BellBrookLabs

Determination of K_m for ATP and Kemptide with Protein Kinase A Enzyme using Transcreener® ADP² FP Assay

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Application Note

Introduction

Transcreener® is a universal, high throughput biochemical assay based on detection of nucleotides, which are formed by thousands of cellular enzymes — many of which catalyze the covalent regulatory reactions that are central to cell signaling and are high value targets in drug discovery. The advantages of the Transcreener® HTS Assay Platform over existing assay methods include the following:

Universality

The detection of invariant nucleotide reaction product means that a single set of detection reagents can be used for all of the enzymes in a family and all acceptor substrates.

Far Red Fluorescence Intensity Detection

Use of far red shifted dyes with a simple relative fluorescence output greatly reduces interference and particulate-based light scattering from fluorescent compounds.

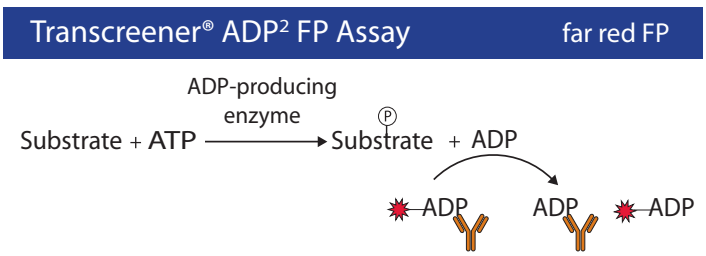
Sensitivity

High affinity antibodies enable robust detection of low levels of substrate conversion (<10%) with less enzyme than other methods.

Assay Principle

The Transcreener ADP² assay is a simple one-step homogenous detection assay. Transcreener ADP Detection Mixture, comprised of an ADP Alexa633 Tracer bound to an ADP² Antibody, is added to an equal volume of enzyme reaction mix. Enzymatically generated ADP displaces the tracer resulting in a decrease in fluorescence polarization. Standard curves are generated using varying concentrations of ATP and ADP to mimic the conversion of ATP to ADP during the course of an enzyme reaction.

Transcreener® ADP² FP Assay Principle



Materials

Instrument: Microplate Reader

Microplates: Corning® 384 Well Low Volume Black Round Bottom PS NBS™ Microplate (Product #3676)

Reagent	Kit/Component Catalog #
Transcreener® ADP² FP Assay	3010-1K
ADP Alexa 633 Tracer	2009
Stop & Detect Buffer B, 10X	2027
ADP² Antibody	2051
5 mM ADP	2052
5 mM ATP	2053
Buffer Components	
500 mM EGTA	Not Provided
1000 mM HEPES	
500 mM $MgCl_2$	
1% Brij-35	
100% DMSO	

Protocol

Enzyme Reaction:
The enzyme was titrated in 7.5 µL reaction volume in the presence of substrate in 4 mM $MgCl_2$, 2 mM EGTA, 50 mM HEPES, pH 7.5, 1% DMSO, and 0.01% Brij-35. 2.5 µL of ATP was added to the enzyme / substrate mix to start the kinase reaction. After incubating at room temperature for a specific period of time, the kinase reaction was stopped by addition of 10 µL of detection mix that consisted of 4nM tracer, 1X Stop and Detect buffer B and ADP² antibody. The concentration of the antibody depended on the ATP concentration and was determined from the linear equation. For 100 µM ATP, the antibody concentration was 109 µg/mL.

Standard Curve Reaction:
Standard curve was set up by adding 10 µL of ATP/ADP mixes in 4 mM $MgCl_2$, 2 mM EGTA, 50 mM HEPES, pH 7.5, 1% DMSO, and 0.01% Brij-35. The total adenine concentration was maintained at 100 µM. After incubating at room temperature for one hour, 10 µL of detection mix was added which consisted of 4 nM tracer, 1X Stop and Detect buffer B and ADP² antibody. The concentration of the antibody depended on the ATP concentration and was determined from the linear equation. For 100 µM ATP, the antibody concentration was 109 µg/mL.

Controls

Free Tracer:
The Free Tracer consists of 20 µL of 0.5X Stop & Detect Buffer B, and 2 nM ADP Alexa633 Tracer.

Buffer Blank:
The Buffer Blank consists of 10 µL of the Kinase buffer plus 10 µL of 1XStop & Detect Buffer B, and 109 µg/mL ADP2 Antibody.

