PAD1 Inhibitor Screening Assay Kit (AMC)

Item No. 701440

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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
701321	PAD Assay Buffer (AMC)	1 vial/10 ml	-20°C
701442	PAD1 (human recombinant) Assay Reagent (AMC)	1 vial/200 µl	-80°C
701323	PAD Substrate (AMC)	1 vial/200 µl	-20°C
701324	PAD Developer (AMC)	1 vial/lyophilized	-20°C
700416	DTT (1M) Assay Reagent	1 vial/1 ml	-20°C
700567	Cl-Amidine Inhibitor Assay Reagent	1 vial/lyophilized	-20°C
400091	Half-Volume 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: 734-971-3640

Email: techserv@caymanchem.com

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 355-365 nm and an emission wavelength of 445-455 nm
2. Adjustable pipettes and a multichannel pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. A 37°C incubator
5. Microcentrifuge tubes and test tubes or vials capable of holding volumes of >3 ml

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 (AMC) provides a convenient method for screening human PAD1 inhibitors. This assay utilizes a fluorescent substrate (Z-Arg-AMC) consisting of an arginine residue, a carboxybenzyl group, and a fluorophore (7-amino-4-methylcoumarin, AMC).[5] Acylation of AMC onto the arginine residue masks the fluorescence of the fluorophore. In the absence of PAD1, the substrate remains unaltered, allowing the developer to release free AMC. In the presence of PAD1, the arginine of the substrate is citrullinated, and while the reaction is quenched by the addition of developer, free AMC is not released. Fluorescence is analyzed with an excitation wavelength of 355-365 nm and an emission wavelength of 445-455 nm. The fluorescent signal is inversely proportional to the amount of citrullination by PAD1.

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 (AMC) - (Item No. 701321)**

   This vial contains 10 ml of 50 mM Tris, pH 7.5, 50 mM NaCl and 10 mM $CaCl_2$. Immediately prior to use, add 50 µl of 1 M DTT and mix. This buffer should be used in the assay and for diluting reagents. After addition of DTT, the buffer should be used within the same day. If not using the entire plate, prepare the required amount of buffer by adding 1 M DTT at a ratio of 1:200.

3. **PAD1 (human recombinant) Assay Reagent (AMC) - (Item No. 701442)**

   This vial contains 200 µl of human recombinant PAD1. Thaw the enzyme on ice, and then gently mix 150 µl of PAD1 with 2.1 ml of PAD Assay Buffer (AMC) in a test tube. The diluted enzyme is stable for four hours on ice. If not using the entire plate, dilute the protein 1:15 in PAD Assay Buffer (AMC), and aliquot the remaining protein for storage at -80°C. Avoid repeated freeze-thaw cycles.

4. **PAD Substrate (AMC) - (Item No. 701323)**

   This vial contains 200 µl of fluorescent substrate. Transfer 150 µl of substrate to a clean 5 ml test tube, and then add 2.85 ml of PAD Assay Buffer (AMC). Protect from light. If not using the entire plate, dilute the substrate 1:20 in PAD Assay Buffer (AMC). Store unused reagent at -20°C.

5. **PAD Developer (AMC) - (Item No. 701324)**

   This vial contains a lyophilized powder. Reconstitute the contents of the vial with 6 ml of Milli-Q water. One vial is enough to assay a 96-well plate. Store unused reagent at -20°C.

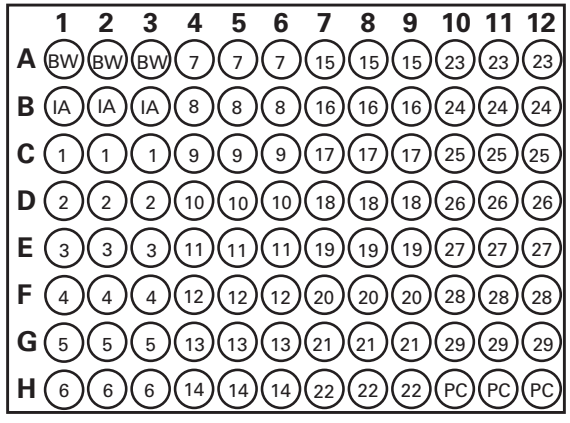
6. **Cl-Amidine Inhibitor Assay Reagent - (Item No. 700567)**

   This vial contains 950 nmol of inhibitor. Reconstitute with 950 µl of PAD Assay Buffer (AMC) to yield a concentration of 1 mM. The final concentration in the assay will be 100 µM. Use within the same day of preparation.

