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™ AMP/GMP Assay is a far-red, competitive fluorescence polarization assay based on the detection of AMP or GMP, and is therefore compatible with any enzyme class that produces either AMP or GMP, including ubiquitin, SUMO, nucleic acid and protein ligases, phosphodiesterases (PDEs), and synthetases. The Transcreener AMP/GMP Assay is a single step, homogenous detection assay enabling the use of unmodified native substrate concentrations of 1 - 1000 µM. The assay provides excellent signal at low substrate conversion, with a $Z' \geq 0.7$ and $\geq 85$ polarization shift (mP) at 10% substrate conversion using 10 µM donor substrate. Reduced development costs and accelerated drug discovery are achieved by utilizing this one simple streamlined screening method.

Figure 1. Transcreener™ AMP/GMP Assay Overview

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

Store reagents at −20°C. Individual reagents tolerate 10 freeze-thaw cycles. Sufficient reagents are provided to complete the protocol when using substrates within a range of 1 to 100 µM. Please contact BellBrook Labs for custom packaging for enzyme reactions with >100 µM cAMP/cGMP/ATP.

AMP/GMP Antibody

A concentrated polyclonal AMP/GMP Antibody is provided in PBS. The concentration of AMP/GMP Antibody needed for a particular target enzyme depends on enzyme assay conditions, particularly nucleotide substrate concentration. To produce the most sensitive and robust assay signal, first perform an AMP/GMP Antibody titration in the buffer system for your enzyme.

AMP/GMP Alexa633 Tracer, 400 nM

The AMP/GMP Alexa633 Tracer is provided at 400 nM in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35.

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 reaction.

500 µM AMP

AMP is not common to all laboratories and therefore is a supplied reagent. AMP is used in the AMP/cAMP and AMP/ATP standard curves.

500 µM GMP

GMP is not common to all laboratories and therefore is a supplied reagent. GMP is used in the GMP/cGMP 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

Necessary enzyme reaction components include enzyme, enzyme co-factors, substrates, MgCl₂, and test compounds. Substrate stock that contains nucleotide impurities, in particular nucleotide monophosphates, will decrease the assay window. Contact BellBrook Labs for suppliers and catalog numbers.
Plate Reader
A fluorescence polarization plate reader configured to measure fluorescence polarization of Alexa Fluor®633 is required. The Transcreener AMP/GMP Assay has been successfully performed on the following instruments: BioTek Synergy 2, BMG Labtech PHERAstar and POLARstar, Molecular Devices Analyst (AD, HT, and GT), Perkin Elmer EnVision, ViewLux, Victor 2V, and Victor 3V, and Tecan Ultra, F500, Safire², and GENios Pro. Contact BellBrook Labs for additional information regarding instrument set up and fluorescence polarization measurements.

Assay Plate
It is important that assay plates 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™ AMP/GMP Assay is designed to fit your enzyme buffer conditions. For optimal results establish the AMP/GMP detection system (e.g., instrument set-up and antibody optimization) before running the enzyme assay. These initial steps will not need to be repeated unless assay reaction conditions change.

- Instrument Set Up
- Antibody Titration
- Enzyme Reaction + AMP/ATP, AMP/cAMP and/or GMP/cGMP Standard Curve
- AMP/GMP Detection

Set up AMP/GMP Detection System

Instrument Settings
Becoming familiar with ideal instrument settings for fluorescence polarization is essential to the success of the Transcreener AMP/GMP Assay.

Verify instrument measures fluorescence polarization
Verify that the instrument can measure fluorescence polarization (not simply fluorescence intensity) of Alexa Fluor®633. Contact BellBrook Labs if you have questions about settings and filter sets for a specific instrument.

Define the maximum mP window for your instrument
Determine high (tracer + antibody) and low (free tracer) polarization values to define the maximum assay window for your specific instrument.

