Development and Validation of a Transcreeper Assay for Detection of AMP- and GMP-Producing Enzymes

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ABSTRACT

Screening of AMP- and GMP-producing enzymes such as phosphodiesterases (PDEs), ligases, and synthetases would be simplified by the ability to directly detect unmodified nucleoside monophosphates. To address this need, we developed polyclonal and monoclonal antibodies that recognize AMP and GMP with nanomolar sensitivity and high selectivity vs. the corresponding triphosphate and 3',5'-cyclic monophosphate nucleotides that serve as substrates for many enzymes in these classes. One of these antibodies was used to develop a Transcreeper® AMP/GMP assay with a far red fluorescence polarization (FP) readout. This polyclonal antibody exhibited extremely high selectivity, with IC_{50} ratios of 6,000 for ATP/AMP, 3,810 for cAMP/AMP, and 6,970 for cGMP/GMP. Standard curves mimicking enzymatic conversion of cAMP, cGMP, and ATP to the corresponding monophosphates yielded Z' values of >0.85 at 10% conversion. The assay reagents were shown to be stable for 24 h at room temperature, both before and after dispensing. The Transcreeper AMP/GMP FP assay was used for enzymatic detection of cGMP- and cAMP-dependent PDEs 4A1A, 3A, and 9A2 and ATP-dependent ligases, acetyl CoA synthetase, and ubiquitin-activating enzyme (UBE1). Shifts of >100 mP were observed in the linear part of the progress curves for all enzymes tested, and the PDE isoforms exhibited the expected substrate and inhibitor selectivity. These studies demonstrate that direct immunodetection

of AMP and GMP is a flexible, robust enzyme assay method for diverse AMP- and GMP-producing enzymes. Moreover, it eliminates many of the shortcomings of other methods including the need for fluorescently labeled substrates, the low signal:background inherent in substrate depletion assays, and the potential for interference with coupling enzymes.

INTRODUCTION

Adenine and guanine nucleotides are interconverted by diverse proteins of therapeutic interest including kinases, phosphodiesterases (PDEs), membrane transporters, DNA-modifying enzymes, and molecular chaperonins. Detection of nucleotide products—usually via radioassay or coupled enzymatic assays—has traditionally served as a convenient generic biochemical assay method for many of these protein families. To avoid the radioactivity, separation steps, and compound interference associated with these methods, we developed an high-throughput screening (HTS) assay platform called Transcreeper™ that enables fluorescent detection of nucleotides in a homogeneous format.¹ The assays rely on highly selective antibodies that are able to distinguish between nucleotides on the basis of a single phosphate group. Signal is generated when nucleotides produced in an enzyme reaction displace a fluorescent tracer from antibody resulting in a change in its fluorescent properties. The Transcreeper assay for ADP has been formatted for ratiometric fluorescent readouts, including fluorescence polarization (FP)² and time-resolved Föster energy transfer (TR-FRET)^{3,4} that minimize compound interference, and has been broadly applied for diverse ATP-utilizing enzymes.^{4–7} Here, we describe

ABBREVIATIONS: EC_{50} or IC_{50} and EC_{85} , quantity of titrated analyte resulting in 50% and 85% of maximum signal, respectively; FP, fluorescence polarization; FPIA, fluorescence polarization immunoassay; mAb1, mouse monoclonal antibody 1; mAb2, mouse monoclonal antibody 2; mP, millipolarization; pAb1, rabbit polyclonal antibody; PDE, phosphodiesterase; TR-FRET, time-resolved Föster energy transfer.

Fig. 1. The Transcreeper AMP/GMP assay principle. The detection reagents are an AMP/GMP antibody and an AMP/GMP-Alexa® Fluor633 tracer. The tracer is displaced from antibody by enzymatically generated AMP or GMP, causing a decrease in polarization.

the development and performance characteristics of an FP-based Transcreeper assay for AMP/GMP and demonstrate its utility for detection of AMP/GMP-generating enzymes (Fig. 1).

AMP- and GMP-Producing Enzymes

AMP and GMP are produced by a number of therapeutically relevant enzyme families including cyclic nucleotide PDEs, ligases, and synthetases. Cyclic nucleotide PDEs are a diverse superfamily of 21 distinct genes in humans encoding as many as 50 splice variants that regulate the signaling activity of cAMP and cGMP by catalyzing their hydrolysis to the corresponding 5' nucleoside monophosphates.⁸ They have been targeted for many therapeutic purposes, most notably central nervous system disorders, inflammation, and hypertension,^{8,9} which led to the fortuitous discovery and subsequent development of PDE5 inhibitors for the treatment of erectile dysfunction.¹⁰