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 wells. It is suggested that each inhibitor be assayed in triplicate and the contents of each well are recorded on the template sheet provided on page 21. It is also recommended that the positive control inhibitor be assayed in triplicate. A typical layout of samples and inhibitors to be measured in triplicate is shown in Figure 1 on page 11.



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 100 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 355-365 nm and an emission wavelength of 445-455 nm.

Performing the Assay

1. **100% Initial Activity Wells -** add 20 µl of diluted PAD1 (human recombinant) Assay Reagent (AMC) and 5 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
2. **Background Wells -** add 20 µl of PAD Assay Buffer (AMC) and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells -** add 20 µl of diluted PAD1 (human recombinant) Assay Reagent (AMC) and 5 µl of inhibitor to three wells. *NOTE: Inhibitors can be dissolved in PAD Assay Buffer (AMC), ≤50% methanol, ≤25% ethanol, or ≤5% DMSO and should be added to the assay in a volume of 5 µl. Increasing the amount of solvent will diminish the sensitivity of this assay. In the event that an appropriate concentration of inhibitor is unknown, it is recommended that several dilutions of the inhibitor are assayed.*
4. **Positive Control Wells** - add 20 µl of diluted PAD1 (human recombinant) Assay Reagent (AMC) and 5 µl of reconstituted Cl-Amidine Inhibitor Assay Reagent to three wells.

Well	PAD1 (human recombinant) Assay Reagent (AMC) (µl)	PAD Assay Buffer (AMC) (µl)	Inhibitor (µl)	Solvent (µl)
100% Initial activity wells	20	-	-	5
Background wells	-	20	-	5
Inhibitor wells	20	-	5	-
Positive Control wells	20	-	5	-

Table 1. Pipetting summary

5. Cover plate with plate cover and incubate for 15 minutes at 37°C.
6. Remove plate cover, and add 25 µl of diluted PAD Assay Substrate (AMC) to all wells being used.
7. Cover plate with plate cover and incubate for 20 minutes at 37°C.
8. Remove plate cover, and add 50 µl of PAD Developer (AMC) to all wells being used.
9. Cover plate with plate cover and incubate for 10 minutes at room temperature.
10. Remove plate cover and read the fluorescence at an excitation wavelength of 355-365 nm and an emission wavelength of 445-455 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of the background, 100% initial activity, positive control, and inhibitor wells.
2. Graph the average fluorescence as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there is 50% inhibition). Inhibition of PAD1 (human recombinant) by Cl-Amidine is shown in Figure 2 (see page 18).

3. If background subtraction is desired, subtract the average fluorescence of the background wells from the average fluorescence of the 100% initial activity and inhibitor wells. Take the absolute values. These are your Corrected values.
4. Determine the percent inhibition or percent initial activity for each inhibitor using one of the following equations:

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

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

NOTE: Use absolute values in the above equations

5. Graph the percent inhibition or percent initial activity as a function of the inhibitor concentration to determine the IC_{50} value.

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. A typical Z´ factor for Cayman's PAD1 Inhibitor Screening Assay Kit (AMC) was determined to be 0.86.

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.

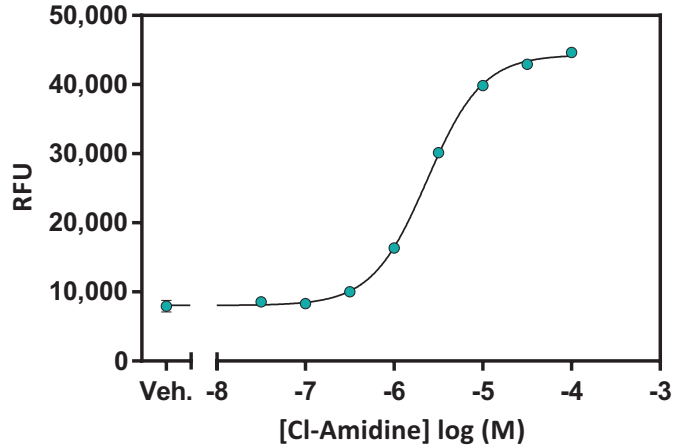


Figure 2. Inhibition of human recombinant PAD1 by Cl-Amidine. "Veh." represents 100% initial activity vehicle control.

