BellBrook Labs

interchim

FAR RED FP

TRANSCREENER®

ADP^2 FP Assay

Technical Manual

New EZ Protocol eliminates antibody optimization step!

v073009

Transcreener® ADP² FP Assay

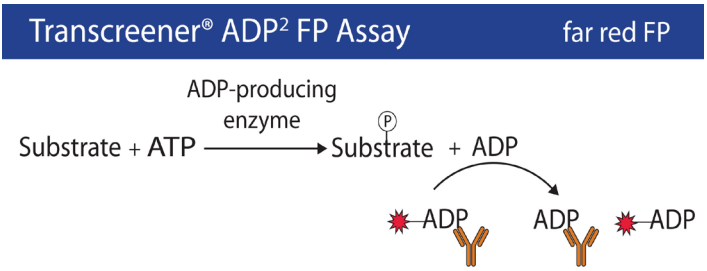
Instructions for Part Numbers 3010-1K and 3010-10K

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 µM ATP). The assay provides excellent signal at low substrate conversion, with a Z′ ≥ 0.7 and ≥ 85 polarization shift (mP) at 10% ATP conversion using 1 µM ATP.

Figure 1. Transcreener® ADP² FP Assay Principle



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. Sufficient reagents are provided to complete up to 1,000 assays with 3010-1K and 10,000 assays with 3010-10K; the exact number is dependent on your enzyme reaction conditions.

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. In section 3.1, a linear relationship is illustrated between [ATP] in the enzyme reaction and the [ADP² Antibody] required for ADP detection. Sufficient antibody is included in the kit to complete 1,000 assays with 3010-1K and 10,000 assays with 3010-10K at an ATP concentration up to 100 µM ATP. Please contact BellBrook Labs for custom packaging for enzyme reactions using > 100 µM ATP.

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 Mg^{2+}-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, $MgCl_2$ or $MnCl_2$, 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 EZ Protocol

The Transcreener ADP² FP Assay is a universal biochemical assay designed for enzymes that produce ADP. It is designed around your initial ATP concentration and enzyme buffer conditions. There are four steps to complete:

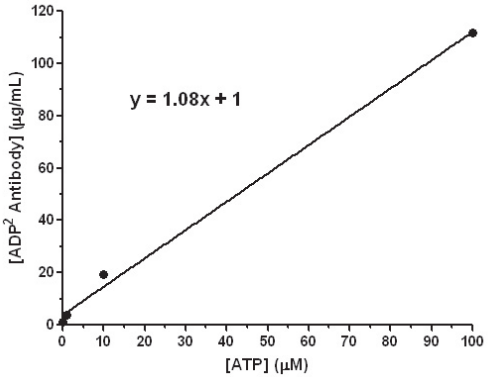
1. Determine antibody concentration
2. Instrument set-up
3. Enzyme titration
4. Detect ADP

Completing these steps will provide optimal ADP detection results. While instrument set-up is not affected by assay conditions, specific instrument settings may be adjusted for optimal results.

3.1 Determine the ADP² Antibody Concentration

The Transcreener 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.

Figure 2. Linear Relationship between [ATP] and [ADP² Antibody]



As shown in Figure 2, the relationship between [ATP] and [ADP² Antibody] is linear. (Though shown for 0.1 µM to 100 µM ATP; the relationship is valid to 1,000 µM ATP.) Therefore the quantity of ADP² Antibody for enzyme reactions that use between 0.1 µM and 1,000 µM ATP can be determined using the 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. We recommend a final volume of 20 µL.

For example, if you are using 3µM ATP in a 10 µl enzyme reaction, the optimal ADP² Antibody concentration in the 1X ADP Detection Mixture (assuming 10 µL of ADP Detection Mixture added to each 10 µL enzyme reaction) would be [1.08 x 3] + 1.0 = 4.24 µg/ml.

Determining your ADP² Antibody concentration using this equation will provide excellent results for most assay conditions. If it does not provide the results you require, please refer to the Appendix for instructions on preparing an ADP² Antibody titration in the buffer system ideal for your enzyme target.

