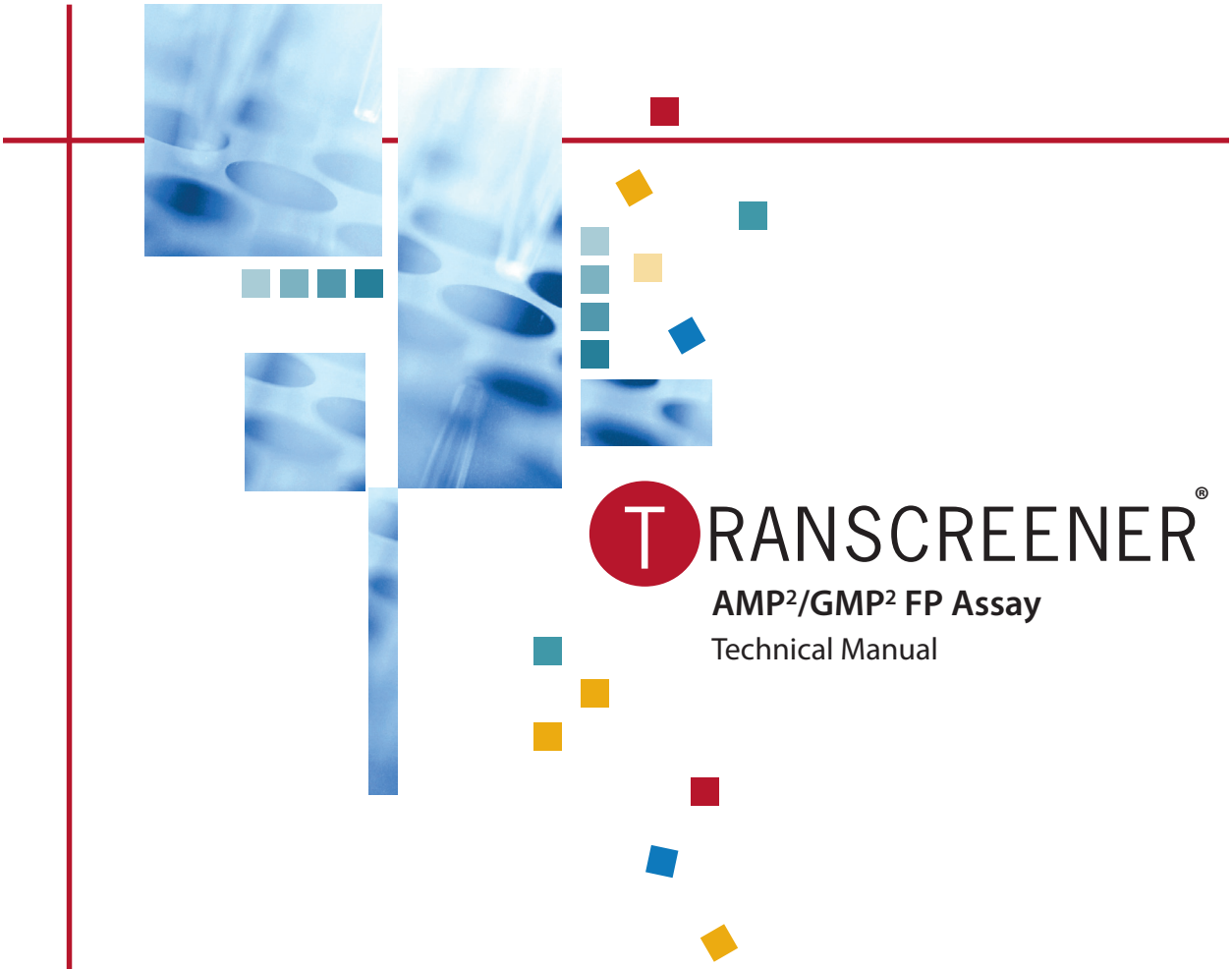
BellBrook Labs

FAR RED FP



TRANSCREENER®

AMP²/GMP² FP Assay

Technical Manual

Instructions for Part Numbers 3015-1K and 3015-10K

TM#131 • Rev 2023-05

Transcreener® AMP²/GMP² FP Assay Technical Manual

Contents

1.0 Introduction

The Transcreener® AMP²/GMP² FP Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). The assay can be used with any enzyme that produce AMP or GMP, regardless of the substrate. Examples of enzymes include ubiquitin, small ubiquitin-related modifiers (SUMO), nucleic acid and protein ligases, phosphodiesterases (PDEs), and synthetases.