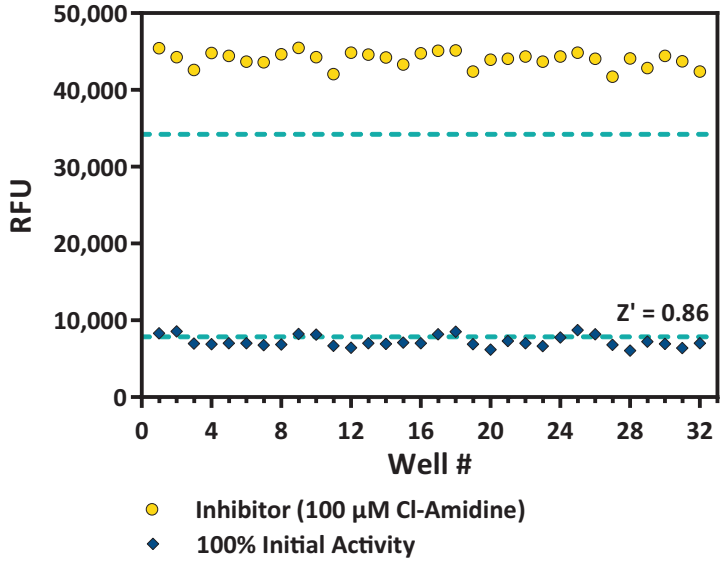


Figure 3. Typical Z´ data for the PAD1 Inhibitor Screening Assay Kit (AMC). Data shown from wells of both positive (circles) and negative (diamonds) controls prepared as described in the kit booklet. The calculated Z´ factor from this experiment was 0.86. The dashed lines correspond to three standard deviations from the mean for each control value.

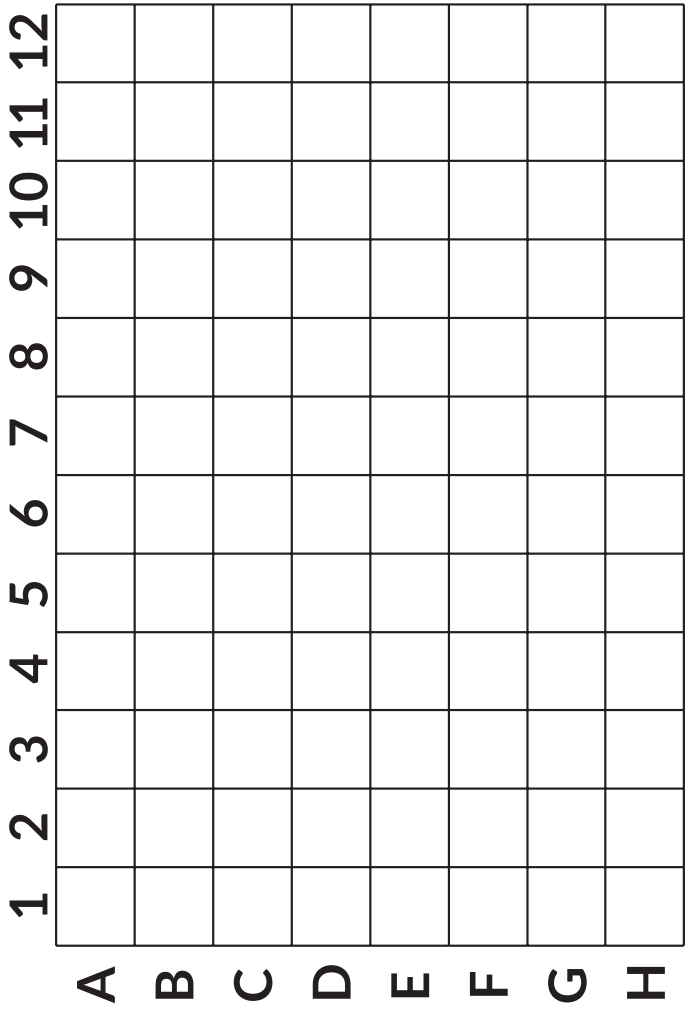
RESOURCES

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
100% Initial activity wells do not show lower fluorescence than background wells	A. Enzyme was not added to the well(s) B. DTT was not added to buffer	A. Make sure to add all of the components to the well(s) B. Make sure to add DTT to buffer just prior to use
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 PAD2	Increase the inhibitor concentration and re-assay
Poor signal	DTT was not added to buffer just prior to use	Make sure to add DTT to buffer just prior to use

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. Wildeman, E. and Pires, M.M. Facile fluorescence-based detection of PAD4-mediated citrullination. *ChemBioChem* **14(8)**, 963-967 (2013).
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.