3.2. Instrument Set-up

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 2 nM ADP Alexa633 Tracer/0.5X Stop & Detect Buffer B with your ADP²

Antibody concentration calculated using the equation of line in Figure 2.

Low Polarization Mixture

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

3.3 Enzyme Titration

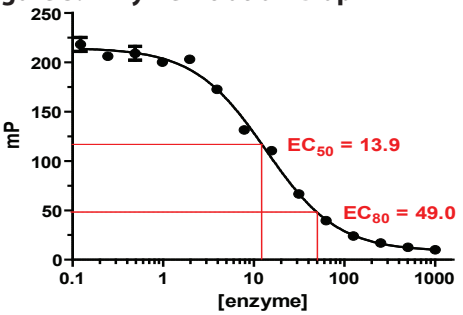
An enzyme titration is performed to identify the optimal enzyme concentration for the Transcreener ADP^2 FP Assay. Choose enzyme buffer conditions and ADP Detection Mixture that are ideal for your enzyme target. Run your enzymatic reaction at its requisite temperature and optimal duration. Quality control of the ADP^2 Antibody is performed in 35 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO (test compound solvent), 0.015% Brij-35 and varying ATP concentrations (0.1 to 1,000 µM).

Enzyme Titration Steps

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 in the presence of both substrate and ATP. To achieve the most robust assay and a large signal, the quantity of enzyme required to produce a 50% - 80% change in polarization signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose response curves. To determine the EC_{80} [enzyme] use the equation below.

$$EC_{80} = (80/(100-80))^{(1/hillslope)} * EC_{50}$$

Figure 3. Enzyme Titration Graph



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

Although optional, an ADP/ATP standard curve can be useful to ensure day to day reproducibility that the assay conditions were performed using initial rates, in addition to being used to calculate inhibitor IC_{50} values. See Appendix for a description of how to run the standard curve.

3.4 ADP Detection

The detection protocol is a single step as shown in Figure 4. 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.

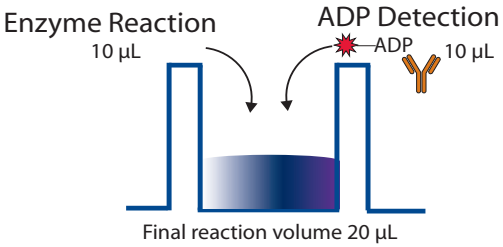


Figure 4. Detection Protocol

Enzyme Reaction (10 µL)

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 µL)

The 1X ADP Detection Mixture is prepared by adding ADP[2] Antibody and ADP Alexa633 Tracer to Stop & Detect Buffer B. Final concentrations should be 4nM ADP Alexa633 Tracer, 1X Stop & Detect Buffer B, and the antibody concentration calculated using the equation of line in Figure 2. Add the 1X ADP Detection Mixture to the enzyme reaction and mix using a plate shaker. Incubate at room temperature (20-25°C) for 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 ADP^2 Antibody and is set to 20 mP.

Without Tracer Control
This sample contains the ADP^2 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 ADP^2 Antibody concentration used in all wells.

Endpoint Assay
The Transcreener ADP^2 FP Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA to stabilize the signal. EDTA stops the enzyme reaction by chelating available $MgCl_2$, which is required for enzyme turnover.

Real-time 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 ADP^2 antibody is greater than 15 minutes, making it difficult to quantitate ADP produced during short term enzyme reactions. Note that the optimal ADP^2 Antibody concentration may change when EDTA is omitted.

4.0 Reagent and Signal Stability
The Transcreener 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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6.0 Appendix

Optimize ADP^2 Antibody Concentration

Using an antibody concentration calculated using equation of line in Figure 2 will produce excellent results for most users. If it does not produce the results you require, we recommend that you perform an ADP^2 Antibody titration in the buffer system ideal for your enzyme target. This will determine the optimal antibody concentration for your assay conditions. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of ADP^2 Antibody. We recommend using the EC_{85} concentration of antibody.