The Transcreener® assay is designed specifically for high-throughput screening (HTS), with a single-addition, mix-and-read format. It offers reagent stability and compatibility with commonly used multimode plate readers. 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® AMP²/GMP² FP Assay provides the following benefits:

- Accommodates ATP/cAMP/cGMP concentrations ranging from1 µM to 1,000 µM.
- Excellent data quality (Z' ≥0.7) and signal (≥100 mP polarization shift) at low substrate conversion using normal reaction conditions.
- Overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by using a single set of assay reagents that detect an invariant product.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

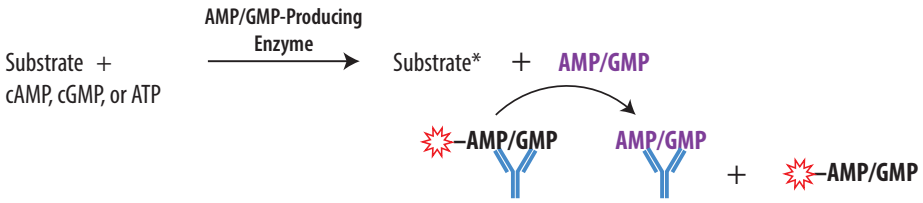


Figure 1. Schematic overview of the Transcreener® AMP²/GMP² FP Assay. The Transcreener® AMP/GMP Detection Mixture contains an AMP/GMP Alexa Fluor® 633 tracer bound to an AMP²/GMP² antibody. AMP/GMP produced by the target enzyme displaces the tracer, which rotates freely, causing a decrease in FP.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® AMP²/GMP² FP Assay	1,000 assays*	3015-1K
	10,000 assays*	3015-10K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 20 µL reaction volumes.

Storage

Store all reagents at –20°C upon receipt.

2.1 Materials Provided

Component	Composition	Notes
AMP²/GMP² Antibody	1.2 mg/mL solution in PBS with 10% glycerol*	The concentration of AMP²/GMP² Antibody needed for an enzyme target is dependent upon the ATP/cAMP/cGMP concentration and buffer conditions in the enzyme reaction (see **Section 4.2**). Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3015-1K) or 10,000 assays (Part # 3015-10K) at an ATP concentration up to 100 µM.
AMP²/GMP² Alexa Fluor® 633 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The final tracer concentration in the 20 µL reaction is 4 nM.
Tris Solution	1 M Tris (pH 7.5)	Tris Solution is used to buffer the Detection Mixture.
AMP	5 mM	AMP is used to create an ATP/AMP or cAMP/AMP standard curve.
GMP	5 mM	GMP is used to create a cGMP/GMP standard curve.

*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration.

2.2 Materials Required but Not Provided

- ***Ultrapure Water***—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.
- ***Enzyme***—Transcreener® AMP/GMP assays are designed for use with purified enzyme preparations. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
- ***Enzyme Buffer Components***—User-supplied enzyme buffer components include enzyme, enzyme cofactors, substrates, and test compounds.
- ***Plate Reader***—A multidetection microplate reader configured to measure FP of the Alexa Fluor® 633 tracer is required. The Transcreener AMP²/GMP² FP Assay has been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4; BMG Labtech PHERAstar Plus and CLARIOstar Plus; Molecular Devices SpectraMax™ Paradigm; Perkin Elmer EnVision and ViewLux; and Tecan Infinite F500, Safire 2™, and M1000.
- ***Assay Plates***—It is important to use assay plates that are entirely black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514). The suggested plate has a square well top that enables easier robotic pipetting and a round bottom that allows good Z′ factors. It has a recommended working volume of 2–20 µL.
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 µL into 384-well plates.

Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of FP instruments.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the FP instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

4.0 Protocol

The Transcreener® AMP²/GMP² FP Assay protocol consists of 4 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 15 µL enzyme reaction and 20 µL complete assay volume at the time that the plates are read. The use of different densities or reaction volumes will require changes in reagent quantities.

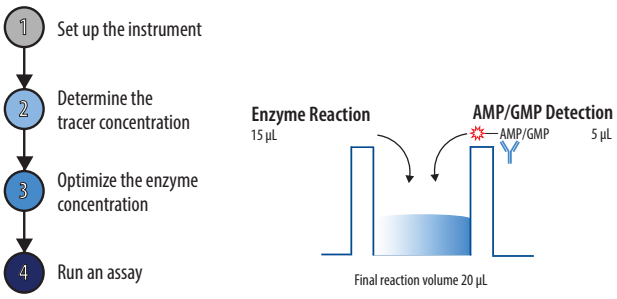


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener® AMP²/GMP² FP Red Assay.

4.1.1 Verify That the Instrument Measures FP

Ensure that the instrument is capable of measuring FP (not simply fluorescence intensity) of Alexa Fluor® 633.

4.1.2 Define the Maximum FP Window for the Instrument

Measuring high (tracer + antibody) and low (free tracer) FP will define the maximum assay window of your specific instrument. Prepare High and Low FP Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use AMP²/GMP² Alexa Fluor® 633 Tracer and Stop & Detect Buffer B at 0.25X concentration in a 20 µL complete assay. This mimics the 4-fold dilution when adding 5 µL of detection mixture to 15 µL of an enzyme reaction. As an example, the 1X AMP²/GMP² Detection Mixture may contain 16 nM tracer. After adding this to the enzyme reaction, the concentration in the 20 µL complete assay would be 4 nM.

High FP Mixture

Prepare the following High FP Mixture as indicated in the table. Pipette 20 µL of the Total High FP Mixture to each well (from the example: 20 µL from 500 µL). Do not further dilute.

Component	Stock Concentration	Complete Assay Concentration	Example: 25 Assays	Your Numbers
AMP²/GMP² Antibody	1.2 mg/mL	20 µg/mL	8.3 µL	
Tris Solution	1 M	12.5 mM	6.3 µL	
AMP²/GMP² Alexa Fluor® 633 Tracer	800 nM	4 nM	2.5 µL	
Water			483 µL	
Total			**500.0 µL**	

The assay window will depend upon your initial ATP/cAMP/cGMP concentration. These volumes can be adjusted for fewer assays and different ATP/cAMP/cGMP concentrations.

Low FP Mixture

Prepare the following Low FP Mixture as indicated in the table. Pipette 20 µL of the Total Low FP Mixture to each well (from the example: 20 µL from 500 µL). Do not further dilute.

Component	Stock Concentration	Complete Assay Concentration	Example: 25 Assays	Your Numbers
Tris Solution	1 M	12.5 mM	6.3 µL	
AMP²/GMP² Alexa Fluor® 633 Tracer	800 nM	4 nM	2.5 µL	
Water			491.2 µL	
Total			**500.0 µL**	

Note: A complete list of instruments and instrument-specific application notes can be found online at: https://www.bellbrooklabs.com/technical-resources/instrument-compatibility Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

Note: The complete assay concentrations with the Stop & Detect Buffers are based on a 20 µL final volume.

4.1.3 Measure the FP

Caution: Contact BellBrook Labs Technical Service for assistance if the assay window is <150 mP.

Subtract the Low FP Mixture readings from the corresponding High FP Mixture readings. The difference between the low and high FP values should be >150 mP.

