



TRANSCREENER
ADP² FP Assay

Technical Manual



Transcreener® ADP² FP Assay

Instructions for Part Numbers 3010-1K and 3010-10K

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1.0 Introduction

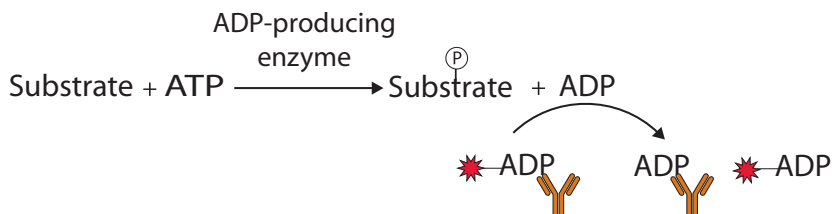
The Transcreener® HTS Assay platform overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by utilizing a single set of assay reagents that detect an invariant product. The generic nature of the Transcreener HTS Assay platform eliminates delays involved in assay development for new HTS targets, and greatly simplifies compound and inhibitor profiling across multiple target families.

The Transcreener® ADP² FP Assay is a far-red, competitive fluorescence polarization (FP) assay based on the detection of ADP and therefore is compatible with any enzyme class that produces ADP, including protein, lipid, and carbohydrate kinases, ATPases, DNA helicases, carboxylases and glutamine synthetase. The Transcreener ADP² Assay is a simple one step homogenous detection assay, and is extremely flexible with regard to ATP concentration (0.1 to 1,000 μ M ATP). The assay provides excellent signal at low substrate conversion, with a Z' \geq 0.7 and \geq 85 polarization shift (mP) at 10% ATP conversion using 1 μ M ATP.

With this assay, modified assay conditions are not required for outstanding results on the PerkinElmer EnVision® microplate reader.

Transcreener® ADP² FP Assay

far red FP



The Transcreener ADP² FP Assay was developed to follow the progress of any enzyme that produces ADP. The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to an ADP² Antibody. The tracer is displaced by ADP, the invariant product generated during the enzyme reaction (Figure 1). The displaced tracer freely rotates leading to a decrease in fluorescence polarization. The assay uses a far red tracer to minimize interference from fluorescent compounds and light scattering.

2.0 Transcreener® ADP² FP Assay Components

Store reagents at -20°C. Individual reagents tolerate 10 freeze-thaw cycles. Sufficient reagents are provided to complete the protocol when using ATP within a range of 0.1 to 100 µM. Please contact BellBrook Labs for custom packaging for enzyme reactions with >100 µM ATP.

ADP² Antibody

A concentrated mouse monoclonal ADP² Antibody is provided in PBS. The concentration of ADP² Antibody needed for an enzyme target is dependent upon the ATP concentration and buffer conditions in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to perform an ADP² Antibody titration in the buffer system ideal for your enzyme target.

ADP Alexa633 Tracer, 400 nM

The ADP Alexa633 Tracer, 400 nM is provided in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The final tracer concentration in the reaction is 2 nM.

Stop & Detect Buffer B, 10X

The Stop & Detect Buffer B, 10X consists of 200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35. The Stop & Detect Buffer B components will stop Mg²⁺-requiring enzyme reactions and aid in the detection and stabilization of the FP signal. Stop & Detect Buffer B is at a 0.5X concentration at the time of polarization measurement. To ensure the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the enzyme reaction.

5 mM ATP

ATP is common to many laboratories, however, if the ATP stock contains impurities, such as ADP, the assay window will be compromised. ATP supplied in this kit can be used for the enzyme reaction and to create the ATP/ADP standard curve. Contact BellBrook Labs for alternate suppliers and catalog numbers.

5 mM ADP

ADP is not common to all laboratories and therefore is a supplied reagent. ADP is used to create the ATP/ADP standard curve.

Materials Required but Not Provided

Ultrapure Water

Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, therefore reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.

Enzyme Buffer Components

The enzyme buffer components supplied by the end-user include enzyme, enzyme buffer, acceptor substrate, MgCl₂ or MnCl₂, EGTA, Brij-35, and test compounds. Contact BellBrook Labs Technical Service for suppliers and catalog numbers.

