BellBrook Labs

Biology at Work

Technical Manual

Product #: 3015-1K and 3015-10K

Technical Manual
V110111

Transcreener® AMP²/GMP² FP Assay Kit

Contents

1.0 Introduction

The Transcreener®AMP²/GMP² FP Assay is a universal biochemical HTS assay for enzymes that produce AMP or GMP, including ubiquitin, SUMO, nucleic acid and protein ligases, phosphodiesterases (PDEs), and synthetases. Enzyme activity is signaled by a decrease in fluorescence polarization as the bound tracer is displaced from the Transcreener® AMP²/GMP² Antibody. The assay is a simple single step mix-and-read format enabling the use of unmodified native substrate concentrations of 1 – 1000 µM. The assay provides excellent signal at low substrate conversion, with a Z' ≥0.7 and ≥100 millipolarization shift (mP) under normal reaction conditions.

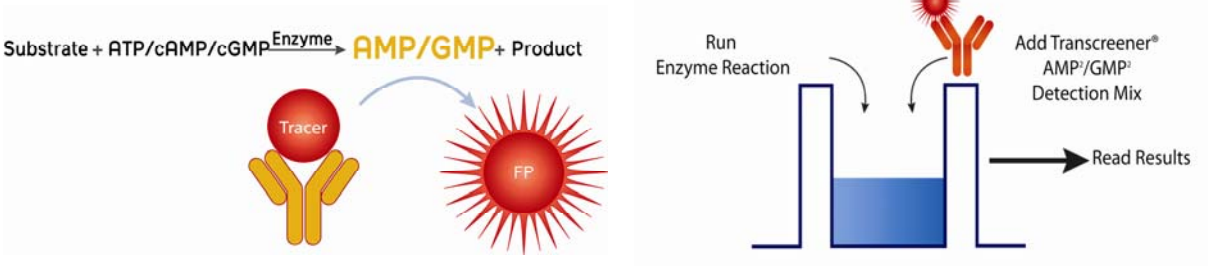


Figure 1. Transcreener® AMP²/GMP² FP Assay Principle (left) with Assay Protocol Schematic (right).

2.0 Transcreener® AMP²/GMP² Assay Components

Materials Provided

The kit contains sufficient reagents for 1,000 wells detecting concentrations up to 100 µM AMP or GMP. Store reagents at –20°C as indicated.

Component	Description
AMP²/GMP² AlexaFluor® 633 Tracer, 800 nM	The AMP²/GMP² AlexaFluor® 633 Tracer is provided at 800 nM in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The AMP²/GMP² AlexaFluor® 633 Tracer is at 4 nM at the time of polarization measurement (20 µL).
AMP²/GMP² Antibody	A concentrated monoclonal AMP²/GMP² Antibody, provided in PBS with 10% glycerol. The concentration of AMP²/GMP² Antibody needed for a particular target enzyme depends on enzyme conditions particularly the initial [AMP] or [GMP].
1M Tris (pH 7.5)	1M Tris (pH 7.5) is used to buffer the Detection Mixture.
5 mM AMP	AMP is used to create an ATP/AMP or cAMP/AMP standard curve.
5 mM GMP	GMP is used to create a cGMP/GMP standard curve.

Materials Required

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 cofactors, substrates, and test compounds.

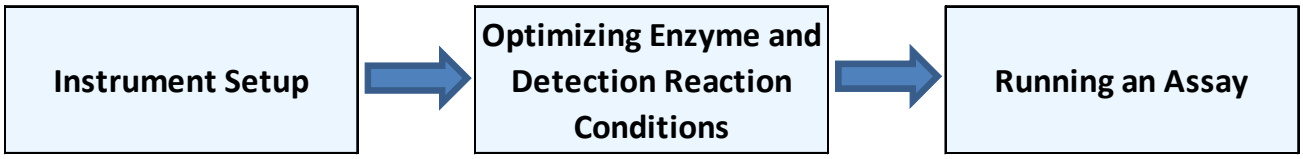
Plate Reader A fluorescence polarization plate reader configured to measure fluorescence polarization of AlexaFluor® 633 is required. The Transcreener® AMP²/GMP² FP Assay using has been successfully performed on the following instruments: BMG Labtech PHERAstar, Perkin Elmer EnVision, and Tecan Safire². NOTE: Contact BellBrook Labs for additional instruments, information regarding instrument set up, and fluorescence polarization measurements.

Assay Plate It is important that assay plates be entirely black with a non-binding surface. We highlyrecommend Corning® 384 well 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 following protocol was developed for 384 well plate format with 15 µL enzyme reactions and 20 µL final volume at the time the plates are read. Use of the assay at different densities and well volumes will require proportional changes in reagent quantities.



