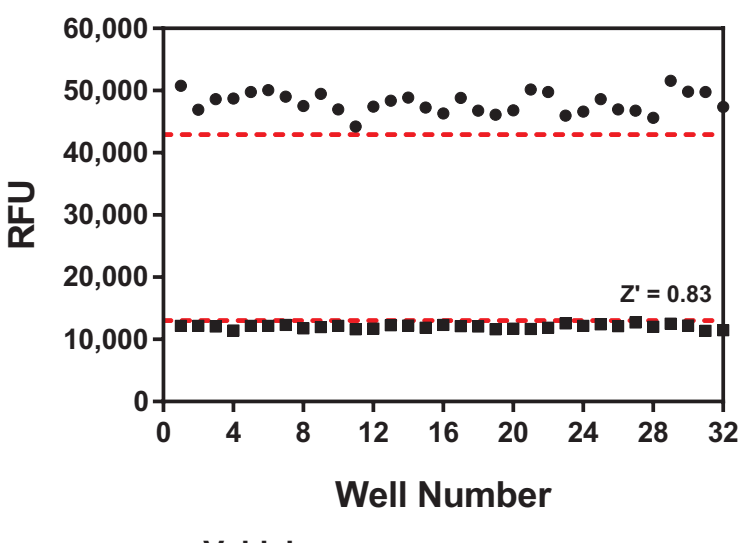


Figure 3. Typical Z´ data for the PAD1 Inhibitor Screening Assay Kit (Ammonia). Data are shown from wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z´ factor from this experiment was 0.83. The red lines correspond to three standard deviations from the mean for each control value.

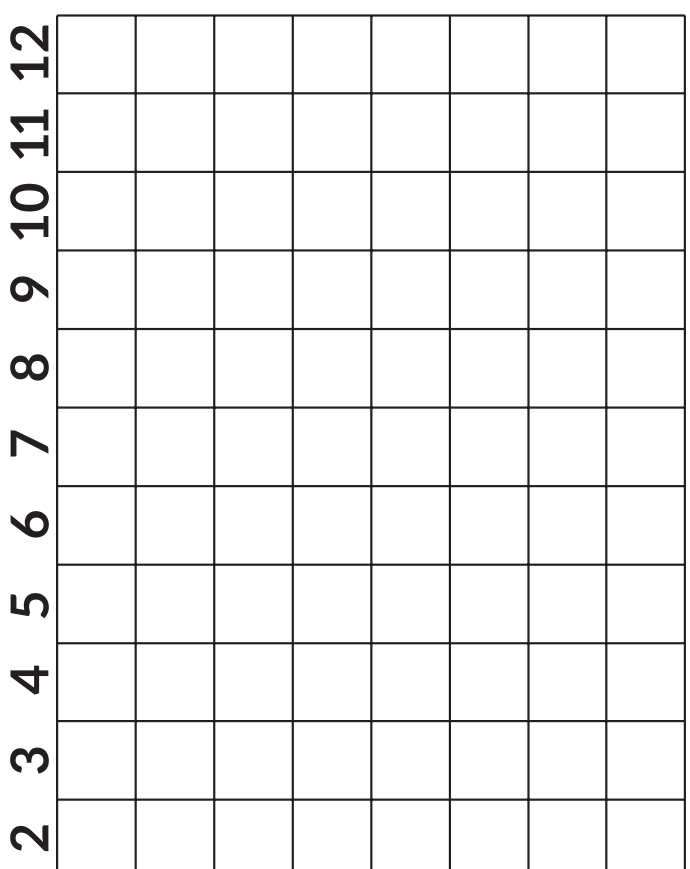
RESOURCES

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 well(s) B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence was detected above background in the inhibitor well(s)	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 well(s)	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. Bicker, K.L. and Thompson, P.R. The protein arginine deiminases: Structure, function, inhibition, and disease. *Bioplymers* **99(2)**, 155-163 (2013).
2. 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).
3. Witalisonk, E.E., Thompson, P.R., and Hofseth, L.J.Protein arginine deiminases and associated citrullination: Physiological functions and diseases associated with dysregulation. *Curr. Drug Targets* **16(7)**, 700-710 (2015).
4. Knuckley, B., Causey, C.P., Jones, J.E., *et al.* Substrate specificity and kinetic studies of PADs 1, 3, and 4 identify potent and selective inhibitors of protein arginine deiminase 3. *Biochem.* **49(23)**, 1-28 (2010).
5. 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).
6. Zhang, J.H., Chung, T.D.Y., and Oldenburg, K.R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of Biomolecular Screening* **4(2)**, 67-73 (1999).



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.