Article

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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.7b03556 • Publication Date (Web): 25 Dec 2017

Direct Detection of Products from *S*-adenosylmethionine-Dependent Enzymes using a Competitive Fluorescence Polarization Assay

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Abstract

S-adenosylmethionine (AdoMet) dependent methyltransferases (MTases) are an essential superfamily of enzymes that catalyze the transfer of a methyl group to several biomolecules. Alterations in the methylation of cellular components crucially impact vital biological processes, making MTases attractive drug targets for treating infectious diseases and diseases caused by overactive human-encoded MTases. Several methods have been developed for monitoring the activity of MTases, but most MTase assays have inherent limitations or are not amenable for high-throughput screening. We describe a universal, competitive fluorescence polarization (FP) assay that directly measures the production of *S*-adenosylhomocysteine (AdoHcy) from MTases. Our developed assay monitors the generation of AdoHcy by displacing a fluorescently labeled AdoHcy molecule complexed to a catalytically inert 5′-methylthioadenosine nucleosidase (MTAN-D198N) variant performed in a mix-and-read format. Producing the fluorescently labeled molecule involves a one-pot synthesis by combining AdoHcy with an amine-reactive rhodamine derivative, which possesses a K_d value of 11.3 ± 0.7 nM to MTAN-D198N. The developed competitive FP assay expresses a limit of detection for AdoHcy of 6 nM, and exhibits a 34-fold preference to AdoHcy in comparison to AdoMet. We demonstrate the utility of the developed assay by performing a pilot screen with the NIH Clinical Collection as well as determining the kinetic parameters of L-histidine methylation for EgtD from *Mycobacterium tuberculosis*. Additionally, the developed assay is applicable to other AdoMet-dependent and ATP-dependent enzymes by detecting adenosine and various adenosine-containing molecules including 5′-methylthioadenosine, AMP and ADP.

INTRODUCTION

S-adenosylmethionine (AdoMet) serves as an essential substrate for a myriad of transfer reactions in bacterial and eukaryotic cells, which include post-translational modification of proteins, epigenetic regulation, polyamine biosynthesis, radical pathways, and quorum sensing [1,2]. In this role, AdoMet is the second most widely used metabolite as a cofactor for enzymatic reactions following ATP [3]. AdoMet-dependent methyltransferases (MTases) are the diverse class of enzymes that assist in the methylation of numerous biomolecular components in cells from all 3 domains of life. MTases catalyze the transfer of a methyl group from AdoMet to an acceptor molecule, generating *S*-adenosylhomocysteine (AdoHcy) and the modified methylated molecule. Additionally, products generated from AdoMet-dependent enzymes including AdoHcy, 5′-methylthioadenosine (MTA), and 5′-deoxyadenosine are known potent inhibitors of enzymes that catalyze methylation reactions, polyamine biosynthesis and AdoMet radical pathways, respectively [4-7]. In some bacteria, the cellular concentrations of products from AdoMet-dependent enzymes are catabolized by MTA/AdoHcy nucleosidase (MTAN) to avoid product inhibition of these vital biochemical processes. Additionally, undesired methylation by MTases in humans leads to epigenetic dysregulation of transcription, which is demonstrated to play an important role in carcinogenesis. More specifically, alterations in the methylation of histones and DNA by MTases result in chromosomal instability and spurious gene expression, consequently leading to tumorigenesis [8,9]. Furthermore, methylation of A2058 of 23S rRNA in the large subunit of the *Escherichia coli* ribosome by MTases have been shown to promote intrinsic resistant to three distinct classes of antibiotics including macrolides, lincosamides and quinupristin [10-12]. For these

reasons, there is great interest in enzymatic characterization of MTase function as well as development of novel inhibitors for MTases. Such compounds could be used as antimicrobials or anticancer therapeutics.

Various methods have been developed to directly and indirectly monitor the activity of MTases, but these lack sensitivity, exhibit inefficiency in medium to high throughput screening, or require radioisotopic labeling of substrates. Several of these assays are dependent on multiple coupled enzymes to process the generated AdoHcy product into a chromophore or luminophore [13-15]. MTAN is a common auxiliary enzyme used in kinetic assays for MTases due to the ability to catalyze the hydrolysis of AdoHcy into an adenine and *S*-ribosylhomocysteine molecule, which assists in eliminating product inhibition for the AdoMet-dependent enzyme. For example, a spectroscopic-based assay developed by Dorgan and coworkers incorporates adenine deaminase to convert the MTAN-generated adenine into hypoxanthine to monitor the activity of a MTase [15]. Early-developed spectroscopic kinetic methods for MTases exhibit inherently low sensitivity when detecting AdoHcy, which becomes problematic for performing kinetic analyses on catalytically slow enzymes. Alternatively, luminescence-based assays developed for MTases have demonstrated to be highly sensitive for detecting generated AdoHcy [14,16,17]. For instance, coupling MTAN, adenine phosphoribosyl transferase, and pyruvate orthophosphate dikinase to produce ATP that is then used by luciferase to generate a luminescent signal was determined to possess an observed limit of detection (LOD) for AdoHcy of 0.1 pmol [18]. An inherent challenge of efficiently screening small molecule

libraries with coupled enzymes is the elevated hit rate of false positives and the requirement for orthogonal assays to validate hits [19].

Assays that directly measure AdoHcy or methylated products improve the adaptability for high-throughput screening by potentially reducing the hit rate of false positives. Immuno-based assays that directly detect methylated histone products have been demonstrated to be feasible for medium to high-throughput screening by successfully identifying inhibitors. For example, such assays have identified inhibitors targeting a G9a MTase, which is a eukaryotic histone lysine MTase [20-22]. Conversely, detecting AdoHcy rather than specific methylated products provides a universal detection mechanism that can be applied to any MTase. For instance, fluorescence polarization (FP) is a widely-used technique that monitors the fractional binding of a fluorescently labeled ligand to an enzyme. Graves and co-workers previously developed a competitive FP immunoassay for detecting generated AdoHcy from MTases, which differentiates AdoHcy from background AdoMet and exhibits a LOD of 0.5 pmol of AdoHcy [23]. Although direct immunodetection of AdoHcy provides several advantages for high-throughput screening of small-molecule libraries, production of high-titer antibodies can be prohibitively expensive.