The current HTS-compatible assay methods for cyclic nucleotide PDEs fall into 2 categories: those that monitor cyclic nucleotide depletion and those that monitor formation of 5'-monophosphates.^{11,12} The substrate depletion assays utilize homogeneous immunoassays for cAMP or cGMP with fluorescent or chemiluminescent readouts (Alpha Screen™, Perkin Elmer, Shelton, CT; HTRF™, CisBio BioAssays, Bedford, MA; HitHunter™, DiscoverX, Fremont, CA); or de-activation of cyclic nucleotide-dependent kinases coupled to luciferase-based ATP detection (PDE Glo™, Promega, Madison, WI). Product formation assays rely on specific binding of fluorescently tagged 5'-monophosphates to cationic beads or enzyme-coupled assays with colorimetric or bioluminescent readouts (PDE Light™, Cambrex Bioscience, Rockland, ME). Though most of these assays use homogeneous detection formats that are well suited to HTS, there are shortcomings. Fluorescently modified cyclic nucleotides are required as substrates for some assays in both categories, and there are limitations on the concentrations that can be used. Substrate depletion assays require high consumption of substrate to generate sufficient signal to background. Enzyme-coupled assay methods are subject to

interference of test compounds with the coupling enzymes, and thus often require counter-screening.

Ligases are a diverse class of biosynthetic enzymes that utilize pyrophosphate bond energy—usually from ATP—to catalyze the joining of 2 molecules, resulting in AMP formation in most cases. Peptide ligases (eg, ubiquitin and SUMO ligases), amino-acyl tRNA synthetases, and acetyl CoA synthetase are representative of the broad range of enzymes that have been targeted for drug development in this class.^{13–16} In many ligase reactions, ATP is used to form an activated adenylate intermediate of one of the substrates, and AMP is released upon ligation to the second substrate. In the case of acetyl CoA synthetase, acetate is adenylated and subsequently ligated to coenzyme A,¹⁷ whereas adenylated ubiquitin is ligated to an active site cysteine in the E1 ligase enzyme as the first step in the transfer to a target protein.¹⁵ Ligases present significant challenges for HTS because of the diversity of substrates. Additionally, many of the substrates are difficult to obtain, which complicates their use as labeled assay reagents. The most common approaches used to date are ELISAs incorporating epitope-tagged ubiquitin and TR-FRET-based assays using labeled ubiquitin and acceptor protein as the donor-acceptor pair.^{18,19} Both of these approaches requires development of new assays for each target protein.

Our success in development of reagents for selective immunodetection of ADP in the presence of excess ATP suggested that a similar approach might also be used for AMP, enabling generic detection of cyclic nucleotide PDEs, ligases, and other challenging targets.²⁰ Transcreeper FP assays are based on a competitive fluorescence polarization immunoassay (FPIA), in which the nucleotide analyte displaces a tracer from antibody resulting in a decreased polarization value.¹ The 2 detection reagents are an antibody and a tracer, comprised of the analyte nucleotide conjugated to a fluor. In order to be useful for measuring enzyme initial velocity, the FPIA must generate a robust response to the enzyme reaction products in the presence of a 10- to 20-fold excess of the substrates. Any response to the substrate nucleotides would be detected as background signal, resulting in a reduced assay window. The method thus requires an antibody that is able to discriminate between purine nucleotides that differ only in their ribose phosphate moieties. Note that the structure of the tracer also affects the selectivity of the FPIA; we have observed selectivity differences of as much as 5-fold using the same antibody with tracers that differ only in their fluor moiety (unpublished results, BBL).

Previously, we produced both polyclonal and monoclonal antibodies for ADP that exhibited selectivities of >100-fold vs. ATP.² Here, we describe the development of polyclonal and monoclonal antibodies and tracer reagents that enable detection of AMP

and GMP with selectivities of >1,000-fold vs. the corresponding enzyme substrates, cAMP, cGMP, ATP, and NAD. The polyclonal antibodies were used to develop a Transcreeper AMP/GMP assay kit with an FP readout, which we have validated for deck and signal stability, enzyme and inhibitor detection, and data quality (Z') using diverse AMP- and GMP-producing enzymes.

MATERIALS AND METHODS

Materials

AMP/GMP polyclonal and monoclonal antibodies were developed at BellBrook Labs using immunogens based on AMP and GMP derivatives conjugated to carrier proteins and purified on IgG-Agarose (Sigma-Aldrich, St. Louis, MO). Alexa Fluor®633 succinimidyl ester (Invitrogen, Carlsbad, CA) was conjugated to AMP and GMP derivatives to produce tracers. Stop and Detect Buffer B at working concentration (0.5×) contains 10 mM HEPES pH 7.5 at room temperature, 0.01% Brij-35, and 20 mM ethylenediaminetetraacetic acid (EDTA).

