A simplified characterization of S-adenosyl-L-methionine-consuming enzymes with 1-Step EZ-MTase: a universal and straightforward coupled-assy for in vitro and in vivo setting

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Methyltransferases use S-adenosyl-L-methionine (SAM) to deposit methyl marks. Many of these epigenetic 'writers' are associated with gene regulation. As cancer etiology is highly correlated with misregulated methylation patterns, methyltransferases are emerging therapeutic targets. Successful assignment of methyltransferases' roles within intricate biological networks relies on (1) the access to enzyme mechanistic insights and (2) the efficient screening of chemical probes against these targets.

To characterize methyltransferases in vitro and in vivo, we report a highly-sensitive one-step deaminase-linked continuous assay where the S-adenosyl-L-homocysteine (SAH) enzyme-product is rapidly and quantitatively catabolized to S-inosyl-L-homocysteine (SIH). To highlight the broad capabilities of this assay, we established enzymatic characteristics of two protein arginine methyltransferases (PRMT5 and PRMT7), a histone-lysine methyltransferase (PKMT) and a sarcosine/dimethylglycine N-methyltransferase (SDMT). Since the coupling deaminase TM0936 displays robust activity over a broad pH-range we determined the pH dependence of SDMT reaction rates. TM0936 reactions are monitored at 263 nm, so a drawback may arise when methyl acceptor substrates absorb within this UV-range. To overcome this limitation, we used an isosteric fluorescent SAM-analog: S-8-aza-adenosyl-L-methionine. Most enzymes tolerated this probe and sustained methyltransfer activity while methylacceptor assays were efficiently monitored through loss of fluorescence at 360 nm. Unlike discontinuous radioative- and antibody-based assays, our assay provides a simple, versatile and affordable approach towards the characterization of methyltransferases. Supported by three logs of linear dynamic range, the 1-Step EZ-MTase can detect methylation rates as low as 2 µM h⁻¹, thus making it possible to quantify low nanomolar concentrations of glycine N-methyltransferase within crude biological samples. With Z⁺-factors above 0.75, this assay is well suited to high-throughput screening and may promote the identification of novel therapeutics.

Introduction

Protein post-translational modifications (PTM) regulate many biochemical processes. For instance, the deposition and removal of histone PTMs, can govern cell fate. Methyl marks are written by methyltransferases (MTases) and fueled by a universal methyl-donor: S-adenosyl-L-methionine (SAM). Small molecule methyltransferases (SMMT) were the first discovered. Further studies identified DNA methyltransferases (DNMT) as key catalysts to edit cytosine at certain Cpg sequences, thus modulating cellular differentiation and transcriptional silencing. Later, protein lysine and arginine methyltransferases (PKMT and PRMT, respectively) emerged as crucial enzymes responsible for histone tail modifications. While methylation of lysine 4 on histone H3 (H3K4me3) is undeniably responsible for activation of transcription,
establishing an universal code to translate PTM’s and their cross talk is still at the early stage of development.\textsuperscript{10,11} Nonetheless, it is evident that erratic methylation patterns are implicated in oncogenesis and tumor progression.\textsuperscript{9,12,13} Overexpression of PKMTs and PRMTs in tumors is correlated with poor clinical prognosis.\textsuperscript{14–18} MTases are emerging cancer targets and they provide a new horizon for biological chemists to enhance the clinical use of personalized therapies.\textsuperscript{19–21}

Current MTase assays rely on the detection of either product of the transferase reactions, methyl marks or S-adenosyl-L-homocysteine (SAH; Fig. S1 and Table S1f). The use of radio-labeled SAM makes it possible to quantify the radioactivity incorporated within the acceptor target. Whether on a DNA strand or a peptide, the methylated product can be separated from methyl-donor via specifically charged filters, solid phase extraction sorbents or liquid chromatography (Fig. S1,† arrows 1 and 2).\textsuperscript{22–25} Methylation can also be detected via antibody-specific recognition combined with fluorescence resonance energy transfer (FRET, AlphalISA; Fig. S1,† arrow 3).\textsuperscript{26,27} Although low detection limits may be reached with these assays, there are major drawbacks to these methods, including: (1) a discontinuous approach limiting the analysis throughput, (2) the elevated costs of radioactive waste treatment along with unstable radioactive SAM and (3) the highly specific immuno-detection may limit the analysis to one single MTase.

On the other hand, SAH detection is well suited to the characterization of a wider range of MTases as SAH is the universal by-product of all transferase reactions. Therefore, multiple assays are based on this detection, either directly or using recombinant coupling enzymes to catabolize SAH and channel it into a metabolite easily detectable (Fig. S1;† arrows 4–12). An additional experimental benefit is that this approach relieves the MTases from product-inhibition.

