Application Note

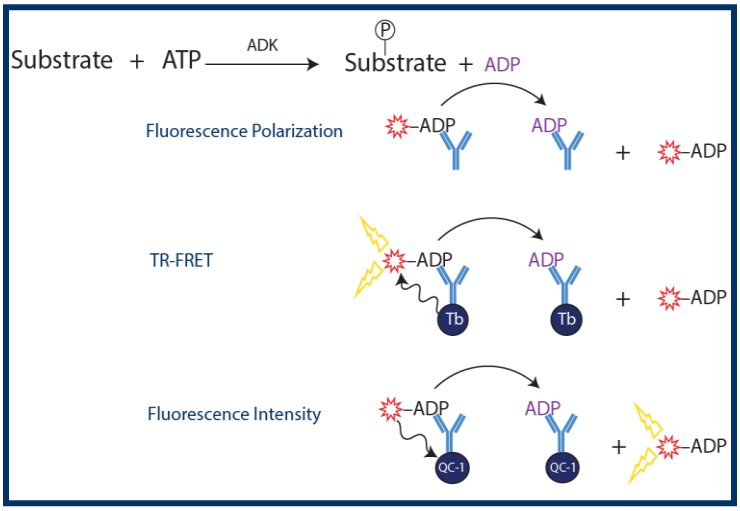
Detection of Adenosine Kinase Activity with the Transcreener® ADP^2 Kinase Assay

BellBrook Labs – Madison, WI

Introduction

Adenosine Kinase (ADK) catalyzes the ATP-dependent phosphorylation of adenosine to AMP; it plays a critical role in regulating intracellular and extracellular adenosine (ADO) levels. This application note describes methods for using the Transcreener ADP^2 Kinase Assay to measure ADK enzymatic activity in a high throughput (384-well) format, e.g., for inhibitor screening and/or inhibitor dose-response measurements. The assay is designed for use with purified ADK; it is not intended for use with cells or cellular lysates.

Transcreener is a universal high throughput biochemical assay platform 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 represent new opportunities for therapeutic intervention. Transcreener assays rely on highly specific antibodies that bind nucleotides, coupled with homogenous fluorescent detection formats suitable for automated HTS applications.



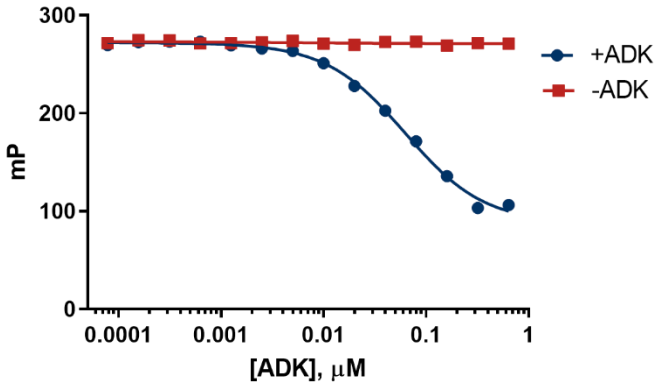


Figure 1. ADK Enzyme Titration with FP Readout

The Transcreener ADP^2 Assay is a far-red, competitive fluorescence immunoassay for direct detection of ADP in a simple mix-and-read format. ADP produced by ADK displaces the ADP tracer from the highly specific ADP antibody resulting in a change in fluorescence. Transcreener ADP^2 Assays are available with fluorescence polarization (FP), fluorescence intensity (FI) and time-resolved Förster resonance energy transfer (TR-FRET) readouts.

Materials

Component	Notes
Transcreener ADP Assay	**Transcreener ADP FP Assay** (BellBrook Labs Cat. #3010) *The results shown are based on the FP Assay. FI & TR-FRET Readouts are also available for purchase. * **Transcreener ADP FI Assay** (BellBrook Labs Cat. #3013) **Transcreener ADP TR-FRET Assay** (BellBrook Labs Cat. #3011)
ATP & ADP	Included in the Transcreener kit. Used to create ATP/ADP standard curve, if desired.
ADK Enzyme	Recombinant human ADK from NOVOCIB Cat. #E-Nov5. If another source is used, the results may be significantly different.
Enzyme Buffer	50 mM Tris (pH 7.5), 50 mM $MgCl_2$, and 0.01% Brij-35
ADO Substrate	10 µM of Adenosine from BellBrook Labs Cat. #2206.
Assay Plates	Corning 384-Well Black Assay Plates BellBrook Labs Cat. #4514 *TR-FRET requires an entirely white plate with a non-binding surface. We suggest Corning 384-Well Plates Cat. #4513.*
Plate Reader	Microplate reader configured to measure FP, FI, or TR-FRET, depending on the assay format used. For the purposes of this application, the CLARIOstar®/Plus from BMG LABTECH was used. Full list of compatible plate readers and setting.
Liquid Handling Devices	Liquid Handling Devices that can accurately dispense sub-microliter volumes into 384-well plates.
Ultrapure Water	Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates & products, reducing assay performance. Use nuclease free water such as Invitrogen Cat. #AM9930