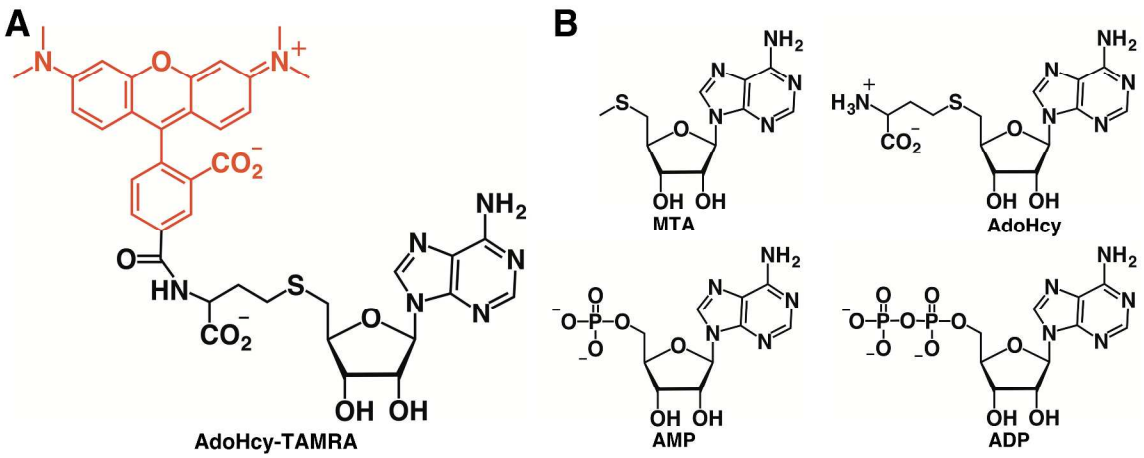

Figure 1: (A) The chemical structure of AdoHcy-TAMRA. (B) The chemical structures of the various adenosine-containing molecules that are quantifiable by the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay.

Here, we present a generic, homogenous, competitive FP assay that allows for the direct quantification of AdoHcy as well as several other adenosine-containing molecules. The concept for the FP assay involves leveraging a *Helicobacter pylori* MTAN variant (HpMTAN-D198N) that prevents catalytic turnover, while allowing the binding of a AdoHcy molecule conjugated to a fluorophore on the α-amino group of the homocysteine moiety. The synthesized fluorescent probe demonstrates a K_d value in the low nM range (AdoHcy-TAMRA, Figure 1A), which allows for a wide range of concentrations to be resolved while performing competitive FP assays [24]. The results from our competitive FP experiments highlight the versatility and sensitivity of measuring various generated products from AdoMet-dependent as well as ATP-dependent enzymes, including AdoHcy, MTA, ADP, and AMP (Figure 1B). Notably, our developed competitive FP method provides an alternative assay for monitoring the activity of MTases that is highly sensitive, cost effective, and suitable for high-throughput screening.

EXPERIMENTAL

Purification of HpMTAN-D198N and Mycobacterium tuberculosis EgtD

The cloning and expression of *Helicobacter pylori* MTAN-D198N and *Mycobacterium tuberculosis* (*Mtb*) EgtD were performed as previously stated [25,26]. An in-depth protocol for cloning and purification for both enzymes can be found in the supplemental section of the manuscript.

Synthesis and Purification of the AdoHcy fluorescent probe

One-pot synthesis of the fluorescent probe was performed by combining AdoHcy (1.3 mg, Sigma) in a 100 mM sodium bicarbonate buffer at pH 9 with 5-carboxytetramethylrhodamine succinimidyl ester (SE-TAMRA, 1.0 mg, Setareh Biotech) in 100 % DMSO (Figure S-1). The reaction was incubated overnight at room temperature protected from light to avoid photobleaching of the fluorophore. The resulting product was purified by reverse-phase HPLC (Waters 2487) using a linear gradient consisting of 0.1 % trifluoroacetic acid and acetonitrile on an analytical C18 column. Elution of AdoHcy-TAMRA and AdoHcy were monitored spectroscopically at the absorption maximum of the fluorophore (545 nm) and the adenine moiety of AdoHcy (260 nm) using a dual wavelength detector (Waters 2487 Dual λ Absorbance Detector). Eluted compounds demonstrating absorption at both wavelengths were collected and further characterized using ESI-MS (Finnigan LC-Q-Deca) in positive ion mode.

Equilibrium Binding and Competitive FP Assays with AdoHcy-TAMRA

All FP assays were conducted using a Biotek Synergy H4 Hybrid Reader to measure the parallel ($F_{||}$) and perpendicular ($F_{\perp}$) fluorescence intensities, which were then used to

calculate the mP value (Equation 1) [27]. Samples for performing the equilibrium binding or competitive FP experiments were incubated for 10 minutes at ambient temperature prior to measuring the fluorescence intensities. Equilibrium binding experiments to determine the K_d value for the HpMTAN-D198N/AdoHcy-TAMRA complex were performed using 5 nM of AdoHcy-TAMRA with varying concentrations of HpMTAN-D198N in 50 mM HEPES at pH 7.5. The resulting data were fitted to a one site - total binding equation in Prism 7 (GraphPad Software, San Diego, CA) where mP_{max} represents the maximum fluorescent polarization signal, NS is the slope for nonspecific binding of the fluorescent probe, and mP_{min} is the minimum fluorescent polarization signal (Equation 2).