High polarization mixture (antibody + tracer)
Prepare 20 µg/mL AMP/GMP Antibody and 2 nM AMP/GMP Alexa633 Tracer in 0.5X Stop & Detect Buffer B.
**Low polarization mixture (tracer alone)**

Prepare 2 nM AMP/GMP Alexa633 Tracer in 0.5X Stop & Detect Buffer B.

**Measure the fluorescence polarization**

The low and high polarization values should differ by >175 mP. If the assay window is <175 mP please contact BellBrook Labs.

**Optimize AMP/GMP Antibody Concentration**

The Transcreener ADP Assay requires detection of AMP/GMP in the presence of excess cAMP/cGMP/ATP (assuming initial velocity enzyme reaction conditions) using an antibody with a finite selectivity for the monophosphate vs. the substrate. The concentration of AMP/GMP Antibody determines the total assay window and the AMP/GMP detection range, and the amount needed is dependent upon the cAMP/cGMP/ATP concentration in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to perform an AMP/GMP Antibody titration in the buffer system ideal for your enzyme target.

To determine the optimal antibody concentration, titrate the AMP/GMP 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 AMP/GMP Antibody. We recommend using the EC$_{85}$ antibody concentration.

**Titrate AMP/GMP Antibody in 1X Stop & Detect Buffer B**

Prepare 4 nM AMP/GMP Alexa633 Tracer in 1X Stop & Detect Buffer B with and without AMP/GMP Antibody (1 mg/mL). Dispense 20 µL of the mixture (with antibody) into the 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, transfer this amount to column 2 and continue serial antibody titration across the plate (to column 24).

**Add Enzyme Reaction Mix (containing nucleotide substrate)**

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

**Fluorescence Polarization Reader Calibration Controls**

Add these controls to the plate and use them to calibrate the fluorescence polarization plate reader.

**Without Antibody (Free Tracer) Control**

This control is used to set the lower limit polarization value to 20 mP. It contains 2 nM AMP/GMP Alexa633 Tracer in 0.5X enzyme reaction mix and 0.5X Stop & Detect Buffer B (without antibody).

**Without Tracer Control**

This control is used for background subtraction. It contains antibody in 0.5X enzyme reaction mix and 0.5X Stop & Detect Buffer B (without tracer).
Plot mp vs. log of AMP/GMP 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.

\[
EC_{85} = \left(\frac{85}{(100-85)}\right)^{1/hillslope} \times EC_{50}
\]

**Figure 2. AMP/GMP Antibody Titrations at Various ATP Concentrations**

Figure 2. AMP/GMP Antibody titrations for different ATP Concentrations. The final 20 µL assay volume consisted of 35 mM HEPES (pH 7.5), 5 mM MgCl₂, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 20 mM EDTA, 2 nM AMP/GMP Alexa633 Tracer, nucleotide, and AMP/GMP Antibody (n=3).

**Detect AMP/GMP in an Enzyme Reaction**

The Transcreener AMP/GMP Assay is a universal biochemical assay designed for enzymes that produce AMP or GMP. In the one-step detection protocol, 10 µL AMP/GMP Detection Mixture is added to the 10 µL enzyme reaction then mixed and incubated for 1 hour. The enzyme reaction components (including ATP, cAMP, or cGMP) and the AMP/GMP Detection Mixture are 0.5X in the final 20 µL volume.

**Figure 3. Protocol**
Enzyme Reaction Conditions
Choose enzyme buffer conditions and AMP/GMP 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 AMP/GMP Assay. Quality control of the Transcreener AMP/GMP 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 cAMP/cGMP/ATP concentrations (1 to 1,000 µM).

Enzyme Assay Controls
The enzyme reaction controls define the limits of the enzyme assay.

0% cAMP/cGMP/ATP Conversion Control
This control consists of the AMP/GMP Detection Mixture, the enzyme reaction components (without enzyme), and 100% cAMP/cGMP/ATP (0% AMP/GMP). This control defines the upper limit of the assay window.