Methods

We describe methods for four common procedures for enzymatic assays in a drug discovery setting:

Performing an enzyme titration

1. Performing an enzyme titration to determine the appropriate concentration for a good assay signal

Generating a Standard Curve

2. Generating a standard curve to enable conversion of the assay signal (mP) to amount of product formed

Generating a Dose Response Curve

3. Generating a dose response (DR) curve for an inhibitor and determining the IC_{50}

Determining Z'

4. Determining Z', a parameter that combines dynamic range and signal variability to provide a measure assay robustness

Depending on your goals, it may not be necessary to perform every procedure. For example, if you are screening compounds at a single concentration, you would only need to perform an enzyme titration and determine Z'; screening hits are generally defined by the change in assay signal, and therefore conversion to product formation is unnecessary. Alternatively, if you already have inhibitors in hand and want to measure potency, a standard curve would be required to get an accurate IC_{50}, but a Z' measurement would not be necessary as it is primarily relevant to screening.

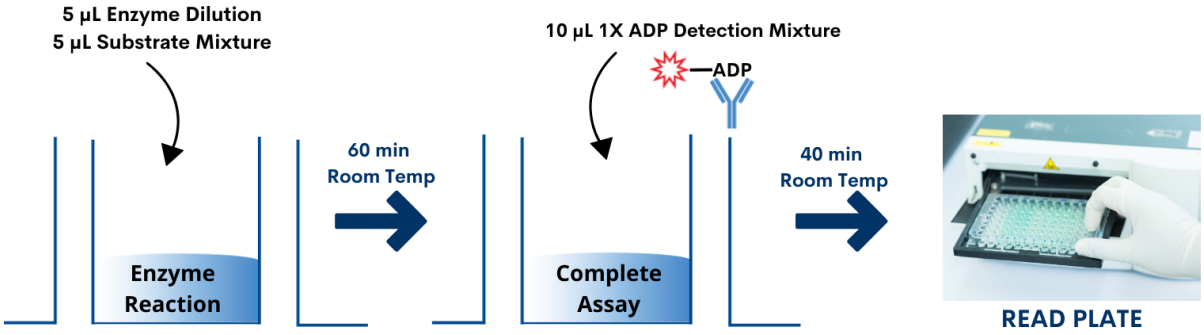


Figure 2. Schematic of assay steps. An optimized concentration of the enzyme is diluted and then incubated with its substrate. The 1X ADP Detection Mix is then added and a final incubation occurs prior to reading.

	10 µL Enzyme Reaction Components	
Component	**Working Stock Conc.**	**Conc. in 10 µL Enzyme Reaction**
ADK Enzyme	Varies Per Distributor	Determined Experimentally
Tris HCl	50 mM	50 mM
Brij-35	0.02%	0.01%
ATP	200 µM	100 µM
Adenosine	20 µM	10 µM
$MgCl_2$	10 mM	5 mM

Table 1. ADK Final Enzyme Reaction Conditions

	1X ADP Detection Mixture – Add 10 µL Per Well					
	FP		FI		TR-FRET	
Component	**Detection Mix Conc.**	**Complete Assay**	**Detection Mix Conc.**	**Complete Assay**	**Detection Mix Conc.**	**Complete Assay**
Stop & Detect Buffer B	1X	0.5X	1X	0.5X	1X	0.5X
ADP Tracer	4 nM	2 nM	8 nM	4 nM	1000 nM	500 nM
ADP Antibody	40 µg/mL	20 µg/mL	40 µg/mL	20 µg/mL	8 nM	4 nM

Table 2. 1X ADP Detection Mixture Concentrations for FP, FI, & TR-FRET Assay Formats

Tables 1 & 2: List of final concentrations of each component in the assay. Please note that the concentrations for the enzyme reaction are based on a 10 µL final volume while the complete assay concentrations with the Stop and Detect Buffers are based on a 20 µL final volume. For the purposes of this application note, concentrations of 100 µM ATP and 10 µM ADO were used; the ADP antibody and ADK concentrations were optimized for these conditions. The Transcreener ADP[2] Assay can accommodate concentrations from 0.1 µM to 1000 µM, but the ADP antibody and ADK concentrations will need to be optimized for the specific conditions chosen. The published K_m values for ATP and ADO with human ADK are 1.82 µM and 11.4 µM, respectively.[1]

1 Performing ADK Enzyme Titration

We have optimized the Transcreener ADP^2 Assay for this application using human ADK from external vendor (NOVOCIB #E-Nov5). Because the activity of the enzyme will vary from prep to prep, we highly recommend performing an enzyme titration to ensure that the reaction is within the initial velocity region (<20% conversion of substrate to product) and that the signal is within the dynamic range for the optimized Transcreener ADP^2 Assay conditions.