Equation 1:

$$mP = 1000 \times \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + F_{\perp})}$$

Equation 2:

$$mP = \left(\frac{mP_{max} \times [Protein]}{K_d + [Protein]}\right) + (NS \times [Protein]) + mP_{min}$$

The competitive FP assays were conducted using a concentration of AdoHcy-TAMRA at the K_d value and a concentration of HpMTAN-D198N that allows for 70 – 80 % enzyme-probe complex. Therefore, the competitive FP experiments involved using a concentration of 11.3 nM for AdoHcy-TAMRA and 50 nM for HpMTAN-D198N with varying concentrations of ATP, ADP, AMP, AdoHcy, AdoMet, or MTA. Determination of the K_i values of all adenosine-containing compounds were fitted to a one site – fit K_i (equation 3) from Prism 7 (GraphPad Software, San Diego, CA).

Investigating the effects of background AdoMet was assessed by generating multiple displacement curves of AdoHcy in the presence of various concentrations of

AdoMet. Additionally, the reproducibility for the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay was determined by measuring 13 replicates of positive and negative reactions, which were used to determine the Z′ value (Equation 4) [28].

Equation 3:

$$\mathrm{mP} = \mathrm{mP_{min}} + \frac{(\mathrm{mP_{max}} - \mathrm{mP_{min}})}{(1 + 10^{[\mathrm{Ligand}] - \mathrm{Log\,EC_{50}}})}$$

$$\mathrm{Log\,EC_{50}} = \mathrm{Log}\,(10^{\mathrm{Log}\,K_i} \times \left(1 + \frac{[\mathrm{Probe}]}{K_d}\right))$$

Equation 4:

$$Z' = 1 - \frac{3\sigma_P + 3\sigma_N}{|\mu_P - \mu_N|}$$

Kinetic Characterization of EgtD and Alkaline Phosphatase using HpMTAN-D198N/AdoHcy-TAMRA Competitive FP assay

Determining the K_m^{app} of L-histidine for the purified EgtD using the developed competitive FP assay was performed at 25 °C. The assay components consisted of 50 nM HpMTAN-D198N, 11.3 nM AdoHcy-TAMRA, and 5 nM EgtD in 25 mM TRIS at pH 8.0. A stock solution of L-histidine was serially diluted in the kinetic reactions to provide a concentration range from 0.05 to 3.00 mM. The kinetic reactions were initiated by the addition of 500 nM of AdoMet, and the rate of catalysis of EgtD was continuously monitored by observing a decrease in the mP value. Conversion of the observed initial velocities into [AdoHcy]/minute was calculated using a standard curve of AdoHcy in the presence of 500 nM AdoMet (Figure S-2). The resulting data were fitted to the Michaelis-Menten equation using Prism 7 for calculating K_m^{app} of L-histidine for EgtD (Equation 5).

Equation 5:

$$V_o = \frac{V_{max} \times [Histidine]}{K_m + [Histidine]}$$

Kinetic characterization of ATP hydrolysis by Calf Intestinal Alkaline Phosphatase (CIAP, New England Biolabs) was performed similarly to the kinetic analysis of EgtD. Briefly, an assay mixture consisting of 50 nM HpMTAN-D198N, 11.3 nM AdyHcy-TAMRA, and 0.25 U/mL of CIAP in 25 mM TRIS at pH 8.0 were initiated by adding ATP at varying concentrations ranging from 0.1 to 20 μM. Steady-state analysis of the resulting data was fitted to a substrate-inhibition equation using Prism 7 (Equation 6).

Equation 6:

$$V_o = \frac{V_{max} \times [ATP]}{K_m + [ATP]\frac{1 + [ATP]}{K_i}}$$

Pilot Screen and Hit Validation of NIH Clinical Collection with EgtD

The pilot screen involved screening EgtD against the NIH Clinical Collection, which contains 446 compounds that were dissolved in 100 % DMSO to provide a stock concentration of 10 mM for each compound. Briefly, screening the NIH Clinical Collection consisted of adding 48.5 μL of an assay mixture (51.5 nM HpMTAN-D198N, 11.6 nM AdoHcy-TAMRA, 11.1 nM EgtD, 773 μM L-histidine, and 51.5 mM TRIS at pH 8.0) to a 384-black well plate, followed by the addition 0.5 μL of each tested compound (100 μM, and 1 %DMSO) to each well. The reactions were initiated with 1 μL of AdoMet (500 nM) and the rate of all reactions for EgtD were monitored continuously. Screening hits were validated assessing the inhibitor dose-response to EgtD by serially diluting the compound of interest and performing the assay as previously described. Additionally, the robustness and reproducibility of the developed competitive FP assay

for monitoring the activity of EgtD was assessed by calculating the Z′ value (Equation 3) using 13 positive and negative replicates.

RESULTS AND DISCUSSION

Design and Synthesis of AdoHcy-TAMRA

Previously, synthesized AdoHcy and AdoMet analogues possessed a fluorophore on the adenine or 5'-thio moieties [23,29,30], but this was avoided in the design of the fluorescent probe for HpMTAN-D198N. The active site cavity of HpMTAN is compact, and accommodation of a fluorophore conjugated to either of the aforementioned moieties would potentially disrupt essential interactions between the fluorescent probe and residues in the MTAN active site cavity. Conversely, examination of the crystal structure for HpMTAN-D198N complexed with AdoHcy illustrates that the α-amino moiety of AdoHcy is not buried in the active site, but is positioned at the protein surface (Figure 2) [25]. We envisioned that placement of a fluorophore on the α-amino moiety of AdoHcy would maintain essential interactions for the fluorescently labeled ligand within the active site cavity and promote high-affinity binding of the fluorescent probe. The synthetic approach for AdoHcy-TAMRA involved using nucleophilic acyl substitution in a one-pot synthesis by combining AdoHcy with SE-TAMRA, which is a rhodamine derivative containing an amine-reactive moiety (Figure S-1). In slightly basic conditions, the α-amino group of AdoHcy attacks the electrophilic center of the succinimidyl ester linkage of SE-TAMRA, resulting in the formation of an amide linkage between TAMRA and AdoHcy. The synthesized AdoHcy-TAMRA probe was separated from the reaction mixture using reverse phase HPLC (Figure S-3), and the appropriate eluting peak was

immediately validated by ESI-MS providing the expected mass of 797.69 Da (Figure S-4).