Many assays have been developed to detect SAH. For instance, bacterial S-adenosyl-L-homocysteine nucleosidase (MTAN, E.C. 3.2.2.29) generates adenine and S-(5-deoxy-\textalpha-ribos-5-yl)-L-homocysteine (SRH). Adenine can either be detected continuously by (1) luminescence at 570 nm through efficient conversion into AMP and ATP using adenine phosphoribosyltransferase (APRT, E.C. 2.4.2.7), pyruvate phosphate dikinase (PPDK, E.C. 2.7.9.11) and firefly luciferase (FLUC, E.C. 1.13.12.7; Fig. S1;† arrow 4)\textsuperscript{28–29} or (2) decrease of absorbance at 265 nm following deamination into hypoxanthine (Hx; Fig. S1;† arrow 5) using adenosine deaminase (ADA, E.C. 3.5.4.2).\textsuperscript{30,31} Meanwhile, S-ribosylhomocysteinase (LuxS, E.C. 4.4.1.21) catabolizes SRH into \textgamma-homocysteine (Hcy-SH) for further detection of free-thiol with Ellman’s reagent at 412 nm (Fig. S1,† arrow 6).\textsuperscript{32} Likewise, recombinant S-adenosyl-L-homocysteine hydrolase (SAHH, E.C. 3.3.1.1) may utilize SAH to generate Hcy-SH, later detected with thiol-sensitive reagents (e.g. ThioGlo®-1; Fig. S1,† arrow 7).\textsuperscript{33,34} In presence of ATP and adenosine kinase (AK, E.C. 2.7.1.20), the adenosine product of the SAHH reaction, is phosphorylated to AMP and further detected with a specific antibody (Fig. S1,† arrow 8).\textsuperscript{35} The remaining ATP from this kinase reaction can also be quantified by KinaseGlo® reagent (luminescence; Fig. S1,† arrow 9).\textsuperscript{36} Another approach, involving PPDK and FLUC, allows for continuous monitoring of SAH through recording of light output (Fig. S1,† arrow 10).\textsuperscript{37} A universal, yet discontinuous detection of SAH, based on competitive fluorescence polarization immunoassay (FPIA), was also achieved.\textsuperscript{38}

Finally, a very early report described a continuous assay involving a single coupling enzyme.\textsuperscript{39} The conversion of SAH into S-inosyl-L-homocysteine (SIH) was implemented to characterize the rat liver catechol O-methyltransferase through monitoring of absorbance at 265 nm (Fig. 1A and B). Isolation of the deaminase from Aspergillus oryzae is likely the major drawback in using the assay,\textsuperscript{40} so the method has not been used since 1973. However, recent efforts towards the annotation of enzyme function have predicted the SAH-deaminase activity of TM0936 from Thermotoga maritima (Fig. 1A).\textsuperscript{41} Further reports have described additional members of this enzyme family, none of which are able to catabolize SAM.\textsuperscript{42–43}

A coupled assay relying on one single enzyme is a clear asset, thus we took advantage of TM0936. This SAH-deaminase is a robust and efficient catalyst.\textsuperscript{44–45} By converting SAH into SIH, the enzyme relieves MTases from product inhibition.\textsuperscript{29,46} We coupled this catalyst to several MTase families, including two PRMTs (PRMT5 and PRMT7), a PKMT (DIM-5) and a SMNT (i.e. sarcosine/dimethylglycine N-methyltransferase; SDMT). Here, we demonstrate that this assay can provide efficient measurement of kinetic parameters well suited to high-throughput screening (HTS). Furthermore, we measured the inhibition value of sinefungin against SDMT, thus establishing the compatibility between sinefungin and TM0936 and demonstrating its use for inhibitor screening.

TM0936 is a strong catalyst and pH variations only affected the deaminase activity to a small extent; the decrease of absorbance at 263 nm was monitored accurately across a 5-unit pH range (5.0–10.0). Thus, in a technical \textit{tour de force}, we established the pH dependence of SDMT reaction rates. Likewise, variations in salt concentration had no effect onto TM0936 activity, and we quantified the impact of ionic strength onto the affinity between histone tails and their MTase target. Conscious that a UV-mode of detection may limit the applications of this assay, we synthesized a fluorescent SAM analog. In most cases, the S-8-aza-adenosine-\textgamma-methionine (8-aza-SAM) was a good substrate for MTases. As TM0936 efficiently converted 8-aza-SAM into the non-fluorescent product 8-aza-SIH, PRMT7 activity was monitored through a decrease of fluorescence emission at 360 nm.

Finally, taking advantage of a low limit of SAH-detection (2 \textmu M h\textsuperscript{-1}), we successfully quantified glycine N-methyltransferase (GNMT) activity within rat liver extracts, demonstrating the \textit{in vivo} applicability of this assay. Unlike other techniques, the continuous detection of methyltransferase with 1-Step EZ-MTase is compatible with adenine, phosphorylated adenosines and reactive thiol species (e.g. glutathione, homocysteine and cysteine) often present in crude biological samples. Thus, this novel assay allows for fast, simple and accurate measurement of GNMT activity, while overcoming limitations of interference observed in previous formats. Importantly, measurements can be detected in as little as 30 \mu g of tissue protein, suggesting applicability even for biologic samples where protein yield is limited.
FLUC) is a tedious process and batch-to-batch reproducibility encountered limitations (Table S1). These parameters are in good agreement with previous reports describing the homologous reaction using 8-aza-adenosyl (8-aza-A) was monitored at both 292 and 282 nm (black and white circles, respectively).

Results and discussion

The deaminase TM0936 is a prime-choice candidate for MTase assays

We used a two-step purification of TM0936 as a polyhistidine-tagged protein expressed in bacteria. The high-purity enzyme from the hyperthermophile Thermotoga maritima can sustain multiple freeze/thaw cycles, it remains unable to catabolize SAM and it displays an excellent reactivity towards SAH at room temperature (Fig. S2A†). The preparation of four recombinant enzymes (MTAN, APRT, PPDK, FLUC) is a tedious process and batch-to-batch reproducibility may be an issue. Furthermore, the coupling buffer requires 5-phospho-β-D-ribofuranosyl-1-pyrophosphate (PRPP), a highly unstable and expensive substrate to fuel APRT. Likewise, the presence of adenine and phosphorylated adenosine species within crude biological samples preclude the use of luciferase-based assays for the in vivo settings. Therefore, based on the TM0936 performance, we developed the 1-Step EZ-MTase assay to provide a simple and robust method for measuring SAM consumption.