HEPES and EGTA were from Acros (Geel, Belgium), part numbers 17257-2500 and 409911000. Magnesium chloride and sodium acetate were from Fisher (Waltham, MA), part numbers M33-500 and BP334-500. Dimethylsulfoxide (DMSO) was from Gaylord Chemical Company (Bogalusa, LA), part number 67-68-5. All nucleotides were from Sigma-Aldrich (St. Louis, MO): 3',5'-cyclic AMP, part# A9501; 3',5'-cyclic GMP, part# G6129; 5'-ATP, part# A7699; 5'-AMP, A1752; 5'-GMP, part# G8377; and Coenzyme A, part# C3019. Human cyclic nucleotide PDEs 4A1A, 3A, and 9A2 were purchased from BPS Bioscience (San Diego, CA), part numbers 60030, 60040, and 60090, respectively. All PDEs were N-terminal GST-tagged; PDE 3A was a truncated protein consisting of amino acids 484 to 1,141. Human ubiquitin-activating enzyme (UBE1), ubiquitin, and E2-conjugating enzyme (UbcH2) were untagged, recombinant proteins from Boston Biochem (Cambridge, MA), part numbers E-305, U100H, and E2-607. S-Acetyl coenzyme A synthetase (*Saccharomyces cerevisiae*) was from Sigma-Aldrich (St. Louis, MO), part number A1765. Rolipram was purchased from Calbiochem (Gibbstown, NJ), part number 557330 and aminophylline, dipyridamole, enoximone, and zardaverine were from Sigma-Aldrich, part numbers A1755, D9766, E1279, and Z3003.

Standard Plate and Instrumentation Settings

Assays were performed in black Corning® 384-Well Flat Bottom Microplates (part# 3654) (Corning, NY) or black Corning 384-Well Round Bottom Low Volume Polystyrene Non-Binding Surface Microplates (part# 3676). Mixing after additions was performed by orbital shaking for 5 min. Unless otherwise noted, 15 to 20 µL assays were equilibrated for 1 h at room temperature before reading the plate on the instrument.

FP measurements utilizing the AMP/GMP-Alexa Fluor®633 tracer were performed on a Tecan Safire²™ plate reader (Grödig, Austria) using 635 nm excitation (LED) and 670 nm emission (20 nm bandwidth) settings. The free tracer reference was set to 20 mP, and the buffer was used as the buffer blank for both the sample and free tracer reference wells.

AMP/GMP Assay Methods

A similar AMP/GMP detection protocol and buffer conditions were used for all experiments. Reactions containing nucleotides and/or enzyme in 10–15 µL volumes were incubated at room temperature in Buffer A (30–40 mM HEPES pH 7.5, 10 mM MgCl₂, 2 mM EGTA, 1.0% DMSO). An equal volume of AMP/GMP Detection Mixture, consisting of antibody and tracer in 1X Stop and Detect Buffer B (20 mM HEPES pH 7.5, 0.02% Brij-35, and 40 mM EDTA), was added and the reactions were allowed to equilibrate for 1 h prior to reading the 384-well plate. AMP/GMP-Alexa Fluor633 tracer was present at a final concentration of 2 nM. Antibodies were used at their optimal concentrations, as determined using one of the two methods described below.

Antibody optimization. The simplest approach used was determination of the EC₈₅ antibody concentration from antibody-tracer equilibrium binding curves generated in the presence of substrate nucleotide (ATP, cAMP, or cGMP) at the concentration to be used during signal measurement. Two-fold, 24-point serial dilutions of pAb1 in 1× Stop and Detect Buffer B (containing 4 nM tracer) covering a concentration range of 3×10^{-5} to 1.0×10^3 µg/mL were added to wells containing an equal volume of nucleotides, in Buffer A, at a concentration representing the initial substrate concentration (eg, 1, 10, 100, and 1,000 µM substrate; Fig. 2). FP was measured after incubation for 1 h at room temperature. Four replicate curves were performed. The EC₈₅ is calculated by using the EC₅₀ and hillslope values, calculated from fitting the equilibrium binding data to a variable slope sigmoidal dose-response curve (GraphPad Prism; GraphPad Software, San Diego, CA), using the equation below.

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

Alternatively, the antibody concentration that produced maximum signal in mock enzyme reactions representing 10% conversion of substrate (initial velocity conditions) was used. Ab titrations were performed as described above in the presence of the initial substrate nucleotide concentration (0% conversion) and in the presence of a 9:1 ratio of substrate:product nucleotide (10% conversion), with the total nucleotide concentration held constant throughout. The difference between the 2 curves was plotted and the Ab concentration that yielded the maximum difference was

Fig. 2. Standard optimization of rabbit polyclonal antibody (pAb1) concentration for 1 to 1,000 μM ATP. EC_{50} values are as follows ($\mu\text{g}/\text{mL}$ pAb1): 1 μM ATP (◆), 9.7; 10 μM ATP (●), 16.0; 100 μM ATP (▲), 25.3; and 1,000 μM ATP (■), 86.6. Values are similar when using cAMP or cGMP for substrate.

selected (Fig. 3). This second approach typically yielded a slightly more sensitive assay with a smaller signal.

Competition binding curves. Nucleotides were tested for their relative ability to displace tracer from antibodies. These experiments were carried out by combination of equal volumes of Buffer A containing nucleotide and detection mixture comprised of antibody at EC_{50} concentration (Fig. 2), 4 nM AMP/GMP tracer, and 1X Stop and Detect Buffer B. Twofold nucleotide serial dilutions were carried out by transferring 10 μL of nucleotide containing mix down a series of wells containing 10 μL of no nucleotide reaction mix. Duplicate curves were performed with FP measured after 1 h at room temperature.