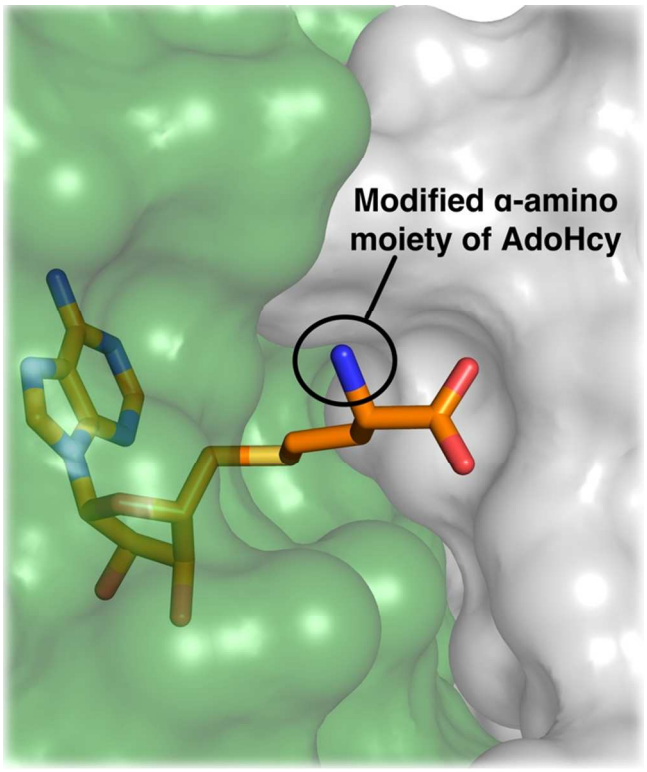

Figure 2: The surface model of the inactive variant HpMTAN-D198N complexed with AdoHcy (PDB: 4OY3) illustrating the rationale for modifying the α-amino moiety of AdoHcy with TAMRA. The enzyme exists as a biological homodimer, which is represented in green and grey. The crystal structure of HpMTAN-D198N complexed with AdoHcy highlights the α-amino moiety positioned at the surface of the protein. Therefore, conjugation of a fluorophore to the α-amino moiety avoids disrupting essential interactions for ligand binding within the compact active site cavity.

Assessing the Affinity of AdoHcy-TAMRA to HpMTAN-D198N

Determining the K_d value for AdoHcy-TAMRA to HpMTAN-D198N was performed in nonstoichiometric conditions by varying the concentration of the inactive variant while maintaining the concentration of the fluorescent probe significantly below the previously measured K_m value of 5.0 μM for AdoHcy [25]. Intriguingly, the generated binding isotherm demonstrates that AdoHcy-TAMRA possesses high affinity to HpMTAN-D198N exhibiting a determined K_d value of 11.3 ± 0.7 nM (Figure 3). Previous kinetic characterization of MTA and AdoHcy for HpMTAN has shown these substrates to

possess low μM affinity by measuring the spectral change of the reaction through formation of adenine [25,31]. More recently, steady-state kinetic analysis using a luciferase-based assay for *Staphylococcus aureus* MTAN indicated negative cooperativity between the two monomers demonstrating K_m values for MTA of 100 nM and 900 nM for the first and second active sight, respectively [32]. The binding isotherm of AdoHcy-TAMRA presented here represents the affinity measured for the first active site binding event, which affords the high sensitivity of the developed FP assay. Fluorescent probes possessing high affinity for their target exhibit several benefits including being cost effective, reduced aggregation of fluorophores, and the capability of resolving a wide range of ligand affinities. A common misconception is that competitive FP assays with tight-binding fluorescently-labeled ligands should be avoided when measuring the potency of inhibitors with intermediate affinity [24]. However, tight-binding fluorescently labeled ligands provide an extended range of inhibitor potency in comparison to moderate affinity fluorescently-labeled ligands. Assessment of the reproducibility of the HpMTAN-D198N/AdoHcy-TAMRA FP assay was determined by calculating the Z′ value using the concentrations of the fluorescent probe and enzyme implemented in the competitive FP assay. The Z′ value is a statistical measure that evaluates the quality of the assay, where calculated values greater than 0.5 demonstrate a suitable assay [28]. The determined Z′ for the developed competitive FP-based assay was determined to be 0.77, validating the reproducibility and dynamic range of the assay.

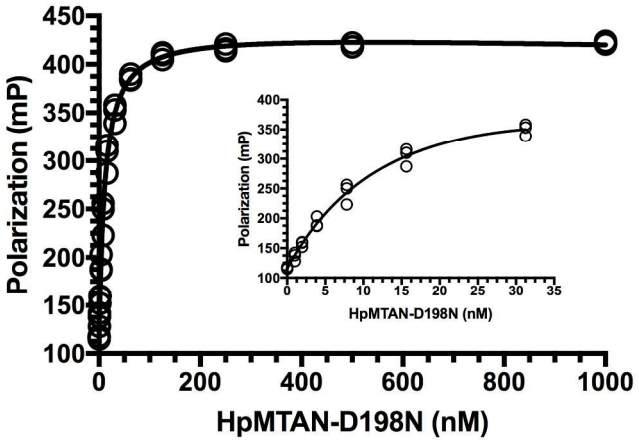

Figure 3: The binding isotherm of AdoHcy-TAMRA to HpMTAN-D198N. The resulting data are presented in triplicates and were fitted to a one-site binding equation (Equation 2). The offset plot represents the linear portion of the binding isotherm. The measured K_d value for AdoHcy-TAMRA to HpMTAN-D198N was determined to be 11.3 ± 0.7 nM.