4.2 Determine the Optimal AMP²/GMP² Antibody Concentration

The antibody 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 AMP²/GMP² Antibody concentrations for nucleotide concentrations up to 1,000 µM (**Table 2**) in a simple buffer system.

Table 1. Optimal concentrations of AMP²/GMP² Antibody in 1X AMP/GMP Detection Mixture for various nucleotide concentrations.

ATP/cAMP/cGMP Concentration in 15 µL Enzyme Reaction	AMP²/GMP² Antibody Concentration in 1X AMP/GMP Detection Mixture
0.1–10 µM	3 µg/mL
11–100 µM	13 µg/mL
101–1,000 µM	110 µg/mL

Note: Contact BellBrook Labs Technical Service for assistance if you require nucleotide concentrations outside this range.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® AMP²/GMP² FP Assay. Use enzyme buffer conditions, substrate, and nucleotide concentrations that are optimal for your target enzyme. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 35 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 1% DMSO (test compound solvent), 0.015% Brij-35, and ATP/cAMP/cGMP. Run your enzymatic reaction at its requisite temperature and time period.

4.3.1 Enzyme Titration Steps

To achieve the most robust assay and a high signal, the quantity of enzyme required to produce a 50–80% change in FP signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 3**). To determine the EC_{80} enzyme concentration, use the following equation:

$$EC_X = (X \div (100 - X))^{(1 \div |\text{hillslope}|)} \times EC_{50}$$

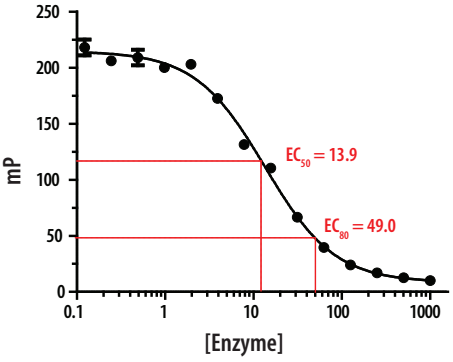


Figure 3. Enzyme titration curve. Titration with the EC_{80} concentration indicated. The EC_{80} may vary based on enzyme lot. Please use C of A for the recommended EC_{80}.

4.3.2 Enzyme Assay Controls

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

Component	Notes
0% ATP/cAMP/cGMP Conversion Control	This control consists of the AMP/GMP Detection Mixture, the enzyme reaction components (without enzyme), and 100% ATP/cAMP/cGMP (0% AMP/GMP). It defines the upper limit of the assay window.
100% ATP/cAMP/cGMP Conversion Control	This control consists of the AMP/GMP Detection Mixture, the enzyme reaction components (without enzyme), and 100% AMP/GMP (0% ATP/cAMP/cGMP). It defines the lower limit of the assay window.
Minus-Nucleotide Control and Minus-Substrate 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/cAMP/cGMP).
AMP/GMP Standard Curve	Although optional, an AMP/GMP standard curve can be useful to ensure day-to-day reproducibility and that the assay conditions were performed using initial rates. It can also be used to calculate product formed and inhibitor IC_{50} values. See **Section 7.1** for a description of how to run the standard curve.
Background Control	Use only 0.5X enzyme reaction conditions and Tris Solution.

4.4 Run an Assay

4.4.1 Experimental Samples

1. Add the enzyme reaction mixture to test compounds and mix on a plate shaker.
2. Start the reaction by adding ATP/cAMP/cGMP, then mix. The final volume of the enzyme reaction mixture should be 15 µL. Incubate at a temperature and time ideal for the enzyme target before adding the AMP/GMP Detection Mixture.
3. Prepare 1X AMP/GMP Detection Mixture as follows:

	ATP/cAMP/cGMP Concentration: Examples			
Component	**0.1–10 µM**	**11–100 µM**	**101–1,000 µM**	**Your Numbers**
AMP²/GMP² Antibody (1.2 mg/mL)	12.5 µL	54.2 µL	458.3 µL	
AMP/GMP Alexa Fluor® 633 Tracer (800 nM)	100 µL	100 µL	100 µL	
Tris Solution (1 M)	250 µL	250 µL	250 µL	
Water	4,637.5 µL	4,595.8 µL	4,191.7 µL	
Total	**5,000 µL**	**5,000 µL**	**5,000 µL**	

Final concentrations in the 1X ADP Detection Mixture should be 50 mM Tris (pH 7.5), 16 nM AMP²/GMP² Alexa Fluor® 633 Tracer, and variable AMP²/GMP² Antibody.

4. Add 5 µL of 1X AMP/GMP Detection Mixture to 15 µL of the enzyme reaction. Mix using a plate shaker.
5. Incubate at room temperature (20–25°C) for at least 90 minutes and measure FP.

4.4.2 AMP/GMP Detection Controls

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

Component	Notes
Minus Antibody (Free Tracer) Control	This control contains the AMP/GMP Alexa Fluor® 633 Tracer without the AMP²/GMP² Antibody and is set to 20 mP.
Minus Tracer Control	This control contains the AMP²/GMP² Antibody without the AMP/GMP Alexa Fluor® 633 Tracer and is used as a sample blank for all wells. It contains the same AMP²/GMP² Antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® AMP²/GMP² FP Assay is designed for endpoint readout.

5.1.2 Real-Time Assay

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 precludes accurate quantitation of AMP/GMP.

5.2 Reagent and Signal Stability

The Transcreener® technology provides a robust and stable assay method to detect AMP/GMP.

5.2.1 Signal Stability

The stability of the FP assay window at 10% substrate conversion was determined after the addition of the AMP/GMP Detection Mixture to the standard samples. The FP assay window at 10% substrate conversion (10 µM) remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FP on the following day, seal the plates to prevent evaporation.

5.2.2 AMP/GMP Detection Mixture Stability

The AMP/GMP Detection Mixture is stable for at least 16 hours at room temperature (20–25°C). If you prepare the AMP/GMP Detection Mixture more than 30 minutes before addition, store it on ice or at 4°C until needed to help decrease the equilibration time.

5.2.3 Stopping the Reaction

We have inhibited the activity of several phosphodiesterases and ubiquitin ligases by the addition of Stop Buffer B, 5X (200 mM HEPES, 0.2% Brij®-35, and 400 mM EDTA [pH 7.5]). This buffer can be purchased separately: Part #2139 (1 mL) or 2140 (10 mL)

6.0 Troubleshooting

Problem	Possible Causes and Solutions
Low selectivity	*Suboptimal antibody concentration* • To achieve maximum sensitivity and assay window, the AMP²/GMP² Antibody concentration must be optimized for each starting ATP/cAMP/cGMP concentration. *ATP/cAMP/cGMP concentration out of range* • Ensure that the starting ATP/cAMP/cGMP concentration is in the range of 1–1,000 µM.
No change in FP observed	*Low antibody/tracer activity* • The tracer and antibody are stable for up to 6 freeze-thaw cycles. For frequent use, aliquot the antibody and tracer and store the aliquots at –20°C. Use a minimum of 20 µL aliquots.
High background signal or change in signal after incubation	*Nonspecific ATP/cAMP/cGMP hydrolysis* • Aliquot the stock solution of nucleotide or prepare it fresh each time to avoid frequent freeze-thaw cycles. *Interference from impurities* • Nuclease contamination in the buffer can cause the assay window to collapse, causing a change in FP. We recommend using nuclease-free water and freshly prepared buffer for each assay. • Some compounds may interfere with the detection mixture, causing a change in signal. • Bovine serum albumin (BSA) at concentrations >1% interferes with the detection reagents. Detergents, such as Brij-35, can be substituted for BSA in the enzyme reaction to prevent nonspecific binding of enzymes and substrates to the plate.