Spectral signature of the SAH deamination reaction catalyzed by TM0936

To determine the precise relationship between absorbance and concentration for the adenosyl to inosyl conversion, we measured the differential extinction coefficient from the reaction catalyzed by TM0936. A nucleoside absorbance spectrum (i.e. maximum absorption wavelength $\lambda_{\text{max}}$, extinction coefficient $\varepsilon$) is pH-dependent, thus reflects the ionization state of this molecule. The adenosyl UV-signature remains unchanged in water under most conditions (neutral species at pH > 3.6; Fig. S2B†); however, the inosyl group may behave differently as it becomes deprotonated in alkaline solutions (pK_a = 8.9).47,48

We established the $\Delta\varepsilon = f(pH)$ relationship for the deamination (Fig 1C). This data-set is a key component of the assay so that MTase reaction rates may be determined accurately across a broad pH-range. Data were fitted to ESI eqn (S2)† where $\Delta\varepsilon_{\text{high pH}} (-4655 \pm 186 \text{ M}^{-1} \text{ cm}^{-1})$ and $\Delta\varepsilon_{\text{low pH}} (-8076 \pm 80 \text{ M}^{-1} \text{ cm}^{-1})$ are the extinction coefficient measured at 263 nm for the deaminase reaction at high and low pH, respectively, and $-pK_a = -8.72 \pm 0.09$ is the logarithm of acid dissociation constant for inosine (lit., 49 $-3890 \text{ M}^{-1} \text{ cm}^{-1}$, $-8270 \text{ M}^{-1} \text{ cm}^{-1}$ and 8.85, respectively).

Fig. 1  The reaction catalyzed by deaminase TM0936 and its use as a coupling enzyme for assay development. (A) The SAH by-product of methyltransferase (MTase) reactions is efficiently converted into its inosyl-derivative (SIH) by the deaminase TM0936. (B) The different spectral signatures between SAH and SIH ($\lambda_{\text{max 1}} = 259$ nm in blue and $\lambda_{\text{max 2}} = 249$ nm in red, respectively) allow continuous monitoring of methyltransfer through UV-detection. The reactions are characterized by a decrease of absorbance at 263 nm (black arrow). (C) The pH-dependence of differential extinction coefficients for the deamination reaction $\Delta\varepsilon = f(pH)$. UV-spectroscopy (scans 220–320 nm) was used to monitor changes in absorbance during the reaction catalyzed by TM0936. The adenosyl (A) to inosyl (I) conversion was monitored at 263 nm (black squares) while the homologous reaction using 8-aza-adenosyl (8-aza-A) was monitored at both 292 and 282 nm (black and white circles, respectively).

\[ \varepsilon_{\text{max}} = 2.2 \pm 0.1 \text{ s}^{-1}, \]

\[ \varepsilon_{\text{max 1}} = 210 \pm 40 \mu\text{M}, \]

\[ \varepsilon_{\text{max 2}} = 12.2 \pm 0.8 \text{ s}^{-1}, \]
Before coupling between enzymes; nearly 50% of SAH-equivalent was lost
cule. The coupling enzymes from the kit do not convert SAH fast
(ADHP) into peroxidase (HRP) to convert 10-acetyl-3,7-dihydroxyphenoxazine
This format allows for higher throughput of data
experiments in 96-well plates compatible with UV detection.

Fig. 2 The drawbacks from a commercial kit. (A) The coupled-assay
for MTase detection (Cayman Chemical, #700150). Through two
enzymatic reactions, SAH is channeled to hypoxanthine; further
oxidation by xanthine oxidase (XO) will produce uric acid and two
molecules of hydrogen peroxide. The peroxide fuels horse radish
peroxidase (HRP) to convert 10-acetyl-3,7-dihydroxyphenoxazine
(ADHP) into fluorescent resorufin. (B) Slow channeling of SAH mole-
cule. The coupling enzymes from the kit do not convert SAH fast
enough and a 10 min lag phase is observed. (C) Channeling of SAH is
not quantitative. A comparison between resorufin and SAH standard
curves (squares and circles, respectively), highlighted the incomplete
channeling between enzymes; nearly 50% of SAH-equivalent was lost
before fluorescence emission.

In addition, similar Δε = f(pH) relationships were estab-
lished at 282 and 292 nm for the 8-aza-adenosine (8-aza-A) to 8-
aza-inosine (8-aza-I) reaction (Fig. 1C). The 8-aza analogs of
SAM/SAH are fluorescent while their inosyl counterparts are not. Thus, 8-aza-SAM may be a valuable substrate for
monitoring MTase activity using a fluorescence mode. Our
experiments provided: Δε_{high pH} (−2026 ± 174 and −3016 ± 119
M⁻¹ cm⁻¹), Δε_{low pH} (−14 975 ± 129 and −10 117 ± 87 M⁻¹
cm⁻¹) and pK_a (7.29 ± 0.03 and 7.30 ± 0.03), at 282 and 292 nm,
respectively.

Kinetic parameters from four methyltransferases using 1-Step
EZ-MTase

To establish a coupled assay that can sample the vast majority
of MTase targets, all having diverse kinetic properties (range of
SAM concentrations: 1–1000 μM; Fig. S3†), we performed
experiments in 96-well plates compatible with UV detection.
This format allows for higher throughput of data vs. a regular
cell-changer spectrophotometer using 1/2–3 mL cuvettes. The
wells accommodate 50–250 μL sample volumes, resulting in an
adjustable optical path so that a linear relationship between
absorbance and concentration is preserved (Fig. S3†). Further-
more, the assay displays a broad range of SAH-detection with
a upper limit set at 10 × 10^3 μM h⁻¹ when using a 4 μM
concentration of TM0936 (i.e. 42 × 10^3 pmol min⁻¹ in a 250 μL
well at pH 8.00; Fig. S4A and B†). Although the cofactor slowly
and partially degrades into SAH, our analytical tool senses
methyltransfer rates as low as 2 μM h⁻¹ (i.e. 8 pmol min⁻¹ in
a 250 μL well at pH 8.00 and 3.5 μM h⁻¹ at pH 9.50; Fig. S4C and
D†).