Standard curves. Nucleotide mixtures mimicking enzymatic conversion of substrate to product (in Buffer A) were added to equal volumes of detection mixture (4 nM tracer, 7.0 $\mu\text{g}/\text{mL}$ antibody, and 1X Stop and Detect Buffer B) thus modeling the standard assay procedure used throughout this study. Nucleotide mixtures contained a constant total nucleotide concentration (1 μM) and the proportion of substrate:product nucleotide was varied to mimic enzymatic conversion over time. Ten microliters of detection mixture was added to 10 μL of nucleotide mixture. In this case, 24 replicate curves were performed representing 0%, 1.0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 25%, 50%, 75%, and 100% conversion of substrate nucleotide. FP was measured after 1 h at ambient temperature.

Deck and signal stability. Deck stability represents the ability of the detection mixture (antibody and tracer in Stop and

Fig. 3. Refined optimization of pAb1 concentration for initial velocity phosphodiesterase (PDE) reactions. (A) Antibody-tracer equilibrium binding curve in the presence of 1 μM cAMP (▲) and 0.9 μM cAMP/0.1 μM AMP (■). (B) Difference plot of the 2 curves in A ($\Delta\text{mP} = \text{mP}_{1\mu\text{M cAMP}} - \text{mP}_{0.9\mu\text{M cAMP}/0.1\mu\text{M AMP}}$). The EC_{50} value was 8.7 $\mu\text{g}/\text{mL}$ for the 1 μM cAMP curve A. The maximal ΔmP value in the difference curve in (B) occurred at an antibody concentration of 3.7 $\mu\text{g}/\text{mL}$.

Detect Buffer B) to remain functional over time when placed on the reagent deck of an automated liquid dispenser. The detection mixture was formulated to working concentration (4 nM tracer, 7.0 $\mu\text{g}/\text{mL}$ antibody, and 1X Stop and Detect Buffer B), placed in translucent containers (subject to ambient light and temperature), and sampled over a 24-h period for generation of AMP/cAMP standard curve data, with a 1-h equilibration period at room temperature. Ten microliters of detection mixture was added to 10 μL of 1 μM AMP/cAMP standard curve (in Buffer A) contained in an assay plate. The 12 point, 1 μM AMP/cAMP standard curves

performed with 4 replicates were used representing 0%, 1.0%, 2.5%, 5.0%, 7.5%, 10%, 12.5%, 15%, 25%, 50%, 75%, and 100% conversion of substrate. Plate stability demonstrates the stability of signal after detection reagents have been added to nucleotides in an assay plate. The detection mixture (same as above) was added to standard curve mixtures in an assay plate and held, sealed at room temperature. FP was measured periodically over 24 h.

Enzyme assay methods. With the exception of the enzyme assays run in continuous mode, all AMP/GMP immunodetection assays were performed using a similar protocol. The AMP/GMP antibodies were used at concentrations that yielded the maximum signal in mock enzyme reactions containing 0.1 μ M AMP or GMP and 0.9 μ M of the corresponding substrate, ATP, cAMP, or cGMP. For rabbit polyclonal antibody (pAb1), this optimal concentration was 3.5 μ g/mL. Change in FP signal was calculated by reference to a negative control. Negative controls included all assay components except the enzyme or with some enzymes, one of the substrates. Background subtraction was performed with mixes containing all assay components except tracer while “free” tracer samples used mixes containing all assay components except antibody (“free” tracer was set to 20 mP). For enzyme reactions run in continuous mode, catalysis was carried out in Buffer A containing 2 nM tracer, 3.5 μ g/mL antibody, and required substrates, and FP was read periodically. Note that when using continuous mode the observed progress curves reflect less than complete equilibration of antibody, tracer, and reaction product, which occurs over a 10- to 15-min period.

Cyclic Nucleotide Phosphodiesterase Assays

Enzyme titrations. Cyclic nucleotide PDEs 4A1A, 3A, and 9A2 were titrated using the same procedure as used for antibody optimization except Stop and Detect Buffer B was omitted therefore excluding EDTA. The 2-fold enzyme dilution series resulted in protein concentrations from 0.03 to 25,000 ng/mL. All isoforms were studied with both 1,000 nM cAMP and cGMP as substrate. FP was measured at convenient time points throughout the 30°C incubations (plates were sealed between measurements). Change in signal was relative to no nucleotide controls and triplicate curves were performed.

Inhibitor studies. PDEs 4A1A and 3A were interrogated with a panel of 5 known PDE inhibitors: rolipram, aminophylline, dipyridamole, enoximone, and zardaverine. Catalysis was carried out in Buffer A. Compounds were titrated across 1.6 ng/mL PDE 4A1A and 4.4 ng/mL PDE3A prior to initiating catalysis with the addition of cAMP or 44.0 ng/mL PDE3A prior to cGMP addition. Reactions (15 μ L) were stopped with the addition of an equal volume containing 4 nM Tracer, 7.0 μ g/mL antibody, and 1X Stop and

Detect Buffer B after 1.5 h at 30°C; equilibrated for 1 h at room temperature; and FP measured. Triplicate curves were generated.

