



TRANSCREENER™
ADP TR-FRET Green
Assay

Technical Manual

Transcreener® ADP TR-FRET Green Assay

Instructions for Part Numbers 3005-1K and 3005-10K

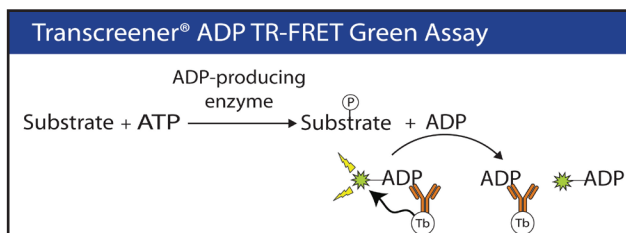
1.0	Introduction	p.2
2.0	Assay Components	p.3
3.0	Protocol	p.4
	Set up ADP Detection System	p.4
	Detect ADP in an Enzyme Reaction	p.6
4.0	Reagent and Signal Stability	p.9

1.0 Introduction

The Transcreener® HTS technology 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 Assay platform will eliminate delays involved in assay development for new HTS targets, and will greatly simplify compound and inhibitor profiling across multiple target families.

The Transcreener® ADP TR-FRET Green Assay is a competitive, time-resolved fluorescence resonance energy transfer (TR-FRET) assay for 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 ADP TR-FRET Green Assay is a simple one-step homogenous detection assay, and is extremely flexible with regard to ATP concentration (from 1 to 100 μM ATP). The assay provides excellent signal at low substrate conversion, with a $Z' \geq 0.5$ at 10% ATP conversion and $Z' \geq 0.7$ at 30% conversion using 10 μM ATP.

Figure 1. Transcreener® ADP TR-FRET Green Assay Principle



The Transcreener ADP TR-FRET Green Assay was developed to follow the progress of any enzyme that produces ADP. The assay measures ADP in a biomolecular competitive assay using a proprietary monoclonal ADP Antibody conjugated to a terbium chelate (ADP Antibody-Tb) and an ADP FAM Tracer. TR-FRET is observed when the lanthanide donor (ADP Antibody-Tb) is bound to the small molecule fluorescein acceptor (ADP FAM Tracer). ADP generated during the enzyme reaction displaces the ADP-FAM Tracer from the ADP Antibody-Tb disrupting TR-FRET (Figure 1). Lanthanide molecules (terbium, europium) have long fluorescence lifetimes. Measuring the FRET signal in a time-gated mode reduces autofluorescent interference of some test compounds, which typically have shorter fluorescent lifetimes. The TR-FRET ratiometric read-out ($\text{FAM}_{520\text{nm}}/\text{Tb}_{495\text{nm}}$) limits fluctuations in signal variability caused by

interwell effects in sample turbidity and reagent volumes.

2.0 Transcreener® ADP TR-FRET Green Assay Components

Store all reagents at -80°C. The ADP Antibody-Tb is sensitive to multiple freeze/thaw cycles. To maintain a robust assay, aliquot the ADP Antibody-Tb after the initial thaw. The ADP FAM Tracer (5 µM), Stop & Detect Buffer A (10X), and 500 µM ADP can withstand >5 freeze/thaw cycles. Sufficient reagents are provided to complete the protocol when using ATP within a range of 1 to 100 µM ATP.

ADP Antibody-Tb, 100X

BellBrook Labs' proprietary monoclonal ADP Antibody has been conjugated to the Lanthascreen™ Terbium Chelate and is supplied at a 100X concentration (400 nM) in 25 mM HEPES (pH 7.5), 137 mM NaCl, 5 mM KCl, and 1 mM Na₂HPO₄.

ADP FAM Tracer, 5 µM

The ADP FAM Tracer consists of a fluorescein conjugated to ADP and is supplied in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35.

Stop & Detect Buffer A, 10X

The TR-FRET Stop & Detect Buffer A (10X) consists of 500 mM HEPES (pH 7.5), 2 M NaCl, 50 mM EDTA, and 0.2% Brij-35. The 1X Stop & Detect Buffer A components will stop the enzyme reaction. To ensure the enzyme reaction is stopped completely, confirm that the EDTA concentration provided is equimolar relative to the magnesium ions present.

500 µM ADP

ADP is not common to all laboratories and therefore is a supplied reagent. ADP is used in 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, ATP, MgCl₂, EGTA, Brij-35, and test compounds. If the ATP stock contains impurities, such as ADP, the assay window will be compromised. Contact BellBrook Labs for suppliers and catalog numbers.