We tested this coupled assay with four unique methyl-
transferases. TM0936 (4 μM) was coupled to these transferases
to establish their kinetic behavior: determination of Michaelis
constant (K_m) and catalytic turnover (k_cat) for either SAM or
acceptor substrate. Using the protein arginine methyltransfer-
ase from Caenorhabditis elegans (CePRMT5) and saturating
levels of SAM (25 μM), we determined the kinetic profile as a
function of the peptide H4L(1–20) concentration (6–180 μM, at
300 nM CePRMT5). The data fit to the Morrison equation gives
a Kiss of 26 ± 2 μM and a k_cat of 32.9 ± 0.8 h⁻¹ (Fig. 3A),
consistent with values we reported using the luciferase-based
assay (lit., K_m = 54 ± 4 μM, k_cat = 28.6 ± 0.7 h⁻¹). Another
member of the PRMT family, PRMT7 from Trypanosoma brucei
(400 nM), was also tested using saturating concentration of
peptide H4L(1–20) (200 μM). Varying SAM concentration (1.5–20.0
μM), reaction rates were determined and plotted against
cofactor concentration. The data were fitted as for CePRMT5,
giving a Kiss of 1.1 ± 0.2 μM and a turnover of 22.3 ± 0.6 h⁻¹.
Likewise, the activity from Neurospora crassa protein lysine
N-methyltransferase (histone H3 lysine-9 specific; NdDIM-5) was
successfully detected at pH = 9.50. Our data (K_m = 0.9 ± 0.1 μM,
k_cat = 30 ± 1 min⁻¹) and reported values (lit., K_m = 7.4 μM,
K_cat = 138 h⁻¹) display a 100-fold difference in the overall
catalytic efficiencies (2000 μM⁻¹ h⁻¹ vs. 19 μM⁻¹ h⁻¹, respecti-
vely). However, we used a H3(1–53) peptide while previous
report mentioned a H3(1–20) peptide harboring a N-terminal
biotinylation; such a modification, vicinal to the reactive lysine-
9, may account for the discrepancy observed. Finally, we
selected sarcosine/dimethylglycine N-methyltransferase from
Galdieria sulphuraria (GsSDMT) to represent members of the
SMMT family. We thought the 1-Step EZ-MTase assay would be
challenged by this enzyme. Indeed, GsSDMT behaves differently
from other MTases we tested: it is a fast enzyme and displays
a high micromolar K_m for SAM (lit., K_m = 144 ± 44 μM,
k_cat = 52 ± 4 min⁻¹). Yet, using a 60 μL reaction volume to
accommodate the elevated SAM levels (50–750 μM), along with
a low enzyme concentration (195 nM), we successfully
determined the GsSDMT kinetic parameters. Both the K_m of
95 ± 18 μM and the k_cat of 42 ± 2 min⁻¹ are in agreement with
the data mentioned earlier.

As we demonstrated with these four examples, the combi-
nation of a single coupling enzyme with a UV-mode of detection
and a 96-well plate format makes the characterization of
methyltransferases simple and straightforward. To further
assist the user in the treatment of her/his experimental data-set,
we developed two exhaustive template spreadsheets “My 1-Step
EZ-MTase Assay (UV) Acceptor Km.xls” and “My 1-Step EZ-MTase
Fig. 3 Monitoring methyltransfer activities using our 1-Step EZ-MTase assay and UV-mode of detection. (A) Application to the protein arginine methyltransferase from CePRMT5. Kinetic parameters using peptide H4(1–20) were determined at pH = 7.60 using 25 μM SAM and 300 nM CePRMT5: $K_m = 26 ± 2 \mu M, k_{cat} = 32.9 ± 0.8 \text{ h}^{-1}$. (B) Application to the protein arginine methyltransferase from Trypanosoma brucei (TbPRMT7). Kinetic parameters using SAM were determined at pH = 7.60 using 200 μM peptide H4(1–20) and 400 nM TbPRMT7: $K_m = 1.1 ± 0.2 \mu M, k_{cat} = 22.3 ± 0.6 \text{ h}^{-1}$. (C) Application to the protein lysine N-methyltransferase (histone H3 lysine-9 specific) from Neurospora crassa (NcDIM-5). Kinetic parameters using peptide H3(1–53) were determined at pH = 9.50 using 25 μM SAM and 7.6 nM NcDIM-5: $K_m = 0.9 ± 0.1 \mu M, k_{cat} = 30 ± 1 \text{ min}^{-1}$. (D) Application to the sarcosine/dimethylglycine N-methyltransferase from Galdieria sulphuraria (GsSDMT). Kinetic parameters using SAM were determined at pH = 7.80 using 100 μM MgCl2, 5 mM sarcosine and 195 nM GsSDMT: $K_m = 95 ± 18 \mu M, k_{cat} = 42 ± 2 \text{ min}^{-1}$. The experiments recorded at 263 nm were all performed using 4 μM final concentration of TM0936. Inserts are Z′-factors determined at multiple MTase concentrations; the lowest transferase concentration displays a $Z′ = 0.5$, a characteristic for a good HTS assay.44

**Assay (UV) CoFactor Km.xlss** (cf. ESI†). These files will help the users in (1) setting-up their experimental conditions (worksheet “Experiment conditions”), (2) importing their raw data (worksheet “Plate reader data”), (3) reporting their key experimental conditions e.g. pH, nature of methyl cofactor being used; worksheet “Coupling enzyme parameters” and (4) computing and displaying the kinetic parameters of their own experiment. The first template mentioned above is pre-loaded with experimental data pertinent to the kinetic behavior of H4(1–20) peptide with TbPRMT7, while the second template contains experimental results leading to the description of 8-aza-SAM substrate with CePRMT5. An overall summary of kinetic parameters from MTases we assayed is represented in Table 1.