Controls without inhibitor along with an intraplate standard curve were used to ensure <20% of substrate was consumed. Control wells containing 20 mM EDTA at time zero were used to demarcate 100% inhibition as wells that contained neither compound nor EDTA established 0% inhibition.

ATP-Utilizing Enzyme Assays

These enzymes were assayed with procedures identical to those used for the PDE titrations except 1,000 nM ATP was used as nucleotide substrate.

S-Acetyl coenzyme A synthetase. S-Acetyl coenzyme A synthetase used 500 μ M sodium acetate and 1 μ M coenzyme A (CoA). S-Acetyl CoA synthetase response was relative to reactions without sodium acetate and CoA. Twofold serial dilutions were begun at 23.4 mU/mL.

Ubiquitin-activating enzyme (UBE1). UBE1 used 30 μ M ubiquitin as substrate and UBE1 activity was referenced to reactions without UBE1. Twofold serial dilutions were begun at 150 nM.

E2-conjugating enzyme (UbcH2). UbcH2 was titrated across 1, 5, and 40 nM UBE1 with 2-fold serial dilutions begun at 2,000 nM. Otherwise, the UbcH2 reactions were run using conditions identical to the UBE1 reactions. Signal was measured after 1.5 h at 30°C and activity correlated to controls minus UBE1 and UbcH2 using 4 replicate curves.

RESULTS

Development and Characterization of Assay Reagents

Polyclonal and monoclonal antibodies were raised in rabbits and mice using several AMP- and GMP-based haptens linked to carrier proteins. A panel of tracers was synthesized by attaching AMP and GMP derivatives to fluors using similar chemistry. The resulting antibodies and tracers were tested in a matrix of FP-based equilibrium binding and competition experiments for high-affinity binding to AMP and GMP, high polarization values when tracer is bound, and minimal antibody cross-reaction with cyclic nucleotides and nucleoside triphosphates. One polyclonal antibody, denoted as pAb1, and an Alexa Fluor633-based tracer, denoted as AMP/GMP-Alexa Fluor633, were chosen for further assay development based on a superior combination of sensitivity, signal magnitude, and selectivity. Two monoclonal antibodies were subsequently generated, using similar NMP-based immunogens, and tested with various tracers. The same AMP/GMP-Alexa Fluor633 tracer was again chosen for further characterization of selectivity.

The selectivity of the 3 antibodies in combination with the common AMP/GMP-Alexa Fluor633 tracer was measured using

competitive binding assays with a panel of nucleotides (Fig. 4); the IC_{50} values determined from these binding curves are shown in Table 1. In these experiments, which are typical of an FPIA, a limiting amount of tracer (2 nM) and near-saturating amounts of antibody were challenged with a panel of purine nucleotides. The polarization decreased in a sigmoidal fashion as the tracer was displaced from antibody by each competing nucleotide. The maximal polarization values observed were ~250 mP for all 3 antibodies, and in most cases competing nucleotides decreased polarization to that of the free tracer—20 to 30 mP—indicating complete displacement. The exception was mAb2, where biphasic binding curves with higher minimal polarization values were observed with some competing nucleotides. The reason for this anomaly has not been explored.

mAb2 exhibited the highest affinity for both AMP and GMP (IC_{50} of 6.7 and 10 nM for AMP and GMP, respectively); the polyclonal, pAb1 had the lowest affinity for both monophosphates (IC_{50} of 100 and 150 nM for AMP and GMP, respectively). Most importantly, all 3 antibodies displayed >200-fold selectivity for detection of AMP vs. ATP or cAMP and of GMP vs. cGMP. We have found that selectivity for product vs. substrate of at least 100-fold is sufficient for development of a high-quality enzyme assay. Cross-reaction was highest with ADP and GDP for all 3 antibodies; however, this nucleotide is not a common substrate for AMP/GMP-producing enzymes other than nonspecific phosphatases.

Because pAb1 was developed earlier than the monoclonals and was found to be suitable in all respects, it was used for development of a commercial Transcreeper AMP/GMP assay kit. The

Fig. 4. Competition binding curves for adenine and guanine nucleotides with AMP/GMP antibodies. (A) Polyclonal antibody pAb1. (B) Monoclonal antibody mAb1. (C) Monoclonal antibody mAb2. Values represent the average of duplicate reactions.

