TR-FRET Based Transcreener AMP²/GMP² Assay for Screening Ligases, Synthetases and Phosphodiesterases

Meera Kumar, Tom Zielinski and Robert G. Lowery
BellBrook Labs, Madison, WI, USA

Abstract

The exploration of more extensive drug target space by pharmaceutical and academic labs is creating a need for HTS compatible methods to detect diverse enzymes. Group transferases, other than kinases, and ligases/synthetases have proven especially difficult for HTS because of the diversity of modified products formed. The Transcreener AMP²/GMP² Assay has helped to meet need this need by enabling detection of any AMP/GMP producing enzyme including ligases, synthetases, and phosphodiesterases while using any precursor substrate, including cAMP, cGMP, ATP, or NAD. Importantly, the assay relies on direct immunodetection of AMP and GMP without using coupling enzymes, which eliminates the need for counter-screening. In response to customer demand, we recently extended the readout options of the Transcreener AMP²/GMP² Assay to include far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) in addition to the original fluorescence polarization based assay. The assay is simple, single step, homogenous and amenable to HTS: run your enzyme reaction, add detection reagents, and read the far red TR-FRET signal on any multimode plate reader. This poster describes the development, optimization and validation of the Transcreener AMP²/GMP² TR-FRET Assay, including detection of different classes of AMP/GMP-producing enzymes. We examined assay performance, including robustness and utility for detection of several pharmacologically relevant AMP or GMP producing enzymes, including PDEs- 3A, 4A1A, and 5; Acyl-Coenzyme A (CoA) and S-acetyl-CoA Synthetases; and SUMO Activating (SAE1/SAE2) Enzymes.

Direct, Homogenous Detection of AMP and GMP with a TR-FRET Readout

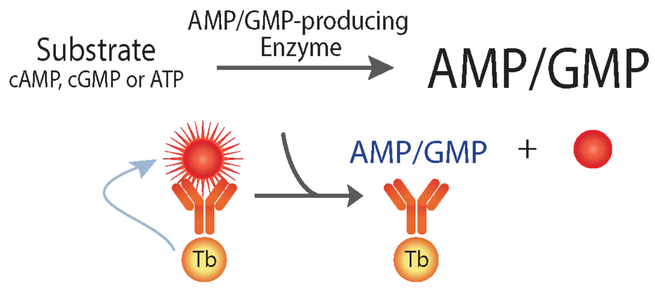
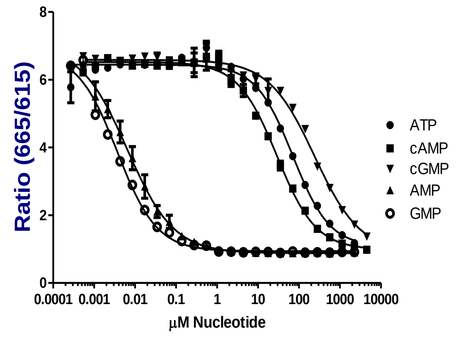


Figure 1. Transcreener AMP²/GMP² TR-FRET Assay. The Transcreener AMP²/GMP² Assay comprises a Hilyte 647 tracer bound to an antibody-Tb conjugate. Excitation of the terbium complex at 330 nm results in energy transfer to the tracer and emission at 665 nm after a time delay. AMP produced by the target enzyme displaces the tracer resulting in a decrease in TR-FRET. The time gated nature of the detection method largely eliminates interference that can result from prompt fluorescence.