3.1 Instrument Set Up

Determination of the optimal instrument settings for fluorescence polarization is essential to the success of the Transcreener® AMP²/GMP² FP Assay. Refer to Table 1 for filter sets and settings for common multimode plate readers. If you are using a different instrument, verify that it can measure fluorescence polarization (not simply fluorescence intensity). Contact BellBrook Labs if you have questions about settings and filter sets for a specific instrument.

PerkinElmer EnVision®		
Mirror	D658fp/D688 dual mirror	Part# 2100-4260
Excitation Filter/Bandwidth	620/40 nm	Part# 2100-5760
Emission Filter(P-pol)/Bandwidth	688/45 nm	Part# 2100-5790
Emission Filter(S-pol)/bandwidth	688/45 nm	Part# 2100-5780
BMG LABTECH PHERAstar Plus (Transcreener FP Optic Module)		
Excitation Filter/Bandwidth	590/50 nm	
Emission A Filter/Bandwidth	675/50 nm	
Emission B Filter/Bandwidth	675 nm	
Tecan Safire2™ (monochromator based)		
Excitation Wavelength/Bandwidth	635/20 nm	
Emission Wavelength/Bandwidth	670/20 nm	
Read-FP Mode		

EnVision is a registered trademark of PerkinElmer. Safire² is a trademark of Tecan.

Table 1. Instrument Filters & Settings. Filter settings and filter part numbers for commonly placed multimode plate readers. A complete list of instruments and instrument-specific application notes can be found online: www.bellbrooklabs.com/transcreener_instrument_validation.html

i. Define the maximum polarization window for your instrument

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

ii. Prepare High and Low polarization mixtures

High Polarization mixture (+ antibody): Add 5 µL of Detection Mixture (containing 80 µg/mL AMP²/GMP² Antibody, 16 nM AMP²/GMP² AlexaFluor® 633 Tracer, and 50 mM Tris (pH 7.5); see Table 3 for an example) to 15 µL enzyme reaction buffer devoid of enzyme and AMP or GMP.

Low Polarization mixture(- antibody): Add 5 µL of Detection Mixture (containing 16 nM AMP²/GMP² AlexaFluor® 633 Tracer and 50 mM Tris (pH 7.5); see Table 3 for an example) to 15 µL enzyme reaction buffer devoid of enzyme and AMP or GMP.

iii. Measure the fluorescence polarization

The low and high polarization values should differ by >175 mP. Note that this measurement gives the maximal *signal window* that the instrument is capable of generating with these reagents. The *assay window* will be less than this, and will depend on how far the AMP/GMP enzyme reaction proceeds. **If the signal window is <175 mP please contact BellBrook Labs for suggestions on optimizing the instrument filters or settings.**

3.2 Optimizing Enzyme and Detection Reaction Conditions

A polarization shift of 60-100 mP units and a Z' value of 0.5 indicates robust assay performance for HTS applications. For initial ATP/cAMP/cGMP concentrations of 0.1 µM, 1 µM and 10 µM, this criteria was achieved when less than 30 nM of the AMP/AMP/GMP was present at detection (Figure 3, page 8). For 100 µM ATP/cAMP/cGMP concentration, this was achieved when 0.5% conversion (500 nM) was present (Figure 3, page 8).

***AMP/GMP enzyme reaction conditions*:** Run your enzymatic reaction with optimal buffer and additives at the requisite temperature. Initial ATP/cAMP/cGMP concentration is critical, as it determines the AMP^2/GMP^2 Antibody concentration (Table 2). The acceptor substrate should be present at a concentration similar or higher than the ATP/cAMP/cGMP concentration to avoid non-linear kinetics resulting from substrate depletion.

Note: BSA at higher concentrations in the enzyme reaction buffer may interfere with the assay resulting in a small assay window. Please contact us via phone (toll-free at 1-866-313-7881) or email (info@bellbrooklabs.com) for more information.

AMP^2/GMP^2 Antibody concentration: This is the only assay component that requires adjustment for different reaction conditions. Its concentration will define the dynamic range of the assay, and it should be adjusted based on the ATP/cAMP/cGMP concentration used in the enzyme reactions. We have determined optimal AMP^2/GMP^2 Antibody concentrations up to 1000 µM (Table 2) in a simple buffer system. If you require nucleotide concentration outside of this range, please contact BellBrook Labs for recommendations.

