Application Note

A Simplified Protocol to Streamline Inhibitor Profiling with the Transcreener® ADP² FP Assay

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Introduction

Transcreener® HTS is a universal, high throughput biochemical assay platform based on the 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 of great value as targets in drug discovery.

The Transcreener® ADP² FP Assay is a far-red, competitive fluorescence polarization (FP) assay based on the detection of ADP, and is a simple, one-step homogenous detection assay. The Transcreener ADP Detection Mixture is comprised of an ADP Alexa633 Tracer bound to an ADP² Antibody. When the Transcreener ADP Detection Mixture is added to an equal volume of enzyme reaction mix, ADP is generated and replaces the ADP Alexa633 Tracer (Figure 1). The displaced ADP Alexa633 Tracer freely rotates leading to a decrease in fluorescence polarization.

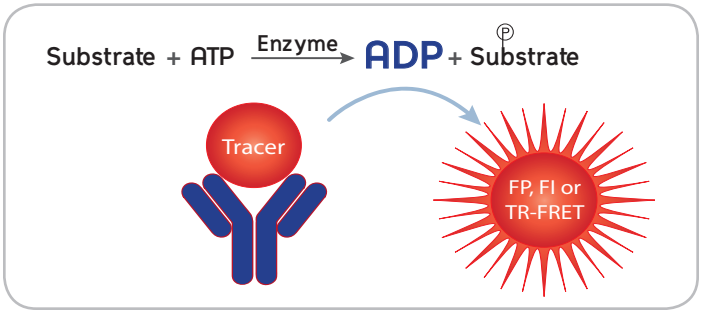


Figure 1. Transcreener® ADP² FP Assay Principle

New research has identified a suggested enzyme concentration range and an IC_{50} Data Conversion Factor, that will enable users to accelerate through the following assay steps:

Step 1) Determine ADP² Antibody Concentration.
Step 2) Perform an Enzyme Titration.
Step 3) Screen Inhibitors.
Step 4) Calculate IC_{50} Values.

This Application Note describes how to streamline the process of inhibitor profiling with the Transcreener® ADP² FP Assay by eliminating the need to experimentally optimize the ADP² Antibody concentration and the need for a standard curve.

Materials and Methods

Instrument: Microplate Reader capable of measuring Fluorescence Polarization.

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

Reagent	Catalog #
Transcreener® ADP² FP Assay	3010-1K
ADP Alexa633 Tracer, 400 nM	2009
Stop & Detect Buffer B, 10X	2027
ADP² Antibody	2051
5 mM ADP	2052
5 mM ATP	2053
Buffer Components:	Not Provided
500 mM EGTA	
1000 mM HEPES	
500 nM $MgCL_2$	
1% Brij-35	
100% DMSO	

Step 1) Determine ADP² Antibody Concentration.

The Transcreener® ADP² FP Assay requires the detection of ADP in the presence of excess ATP (assuming initial velocity enzyme reaction conditions) using an antibody with a finite selectivity for the diphosphate vs. the triphosphate. The concentration of ADP² Antibody determines the total assay window and the ADP detection range, and the amount needed is dependent upon the ATP concentration in the enzyme reaction

To achieve initial rates of reaction, kinase reactions should be carried out at the K_m of ATP and at saturating concentrations of the acceptor substrate. The K_m value of ATP can be obtained from literature or can be determined experimentally using the Transcreener® ADP² FP Assay[1]. Since the the relationship between [ATP] and [ADP² Antibody] is linear, the ADP² Antibody concentration can be estimated using the following equation: y = mx + b; where x = [ATP] (µM) in the 10 µL enzyme reaction, y = [ADP² Antibody] (µg/mL) in the 1X ADP Detection Mixture, m (slope) = 1.08, and b (y-intercept) = 1.0.

For example, the app K_m of ATP for PKA is reported in the literature to be 20 µM[2]. The PKA enzyme titration is carried out in the presence of a saturating amount of Kemptide, the acceptor substrate, and 20 µM ATP. The K_m for Kemptide is reported to be 2 µM[3], so 20 µM Kemptide was selected as a saturating concentration. Therefore, in this equation, the optimal ADP² Antibody concentration would be [1.08 x 20] + 1.0 = 22.6 µg/mL.

Step 2) Perform an Enzyme Titration.

Perform a serial enzyme titration using a buffer formulation that is ideal for your enzyme target. The ideal [enzyme] should be determined using a serial titration of the kinase enzyme in the presence of saturating concentrations of acceptor substrate and ATP.