Quantification of AdoHcy and MTA from AdoMet-dependent enzymes

Adapting the HpMTAN-D198N/AdoHcy-TAMRA complex for quantifying AdoHcy or MTA generated from AdoMet-dependent enzymes is contingent on the sensitivity and capability of differentiating the adenosine-based products from substrates. For instance, assays that monitor MTase activity require high sensitivity due to inherently slow catalytic turnover or avoiding product inhibition at high concentrations of AdoHcy [13,20,33]. We converted the developed HpMTAN-D198N/AdoHcy-TAMRA complex into a competitive FP assay that would directly detect AdoHcy or MTA by monitoring the displacement of AdoHcy-TAMRA from HpMTAN-D198N. The quality of the developed competitive FP assay was determined by generating displacement curves for AdoHcy and MTA to evaluate the affinity and limit of detection (LOD) of these adenosine-based molecules. Analysis of the displacement curves for MTA and AdoHcy demonstrate LOD values measuring up to approximately 10 nM (0.5 pmol) and 6 nM (0.3 pmol) as well as providing calculated K_i values of 24.2 ± 1.2 and 29.5 ± 3.6 nM (Figure 4), respectively. Interestingly, the LOD of AdoHcy for the developed competitive FP assay is notably

better or comparable to previously published spectroscopic, fluorescence-based or FP immunoassays for measuring MTase activity [14,15,23,34]. Several developed assays for MTases require multiple coupled enzymes to manipulate generated products into producing an output signal, which can lower the sensitivity of the assay. Coupling the HpMTAN-D198N/AdoHcy-TAMRA complex directly to AdoMet-dependent enzymes provides a highly sensitive universal assay that immediately detects generated products without the complication of additional auxiliary enzymes. A limitation with the HpMTAN-D198N/AdoHcy-TAMRA FP competitive assay is the narrow linear range for detecting AdoHcy in comparison to previously published AdoHcy quantification-based assays (Figure S-1) [13,14,35]. Previously developed competitive FP immunoassays demonstrate similar limitations in the linear ranges for detecting AdoHcy [23,34], which presents a challenge for obtaining quantifiable kinetic parameters such as K_m and k_{cat}.

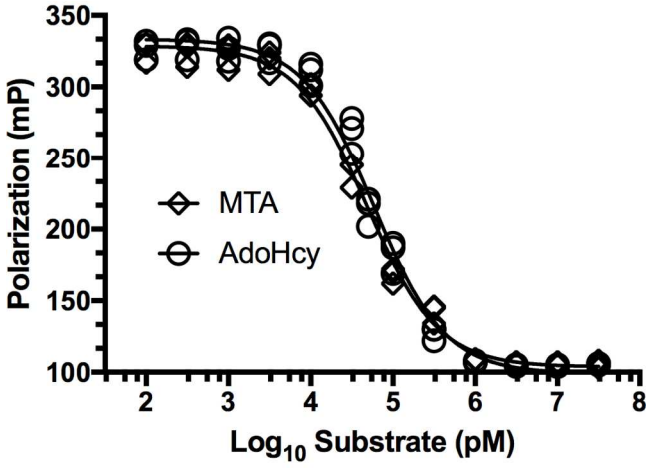

Figure 4: Displacement curves of MTA and AdoHcy using the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay. Triplicates are directly plotted and the resulting data were fitted to equation 3. The determined K_i values for MTA and AdoHcy are 24.2 ± 1.2 and 29.5 ± 3.6 nM, respectively.

Kinetic characterization of AdoMet-dependent enzymes typically requires relatively high concentrations of AdoMet, potentially interfering with the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay. Initially, we assessed the effect of background AdoMet

displacing AdoHcy-TAMRA from HpMTAN-D198N by generating a displacement curve for the AdoHcy analogue. Analysis of the displacement curve for AdoMet presented an unexpected K_i value of 989 ± 160 nM. Comparison of the displacement curves for the adenosine-containing molecules illustrate that MTA and AdoHcy possesses a 34-fold higher affinity to HpMTAN-D198N than AdoMet (Figure 5A). A challenge for coupling the HpMTAN-D198N/AdoHcy-TAMRA complex to monitor the activity of AdoMet-dependent enzymes is the observed moderate LOD for AdoMet, which was determined to be 312 nM. Unfortunately, the effect of low μM concentrations of AdoMet for quantifying AdoHcy/MTA consequently reduces the signal window and slightly increases the LOD of AdoHcy (Figure 5B). The highest AdoMet concentration that the developed competitive FP assay tolerates was determined to be 5 μM, which significantly decreased the signal window to 70 ΔmP. Despite this interference of background AdoMet at higher concentrations, the developed competitive FP assay clearly discriminates between AdoHcy/MTA and AdoMet, resulting in a dynamic range that affords accurate measurement of AdoHcy/MTA production in a workable background of AdoMet.