ATP/cAMP/cGMP Concentration in 15 µL Enzyme Reaction	AMP^2/GMP^2 Antibody Concentration in the AMP/GMP Detection Mixture
0.1 µM to 10 µM	3 µg/mL
11 µM to 100 µM	13 µg/mL
101 µM to 1000 µM	110 µg/mL

Table 2. Optimal concentration of AMP^2/GMP^2 Antibody in the AMP/GMP Detection Mixture for ATP/cAMP/cGMP concentrations up to 1000 µM.

AMP/GMP enzyme concentration: You will need to perform a pilot experiment to determine the optimal enzyme concentration to yield initial velocity conditions (substrate consumption ≤20%) and produce a sufficient assay window. The optimal enzyme concentration should be determined by serial titration using the same conditions that will be used for screening or profiling. We recommend an enzyme concentration that produces 50% to 80% of the maximal change in polarization (EC_{50} to EC_{80}) with a polarization change of at least 100 mP (Figure 2).

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

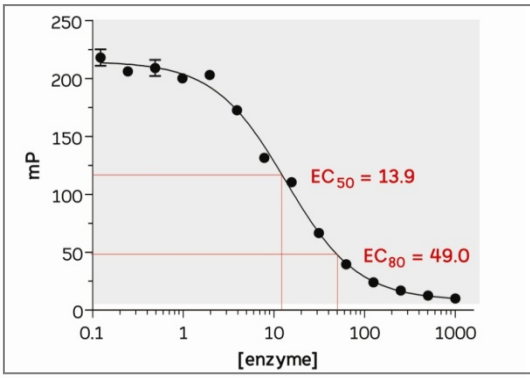


Figure 2. Typical AMP/GMP Enzyme Titration. Based on this curve, the optimal enzyme amount would be 20-40 units.

AMP/GMP Detection Mixture: Prepare on the day of use the amounts of each reagent as shown in Table 3 for 1,000 reactions (384 well density, 5 µL AMP/GMP Detection Mixture per well, 20 µL total reagent volume per well). Adjust quantities proportionately for fewer reactions, different plate densities and well volumes, and the inclusion of Stop Reagents. The AMP/GMP Detection Mixture is stable for at least 16 hours at room temperature (20-25°C); we recommend keeping reagents cold as much of the time as possible.

Note: We have shown that Stop & Detect Buffer B, 5X composed of 200 mM HEPES, 0.2% Brij®-35, and 400 mM EDTA, pH 7.5 (Part # 2139 (1mL) or 2140 (10 mL)) has the ability to stop select ubiquitin ligases and phosphodiesterase enzymes. Please contact us via phone (toll-free at 1-866-313-7881) or email (info@bellbrooklabs.com) for more information.

ATP/cAMP/cGMP Concentration:	0.1 - 10 µM	11 - 100 µM	101- 1000 µM
Component	**Volume**		
Water	4637.5 µL	4595.8 µL	4191.7 µL
1M Tris (pH 7.5)	250 µL	250 µL	250 µL
AMP/GMP AlexaFluor® 633 Tracer, 800 nM	100 µL	100 µL	100 µL
AMP²/GMP² Antibody, 1.2 mg/mL	12.5 µL	54.2 µL	458.3 µL

Table 3. Recipe for AMP/GMP Detection Mixture for 1,000 wells of 20 µL reactions using ATP/cAMP/cGMP concentrations up to 1000 µM. Final concentrations in the AMP/GMP Detection Mixture: 50 mM Tris (pH 7.5), 16 nM AMP²/GMP² AlexaFluor® 633 Tracer and variable AMP²/GMP² Antibody.

3.3 Running an Assay

The Transcreener® AMP^2/GMP^2 Assay is designed for endpoint readout. Following incubation of the enzyme reactions (15 µL) for the requisite time, 5 µL AMP/GMP Detection Mixture is added. Reactions are mixed and allowed to equilibrate at room temperature for 90 minutes before reading.

Assay Steps:

i. ***Run Enzyme Reaction (15 µL).*** Generally, a master mix containing all AMP/GMP enzyme reaction components, except ATP/cAMP/cGMP, is dispensed into wells and reactions are started by adding ATP/cAMP/cGMP. Mix the plate, and incubate at the desired temperature and time.

ii. ***Add AMP/GMP Detection Mixture (5 µL).*** Add 5µL of AMP/GMP Detection Mixture bringing the total volume to 20 µL. Note that the AMP/GMP enzyme reaction components (including ATP/cAMP/cGMP) will be diluted 1.3-fold and the AMP/GMP Detection Mixture will be diluted 4-fold after this addition.

iii. ***Read Plates.*** Allow at least 90 minutes for the detection reaction to equilibrate before reading the plate. Our studies have indicated that the signal is stable (<10% change) for at least 24 hours at room temperature (20-25°C). If polarization measurement is to occur on the following day, seal the plates to prevent evaporation.