Plate Reader

A multidetection microplate reader configured to measure fluorescence polarization of Alexa Fluor®633 is required. The Transcreener ADP² FP Assay has been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4, BMG Labtech PHERAstar and PHERAstar Plus, Molecular Devices Analyst GT, Perkin Elmer EnVision® and ViewLux, and Tecan Infinite® F500, Safire²™, and M1000. Contact BellBrook Labs Technical Service for additional information regarding instrument set-up and fluorescence polarization measurements.

Assay Plates

It is important to use assay plates that are entirely black with a non-binding surface. We recommend Corning® 384 plates (catalog #3676).

Liquid Handling Devices

Use liquid handling devices that can accurately dispense a minimum of 2.5 µL into 384-well plates.

3.0 Protocol

The Transcreener ADP² FP Assay is designed around the customer's initial ATP concentration and enzyme buffer conditions. The ADP Detection system must be established before running the enzyme assay. These steps include instrument set-up and antibody optimization. Completing these initial steps will provide optimal ADP detection results and need not be repeated unless assay reaction conditions change.

Set up ADP Detection System

- Instrument Settings
- Optimize ADP² Antibody Concentration

Detect ADP in an Enzyme Reaction

- Enzyme Reaction Conditions

Set up ADP Detection System

Instrument Settings

Becoming familiar with ideal instrument settings for fluorescence polarization is essential to the success of the Transcreener ADP² FP Assay.

Verify instrument measures fluorescence polarization

Ensure the instrument is capable of measuring fluorescence polarization (not simply fluorescence intensity) of Alexa Fluor®633. Please call BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

Define the maximum mP window for your instrument

Measuring high (tracer + antibody) and low (free tracer) polarization will define the maximum assay window of your specific instrument.

High polarization mixture

Prepare 20 µg/mL ADP² Antibody in 2 nM ADP Alexa633 Tracer/0.5X Stop & Detect Buffer B.

Low polarization mixture

Prepare 2 nM ADP Alexa633 Tracer/0.5X Stop & Detect Buffer B without ADP² Antibody.

Measure the fluorescence polarization

The difference between the low and high polarization values should be >175 mP. If the assay window is <175 mP, please call BellBrook Labs Technical Service.

Optimize ADP² Antibody Concentration

The Transcreeper ADP² FP Assay requires 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 produce the most sensitive and robust assay signal, it is necessary to perform a ADP² Antibody titration in the buffer system ideal for your enzyme target.

To determine the optimal antibody concentration, titrate the ADP² Antibody using the reaction conditions for your enzyme or drug target. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of ADP² Antibody. We recommend using the EC₈₅ concentration of antibody.

Titrate ADP² Antibody in 1X Stop & Detect Buffer B

Prepare 4 nM ADP Alexa633 Tracer in 1X Stop & Detect Buffer B with and without ADP² Antibody (1 mg/mL). Dispense 20 µL of mixture (with antibody) into wells in column 1. Dispense 10 µL of the mixture (without antibody) across a 384-well plate (columns 2-24). Remove 10 µL from column 1 and serially titrate the contents across the plate (to column 24).

Add Enzyme Reaction Buffer (containing ATP)

Prepare your enzyme reaction mixture (include substrate and ATP, but omit enzyme) and add 10 µL to the titrated antibody. Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence polarization.

ADP Detection Controls

These controls are used to calibrate the fluorescence polarization plate reader controls and are added to wells that do not contain enzyme.

Without Antibody (free tracer) Control

This sample contains the ADP Alexa633 Tracer without the ADP² Antibody and is set to 20 mP.

Without Tracer Control

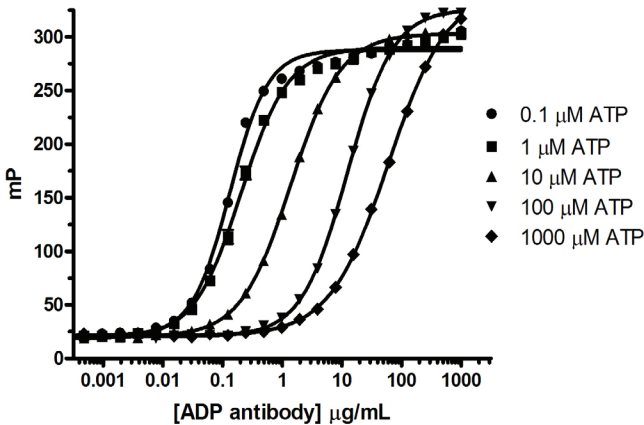
This sample contains the ADP² Antibody without the ADP Alexa633 Tracer and is used as a sample blank for all wells. It is used as a 'buffer blank', but contains the same ADP² Antibody concentration used in all wells.