100% cAMP/cGMP/ATP Conversion Control
This control consists of the AMP/GMP Detection Mixture, enzyme reaction components (without enzyme), and 100% AMP/GMP (0% cAMP/cGMP/ATP). This control defines the lower limit of the assay window.

Without Acceptor Substrate Control
This control consists of the AMP/GMP Detection Mixture, enzyme reaction components (without substrate), and 100% cAMP/cGMP/ATP (0% AMP/GMP). 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 AMP/GMP production.

Without Nucleotide Control
To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of donor (i.e. ATP/cAMP/cGMP) and acceptor substrate.

AMP/cAMP, GMP/cGMP, and AMP/ATP Standard Curves
The standard curves mimic an enzyme reaction (e.g. as ATP concentration decreases, AMP concentration increases; the adenine concentration remains constant). The standard curves allow calculation of the concentration of AMP or GMP produced in the enzyme reaction and therefore the % substrate consumed (% substrate conversion). Prepare a twelve-point curve using concentrations of AMP or GMP and substrate corresponding to 0%, 2%, 4%, 6%, 8%, 10%, 15%, 20%, 30%, 40%, 60%, and 100% cAMP/cGMP/ATP conversion.

Using the reaction conditions for your enzyme, prepare a product stock solution (AMP or GMP) and substrate stock solution (cAMP, cGMP, or ATP). Prepare substrate/product mixes to mimic specific conversion levels. Add 10 µL of each standard to the 384 well plate followed by the addition of 10 µL 1X Stop & Detect Buffer B.
(A) Sample data for 1 µM standard curves are shown in a 20 µL final assay volume consisting of 35 mM HEPES (pH 7.5), 2 mM MgCl₂, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 20 mM EDTA, 2 nM AMP/GMP Alexa633 Tracer, ATP/AMP standards, and 3.5 µg/mL AMP/GMP Antibody. The nucleotide concentration reflects the amount in the enzyme reaction, before the addition of the AMP/GMP Detection Mixture. The data are plotted as mP vs log [AMP/GMP] 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 (n=24).

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

and

\[ Z' = 1 - \left[ \frac{(3*SD_{\text{initial [AMP]}} + 3*SD_{\text{sample}})}{|(mP_{\text{initial [AMP]}} - mP_{\text{sample}})|} \right] \]
The Transcreener AMP/GMP Assay is optimized for the initial velocity region. If intended use is outside the initial velocity region, contact BellBrook Labs for reformatting recommendations.

**Endpoint Assay**
The Transcreener AMP/GMP Assay is designed for an endpoint readout. The Stop & Detect Buffer B contains EDTA to stabilize the signal. EDTA stops the enzyme reaction by chelating MgCl₂, which is required for enzyme turnover.

**Real-time Assay**
You can perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA, but that does contain 0.02% Brij-35. Note that the optimal AMP/GMP 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 AMP and GMP.

**Signal Stability**
The stability of the mP assay window at 10% substrate conversion was determined after the addition of the AMP/GMP Detection Mixture to the standard samples. The mP assay window at 10% substrate conversion (1 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.

**AMP/GMP Detection Mixture Stability**
The AMP/GMP 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 12% DMSO, ethanol, methanol or acetonitrile is used in the enzyme reaction. Contact BellBrook Labs for further reagent compatibility information.
U.S. Patent 7,332,278 and 7,355,010 issued. U.S. Patent Application Nos. 11/353,500 and 11/958,965 and International Patent Application Nos. PCT/US04/002618 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.

Transcreener™ HTS Assay Platform is a patented technology of BellBrook Labs. Transcreener™ is a trademark of BellBrook Labs. AlexaFluor® is a registered trademark of Molecular Probes, Inc (Invitrogen). Corning® is a registered trademark of Corning Incorporated.

©2008 BellBrook Labs. All rights reserved.