**TM0936 activity is resilient to pH variations**

Another key limitation to the luciferase-based assays is their sensitivity in different chemical environments, as the detection is optimum at a very narrow pH value ($\approx 7.7$) and luminescence output is drastically reduced upon pH variations (Fig. S5A and Table S1†). As the pH decreases, so does the green component of the luminescence (Fig. SSB†); light was no longer detected under acidic conditions (pH < 6.0; Fig. S2A†).55 Therefore, key mechanistic insights are undetectable with this assay as it is impossible to describe MTase enzymology over a wide pH-range.

In contrast, TM0936 displays a sustained activity across a broad pH-range (Fig. 4A). With the 1-Step EZ-MTase assay, the transferase rates are the limiting ones at all pHs and absorbance recordings directly relate to methyltransfer. This is a characteristic to consider and take advantage of when developing a coupled enzymatic assay. As support of broad utility of this assay, we harnessed the deaminase activity of TM0936 to probe the enzymatic mechanism from a poorly characterized methyltransferase: sarcosine/dimethylglycine N-methyltransferase (SDMT). SDMT catalyzes a two-step methylation process leading to a key metabolite: betaine. Trimethylglycine is an effective methyl donor involved in the biosynthesis of 1-methionine from l-homocysteine;66 furthermore, under extreme conditions (e.g. high salt concentrations or low temperatures), this molecule stabilizes proteins acting as an osmoprotectant.57 Little mechanistic information is available regarding this enzyme, with a handful of kinetic reports54,58,59 and one single crystal structure of the apo-form of SDMT from *Galdieria sulphuraria*.54,60 In an experimental tour de force, we established the pH-dependence of GsSDMT reaction rates for sarcosine (Dixon plots; Fig. 4B). By reporting $K_m$ and $k_{cat}$ for this substrate

| MTase | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) |
|-------|------------|------------------------|
| CePRMT5 | 6.8 ± 0.3 | 31.9 ± 0.5 |
| H4(1–20) | 26 ± 2 | 32.9 ± 0.8 |
| 8-Aza-SAM | 35 ± 20 | 15 ± 6 |
| TbPRMT7 | 1.1 ± 0.2 | 22.3 ± 0.6 |
| H4(1–20) | 39 ± 3 | 28.2 ± 0.7 |
| 8-Aza-SAM | 15.3 ± 0.7 | 23.0 ± 0.4 |
| NcDIM-5 | 0.68 ± 0.2 | 3.1 |
| H3(1–53) | 0.9 ± 0.1 | 30 ± 1 |
| 8-Aza-SAM | Not a substrate | Not a substrate |
| GsSDMT | 95 ± 18 | 42 ± 2 |
| SAM | 1.7 ± 0.2 | 90 ± 5 |
| Sarcosine | 443 ± 33 | 81 ± 3 |
| 8-Aza-SAM | Not a substrate | Not a substrate |

* From P. Rathert, X. Cheng and A. Jeltsch, *BioTechniques*, 2007, 43, 602, 604, 606.
The log $k_{\text{cat}}$ plotted against pH (Fig. 4B, right; ESI eqn (S9)†) established that reaction rates increased with pH and reached a maximum value at pH $= 6.82$. This plot reports the ionization state of an important residue from the GsSDMT–SAM–sarcosine complex. Our results support our hypothesis that deprotonation of a crucial histidine may enhance the methyltransfer reaction.

One single structure of SDMT has been reported and the apo-form of this enzyme makes it difficult to support our experimental results. However, we successfully superimposed this structure (PDB: 2057) with the SAH–sarcosine complex of the glycine sarcosine N-methyltransferase from Methanohalophilus portucalense (MpGSMT; PDB: 5HIL). Both structures display a good alignment of their N-terminus (Fig. S6;† light shades) and the cofactor binding site was easily identified within this conserved domain. Indeed, SAM interacts with key conserved amino-acids: $\pi$-stacking between adenosine and W115, stabilization of the ribosyl through hydrogen bond with D88 (F141 and N112 from GsSDMT are predicted to be homologous). Likewise, the homocysteyl binding-mode depicted additional groups involved in stabilization of the cofactor. Residues R60, A91 and Q157 from GsSDMT are structurally homologous to R43, A67 and L132 from MpGSMT, respectively, thus we hypothesize their interaction with the homocysteyl moiety from SAM/SAH (Fig. S6†). Although the sarcosine binding site is located within the most divergent region of the structures (dark shades; C-terminus), we identified Y242 (Y206 structural homolog in GsSDMT) as potential candidates involved in sarcosine stabilization via hydrogen bond with the carboxylate tail. Furthermore, the histidine H162 from GsSDMT, also present in MpGSMT (H138), may influence methyltransfer rate since it is equidistant from both nitrogen and sulfur reactive centers from sarcosine and SAH, respectively (Fig. S6†).

The conclusive assignment of our experimental $pK_a$ values to definite residues or chemical groups requires further work, such as site-directed mutagenesis of GsSDMT. Yet, establishing this data set is a first step towards the elucidation of a more elaborate enzymatic mechanism.
making FLUC-based assays more difficult. Importantly, TM0936 was not affected by increasing levels of salt. In our hands, the deaminase steadily catabolized adenosine (10 μM), even at sodium chloride concentrations as high as 2 M (Fig. 4C). This property, specific to TM0936, may facilitate future experimental set-up.