7.0 Appendix

7.1 Standard Curves

A standard curve (**Figure 5**) is required to convert mP values to product formation (AMP or GMP) for quantitative data analysis. Because the Transcreener® AMP²/GMP² FP Assay relies on a competitive binding reaction, the response is nonlinear, and therefore the signal is not directly proportional to reaction progress.

The wells for the standard curve should contain all AMP/GMP reaction components except the enzyme and receive AMP/GMP Detection Mixture. The standard curve mimics an enzyme reaction (as ATP/cAMP/cGMP concentration decreases, AMP/GMP concentration increases); the total nucleotide concentration remains constant. The standard curve allows calculation of the concentration of AMP/GMP produced in the enzyme reaction and, therefore, the % ATP/cAMP/cGMP consumed (% conversion). In this example, a 12-point standard curve was prepared using the concentrations shown in **Table 2**. Commonly, 8- to 12-point standard curves are used.

Table 2. Concentrations of ATP/AMP to prepare a 12-point standard curve.

% Conv.	ATP (µM)	AMP (µM)
100	0	100
50	50	50
30	70	30
15	85	15
10	90	10
7.5	92.5	7.5
5	95	5
3	97	3
2	98	2
1	99	1
0.5	99.5	0.5
0	100	0

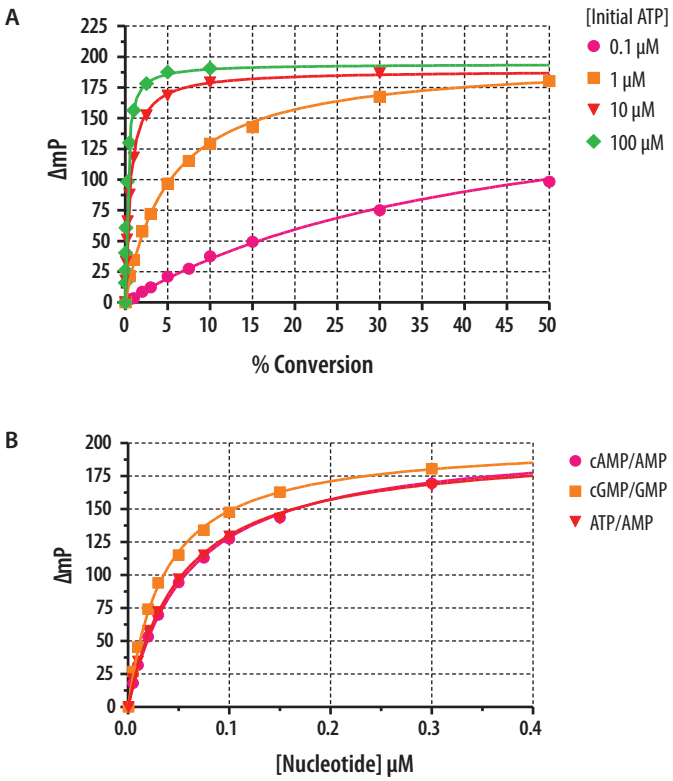


Figure 4. Standard curves. **A)** Sample data for standard curves starting at initial ATP concentrations of 0.1, 1, 10, and 100 µM are shown (ATP concentrations in the 15 µL mock enzyme reaction before the addition of 1X AMP²/GMP² Detection Mixture). A polarization shift of 60–100 mP and a Z′ value of 0.5 indicates robust assay performance for HTS applications. For an initial ATP concentration of 0.1 µM, these criteria were achieved when less than 30 nM of AMP was present. For 100 µM ATP, this was achieved when 0.5% conversion (500 nM AMP) was present. **B)** Sample data for 1 µM standard curves are shown for the indicated nucleotides. The nucleotide concentration reflects the amount in the enzyme reaction before the addition of the 1X AMP/GMP Detection Mixture. The data are plotted as ΔmP vs. [AMP/GMP] using Michaelis-Menten curve fitting.

Use the following equations to calculate the Z′ factor:

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

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

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