Serially titrate the enzyme in a volume of 7.5 µL in the kinase buffer containing the acceptor substrate. Add 2.5 µL of ATP to jump start the kinase reaction (10 µL total volume). Mix the plate on a plate shaker, cover with a plate seal, and incubate for one hour at room temperature (20-25°C). Note, the temperature and length of the kinase reaction will depend upon the enzyme and its turnover time. At the end of the incubation time, add the 1X Stop and Detect Buffer B, the 4nM ADP Alexa633 Tracer and the ADP² Antibody concentration (calculated in Step 1 to be 22.6 µg/mL for this example). Mix using a plate shaker, incubate at room temperature (20-25°C) for 1 hour, and measure fluorescence polarization.

A PKA titration, performed in the presence of 20 µM Kemptide and 20 µM ATP, is shown in Figure 2.

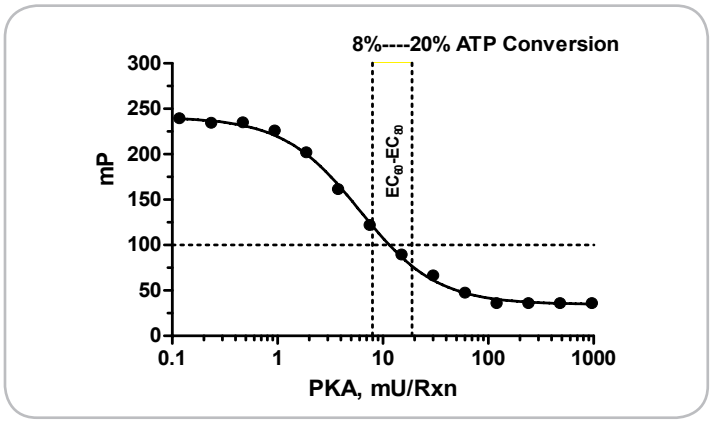


Figure 2. Enzyme Titration to Identify Optimal Enzyme Concentration

To achieve the most robust assay and a large signal, the quantity of enzyme required to produce a 60% - 80% change in polarization signal is ideal (EC_{60}-EC_{80}) for screening of large compound libraries and generating inhibitor dose response curves. EC_{60}-EC_{80} values can be calculated directly from GraphPad Prism® using "log agonist versus response, find EC anything" under non-linear regression. The ECF [enzyme] can also be calculated using the equation below:

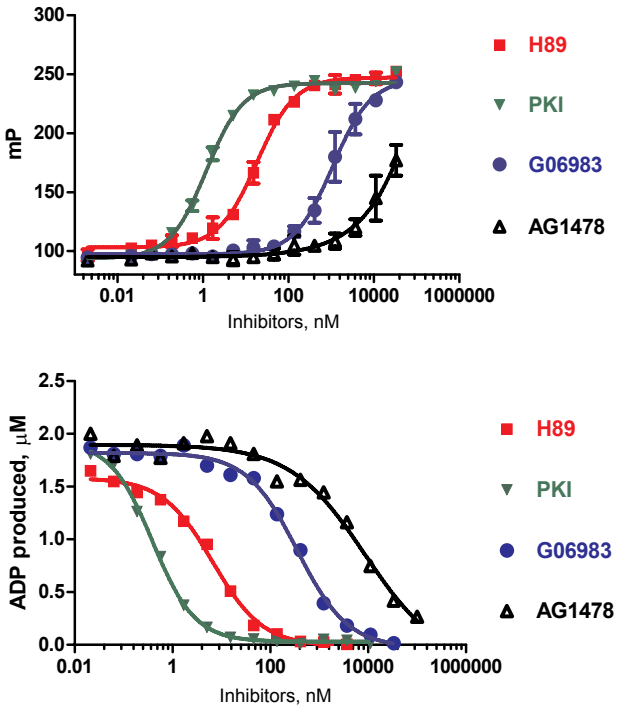
$$EC_F=(F/(100-F))^{(1/hillslope)}*EC_{50}$$

The EC_{60}-EC_{80} range represents an optimal enzyme range because a) it results in a very good assay window (>100mP), b) it is within initial velocity conditions (8-20% ATP conversion), and c) inhibitor potencies can be determined directly from raw polarization data in this range (see Step 4).

Step 3) Screen Inhibitors.

Perform an inhibitor titration in the presence of EC_{60}-EC_{80} concentration of enzyme (as determined in Step 2), saturating concentration of acceptor substrate, and ATP concentration (calculated in Step 1). The inhibitor is serially titrated in 7.5 µL in the presence of the acceptor substrate and the EC_{60}-EC_{80} concentration of enzyme. Pre-incubate the inhibitor with the enzyme and substrate for 15 minutes at room temperature. Add 2.5 µL of ATP to jump start the kinase reaction (10 µL total volume). Mix the plate on a plate shaker, cover with a plate seal, and incubate for one hour at room temperature (20-25°C). At the end of the incubation time, add the 1X Stop and Detect Buffer B, the 4nM ADP Alexa633 Tracer and the ADP² Antibody concentration (calculated in Step 1 to be 22.6 µg/mL for this example). Mix using a plate shaker, incubate at room temperature (20-25°C) for 1 hour, and measure fluorescence polarization.