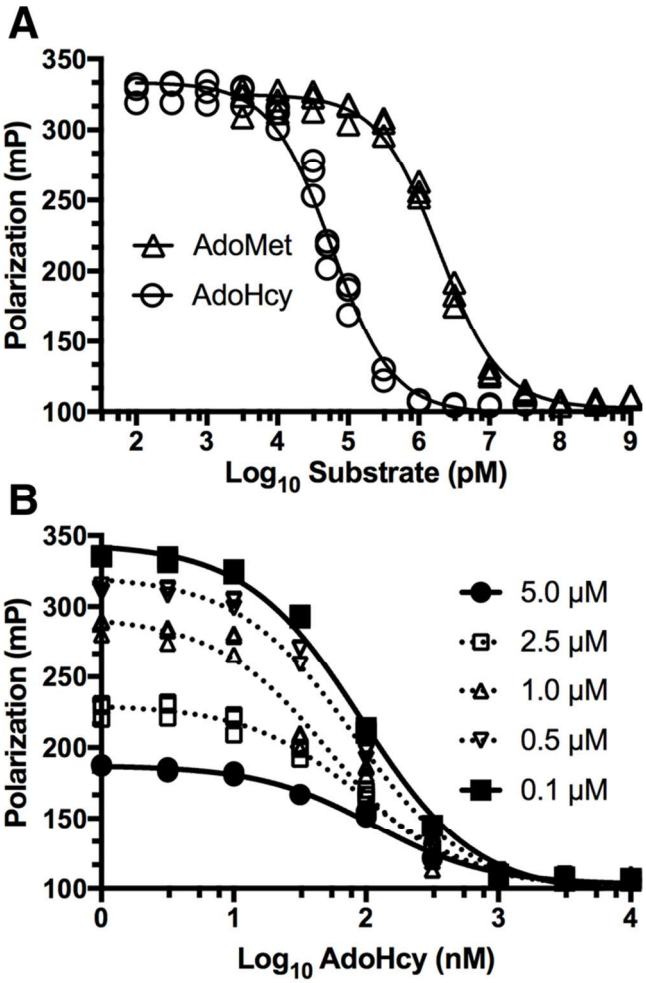

Figure 5: Displacement curves of AdoMet and AdoHcy. Triplicates are demonstrated in all plots and data were fitted to equation 3. (A) Generated displacement curves of AdoMet and AdoHcy by the developed competitive FP assay highlighting the differentiation of the two adenosine-containing molecules. The determined K_i values for AdoMet and AdoHcy were 989 ± 160 nM and 29.5 ± 3.6 nM, respectively. (B) Displacement curves of AdoHcy in the presence of varying concentrations of AdoMet. The effect of higher concentrations of AdoMet consequently increases the LOD of AdoHcy and decreases the signal window for the developed competitive FP assay.

Kinetic Characterization and Pilot Screen of EgtD

Initially, the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay was used to examine the kinetic parameters of L-histidine methylation by an AdoMet-dependent MTase enzyme, EgtD, encoded by *Mtb* using non-saturating steady-state kinetics. EgtD catalyzes the initial step in the biosynthesis of ergothioneine by catalyzing the trimethylation of the α-amino acid moiety of L-histidine (Figure 6A) [26,36]. Previously,

determination of the affinity for L-histidine and AdoMet to *Mycobacterium smegmatis* EgtD using isothermal calorimetry showed K_d values of 290 ± 14 and 270 ± 20 μM [36], respectively. Kinetic characterization of L-histidine by EgtD at non-saturating AdoMet concentrations using the developed competitive FP assay demonstrated a K_m^{app} value of 330 ± 35 μM (Figure 6B), which is within error of the previously determined K_d value for the homolog. Pseudo first-order kinetics was not performed because of the interference of background AdoMet at higher concentrations; however, steady-state kinetics can be conducted to approximate the K_m value for substrates using the developed competitive FP assay. Once the kinetic parameters for EgtD were determined, the assay conditions were optimized to maximize the dynamic range of the competitive FP assay to perform high-throughput screening. Validating the suitability of performing high-throughput screening with the optimized assay condition was evaluated by calculating the Z′ value, resulting in an acceptable Z′ of 0.56.

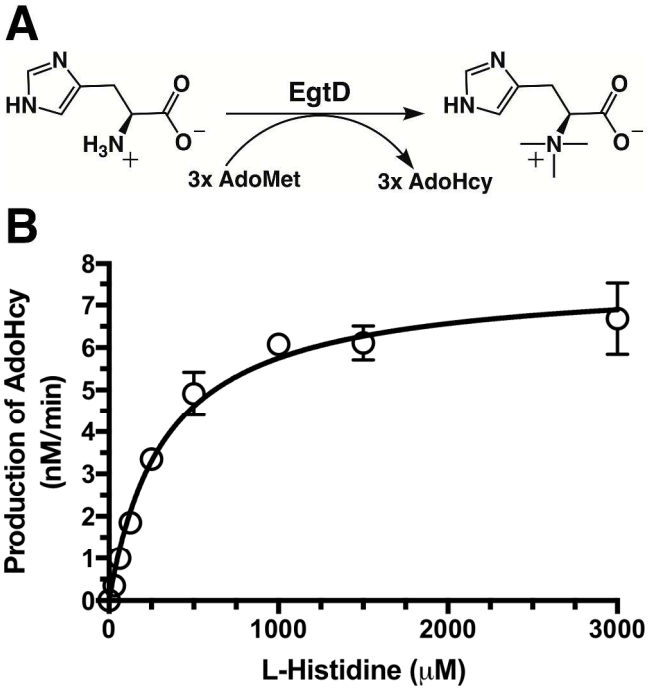

Figure 6: (A) The enzymatic reaction for EgtD, which catalyzes the trimethylation of the α-amino acid group of L-histidine. (B) The generated Michaelis-Menten curve for L-histidine to EgtD using

the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay. The data were plotted as the mean with the standard deviation of the three independent experiments. The determined K_m^{app} for L-histidine to EgtD was 330 ± 35 μM.

As a proof of concept, EgtD was screened against the NIH Clinical Collection to identify potential inhibitors as well as demonstrate the capability of performing high-throughput screening with the developed competitive FP assay. Previously, EgtD has been shown to be required for the biosynthesis of ergothioneine as well as being essential for the growth of *Mtb* in murine macrophages and animal models [37-39]. Analysis of the 446 compounds screened from the NIH clinical collection yielded a hit cut-off of 30.1 % activity of EgtD, which produced an initial hit rate of 4.3 % (19 compounds, Figure 7A). The hit cut-off was determined by calculating 3 standard deviations from the average of the entire screening data. Notably, the design of the developed competitive FP assay provides an advantage when performing high-throughput screening by easily identifying and reducing the hit rate of interfering compounds with the assay. As stated previously, several current available methods for MTases require multiple coupling enzymes, which complicates high-throughput screening by increasing the number of false positives. The developed competitive FP assay simplifies high-throughput screening for MTases by only requiring the HpMTAN-D198N/AdoHcy-TAMRA complex, and easily identifying compounds interfering with the assay by observing an initial decrease in the signal window. Inspection of the high-throughput screening data demonstrated 3 compounds interfering with the competitive FP assay by either significantly decreasing the signal window or containing similar fluorescent properties as the fluorophore. The preliminary 19 hits from the primary screen were validated by determining if the observed decrease in activity was reproducible and if the small molecules exhibited a dose-response relationship to EgtD. Out of the 19 hits, only 6 compounds reproduced a decrease in activity below the hit cut-

off as well as demonstrated a dose-response to EgtD (Figure 7B), concluding a final hit rate of 1.4 % for screening of the NIH clinical collection. A potential challenge for screening AdoMet-dependent MTases containing a K_m value greater than 2.5 μM of AdoMet will be experiencing an initial increase in hit rates from identifying compounds that bind weakly to the enzyme of interest. Indeed, it is commonly understood that the choice of concentrations for substrates relative to an enzyme's inherent K_m values can alter screening results [40]. The presented screening results for EgtD initially identified 13 false positives, which we attributed to performing the assay with an AdoMet concentration significantly below the previously determined K_d value. Despite the limitations of interfering background AdoMet at μM concentrations, the developed competitive FP assay identified and validated 6 inhibitors from the NIH Clinical Collection that demonstrate moderate affinity for EgtD.