Control Reactions

When screening or profiling inhibitors, two types of controls are needed at a minimum: a) negative ("no enzyme") control wells to calculate the decrease in polarization caused by enzyme activity; this control defines the upper limit (highest polarization value) of the assay window b) positive ("no inhibitor") control wells lacking any test compounds to determine the full activity of the enzyme being screened. This control defines the lower limit of the assay window. Z' values are usually calculated from these two types of controls.

Standard Curves

A standard curve (Figure 3, page 8) is needed to convert polarization values to product formation (AMP or GMP) for quantitative data analysis. (Note that because the Transcreener® AMP^2/GMP^2 Assay relies on a competitive binding reaction, the response is non-linear, so the signal is not directly proportional to reaction progress). Whether to use a standard curve is a matter of choice. Many Transcreener® users do not use one for screening purposes, because the criteria for a hit are based on statistical analysis of the raw data. However, if quantitative enzyme turnover information is required for Michaelis-Menten kinetic analysis, a standard curve will be needed.

The wells for the standard curve should contain all AMP/GMP reaction components **except the enzyme** and receive AMP/GMP Detection Mixture. The curve is constructed to mimic an enzyme reaction: starting at the ATP/cAMP/cGMP concentration used for the screening reactions, ATP/cAMP/cGMP is dcreased in increments and the AMP/AMP/GMP concentration is increased proportionately, keeping the sum of their concentrations [ATP/cAMP/cGMP + AMP/AMP/GMP] constant. We recommend using a twelve-point curve with concentrations of ATP/cAMP/cGMP and AMP/AMP/GMP corresponding to 0%, 0.5%, 1%, 2%, 3%, 5%, 7.5%, 10%, 15%, 30%, 50%, and 100% ATP/cAMP/cGMP conversion. Allow 90 minutes incubation prior to polarization measurement for the coupling enzyme reaction to reach completion.

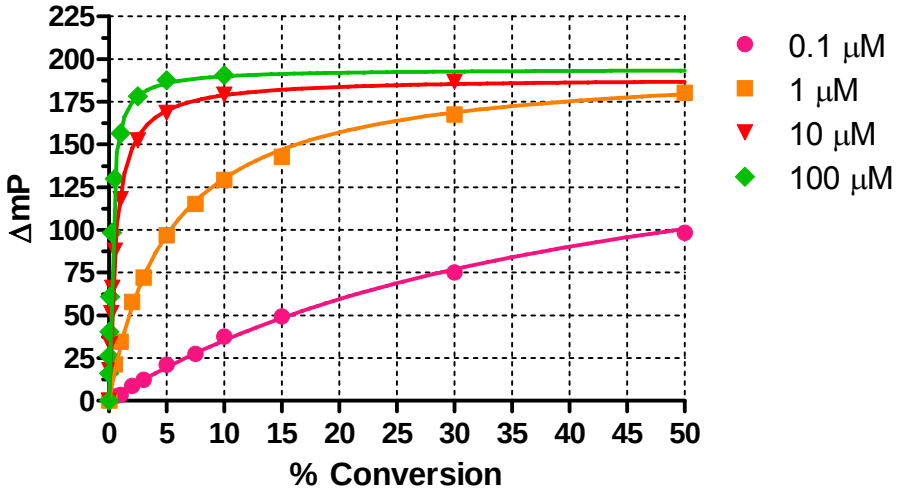


Figure 3. ATP/AMP Standard Curves. Sample data for standard curves starting at initial ATP concentrations of 0.1, 1, 10, and 100 µM are shown (ATP concentrations in the 15 µL mock enzyme reaction before the addition of Detection reagents). A polarization shift of 60-100 mP units and a Z' value of 0.5 indicates robust assay performance for HTS applications. For initial ATP/cAMP/cGMP concentrations of 0.1 µM, 1 µM and 10 µM, these criteria were achieved when less than 30 nM of the AMP/AMP/GMP was present. For 100 µM ATP/cAMP/cGMP concentration, this was achieved when 0.5% conversion (500 nM) was present.