Final concentration of components in 20 µL reaction:
2 mM MgCl2, 1 mM EGTA, 25 mM HEPES (pH 7.5), 0.5% DMSO, 0.005% Brij-35, 0.5X Stop & Detect Buffer B (25 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM EDTA, and 0.01% Brij-35), 2 nM ADP Alexa633 Tracer, and 109 µg/mL

Initial Reaction Kinetics

An appropriate working concentration of PKA was determined by running an enzyme titration in the presence of saturating concentrations of Kemptide and ATP. This experiment was run to determine the initial rates of the enzyme. PKA was titrated in the presence of 100 µM Kemptide and 100 µM ATP in a total reaction volume of 10 µL. The reactions were stopped at different time intervals, for a period of one hour by adding 1X Stop and Detect Buffer B with 4 nM Alexa 633 tracer and antibody concentration at 109 µg/mL as determined from the linear equation in the technical manual. A standard curve of 100 µM ATP/ADP was run in the same plate. The raw mP values were converted to ADP based on the standard curve. The data indicated that 6 mU/Rxn of PKA was sufficient to produce a ΔmP 145 and produced 6 µM ADP at the end of one hour, which corresponds to less than 10% conversion of ADP.

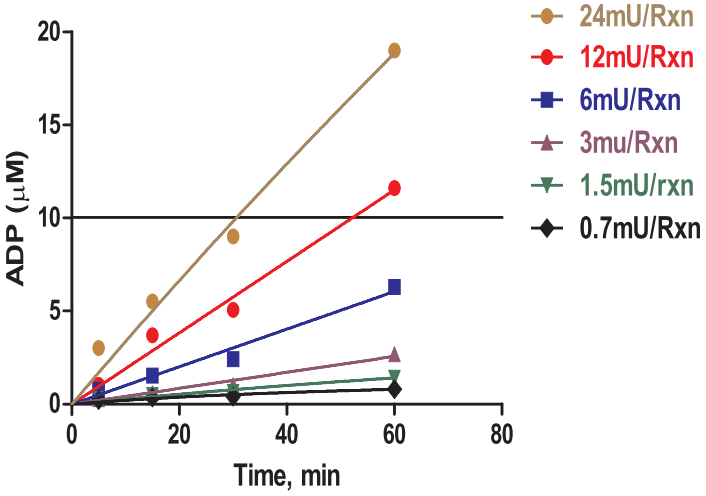


Figure 1. Initial rates of PKA enzyme in triplicates of 20 µL reactions containing varying amounts of PKA, 100 µM ATP and 100 µM Kemptide showing the amount of product formed with time.

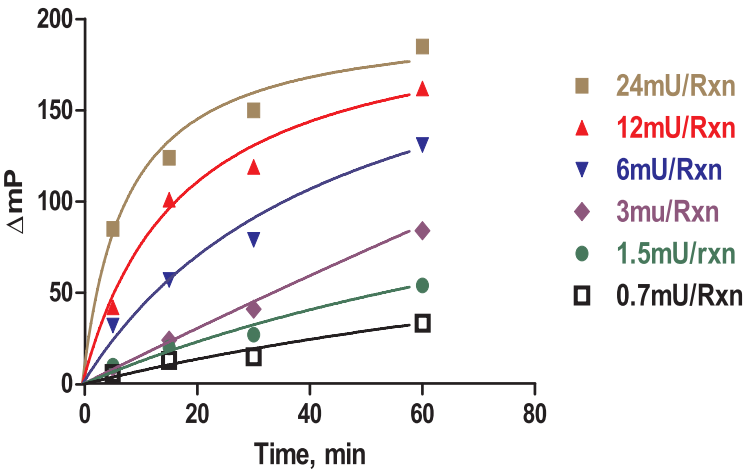


Figure 2. Initial rates of PKA enzyme in triplicates of 20 µL reactions containing varying amounts of PKA, 100 µM ATP and 100 µM Kemptide showing the change in polarization with time.

K_m Determinations

K_m values were determined for Kemptide and ATP using the Transcreener ADP² FP assay. The K_m of Kemptide was determined using saturating concentrations of ATP and varying the acceptor substrate, Kemptide concentration. 10 µL enzyme reactions containing increasing concentrations of kemptide , 6 mU of PKA/reaction and 100 µM ATP was run. The reactions were stopped at time-0, 5, 15, 30, 45 and 60 minutes by adding 10 µL of 1X stop and Detect buffer B with 4nM tracer and 109 µg/mL of ADP² antibody as determined from the linear equation. A 100 µM ATP/ADP standard curve was run on the same plate. The raw mP values were converted to ADP based on the standard curve. The initial reaction velocities (V) were calculated for each substrate concentration by finding the slopes of product formed vs. time during the first 60 minutes of the reaction. K_m was determined by plotting the (V) vs. substrate concentration and applying non linear regression analysis using the Michaelis –Menton equation. The K_m of Kemptide was determined to be 2µM.