To test the utility of this new assay in varied ionic conditions we measured histone methyltransferase activity. Histone tails are positively charged under physiological conditions, as they are enriched in lysine and arginine residues; this electrostatic property may partially account for binding of these substrates onto MTase target. Using \( \text{TbPRMT7} \), we determined the kinetic behavior for H4(1-20) peptide at four phosphate concentrations \((pH = 7.60, 25-100 \text{ mM})\). The initial rates were plotted against peptide concentrations (Fig. 4D, left). The data fit to the Michaelis–Menten equation \((\text{ESI eqn (S5)})\) gives four \(K_m\) values (μM) of 39 ± 3, 93 ± 5, 212 ± 7, 413 ± 8 at 25, 50, 75 and 100 mM phosphate concentrations, respectively (Fig. 4D, right). As the buffer concentration increased, so did the \(K_m\) value for the histone peptide, thus reflecting a loss of affinity between substrate and PRMT7. Our data are in good agreement with the isothermal titration calorimetry experiments performed with the same PRMT7/peptide pair. While a 20-fold decrease in affinity between peptide and PRMT7 was observed when varying salt concentration from 20 to 150 mM, a 300 mM salt concentration precluded binding event (lit., \(K_d = 400 \text{ 000 \mu M}\)).

1-Step EZ-MTase is well suited for high-throughput screening

Our analytical tool provides an alternative to overcome major drawbacks from previous MTase assays (Fig. S1†); methods involved as much as four coupling enzymes, and the single deaminase activity from 1-Step EZ-MTase may decrease the risk for off-target inhibition and apparition of false positives. A high sensitivity and wide dynamic range of detection make this assay very competitive. Methyltransferase rates as low as 2 μM h⁻¹ are detectable and the use of nonomolar MTase concentrations is also achieved, important as many eukaryotic MTases are difficult to purify in quantity sufficient for other assays (Fig. 5A and B). Very good values of screening window coefficient (Z’-factor) are obtained and always above 0.50, reaching maximum values of 0.87, 0.93, 0.80 and 0.92 for CePRMT5, TbPRMT7, NcDM-5 and GsSDMT, respectively (Fig. 3; inserts).\(^{44}\) Such high values reflect the overall quality of the 1-Step EZ-MTase assay. Our analytical tool is well suited for HTS, with a signal displaying low variability and a good separation from background (e.g. SAM decomposition; Fig. S4E†).

Sinefungin is a potent, yet non-selective inhibitor of MTases, and frequently is used as a positive control during inhibitor screening. Sinefungin is a known inhibitor of the SAHH enzyme,\(^{36,65}\) while MTAN catabolizes sinefungin into adenine (Fig. 6A and Table S1†). Thus, most enzyme-coupled assays for MTase analysis are incompatible with this molecule (Fig. S1† arrows 4–10). We evaluated the possibility to use sinefungin within the 1-Step EZ-MTase assay and established the reactivity profile between TM0936 and this chemical. Unlike other coupling enzymes, TM0936 displayed moderate reactivity towards this inhibitor and deamination of sinefungin was slower at \(pH = 6.80\) than at \(pH = 8.00\) (Fig. 6B). Indeed, the coupling enzyme deaminates the inhibitor very slowly under acidic conditions \((pH \leq 6.80, 125 \text{ nM min}^{-1})\); Fig. 6C). As pH increases, so does the rate of deamination. The \(-\text{NH}_2^{+}\) group from sinefungin is deprotonated into \(-\text{NH}_3^{+}\) thus becoming a lesser mimic of the CH₂-S⁻ group from SAM \((pH = 8.00, 760 \text{ nM min}^{-1})\); Fig. 6C). This observation is in good agreement with TM0936 inability to catabolize SAM.

Taking advantage of this substrate selectivity, we measured \((pH = 6.80)\) the inhibition constant \((K_I)\) for sinefungin against GsSDMT (Fig. 6D). Under our experimental conditions, sinefungin was a potent inhibitor of the reaction between sarcosine and SAM catalyzed by GsSDMT \((K_I = 1.8 \pm 0.4 \text{ μM})\). This result demonstrated the compatibility of our enzyme-coupled assay with sinefungin.

8-Aza-SAM and its application to the 1-Step EZ-MTase assay

A second mode of detection, complementary to the current UV-based assay, may present advantages. The use of a fluorescent cofactor analog may improve detection specificity compared with UV absorption. 8-Aza-adenosine is an isosteric and fluororescent analog of the nucleoside. When excited at 282 nm, this probe exhibits a strong fluorescence signature with a maximum at 360 nm.\(^{58}\) This characteristic is not shared with the 8-aza-adenosine and this deaminated product is a weak fluorophore.\(^{58}\) Several SAM analogs, including the 8-aza modification, are biologically active and display affinity with the SAM-III riboswitch, the EcoRI methyltransferase and other MTases.\(^{64,66}\) Therefore, we anticipated this probe may be used in place of the natural cofactor.

We successfully resynthesized 8-aza-SAM through reaction between 8-aza-ATP and 1-methionine.\(^{68}\) SAM isomerizes readily at the sulfonium center and only the \((S)\)-epimer is biologically active. Thus, we performed a short reaction at 35 °C [enzyme : triphosphate, 1 : 400] to limit such epimerization and yield 8-aza-SAM (65% yield, 5% \((R)\)-epimer; cf. ESI† NMR analysis). Although 8-aza-ATP is commercially available (TriLink Biotechnologies, \#N-1004), this molecule is cost prohibitive for this assay. Therefore, we synthesized this chemical through multiple phosphorylation of the more affordable 8-aza-adenosine. This approach is reminiscent of a strategy we previously applied towards the preparation of a C-P lyase inhibitor.\(^{67}\) Performed on a 50 mg scale using a 2 mL tube, the one-pot synthesis provides an easy access to high quantities of pure 8-aza-ATP (95%; cf. ESI† Enzymatic syntheses).