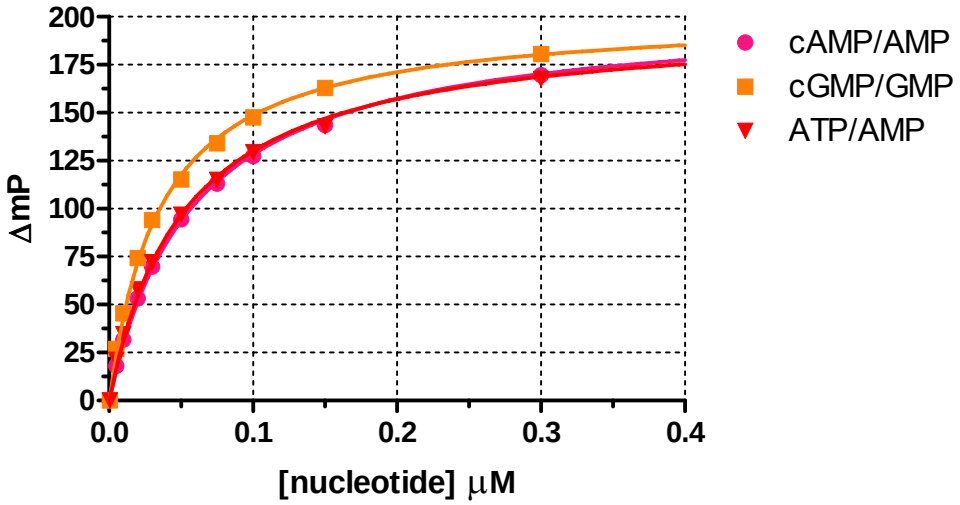


Figure 4. Standard Curves. Sample data for 1 µM standard curves are shown consisting of 50mM Tris (pH 7.5), 0.01% Brij® 35, 1% DMSO and nucleotide standards. The Detection mixture consisted of 3 µg/mL AMP²/GMP² Antibody, 16 nM AMP²/GMP² AlexaFluor® 633 Tracer, and 50 mM Tris (pH 7.5). The nucleotide concentration reflects the amount in the enzyme reaction before the addition of the AMP/GMP Detection Mixture. The data are plotted as ΔmP vs [AMP/GMP] using Michaelis-Menten curve fitting.

4.0 FAQs

How sensitive is the assay?

The lower limit of AMP/GMP detection is dependent on the starting concentration of ATP/cAMP/cGMP in the enzymatic reaction. The assay becomes more sensitive with decreasing ATP/cAMP/cGMP concentration. For example, the lower limit of detection (LLD) for AMP/GMP in enzyme reactions utilizing 100 µM ATP is 0.025 µM AMP, whereas reactions containing 0.1 µM ATP has an AMP LLD of 0.003 µM. LLD is defined as the minimum amount of AMP/GMP that generates a Z'>0.

What can I use to stop my reaction? The activity of several Phosphodiesterases and Ubiquitin Ligases have been inhibited by the addition of Stop Buffer B (200 mM HEPES, 0.2% Brij®-35, and 400 mM EDTA, pH 7.5 - Part # 2027 (1mL) or 2032 (10 mL)) by quenching $MgCl_2$ with EDTA.

Can this assay be run in continuous mode? This assay can be performed in real time by eliminating stop reagents and including the AMP/GMP Detection Mixture components (antibody and tracer) in the enzyme reaction. However, this mode should only be used for relative activity comparisons because the extended signal equilibration time (1.5 hours) precludes accurate quantitation of AMP/GMP.

Does BSA interfere with the assay?

Bovine Serum Albumin (BSA) interferes with the detection reagents and should be avoided. Detergent such as Brij-35 can be substituted in the enzyme reaction to prevent non-specific binding of enzymes and substrates to the plate.

Why does my signal (mP units) change after addition of detection mix followed by the recommended incubation period?

- Enzyme reaction is not stopped by the Stop & Detect Buffer included in the kit. User will need to determine an alternative stop mix depending upon their target enzyme`s cofactors and modulator requirements.
- Nuclease contamination in the buffer can cause the window to collapse causing a change in mP units. We recommend using nuclease free water and buffer that has been prepared fresh.
- Non specific hydrolysis of ATP, cAMP, or cGMP may also cause signal change. Aliquot your nucleotide or make it fresh each time to avoid frequent freeze thaw cycles.
- There is a rare possibility of compound interference with the detection mix may lead to change in signal.

5.0 References.

Klink, T., Staeben, M., Twesten, K., Kopp, A., Kumar, M., Schall Dunn, R., Pinchard, C., Kleman, K., Klumpp, M., Lowery, R. Development and Validation of a Generic Fluorescent Methyltransferase Activity Assay Based on the Transcreener® AMP/GMP Assay. *Journal of Biomolecular Screening* **2011**, accepted for publication.

Staeben M, Kleman-Leyer KM, Kopp AL, Westermeyer TA, Lowery RG. Development and Validation of a Transcreener Assay for Detection of AMP- and GMP Producing Enzymes. Assay Drug Dev Technol. 2010 Feb 17.

Terms & Conditions

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