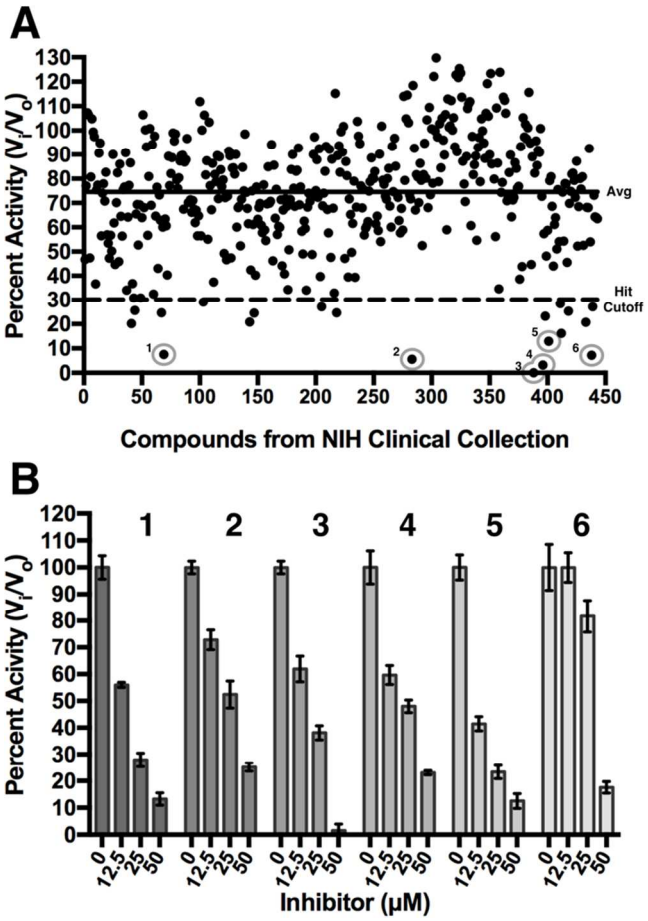

Figure 7: (A) Graphical representation of EgtD screened with the NIH clinical collection. The plotted solid line represents the average of the entire screening data. Whereas, the plotted dashed line represents the hit cut-off, which was determined by taking the 3 standard deviation units from the average. Any compound that falls below the hit cut-off represents a preliminary hit. Initially, 19 compounds were identified, but after validating only 6 compounds were confirmed as hits. These 6 compounds are circled on the presented plot. (B) The dose-dependence assessment of the 6 confirmed hits form the high-throughput screen of EgtD with the NIH clinical collection.

Further Applications of the HpMTAN-D198N/AdoHcy-TAMRA Competitive FP Assay

We further explored applications of the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay for quantifying additional adenosine-based molecules to expand the versatility of the developed assay. In addition to catalyzing AdoHcy and MTA hydrolysis, it has been shown that MTAN hydrolyzes the *N*-ribosidic bond for a variety of adenosine-containing molecules with various substituents on the 5' position [41].

Intriguingly, it was serendipitously observed that the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay detects AMP and ADP at moderate concentrations, while ATP is unable to displace AdoHcy-TAMRA (Figure 8A). The capability of differentiating AMP and ADP from ATP suggests that the developed competitive FP method is an alternative assay for measuring the activity of ATP-dependent enzymes. Analysis of the displacement curves for AMP and ADP provide measured K_i values of 933 ± 66 and 15.2 ± 1.3 μM, respectively. The determined affinities of the adenosine-containing molecules illustrate that the developed competitive FP assay is more efficient at monitoring enzymes that produce ADP. Currently, several assays exist commercially to monitor the activity of kinases [42]. Most of these assays do not tolerate a high background concentration of ATP [43], whereas the developed competitive FP assay tolerates up to 100 mM ATP. Our developed competitive FP assay allows for more than one order of magnitude higher ATP concentration to be tolerated in comparison to standard kinase assays. The advantages of tolerating a wide range of ATP concentrations is that it can easily determine and accommodate a diverse range of K_m values for ATP exhibited by kinases as well as allow for ATP competitiveness studies [43].