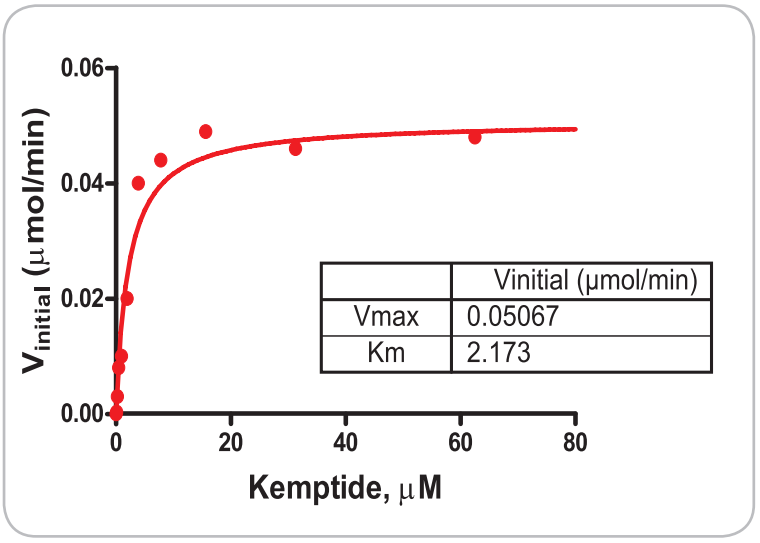


Figure 3. The plot shows Michaelis-Menten non linear regression analysis of initial rates vs peptide concentartion. Reactions were triplicates of 20µL reactions with 6 mU PKA/Rxn, 100 µM ATP with increasing concentrations of Kemptide.

The Km of ATP was determined using saturating concentrations of Kemptide and varying the ATP concentration. 10 µL enzyme reactions containing different ATP amounts , 6 mU of PKA/reaction and 100 µM Kemptide was run. The reactions were stopped at time- 0, 5, 15, 30, 45 and 60 minutes by adding 10 µL of 1X stop and Detect buffer B with 4 nM tracer and ADP2 antibody, whose concentration depended on the amount of ATP used for each point and was determined from the linear equation. Standard curves for each of the ATP concentration was run on the same plate to convert the raw mP values to determine the amount of ADP formed. The initial reaction velocities (V) were calculated for each substrate concentration by finding the slopes of product formed vs. time during the first 60 minutes of the reaction. Km was determined by plotting the (V) vs. substrate concentration and applying non linear regression analysis using the Michaelis –Menton equation. The Km of ATP was determined to be 23µM.

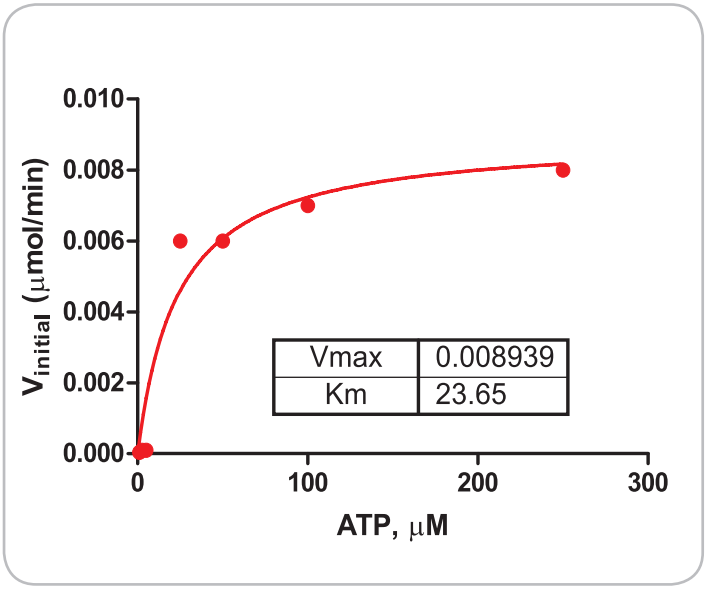


Figure 4. The plot shows Michaelis-Menten non linear regression analysis of initial rates vs ATP concentartion. Reactions were triplicates of 20µL reactions with 6 mU PKA/Rxn, 100 µM Kemptide with increasing concentrations of ATP.

Summary

Km of Kemptide was determined to be 2 µM.

Km of ATP was determined to be 23 µM.

For inhibitor screening we recommend using the Km of ATP and saturating concentrations of acceptor substrate.

Additional Information

Related Products

Transcreener® ADP² FP Assay	3010-1K
Transcreener® ADP² TR-FRET Red Assay	3011-1K
Transcreener® ADP² FI Assay	3013-1K
Transcreener® AMP/GMP Assay	3006-1K
Transcreener® UDP Assay	3007-1K
Transcreener® GDP FP Assay	3009-1K
Transcreener® GDP FI Assay	3014-1K
Transcreener® AMP²/GMP² Assay	3015-1K
Transcreener® UDP² Assay	3018-1K
Transcreener® EPIGEN Methyltransferase Assay	3017-1K

Technical Information

For technical information, please contact
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Ordering Information

Please contact BellBrook Labs for product pricing.
Custom quotes are available for orders of 10,000 wells or more.

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Fax orders:
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