Titrate ADP^2 Antibody in 1X Stop & Detect Buffer B

Prepare 4 nM ADP Alexa633 Tracer in 1X Stop & Detect Buffer B with and without ADP^2 Antibody (2 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)

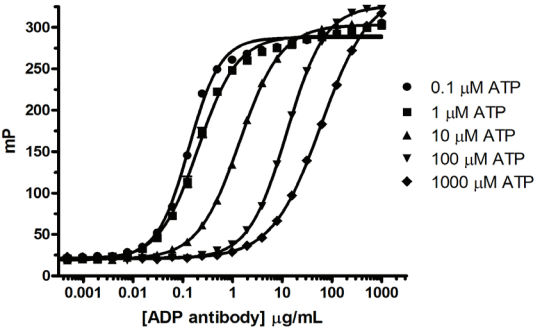
Prepare your enzyme reaction mixture (include substrate and ATP, but omit enzyme) and add 10 µL to the titrated antibody. Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence polarization.

Plot mP vs. log of ADP^2 Antibody Concentration and Calculate the EC_{85}

The antibody concentration at the EC_{85} is often used as a good compromise between sensitivity and maximal polarization value. The EC_{85} is determined by inputting the EC_{50} and hillslope values from a sigmoidal dose response curve fit into the equation below.

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

Figure 5. ADP² Antibody Titrations at Various ATP Concentrations

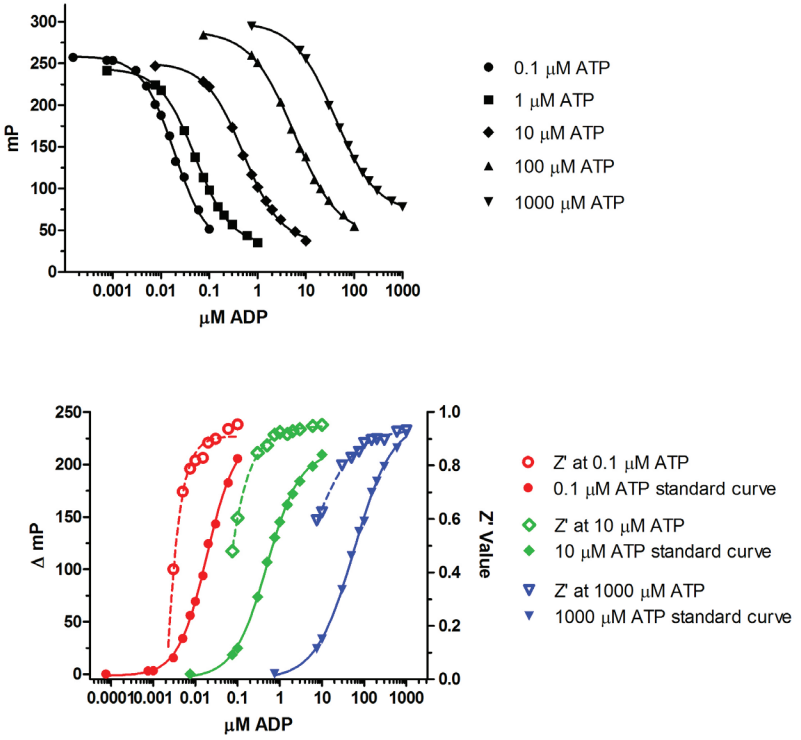


The final 20 µL assay volume consisted of 2 nM ADP Alexa633 Tracer , 0.5X Stop & Detect Buffer B, and 0.5X enzyme reaction mixture (35 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.015% Brij-35, and ATP) and ADP² Antibody (n=3).

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%, 0.5%, 1%, 2%, 3%, 5%, 7.5%, 10%, 15%, 25%, 50%, and 100% ATP conversion.

Figure 6. 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 35 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.015% 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

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

U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 applied. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. BellBrook Labs LLC will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, BellBrook Labs LLC is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, BellBrook Labs LLC, 5500 Nobel Drive, Suite 250, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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