1. Prepare Enzyme Buffer containing 50 mM Tris HCl and 0.02% Brij-35.
2. Dilute ADK to desired concentration in Enzyme Buffer and add 5 µL to the target wells. This example utilized a concentration of 10.24 µM ADK which then underwent a two-fold dilution down the plate for the purposes of generating an enzyme titration.
3. Following ADK addition, add 5 µL of Substrate Mixture containing 200 µM ATP, 20 µM Adenosine, 50 mM Tris and 10 mM $MgCl_2$.
4. Mix for 40 seconds, cover the plate with a plate sealer and allow the ADK reaction to incubate for 60 min at room temperature.

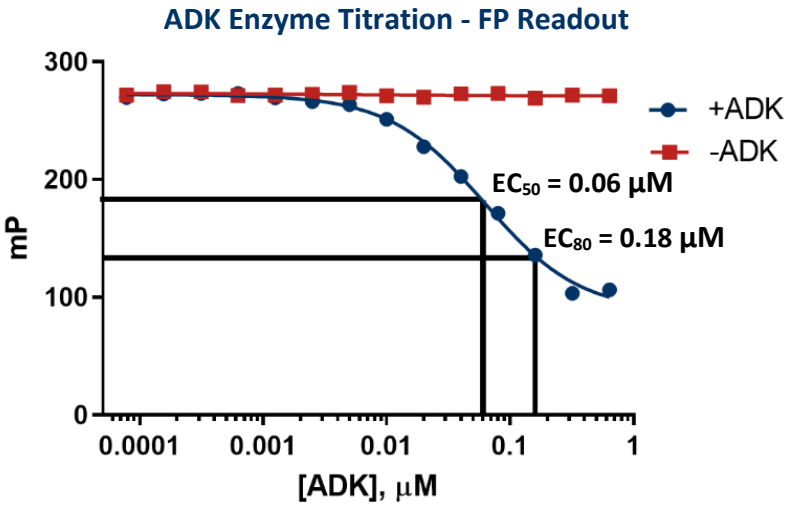


Figure 3. ADK Enzyme Titration with FP Readout. EC_{50} = 0.06 µM & EC_{80} = 0.18 µM

5. Following the 60 min ADK reaction, add 10 µL of the 1X ADP Detection Mixture to the 10 µL of the enzyme reaction. The 1X ADP Detection Mixture varies depending on the readout mode. For example, the 1X ADP Detection Mixture for FP would include 1X Stop and Detect Buffer B, 4 nM of ADP AlexaFluor633 Tracer and 40 µg/mL ADP^2 Antibody. Table 2 lists 1X ADP Detection Mix concentrations for each readout mode & the corresponding 20 µL complete assay concentrations.
6. Finally, mix for 40 seconds, cover the plate with a plate sealer and incubate for 40 minutes to allow the 20 µL complete assay to equilibrate before measuring the Fluorescence Polarization in the instrument of choice.