Table 1. Competitor Binding Isotherm IC₅₀ Values (μM)—Antibody Comparison

	GMP	GDP	GTP	cGMP	AMP	ADP	ATP	cAMP
pAb1	0.152	3.69	87.9	1,060	0.100	37.4	600	381
mAb1	0.038	0.273	3.71	511	0.075	0.516	15.5	193
mAb2	0.010	0.496	14.5	170	0.0067	5.09	135	21.7

selectivity profile of pAb1 with the nucleotides that will be used as substrates in most enzyme reactions expressed as the inverse ratio of their IC₅₀ values were as follows: AMP vs. ATP, 6,000; AMP vs. cAMP, 3,810; and GMP vs. cGMP, 6,970. In separate experiments to assess the utility of the assay for NAD-dependent ligases, the pAb1 exhibited >400-fold selectivity for AMP vs. NAD (data not shown).

In a competitive FPIA, the antibody concentration is the main variable controlling the total polarization shift and the detection range of the assay. The use of subsaturating antibody allows more sensitive detection but yields a submaximal initial signal, whereas the use of higher antibody results in higher initial signal, but also decreases sensitivity because more analyte is required to displace the tracer. The simplest approach is to use the EC₈₅ concentration derived from an antibody-tracer binding curve with substrate present at the concentration to be used in the enzyme assay. In this case, however, we went a step further and optimized the antibody concentration to calibrate the dynamic range of the assay for the amount of product formation expected in the planned enzyme assays. An example of how this was done for cAMP PDEs is shown in Figure 3. Antibody binding curves were generated in the presence of 1 μM cAMP and 0.9 μM cAMP/0.1 μM AMP, conditions representing the initial and final conditions of a PDE reaction run under initial velocity conditions (Fig. 3A). The difference between the 2 binding curves was then plotted to identify the antibody concentration that would yield the maximal polarization shift—approximately 100 mP—for such a reaction (Fig. 3B). Similar analyses were carried out to optimize antibody for cGMP PDEs and ATP-dependent ligases. Optimal antibody concentrations of 3.3, 3.2, and 4.0 μg/mL were determined in this fashion for 10% conversion of cAMP, cGMP, and ATP to the corresponding monophosphate, respectively. Because the values were so similar, the average antibody concentration of 3.5 μg/mL was used for subsequent experiments with all enzyme types.

In practice, it is rarely possible to control the progress of enzymatic reactions to an exact endpoint. To allow quantification of AMP or GMP over a range of concentrations, standard curves mimicking enzyme reactions were generated (Fig. 5). Substrate and product nucleotides were added in amounts representing points on a reaction progress curve: increasing amounts of AMP

or GMP were added and cAMP, cGMP, or ATP was decreased proportionately, with the total nucleotide concentration held constant. The 3 curves were almost identical because of the similar IC₅₀ values for AMP and GMP and the very low level of antibody cross-reactivity with all 3 substrate nucleotides. As with all competitive binding assays, the signal response is nonlinear with product formation. As expected from the antibody optimization experiment (Fig. 3B), 10% substrate conversion resulted in a shift of close to 100 mP; the total shift observed at 100% substrate conversion was at or near 150 mP. The standard curves were performed in 24-point replicates to allow calculation of Z' values for each % conversion. At the lower limit of detection of 10 nM AMP or GMP, which is equivalent to 1% reaction progress, the Z' values for conversion of cAMP, cGMP, or ATP to the corresponding monophosphates were 0.65, 0.39, and 0.54, respectively. For 10% conversion of cAMP, cGMP, or ATP, the respective Z' values were 0.90, 0.86, and 0.87.

To assess the suitability of the Transcreeper AMP/GMP assay for an automated HTS environment, we tested the stability of the reagents before (deck stability) and after (signal stability) dispensing (Fig. 6). To measure deck stability, pAb1 and tracer were

Fig. 5. Standard curves. Demonstrated here are mock reaction progress curves representing: 0%, 1.0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 25%, 50%, 75%, and 100% conversion of substrate nucleotide. AMP/cAMP standard curve (■); GMP/cGMP standard curve (▲); AMP/ATP standard curve (●).

Fig. 6. Stability of AMP detection reagents on the liquid handling deck and assay signal in the plate. (A) 1× Detection mixture was formulated and held in ambient light and temperature for 1 (●), 2 (■), 5 (▲), 8 (◆), and 24 (x) h after which an equal volume was added to standard curve aliquots and fluorescence polarization (FP) measured. (B) In-plate signal stability was tested by measuring the signal over time of a standard curve (same as in A) and detection reagents plated and held, sealed, at ambient temperature for 1 (●), 2 (■), 6 (▲), 8 (◆), and 24 (x) h.

mixed, stored at room temperature, and dispensed at intervals into wells containing standard curves mimicking conversion of 1 μ M cAMP to AMP. The standard curves are superimposed (Fig. 6A), demonstrating that the reagents are stable at room temperature for at least 24 h prior to dispensing. Signal stability was measured by intermittently reading a plate containing a similar cAMP/AMP standard curve and kept at room temperature. These standard curves also superimpose (Fig. 6B), demonstrating that the FP assay signal is stable for at least 24 h.