Plate Reader

A plate reader configured to measure time-resolved fluorescence resonance transfer (TR-FRET) is required. The Transcreener ADP TR-FRET Green Assay has been successfully used on both filter-based and monochromator-based instruments. A description of instrument settings compatible with TR-FRET (Tecan Ultra and GENios Pro, Molecular Devices (LJL) Analyst HT, Perkin Elmer EnVision, and BMG LABTECH PHERAstar) can be found at <http://www.invitrogen.com>. Suitable excitation and emission filters can be purchased from Chroma Technology Corporation (Part Numbers

PV002 and PV003). Filter sets were Ex_{340nm} (bw = 35 nm), Em_{495nm} (bw = 10 nm), Em_{520nm} (bw = 25 nm). Tecan Ultra and Tecan Safire²™ settings included a 100 µsec delay, 100-200 µsec integration time, 20 flashes at 30°C. Contact BellBrook Labs for additional information regarding instrument set up and TR-FRET measurements.

Assay Plates

Recommended plates are Corning® white (catalog # 3673) or black (catalog # 3676) 384-well, round bottom, low volume, polystyrene, non-binding surface plates. White plates often show better performance using monochromator-based plate readers

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

- Instrument Set Up
- Tracer Titration
- Enzyme Reaction + ADP/ATP Standard Curve
- ADP Detection

Set up ADP Detection System

Instrument Settings

Becoming familiar with ideal instrument settings for time resolved fluorescence resonance energy transfer is essential to the success of the Transcreener ADP TR-FRET Green Assay.

Verify instrument measures TR-FRET.

Ensure the instrument is capable of measuring time resolved fluorescence resonance energy transfer (not simply FRET). Both the fluorescein signal (520nm) and the terbium signal (495) are read in a time-gated mode. The TR-FRET ratiometric readout (520nm/495nm) provides signal stability. Please call BellBrook Labs for settings and filter sets for specific instruments used for the Lanthascreen technology.

Define the maximum TR-FRET window for your instrument.

Measuring high (tracer + antibody) and low (free tracer) TR-FRET values will define the assay window of your specific instrument.

High TR-FRET mixture.

Prepare 20 nM ADP FAM Tracer in 0.5X ADP Antibody-Tb/0.5X Stop & Detect Buffer A.

Low TR-FRET mixture.

Prepare 0.5X ADP Antibody-Tb/0.5X Stop & Detect Buffer A without ADP FAM Tracer.

Measure TR-FRET.

The difference between the low and high 520/495 ratio should be >1.0. Please call BellBrook Labs to troubleshoot current instrument settings.

Optimize ADP FAM Tracer Concentration

The Transcreeper ADP TR-FRET Green 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 ADP FAM Tracer 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 an ADP FAM Tracer in the buffer system ideal for your enzyme target. We recommend using the EC_{50} concentration of ADP FAM Tracer.

Titrate ADP FAM Tracer in 1X Stop & Detect Buffer A

Prepare a 1X ADP Antibody-Tb in 1X Stop & Detect Buffer A with and without ADP FAM Tracer (250 nM). Dispense 20 μ L of mixture (with ADP FAM Tracer) into wells in column 1. Dispense 10 μ L of the mixture (without ADP FAM Tracer) across a 384-well plate (columns 2-24). Remove 10 μ L from column 1 and perform 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 μ L to the titrated tracer. Mix the plate, equilibrate at room temperature (1 hour), and measure TR-FRET.

ADP Detection Controls

These controls identify background signal and are used to establish the TR-FRET signal in the absence of one of the fluorescent probes.

Without Ab (free tracer) Control

This sample contains the ADP FAM Tracer without the ADP Antibody-Tb.

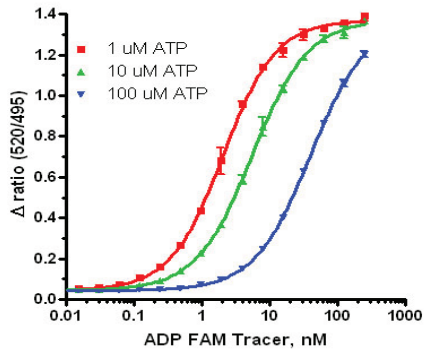
Without Tracer Control

This sample contains the ADP Antibody-Tb without the ADP FAM Tracer.

Plot ADP FAM Tracer concentration vs. TR-FRET ratio (520 nm/495 nm) on a log scale

To produce a signal that is both sensitive and robust, the EC_{50} concentration of ADP FAM Tracer is recommended. Graph data using sigmoidal dose response curve fit.

Figure 2. ADP FAM Tracer Titration for Various ATP Concentrations.

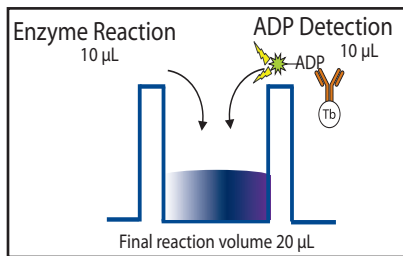


The final 20 μL assay volume was generated using 0.5X ADP Antibody-Tb, 0.5X Stop & Detect buffer A, and 0.5X enzyme reaction mixture, which was 50 mM HEPES (pH 7.5), 4 mM MgCl_2 , 2 mM EGTA, 1% DMSO, 0.01% Brij-35 and 1 μM , 10 μM , and 100 μM ATP.