Plot mP vs. log of ADP² Antibody concentration and calculate the EC₈₅

The antibody concentration at the EC₈₅ is often used as a good compromise between sensitivity and maximal polarization value. The EC₈₅ is determined by inputting the EC₅₀ and hillslope values from a sigmoidal dose response curve fit into the equation below.

$$EC_{85} = ((85/(100-85))^{1/\text{hillslope}}) * EC_{50}$$

Figure 2. ADP² Antibody Titrations at Various ATP Concentrations

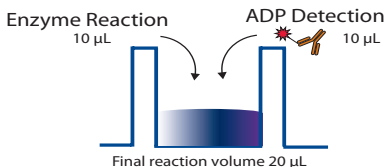


The final 20 μL assay volume consisted of 2 nM ADP Alexa633 Tracer, 0.5X Stop & Detect Buffer B, and 0.5X enzyme reaction mixture (50 mM HEPES (pH 7.5), 2 mM MgCl₂, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 20 mM EDTA, ATP, and ADP² Antibody) (n=3).

Detect ADP in an Enzyme Reaction

The Transcreeper ADP² FP Assay is a universal biochemical assay designed for enzymes that produce ADP. In the one-step detection protocol, 10 μL ADP Detection Mixture is added to the 10 μL enzyme reaction then mixed and incubated for 1 hour. The enzyme reaction components (including ATP) and the ADP Detection Mixture are 0.5X in the final 20 μL.

Figure 3. Protocol



Enzyme Reaction (10 μ L)

Add the enzyme reaction mixture to test compounds and mix on plate shaker. Start the reaction by adding ATP and mix. Incubate at temperature and time ideal for enzyme target before addition of the ADP Detection Mixture.

ADP Detection Mixture (10 μ L)

The 1X ADP Detection Mixture is prepared by adding ADP² Antibody (2*EC₈₅) and 4nM ADP Alexa633 Tracer in 1X Stop & Detect Buffer B. Add the 1X ADP Detection Mixture and mix on plate shaker. Incubate at room temperature (20-25°C) for 1 hour, and measure fluorescence polarization.

Enzyme Reaction Conditions

Choose enzyme buffer conditions and ADP Detection Mixture that are ideal for your enzyme target. Perform your enzymatic reaction at its requisite temperature and optimal duration. An enzyme titration can be performed to identify the optimal enzyme concentration for the Transcreener ADP² FP Assay. Quality control of the ADP² Antibody is performed in 50 mM HEPES (pH 7.5), 4 mM MgCl₂, 2 mM EGTA, 1% DMSO (test compound solvent), 0.01% Brij-35 and varying ATP concentrations (0.1 to 1000 μ M).

Enzyme Assay Controls

The enzyme reaction controls define the limits of the enzyme assay.

0% ATP Conversion Control

This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme), and 100% ATP (0% ADP). This control defines the upper limit of the assay window.

100% ATP Conversion Control

This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme) and 100% ADP (0% ATP). This control defines the lower limit of the assay window.