Fig. 7. Cyclic nucleotide phosphodiesterase (PDE) assays. (A) Time course of cAMP-dependent reactions using 1.6 ng/mL 4A1A (■), 3.3 ng/mL 3A (▲), and 24.0 ng/mL 9A2 (●). (B) Time course of cGMP-dependent reactions using the same enzyme concentrations as A.

Cyclic Nucleotide Phosphodiesterase Detection and Inhibition

The Transcreeper AMP/GMP Assay was first tested for enzyme detection using human cyclic nucleotide PDEs 4A1A, 3A, and 9A2 and known PDE inhibitors. Titrations of all 3 enzymes were performed in the presence of both cAMP and cGMP and continuously monitored over time. Representative progress curves are shown in Figure 7A and 7B. As reported earlier, PDE4A1A showed strict dependence on cAMP as substrate, whereas PDE3A and PDE9A2 were able to utilize either substrate, but exhibited their characteristic preferences for cAMP and cGMP, respectively.⁸ For each isoform, a polarization change of at least 100 mP was observed during the linear phase of the reaction when the optimal substrate was used.

Fig. 8. Select inhibitor curves from *Table 2* data set. Rolipram inhibitor curves using cAMP as substrate: isoform 4A1A (●) and 3a (▲).

A panel of known PDE inhibitors was used to assess the ability to quantify enzyme inhibition with the assay using PDE4A1A and PDE3A. Representative inhibition curves for rolipram, a PDE4-specific inhibitor, are shown in *Figure 8*. IC₅₀ values for the full panel of inhibitors are given in *Table 2*, with values generally consistent with literature values, with the exception of rolipram inhibition of PDE4A1A whose IC₅₀ was determined to be 0.1 μM relative to reported values of 1–2 μM.^{8–15} Standard curves like the one shown in *Figure 5* were used to convert raw polarization data to product (AMP or GMP) for calculation of IC₅₀ values. Considering the data for cAMP-dependent activity first, rolipram, a PDE4-selective inhibitor, exhibited an IC₅₀ of 100 nM for PDE4A1A and almost 1 mM with PDE3A. Conversely, the PDE3-selective inhibitor enoximone was >4-fold more potent with PDE3A than with PDE4A1A. The mixed PDE3/4 inhibitor zadaverine inhibited both isoforms at submicromolar concentrations, but was almost 10-fold more potent with PDE4A1, with an IC₅₀ lower

than that observed for rolipram. Dipyridamole, which is considered to be a selective PDE5 inhibitor, had low micromolar potency with PDE4A1A, and no detectable inhibition of PDE3A, whereas aminophylline, a nonselective PDE inhibitor, had relatively low potency with both isoforms.

ATP-Utilizing Enzymes: Acetyl CoA Synthetase and Ubiquitin-Activating Enzyme UBE1

To assess the utility of the Transcreener AMP/GMP assay for detection of ligases, we titrated acetyl CoA synthetase and the ubiquitin ligase, UBE1, in the presence of 1 μM ATP and the appropriate co-substrates and continuously monitored AMP formation relative to control reactions lacking acetate and UBE1, respectively. Note that AMP formation by UBE1 was also strictly dependent on the presence of ubiquitin (data not shown). Polarization decreased over time at rates that correlated with the enzyme concentration, as shown in *Figure 9A*. Because UBE1 catalyzes an autoligation, the amount of AMP produced—and thus the maximal signal—was limited by the amount of enzyme, which was present at relatively low concentrations. Thus in this experiment we were measuring a single enzymatic turnover, which resulted in the step-like pattern in the progress curves (*Fig. 9A*). In contrast, the acetyl CoA synthetase reactions were not limited either by substrate or by enzyme, but rather by the dynamic range of the assay, as reflected in the more typical fan-like pattern of the progress curves (*Fig. 9B*).

To demonstrate the ability to measure steady-state enzymatic turnover by UBE1, we titrated catalytic amounts of UBE1 with UbcH2, the protein to which it transfers its covalently bound ubiquitin. In a 1.5-h reaction, the polarization change observed for 5 nM E1 alone was 25 mP and it increased to >100 mP when 125 nM UbcH2 was added (*Fig. 10*). The UbcH2 dependence is consistent with multiple cycles of ubiquitin transfer from E1 to UbcH2 with the concomitant formation of AMP in the UBE1 activation step. These results suggest that monitoring the formation of AMP may serve as a generic method for measuring ubiquitination and other types of

peptide ligation reactions such as sumoylation and neddylation.