An inhibitor titration of selected compounds (H89, PKI, G06983, and AG1478), performed in the presence of the EC_{60}-EC_{80} concentration of PKA, 20 µM Kemptide, and 20 µM ATP, is shown in Figure 3. IC_{50} values from raw and converted data are shown below.



	H89	PKI	G06983	AG1478
IC_{50} Raw Data (nM)	18.35	1.199	1082	>10,000
IC_{50} Converted Data (nM)	6.652	0.3899	355.9	8616

Figure 3. Inhibitor Dose Response Data

Step 4) Calculate IC_{50} Values

To determine the IC_{50} value of an inhibitor in terms of product formed, it is traditional to run a standard curve in each plate and interpolate the amount of product formed from the standard curve. The IC_{50} value derived when plotted using ADP formed is generally lower than the IC_{50} value obtained when plotted using raw data. Our research reveals that, within the defined assay response range (EC_{60}-EC_{80}), there is a direct correlation between the IC_{50} value of raw data and the IC_{50} value of converted data.

Using six different enzymes and a total of four inhibitors each (at EC_{60}-EC_{80} concentration of their enzyme), inhibitor titrations were run and the IC_{50} values obtained from the raw data (mP units) were compared to the IC_{50} values obtained when the data was converted into product formed (using a standard curve). The correlation factor of 2.5 corresponds to the slope of the line in Figure 4. Note, this correlation factor of 2.5 will only be valid when the enzyme concentration falls within the EC_{60}-EC_{80} range. Researchers can

now directly derive the IC_{50} value of the converted data by dividing the IC_{50} of raw data by a factor of 2.5 (as shown in the equation below) and bypass the need to generate standard curves.

$$\frac{IC_{50}\ (\text{Raw data})}{2.5} = IC_{50}\ (\text{Converted data})$$

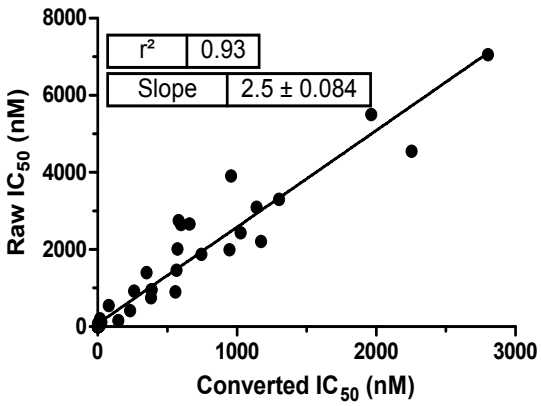


Figure 4. IC_{50} Correlation of Raw Data and Converted Data

Conclusions

This application note demonstrates a streamlined approach to profile kinase inhibitors using the Transcreener® ADP² FP Assay. This study illustrated an improved procedure for the screening of inhibitors by using a quickly-calculated antibody concentration, a standard EC_{60}-EC_{80} target enzyme concentration, and an IC_{50} Data Correlation Factor. All of these improvements to the Transcreener ADP² FP Assay protocol combine to accelerate the process of inhibitor profiling.

References & Notes

1. Kleman-Leyer K.M, Klink T.A, Kopp A.L, Westermeyer T.A, Koeff M.D, Larson B.R, Worzella T.J, Pinchard C.A, Van de Kar S.A.T, Zaman G.J.R, Hornberg J.J, Lowery R.G: Characterization and optimization of a red-shifted fluorescence polarization ADP detection assay. Assay and Drug Dev Tech 2009; 7: 56-67.
2. Card A, Caldwell A, Min H, Lokchander B, Xi H, Sciabola S, Kamath A.V, Clugston S.L, Tschantz W.R, Wang L, Moshinsky D. J : High-Throughput biochemical kinase selectivity assays: panel development and screening applications. J Biomol Screen 2009 Jan;14(1):31-42.
3. Miick S. M, Jalali S, Dwyer B.P, Havens J, Thomas D, Jimenez M.A, Simpson M.T, Zile B, Huss K.L, Campbell R.M: Development of a Microplate-Based, Electrophoretic Fluorescent Protein Kinase A Assay: Comparison with Filter-Binding and Fluorescence Polarization Assay Formats. J Biomol Screen June 2005 10: 329-338.

Additional Information

Ordering Information

Please visit www.bellbrooklabs.com or contact BellBrook Labs for pricing for the Transcreener® ADP² FP Assay. Custom quotes are available for bulk orders.

Phone Orders:
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866.3137881

Fax Orders:
608.441.2967

Email Orders:
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Transcreener® UDP Assay	3007-1K
Transcreener® GDP FP Assay	3009-1K
Transcreener® GDP FI Assay	3014-1K

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