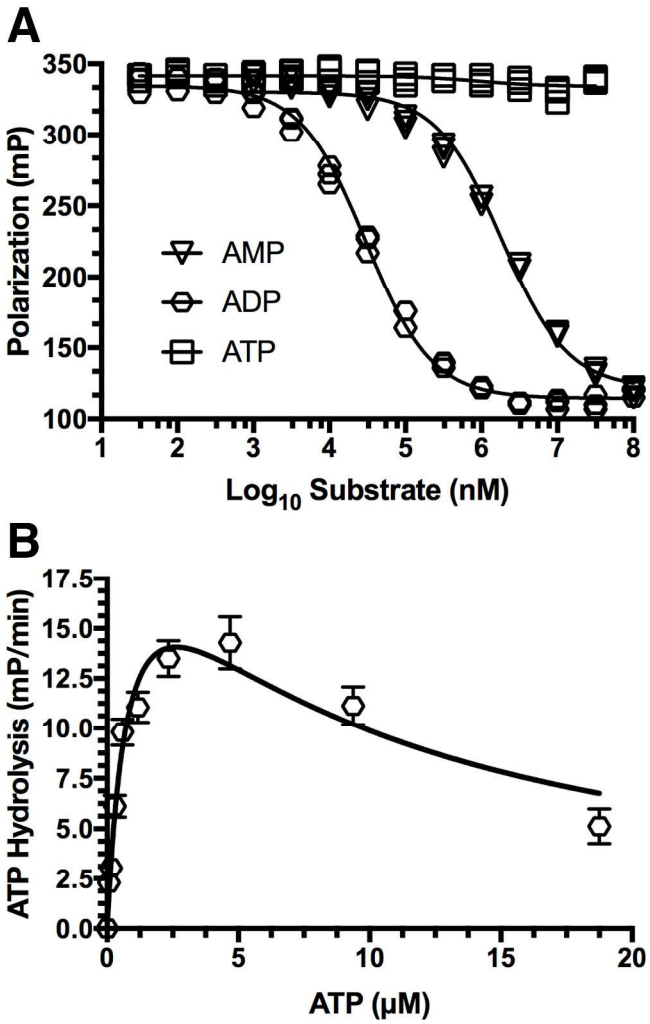

Figure 8: (A) Generated displacement curves of ATP, ADP and AMP using the HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay. Triplicates are demonstrated in all plots and data were fitted to equation 3. The determined K_i values for ADP and AMP were 15.2 ± 1.3 and 933 ± 67 μM, respectively. (B) Michaelis-Menten plot of alkaline phosphatase exhibiting the known substrate inhibition curve. The resulting data were fitted to equation 6 resulting in K_m values of 0.9 ± 0.2 μM and K_i value of 7.6 ± 1.8 μM for ATP, respectively.

As a proof of concept, we examined the kinetic parameters of ATP hydrolysis by CIAP using the developed HpMTAN-D198N/AdoHcy-TAMRA competitive FP assay. Previously, Michaelis-Menten kinetics performed on CIAP using a molybdenum blue based assay exhibited substrate inhibition of ATP at low μM concentrations, which resulted in a K_m value of 20.5 μM and a K_i value of 19.0 μM [44]. Steady-state analysis of CIAP using the developed competitive FP assay demonstrates a K_m value of 0.9 ± 0.2 μM

and K_i value of 7.6 ± 1.8 μM for substrate inhibition of ATP (Figure 8B). The 8-fold difference in the determined K_i in comparison to the previously published value we attribute to the assay reaction not containing an equivalent concentration of magnesium chloride, which is known to contribute to substrate inhibition of ATP [44]. The lower K_m and K_i values measured for ATP is likely a result of the developed competitive FP assay containing higher sensitivity in comparison to the molybdenum blue based assay. The previous determined LOD for measuring free inorganic phosphate using the molybdenum blue based assay was determined to be 25 μM [45]. Additionally, it is well known that CIAP sequentially hydrolyzes the inorganic phosphate groups from ATP [46], thereby creating a mixture of analytes with differing affinities to MTAN. At any point in time, the results exhibit an overall increase in adenosine nucleotides and adenosine, but, in this specific application, the developed competitive FP assay cannot distinguish between ADP, AMP, or adenosine. The LOD demonstrated by the developed competitive FP assay for ADP was determined to be 0.5 μM, providing a 50-fold increase in sensitivity in comparison to the molybdenum blue based assay. Alternatively, adenosine is structurally analogous to MTA and 5′-deoxyadenosine, which are hydrolyzed by MTAN to avoid product inhibition of polyamine biosynthesis and AdoMet radical pathways, respectfully [47]. Since the developed competitive FP assay demonstrates a LOD of 10 nM for MTA, it is likely that adenosine possesses similar affinity to HpMTAN-D198N and would provide a 2500-fold increase in sensitivity in comparison to the molybdenum blue assay. Ultimately, most applications for this assay will be toward enzymes with a known single adenosine-containing product, and the observed lack of discrimination specific to the coupled alkaline phosphatase assay would not be relevant in the other applications.

CONCLUSION

The work presented describes an alternative method for universally detecting generated AdoHcy from MTases that is highly sensitive, amenable for high-throughput screening and performed in a simple mix-and-read format. Besides monitoring the activity of MTases, our developed competitive FP assay is applicable to several AdoMet-dependent and ATP-dependent enzymes because of the promiscuity of MTAN binding various adenosine-containing molecules. Additionally, our developed FP assay allows for a feasible method for high-throughput screening as well as determination of binding constants for ligands to the various homologs of MTAN.

ACKNOWLEDGMENTS

A portion of this research was also supported by the_National Institute of Allergy and Infectious Disease (NIAID)/National Institutes of Health (NIH) to D.R.R (AI105084).

SUPPORTING INFORMATION

Cloning and purification of HpMTAN-D198N and EgtD, one-pot synthetic scheme for AdoHcy-TAMRA (Figure S-1), standard curve of AdoHcy with 500 nM AdoMet (Figure S-2), HPLC chromatogram for the purification of AdoHcy-TAMRA (Figure S-3), and the mass spectrum of purified AdoHcy-TAMRA (Figure S-4).

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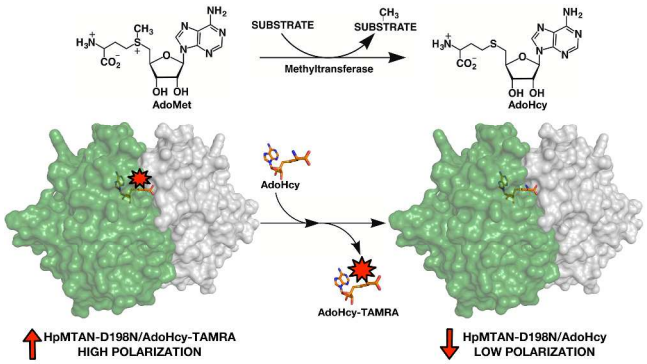
SUBSTRATE
CH3
SUBSTRATE
Methyltransferase
AdoMet
AdoHcy
AdoHcy
AdoHcy-TAMRA
HpMTAN-D198N/AdoHcy-TAMRA
HIGH POLARIZATION
HpMTAN-D198N/AdoHcy
LOW POLARIZATION

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