DISCUSSION

In developing a number of different FPIAs for measuring enzymatic nucleotide interconversion (Kleman-Leyer et al.²; and unpublished

Table 2. Phosphodiesterase Inhibitor Potencies (IC ₅₀ s, μM)					
	PDE4A1A cAMP Hydrolysis		PDE3A cAMP Hydrolysis		PDE3A cGMP Hydrolysis
	Observed	Literature	Observed	Literature	
Rolipram	0.1	0.08–2 ^{22,23,26,27}	864	83–110 ²⁷	>1,000
Aminophylline	166		182		726
Dipyridamole	5.7		>1,000		>1,000
Enoximone	12.6	1–160 ^{21,24,25,26}	2.8	1.8–10 ^{8,24,25,27}	13.5
Zardaverine	0.07	0.2–3 ^{21,26}	0.6	0.6–3 ^{21,26}	6.0

Fig. 9. ATP-utilizing enzyme assays. *S*-Acetyl coenzyme A synthetase and ubiquitin-activating enzyme (UBE1) were assayed. (A) UBE1 titration: 1.2 (+), 2.3 (Δ), 4.7 (□), 9 (○), 19 (◆), 38 (●), 75 (▲), and 150 (■) nM. (B) *S*-Acetyl coenzyme A synthetase titration: 0.02 (+), 0.05 (Δ), 0.1 (□), 0.2 (○), 0.4 (◆), 0.7 (●), 1.5 (▲), and 2.9 (■) mU/mL.

results by Lowery et al.), BBL, we have found that robust detection of initial velocity requires a product-specific antibody exhibiting <1% cross-reactivity with substrates. In this study, we have demonstrated that antibodies can be generated that recognize AMP and GMP equally well and exhibit well under 1% cross-reactivity with the nucleotides most often used as substrates by AMP- and GMP-forming enzymes, including cAMP and cGMP, ATP, and NAD.

The 10^3 - to 10^4 -fold levels of analyte discrimination are impressive given the small size of the nucleotide haptens and the fact that they differ from related molecules only in their ribose phosphate moieties. For comparison, the FPIA reagents

Fig. 10. E2-conjugating enzyme (UbcH2) assays. UbcH2 enzyme was titrated across 1 nM, 5 nM, and 40 nM UBE1. Plotted is the signal generated (1 h at 30°C) with the optimal UbcH2 concentration for respective UBE1 concentrations (■); the signal due to corresponding UBE1 quantities without UbcH2 (□); and the signal with this same concentration UbcH2 absent UBE1 (○).

we have developed for detection of ADP and UDP discriminate from their respective substrates, ATP and UDP-glucuronic acid, with selectivities of 100- to 200-fold. From a practical point of view, the high degree of selectivity observed in this case means that less adjustment of antibody concentration is necessary to accommodate the different substrates and their varying concentrations that are used for diverse AMP- and GMP-generating enzymes. In this regard, it should be noted that while the antibodies discriminate exquisitely with respect to products vs. substrates for key enzyme families, their recognition of both guanine and adenine monophosphates would limit their usefulness in cell extracts. Thus, we focused on studies with purified enzymes.

In addition to the required selectivity, the AMP/GMP polyclonal antibody and AMP/GMP-Alexa Fluor633 tracer exhibited the other key elements required for robust HTS detection with an FPIA, including shifts of >100 mP for detection of product in the presence of excess substrate (Figs. 3 and 4) and stability at room temperature both before and after addition to reactions (Fig. 6). Furthermore, the use of a far-red-shifted fluorophore (Alexa Fluor633) provides the benefit of lower assay interference from compound fluorescence and light scatter.^{28,29} The combined properties of the detection reagents are reflected in the Z' values observed in standard curves mimicking enzyme reactions (Fig. 5). Z' values >0.7, which is generally considered quite good, were observed at levels of substrate conversion of 2.5% or greater, which is well within the initial velocity range. While we focused initially on FP as a detection mode because it is the simplest format for a competitive binding assay, it is likely that the assay

could be configured for TR-FRET to yield similar data quality and robustness.

Three cyclic nucleotide PDEs and 2 ATP-dependent ligases, one a peptide ligase and the other an acid-thiol ligase, were used to demonstrate the diverse types of enzymes that can be detected using the Transcreeper AMP/GMP assay and the difficulties with existing assay methods that can be overcome. Time, enzyme, and substrate dependence of the signal was observed in all cases (Figs. 7 and 9) as well as the expected substrate and inhibitor selectivity for the PDEs (Fig. 7 and Table 2). Direct immunodetection of AMP as a PDE assay method eliminates the shortcomings of other assay methods including the need for fluorescently labeled substrates, the signal:background problems inherent in substrate depletion assays, and the potential for interference with coupling enzymes. With respect to the ligases, the ability to monitor AMP formation may provide the basis of a very broadly applicable generic assay method for this diverse class of enzymes. Moreover, our results suggest that monitoring AMP formation can be used to probe steady-state flux through any of the multienzyme peptide ligation cascades that are tightly linked to ATP hydrolysis. Ongoing studies with SUMO ligases in our laboratory support this hypothesis.

The purinome, the enzymes that utilize purine nucleotides as substrates or cofactors, comprises >13% of the human genome.³⁰ Combined with the Transcreeper assays for ADP and GDP, the AMP/GMP assay will enable the facile interrogation of a significant fraction of the enzymes of the purinome, both for therapeutic intervention and for off-target effects.

AUTHOR DISCLOSURE STATEMENT

All authors are employed by BellBrook Labs (Madison, WI).

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