Highly Selective Recognition of AMP and GMP vs. Related Nucleotides

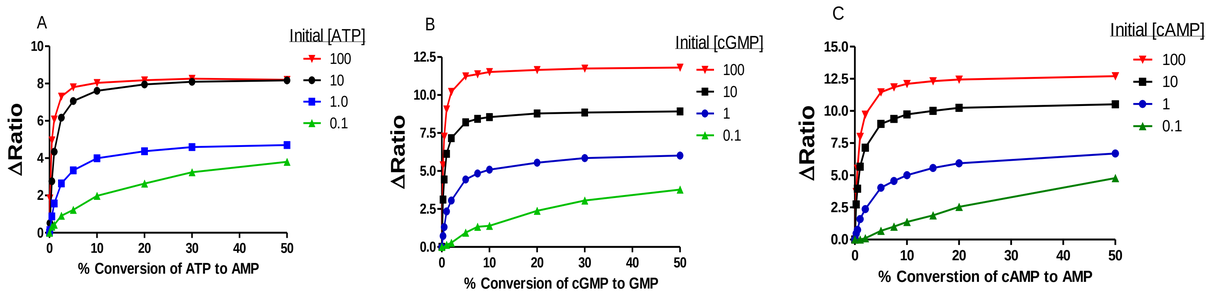


Selectivity	
AMP vs. ATP	8563
AMP vs. cAMP	3516
GMP vs. cGMP	27326

	ATP	cAMP	AMP	cGMP	GMP
IC_{50} (µM)	51.36	21.09	0.006	163.9	0.0032

Figure 2. Competition Curves with Various nucleotides. Nucleotides (ATP, cAMP, cGMP, AMP and GMP) were titrated in in 50 mM Tris Buffer and 5 mM $MgCl_2$. An equal volume of detection reagents comprising of 4 nM AMP²/GMP² Hilyte 647 tracer and 4 nM AMP²/GMP² -Tb antibody was added and mixed well. The plate was read after 90 minutes of incubation. Table: Selectivity is expressed as the inverse ratio of the IC_{50} values for competitive displacement of tracer by AMP or GMP vs. other nucleotides (see inset below graph).

Sensitive and Robust AMP and GMP Detection with Tunable Dynamic Range



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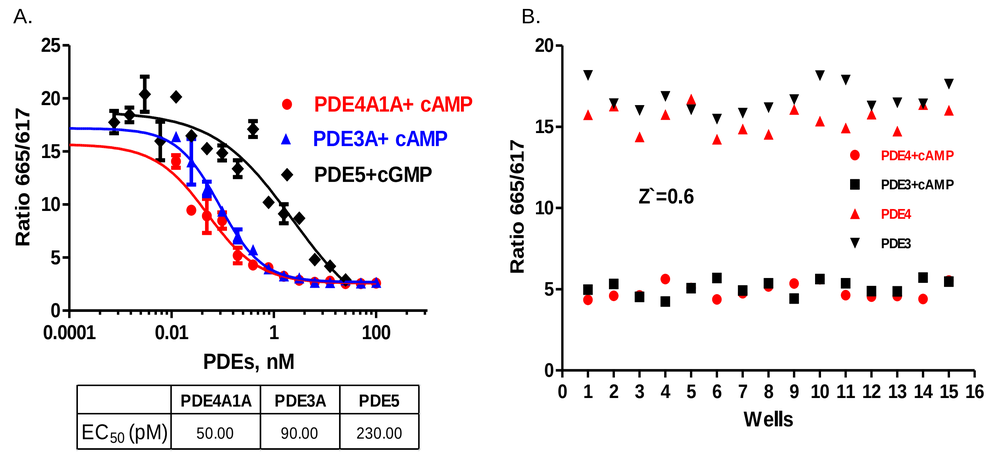
	0.1 µM Standard Curve		1.0 µM Standard Curve		10 µM Standard Curve		10 µM Standard Curve	
	Z' at 30% Conversion	LLD (nM)	Z' at 10% Conversion	LLD (nM)	Z' at 10% Conversion	LLD (nM)	Z' at 10% Conversion	LLD (nM)
ATP/AMP	0.8	5	0.9	5	0.9	50	0.9	250
cGMP/GMP	0.7	7.5	0.7	10	0.8	50	0.9	250
cAMP/AMP	0.7	7.5	0.7	10	0.8	50	0.9	250

Figure 3. Standard curves mimicking the conversion of substrates (ATP, cAMP, cGMP) to AMP or GMP. ATP to AMP (A), cGMP to GMP (B) and cAMP to AMP (C) standard curves for initial substrate concentrations of 0.1µM to 100µM. All assays were performed in 384-well plates (n=12) and read on the Perkin Elmer EnVision multimode plate reader. ΔRatio is the change in ratio from 0%. D. Z' values for initial velocity detection (10% conversion for 1µM, 10µM and 100µM ATP/ADP standard curves, 30% for 0.1 µM) and lower limits of detection. *LLD=Lower Limit of Detection, defined as the concentration of ADP that generates Z'>0.

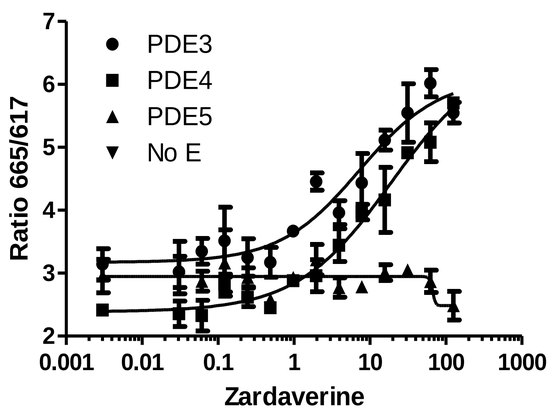
Detection of Cyclic Nucleotide Phosphodiesterases



	PDE4A1A	PDE3A	PDE5
EC_{50} (pM)	50.00	90.00	230.00

Figure 4. Titration of PDEs. A. PDE3A and PDE4A1A were titrated in 15 µL in the presence of 1 µM cAMP. PDE5 was titrated in the presence of 1 µM cGMP. Reaction was run at 37°C for 30 minutes. Control reactions lacked cyclic nucleotides. B. Z` determination for PDE3A and PDE4A1A using 100 pM of both enzymes with 16 replicates.