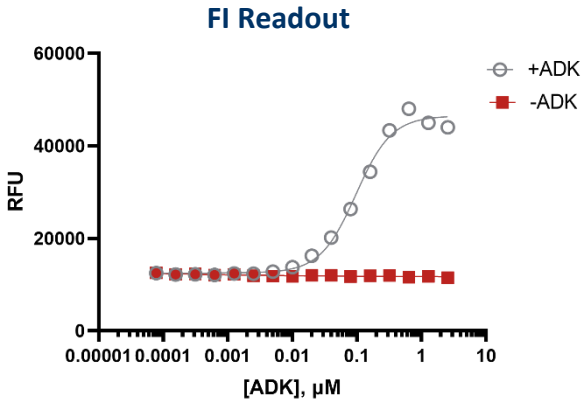


Figure 4. ADK Enzyme Titration with FI Readout

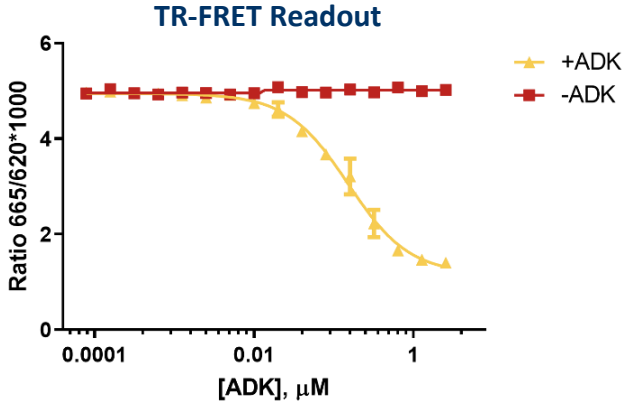


Figure 5. ADK Enzyme Titration with TR-FRET Readout

2 Generating ATP/ADP Standard Curve

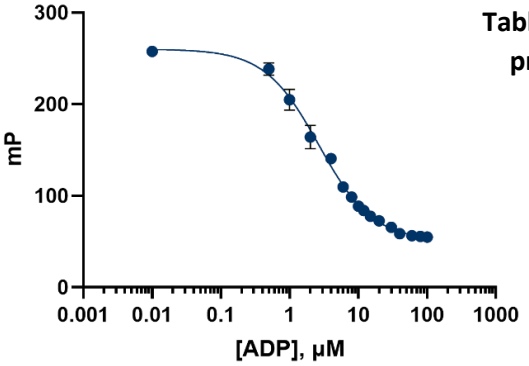
For quantitative measurements of ADK enzyme activity, a standard curve is used to convert the polarization signal from the Transcreener ADP^2 Assay to the amount of ADP produced. The standard curve essentially mimics the enzyme conversion of ATP to ADP; starting at the initial ATP concentration, ATP is sequentially decreased, and ADP is increased proportionally.

1. Prepare Reaction Buffer containing 2.5 mM $MgCl_2$, 0.01% Brij-35, 50 mM Tris HCl, 10 µM Adenosine.
2. Dilute 5 mM ATP and ADP to 100 µM in Reaction Buffer. The volume for both dilutions should be based on the total amount of volume to be utilized during preparation of the ATP/ADP percent conversion solutions.
3. Starting from 100 µM of ADP, add proportional values in separate microcentrifuge tubes to generate the desired percent conversions utilized in the standard curve. An example of the dilution scheme can be found in the figure below. Once all dilutions are completed, add 10 µL of each solution to the respective wells.
4. Afterwards, add 10 µL of 1X ADP Detection Mixture. The 1X ADP Detection Mixture varies for the readout mode used. Table 2 lists the 1X ADP Detection Mixtures for each readout.
5. Finally, mix for 40 seconds, before measuring the Fluorescence Polarization in the instrument of choice.

% Conv.	ATP (µM)	ADP (µM)
100	0	100
80	20	80
60	40	60
40	60	40
30	70	30
20	80	20
15	85	15
12	88	12
10	90	10
8.0	92	8.0
6.0	94	6.0
4.0	96	4.0
2.0	98	2.0
1.0	99	1.0
0.5	99.5	0.5
0	100	0

Table 3. Concentrations of ATP/ADP to prepare a 16-point standard curve

Figure 6. ADP/ATP Standard Curve



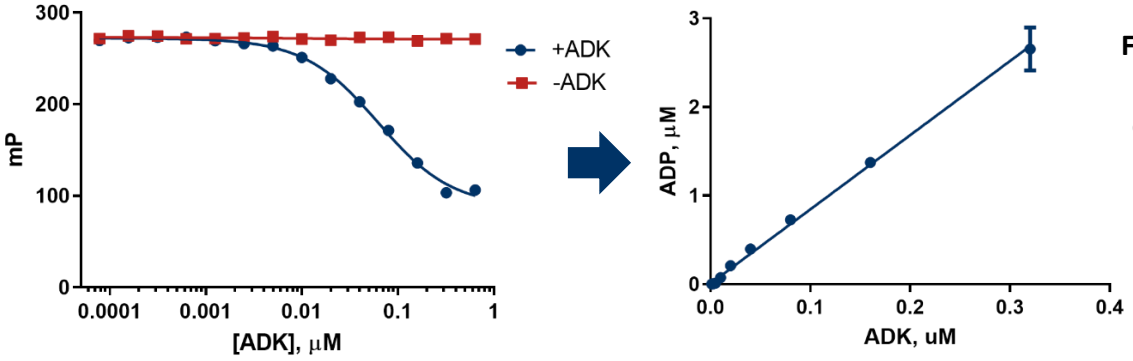
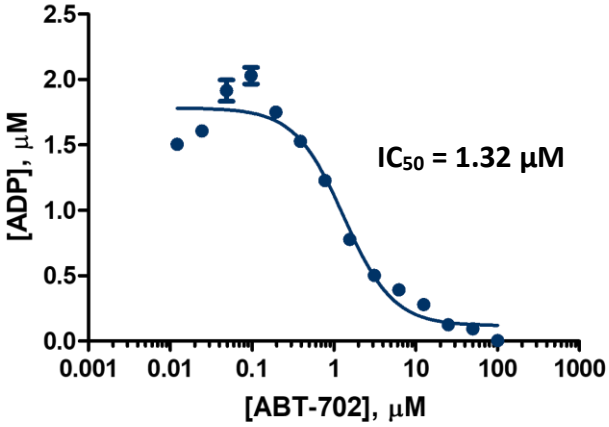