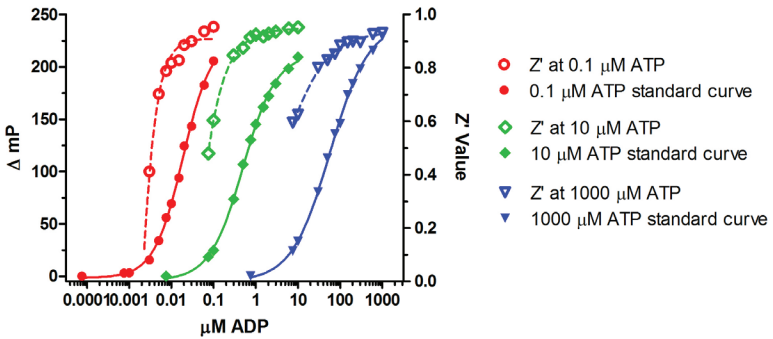
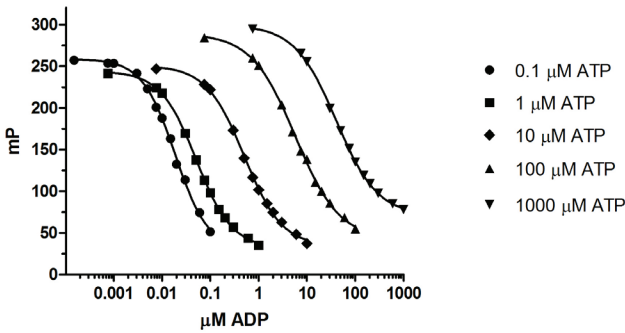
Without Nucleotide Control

To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of nucleotide (i.e. ATP) and acceptor substrate.

ADP/ATP Standard Curve

The standard curve mimics an enzyme reaction (as ATP concentration decreases, ADP concentration increases); the adenine concentration remains constant. The ADP/ATP standard curve allows calculation of the concentration of ADP produced in the enzyme reaction and therefore the % ATP consumed (% ATP conversion). Prepare a twelve-point curve using concentrations of ADP and ATP corresponding to 0%, 2%, 4%, 6%, 8%, 10%, 15%, 20%, 30%, 40%, 60%, and 100% ATP conversion.

Figure 4. ADP/ATP Standard Curves



A) Sample data for 0.1 μM , 1 μM , 10 μM , 100 μM , and 1000 μM ADP/ATP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the ADP Detection Mixture. Curves are obtained in a final 20 μL assay volume consisting of 50 mM HEPES (pH 7.5), 2 mM MgCl_2 , 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 20 mM EDTA, 2 nM ADP Alexa633 Tracer, ADP/ATP standards, and ADP Antibody (EC_{85} concentration) ($n=24$). The data are plotted as mP vs log [ADP] using four-parameter nonlinear regression curve fitting. Alternatively a two-phase exponential decay and nonlinear regression can be used to present the data (GraphPad Prism). B) Excellent Z' values are obtained at <10% ATP conversion for the range of ATP concentrations. Shown are 0.1 μM , 10 μM , and 1,000 μM ATP standard curves.

$$\Delta mP = mP_{\text{initial [ATP]}} - mP_{\text{sample}}$$

and

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

Endpoint Assay

The Transcreeper ADP² FP Assay is designed for endpoint readout. The Stop & Detect Buffer B, 10X contains EDTA to stabilize the signal. EDTA stops the enzyme reaction by chelating available MgCl₂, which is required for enzyme turnover.

Realtime Assay

The end-user may perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. However, the equilibration time for the ADP tracer and ADP² antibody is greater than 15 minutes, making it difficult to quantitate ADP produced during short term enzyme reactions. Note that the optimal ADP² Antibody concentration may change when EDTA is omitted.

4.0 Reagent and Signal Stability

The Transcreeper technology provides the end-user with a robust and stable assay method to detect ADP.

Signal Stability

The stability of the mP assay window at 10% substrate conversion was determined after the addition of the ADP Detection Mixture to the standard samples. The mP assay window at 10% substrate conversion (10 μM) remained constant (< 10% change) for at least 24 hours at room temperature (20-25°C). If plates are to be read the following day, they should first be sealed to prevent evaporation.

ADP Detection Mixture Stability

The ADP Detection Mixture is stable for at least 24 hours at room temperature (20-25°C) before adding to the enzyme reaction (i.e. stored on the liquid handling deck).

Solvent Compatibility

The mP assay window at 10% substrate conversion (10 μM ATP) remains constant (< 10% change) when up to 10% DMSO, DMF, ethanol, acetonitrile, ethanol, or methanol are used in the enzyme reaction. Contact BellBrook Labs for further reagent compatibility information.

5.0 References

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Huss KL, Blonigen PE, Campbell RM: Development of a Transcreener™ Kinase Assay for Protein Kinase A and Demonstration of Concordance of Data with a Filter-Binding Assay Format. *J Biomol Screen.* 2007;12(4):578-584.

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