Inhibitor Selectivity Profiling with PDEs



	PDE3A	PDE4A1A	PDE5
IC_{50} (µM)	7.337	18.74	ND

Figure 5. Dose response curves for zardaverine with three PDEs: Zardaverine was titrated in the presence of 1 µM cAMP/cGMP in 15 µL reaction with either 100 pM PDE3, PDE4A1 or 1 nM PDE5. The reaction was carried out at 37°C for 30 min. The reactions were stopped by the addition of 5 µL of Detection Mixture comprising of 5 nM AMP²/GMP² tracer and 4 nM AMP²/GMP² –Tb antibody. The plates were read in Envision after 90 minutes and the data was analyzed using Graph Pad prism using 4-parameter fit.

Detection of CoA-Dependent Synthetases

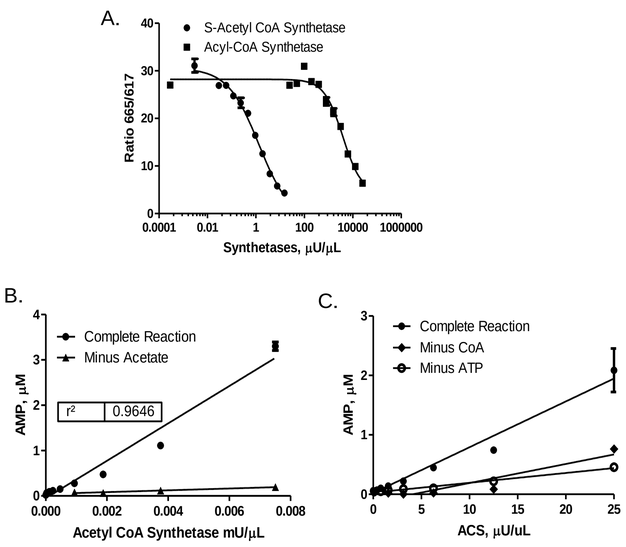


Figure 6. Titration of Acyl CoA Synthetase and S-Acetyl CoA Synthetases: A. Titration of both enzymes, showing TR-FRET signal, with 1 µM COA and 10 µM ATP and 10 µM Sodium Oleate or 10 µM Sodium Acetate respectively. B. Standard curves (Fig 3) were used to convert TR-FRET ratios to AMP formation, demonstrating linearity of assa response.

Detection of SUMO Ligase

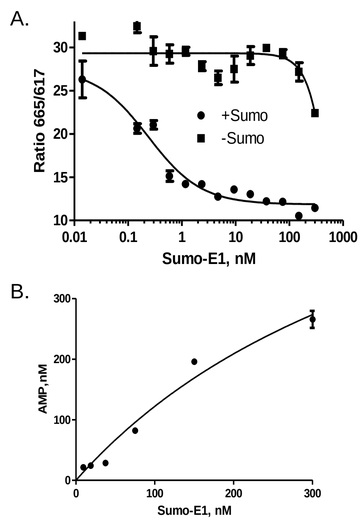


Figure 7. Titration of SUMO E1 Activation enzyme: A. TR-FRET readout for SUMO E1 Activation enzyme in the presence of 5 µM SUMO-1 and 10 µM ATP. Reactions were carried out in kinetic mode and data represents reactions run to completion at 12 hours. B. AMP formation is stoichiometric with SUMO E1 concentration, as would be expected based on the reaction mechanism.

Conclusions

- **The Transcreener AMP²/GMP² TR-FRET Assay is the only HTS method that allows direct detection of unmodified AMP and GMP.**
- **The assay relies on an antibody with >1,000-fold selectivity for AMP/GMP vs. ATP, cAMP and cGMP.**
- **The assay is extremely sensitive and flexible, with a LLD of 6-8 nM for AMP or GMP and a dynamic range that can be tuned for substrate concentrations from 0.1 to 100 µM.**
- **The high selectivity for AMP and GMP allows detection of diverse enzymes, including cyclic nucleotide PDEs, CoA-dependent synthetases and peptide ligases.**

BellBrook Labs, 5500 Nobel Drive, Suite 250, Madison, WI 53711
866.313.7881 or 608.443.2400