Figure 7. Assay shows linearity when raw data is converted to ADP formed using standard curve & enzyme titration.

3 Generating Dose Response Curve

1. Dilute ADK in 50 mM Tris HCl and 0.02% Brij-35 to the EC_{80} concentration determined in the enzyme titration.
2. Add 5 µL of the diluted ADK to the test compounds at the desired concentration. The total volume of this reaction is 5 µL. Mix for 40 seconds, cover the plate with a plate sealer and incubate for 30 minutes at room temperature.
3. Following the incubation, add 5 µL of Substrate Mixture containing 200 µM ATP, 20 µM Adenosine, 50 mM Tris and 10 mM $MgCl_2$.
4. Mix for 40 seconds, cover the plate with a plate sealer and allow the ADK reaction to incubate for 60 minutes at room temperature.
5. Following the 60 min ADK reaction, add 10 µL of 1X ADP Detection Mixture. The 1X ADP Detection Mixture varies for the readout mode used. Table 2 lists the 1X ADP Detection Mixtures for each readout.
6. Mix the plate for 40 seconds, cover it with a plate sealer and allow it to incubate for 40 minutes before measuring the Fluorescence Polarization in the instrument of choice.

Figure 8. ABT-702 DRC utilizing 0.17 µM of ADK



4 Determining Z'

1. Complete assay is performed with and without enzyme with 16 replicates.
2. Z' is then calculated based on the following formula:

Equation To Calculate Z':

$$Z' = 1 - \frac{[(3 \times SD_{initial\ [ATP]}) + (3 \times SD_{sample})]}{|\ (mP_{initial\ [ATP]}) - (mP_{sample})\ |}$$

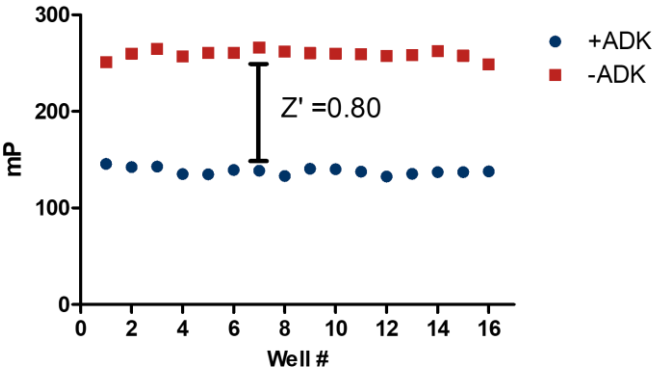


Figure 9. Z' = 0.80. Robust Data like this is vital for sizeable high throughput screens

Summary

This application note demonstrates how to screen for ADK enzyme activity using the Transcreener® ADP^2 Assay. This study provided the procedure required to perform the assay, as well as additional data that confirms the robustness of the assay. Following the Transcreener ADP^2 Assay protocol accelerates the process of inhibitor profiling.

References & Notes

1. de Oliveira, R.R., Morales-Neto, R., Rocco, S.A. et al. Adenosine Kinase couples sensing of cellular potassium depletion to purine metabolism. Sci Rep 8, 11988 (2018). https://doi.org/10.1038/s41598-018-30418-5

Ordering Information

Please visit www.bellbrooklabs.com or contact BellBrook Labs for assay pricing. Custom quotes are available for bulk orders.

Email Orders: info@bellbrooklabs.com

Phone Orders: (608) 443-2400

Toll-Free: (866) 313-7881

Fax Orders: (608) 441-2967

Technical Information

For Technical Information, please contact one of our BellBrook scientists:

Email: info@bellbrooklabs.com

Telephone: (608) 443-2400

Toll-Free: (866) 313-7881