We further assayed the reactivity of 8-aza-SAM towards our methyltransferases. This analog was well tolerated by our candidates since only NcDM-5 was unable to process this cofactor. Likewise, the 8-aza-SAH product of methyltransfer reactions \((\lambda_{\text{max}} = 280 \text{ nm})\) was successfully converted into the deaminated 8-aza-SIH \((\lambda_{\text{max}} = 254 \text{ nm})\); Fig. 5C). In fact, the deamination of adenosine by TM0936 is 10-fold slower than that of its 8-aza analog (Fig. 4A). Using the UV-based assay, we determined the 8-aza-SAM kinetic parameters with CePRMT5 at saturating concentration of H4 peptide (Fig. 5D). Turnover was...
moderately affected and the enzyme processed SAM twice as fast as its 8-aza-analog ($k_{cat} = 31.9 \pm 0.5$ h\(^{-1}\) vs. $15 \pm 6$ h\(^{-1}\), respectively; Fig. 5B and D). The catalytic efficiency for 8-aza-SAM was one order of magnitude lower than that of the natural cofactor (120 M\(^{-1}\) s\(^{-1}\) and 1300 M\(^{-1}\) s\(^{-1}\), respectively). Unlike SAM, the 8-aza analog displayed significant substrate inhibition ($K_i = 67 \pm 42$ µM; Fig. 5D). When assayed against either *Th*PRMT7 or *Gs*SDMT, this inhibitory behavior of the analog was not observed (Table 1).

With the relevance of 8-aza-SAM validated, we then calibrated the fluorescence signal at 360 nm for the 8-aza-adenosine (8-aza-A) to 8-aza-inosine (8-aza-I) reaction using two excitation wavelengths (*i.e.* 282 and 292 nm). The 282 nm excitation provided a higher limit of detection for 8-aza-A and
Applicability to biological samples: detection of GNMT activity

Glycine N-methyltransferase (GNMT) is a key component of SAM homeostasis. As a methionine-rich diet replenishes the SAM pool, the increasing concentration of methyl donor inhibits the 5,10-methylene-tetrahydrofolate reductase, thus impairing the 5-methyltetrahydrofolate (5-CH3-THF) synthesis. GNMT is a folate-binding protein and 5-CH3-THF is deleterious to its activity.69,69 Within this feedback mechanism, the alleviating GNMT inhibition promotes SAM consumption through sarcosine synthesis.79 GNMT establishes the cross talk between the one carbon folate pathway and the methionine cycle, thereby maintaining a healthy SAM/SAH ratio, which is indicative of methylator potential.74,75

Development of improved assays for probing GNMT activity within biological samples is important to accelerate understanding of the interplay among metabolic pathways, energetics, epigenetics and cancer metabolism.73–75 However, classical methods using tritiated SAM, and the detection of radioactive sarcosine within crude protein extracts is a tedious and discontinuous approach. Furthermore, valuable tissue samples from animal studies may be limited and the requirement for large amounts of extract (e.g. 250 μg total protein) may be challenging when using this method.72 To overcome these drawbacks, we optimized the 1-Step EZ-MTase platform and made it compatible with biological samples. Using protein extracts from rat liver, we successfully quantitated GNMT activity within these crude biological samples (Fig. 8).

The deamination reaction catalyzed by TM0936 only occurs with SAH, methylthioadenosine and adenosine.44 The substrate specificity of our coupling enzyme makes it compatible with the highly complex content of biological samples. Luciferase-based assays (Fig. S1, arrows 4, 9 and 10) are not suited for such a type of sample where adenine and phosphorlated adenosine species generate high background signal. Likewise, endogenous thiol species (e.g. glutathione, homocysteine, cysteine residues from proteins) preclude the continuous detection of GNMT activity through efficient derivatization of homocysteine (e.g. Ellman’s reagent, ThioGlo®-1; Fig. S1, arrows 6 and 7). In addition to reacting with these thiol species, the 5,5′-dithio-bis-(2-nitrobenzonic acid) completely inactivates the methyltransferase upon reaction with its cysteine residues.76

In our hand, GNMT activity was not affected by the tissue lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH = 7.4, 1% Triton X-100, 1 mM orthovanadate, 1 mM EDTA, 10 mM NaF, 1× protease inhibitor cocktail, and 1 mM PMSF), thus establishing the compatibility between this universal reagent and the 1-Step EZ-MTase (Fig. 8A and B). With its low 2 μM h−1 SAH-detection limit (Fig. S4D†), our analytical tool successfully senses methyltransfer catalyzed by GNMT. At 75 μM SAM concentration, endogenous glycine was not sufficient for sarcosine synthesis catalyzed by rat GNMT (Fig. S7†). Upon addition of saturating glycine (20 mM), a 2.28-fold increase of methyltransfer rate was observed (Fig. S7A† 2.38-fold increase as monitored with the radioactive assay, Fig. S7B†). Our observations are in good agreement with the estimated 50–100 μM endogenous glycine concentration (2.37 mM in rat liver)77 and a Km value for glycine of 130 μM.78

The decrease of absorbance at 263 nm is concentration dependent and as low as 30 μg of total protein were sufficient to monitor sarcosine synthesis over an extended period (Fig. 8C). Our platform surpasses the performance of the tedious radioactive assay (Fig. 8D) by only requiring a fraction of fluorescence signal was most intense (~2-fold; Fig. 7A). Plotted against 8-aza-A concentrations (0–25 μM), the variation of fluorescence (∆FLUO) observed over deaminase reaction fits a polynomial equation (ESI eqn (S10) and Table S2†). Furthermore, experiments performed at various pHs (5.00–9.50) support the weak fluorescence properties of 8-aza-I. The deaminated product is a poor fluorophore, it does not emit light under acidic conditions and its fluorescence is 10-fold weaker than that of 8-aza-A at pH = 9.50 (35 RLU μM−1 vs. 380 RLU μM−1; Fig. 7A, insert). Thus, at the early stage of methyltransfer reaction (i.e. less than 10% of 8-aza-SAM consumed), the 8-aza-SIH contribution to the fluorescence is negligible and accounts for less than 1% of the signal.