Detect ADP in an Enzyme Reaction

The Transcreeper ADP Assay is a universal biochemical assay designed for enzymes that produce ADP. In the one-step detection protocol, the 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.



Prepare Enzyme Reaction (10 μL)

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

Add ADP Detection Mixture (10 μL)

The 1X ADP Detection Mixture is prepared by adding ADP FAM Tracer (2^*EC_{50}) and 1X ADP Antibody-Tb in 1X Stop & Detect Buffer A. Add the ADP Detection Mixture and mix on plate shaker. Incubate at room temperature (20-25°C) for 1 hour, and measure TR-FRET.

Enzyme Reaction Conditions

Choose enzyme buffer conditions and ADP Detection Mixture that are ideal for each enzyme target. The enzyme reaction can be performed at any reaction temperature for any incubation time. An enzyme titration can be performed to identify the optimal enzyme concentration for the Transcreener ADP TR-FRET Green Assay. Quality control of the Transcreener ADP TR-FRET Green Assay components 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 (1 to 100 μM).

Enzyme Assay Controls

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

0% ATP Conversion Control (Without Enzyme)

This control consists of the ADP Antibody-Tb and ADP FAM Tracer complex enzyme reaction components and 100% ATP (0% ADP). This control defines the upper limit of the assay window.

100% ATP Conversion Control (Without Enzyme)

This control consists of the ADP Detection Mixture, the enzyme reaction components, and 100% ADP (0% ATP). This control defines the lower limit of the assay window.

Without Acceptor Substrate Control

This control consists of the ADP Detection Mixture, the enzyme reaction components, and 100% ATP (0% ADP). Because some enzymes show activity in the absence of acceptor substrate, this control should not be used to define the upper limit of the assay window. However, this control can be used to monitor substrate-independent ADP production.

Without Nucleotide Control

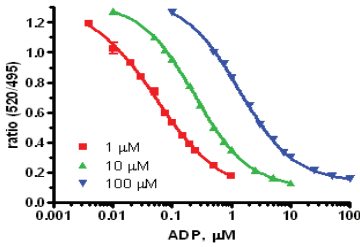
To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of nucleotide (i.e. ATP) and acceptor substrate.

ADP/ATP Standard Curve

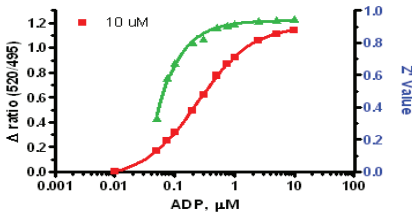
The standard curve mimics an enzyme reaction (as ATP concentration decreases, ADP concentration increases; the adenine concentration remains constant). By graphing the TR-FRET ratio (520 nm/495 nm) vs μM ADP, the TR-FRET ratio can be correlated to the concentration of ADP generated (and then to % ATP conversion). Prepare a twelve-point curve using concentrations of ADP and ATP corresponding to 0%, 0.5%, 1%, 2%, 4%, 6%, 8%, 10%, 20%, 30%, 50%, and 100% ATP conversion.

Figure 4. ADP/ATP Standard Curves.

A.



B.



The nucleotide concentration (1 μM , 10 μM , and 100 μM) reflects the amount in the enzyme reaction, prior to the addition of the ADP Detection Mixture. Data are shown 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, 10 mM EDTA, 400 mM NaCl, 2 nM ADP Antibody-Tb, ADP/ATP standards, and ADP FAM Tracer (EC_{50} concentration) ($n=24$). Data are shown as TR-FRET ratio 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 10 μM ATP standard curve; $\Delta\text{TR-FRET}$ from raw TR-FRET values.

$$\Delta\text{Ratio} = \text{Ratio}_{\text{initial [ATP]}} - \text{Ratio}_{\text{sample}}$$

and

$$Z' = 1 - [(3 * \text{SD}_{\text{initial [ATP]}} + 3 * \text{SD}_{\text{sample}}) / |(\text{Ratio}_{\text{initial [ATP]}} - \text{Ratio}_{\text{sample}})|].$$

Endpoint Assay

The Transcreeper ADP TR-FRET Green Assay is designed for endpoint readout. The Stop & Detect Buffer A, 10X contains EDTA to stabilize the signal. EDTA stops the enzyme reaction by chelating available MgCl_2 , which is required for enzyme turnover.

Realtime Assay

The end-user may perform kinetic experiments by substituting the Stop & Detect Buffer A, 10X (provided) with a preferred detection buffer (prepared by the user without EDTA). Note: depending on the other reaction components, optimal ADP FAM Tracer 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 TR-FRET signal was measured after the addition of the ADP Detection Mixture. The TR-FRET signal remained steady (within 3 standard deviations of the control mean) for >5 hours at room temperature (20-25°C).

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 assay window at 10% substrate conversion (10 μ M ATP) remains constant (< 10% change) when up to 6% DMSO or DMF, and up to 12% acetonitrile, ethanol or methanol is used in the enzyme reaction. Contact BellBrook Labs for further reagent compatibility information.

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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