To establish the utility of a fluorescence mode of detection (Table S1†), we measured kinetic parameters for the transfer reaction catalyzed by TbPRMT7 using 8-aza-SAM and various concentration of H4(1–20) peptide (Fig. 7B). The data fit to the Michaelis–Menten equation (ESI eqn (S5)†) gives Km and kcatal values of 71 ± 8 μM and 39 ± 2 h−1, respectively (Fig. 7B). Similar to the UV-mode of detection and to facilitate data analysis, we developed a third template spreadsheet “My 1-Step EZ-MTase Assay (FLUO) Acceptor Km.xlsx” (cf. ESI†). This file contains the calibration curves for the 8-aza-adenosyl to 8-aza-inosyl reaction at various pHs and is pre-loaded with experimental data pertinent to the kinetic behavior of H4(1–20) peptide with TbPRMT7 using 8-aza-SAM cofactor.

Fig. 7 The use of 8-aza-SAM and a fluorescence-mode of detection within the 1-Step EZ-MTase assay. (A) Calibration curves for the deamination of 8-aza-adenosine by TM0936. The deamination reactions using 8-aza-A (0–25 μM) were monitored through light emission at 360 nm with both 282 and 292 nm excitation wavelengths (white and black circles, respectively). Experiments were carried out at pH = 5.00 (black curve fit) and pH = 9.50 (red curve fit). The reaction product, 8-aza-inosine (8-aza-I), is a poor fluorophore: it does not emit light under acidic conditions and its fluorescence is 10-fold weaker than that of the 8-aza-A substrate at pH = 9.50 (insert). (B) Kinetic behavior for H4(1–20) peptide against TbPRMT7 using 8-aza-SAM cofactor. The Km and kcatal for H4 peptide were determined using the fluorescence-mode of detection.
The methyltransfer (MT) catalyzed by rat GNMT is detected through loss of absorbance at 263 nm. The MT rates (black bars) and the 1-Step EZ-MTase coupled assay (UV; grey bars). The e

within rat liver extracts. Two tissue samples from a same liver were prepared (1 and 2). GNMT activity was measured with both the radioactive (R; tritiated sarcosine product of the GNMT catalyzed reaction is isolated through solid phase extraction with charcoal. (E) GNMT activities measured within rat liver extracts. Increasing volumes of extract (6–24 μL) were mixed at pH 8.00 with SAM, glycine and TM0936 (75 μM, 20 mM and 4 μM, respectively). The methyltransfer (MT) catalyzed by rat GNMT is detected through loss of absorbance at 263 nm. The MT rates (μM h⁻¹) are proportional to liver extract volumes (insert). (D) The traditional discontinuous and radioactive assay for detection of GNMT activity. Using radioactive SAM, the tritiated sarcosine product of the GNMT catalyzed reaction is isolated through solid phase extraction with charcoal. (E) GNMT activities measured within rat liver extracts. Two tissue samples from a same liver were prepared (1 and 2). GNMT activity was measured with both the radioactive (R; black bars) and the 1-Step EZ-MTase coupled assay (UV; grey bars). The effect of three freeze–thaw cycles from a same liver were prepared (1 and 2). GNMT activity was measured with both the radioactive (R; black bars) and the 1-Step EZ-MTase coupled assay (UV; grey bars).

To facilitate the quantitation of GNMT activity within crude biological samples, we developed a customized template spreadsheet “MY 1-Step EZ-MTase Assay (UV) GNMT Activity BioSample.xlsx” (cf. ESI†). This file provides a framework for the user to determine GNMT activity expressed in μM h⁻¹ and pmol min⁻¹ mg⁻¹ after importing raw data from a plate reader.

**Conclusion**

Epigenetic modifications catalyzed by methyltransferases play a central role in gene transcription and parental imprinting. A correlation between dysregulation of methylation patterns and occurrence of human diseases (e.g. cancer, diabetes) is becoming more obvious. Several methyltransferases are now validated therapeutic targets. The regulation of these enzymatic activities by specific and potent inhibitors may offer new opportunities for patients. To promote our understanding of methyltransferases and the discovery of new chemotherapeutics, we have developed a simple and straightforward assay to study this class of enzymes. Unlike anything else available, this analytical tool harnesses the power of one single protein: the SAH-deaminase TM0936. The coupling enzyme is an easily accessible and robust catalyst that allows for quick and facile determination of enzymatic rates through monitoring of absorbance at 263 nm. We demonstrate its utility by coupling it to a panel of four enzymes, including lysine and arginine methyltransferases. A 96-well plate format allows high-throughput performance, and the high Z' factors reflect the overall quality of this assay. Furthermore, sinefungin is compatible with this coupled assay, suggesting this platform may have a significant impact on the identification of new inhibitors using high-throughput screening. We implemented a second mode of detection, providing users with the option to detect methyltransfer through monitoring the loss of fluorescence at 360 nm. This approach relies on 8-aza-SAM, a fluorescent analog of the universal methyl donor. We established the relevance of this alternative cofactor and determine kinetic properties of the PRMT7 from Trypanosoma brucei using H4(1–20) peptide. Our analytical tool detects methyltransfer rates as low as 2 μM h⁻¹ and is able to sense low nanomolar concentrations of GNMT within crude biological samples. Overall, the 1-Step EZ-MTase surpasses the performances of other techniques while retaining a compact and simple format with broad applicability (Table S1†). Finally, to further reduce data processing, we provide the users with worksheets in which absorbance/fluorescence recordings can be imported and the templates calculate key kinetic parameters of methyltransferases (Km, kcat, activity and curve fit).
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