Site-selective and Re-writable labeling of DNA through Enzymatic, Reversible and Click Chemistries.

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For Table of Contents Only

Synopsis: We present a method using MTases, acyl hydrazone and click chemistry to site-selectively tag, un-tag and re-write on DNA. Sequential labeling of DNA or sorting PCR DNA demonstrate its versatility.
Site-selective and Re-writable labeling of DNA through Enzymatic, Reversible and Click Chemistries.

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ABSTRACT: Current methods for bioconjugation rely on the introduction of stable linkers that lack the required versatility to perform sequential functionalizations. However, sequential manipulations are an increasing requirement in chemical biology because they can underpin multiple analyses of the same sample to provide a wider understanding of cell behavior. Here, we present a new method to site-selectively write, remove and re-write chemical functionality to a biomolecule, DNA in this case. Our method combines the precision and robustness of methyltransferase-directed labeling with the reversibility of acyl hydrazones and the efficiency of click chemistry. Underpinning the method is a new S-adenosyl-L-methionine derivative to site-selectively label DNA with a bifunctional chemical handle containing an acyl hydrazone-linker and a terminal azide. Functional tags are conjugated via the azide, and can be removed (i.e. un-tagged) when needed at the acyl hydrazone via exchange with hydroxyl amine. The formed hydrazide-labeled DNA is a versatile intermediate that can be either re-written to reset the original chemical handle, or covalently reacted with a permanent tag. This ability to write, tag, un-tag and permanently tag DNA is exploited to sequentially introduce two fluorescent dyes on DNA. Finally, we demonstrate the potential of the method by developing a protocol to sort labeled DNA using magnetic beads, with subsequent amplification of the sorted DNA sample for further analysis. The presented method opens new avenues for site-selective bioconjugation and should underpin integrative approaches in chemical biology where sequential functionalizations of the same sample are required.

Introduction

Established and emerging approaches for studying biomolecules rely on their conjugation with chemical groups or functional tags (Figure 1A).1–4 The introduced functionalities enable manipulations that go from simple extraction and purification from complex mixtures to advanced analytical studies. This way bioconjugation has enabled research into post-translational modifications and the biological processes where these biomolecules are involved and, more recently, the exploitation of these molecules for application.5–10 Critically, being able to introduce these chemical handles in a precise location of the biomolecule minimizes the impact that bioconjugation has on secondary structure and activity, and can throw light on modifications and regulation of these biomolecules. Common methods for site-selective functionalization of these biomolecules include introducing site-specific mutations or the development of selective chemical handles that discriminate similar reacting groups within these molecules.11–17

In recent years, there has been a growing interest in the use of chemoenzymatic methods to label biomolecules.18–20 These methods not only introduce site-selective chemical modifications but can label these biomolecules in complex mixtures, such as living cells or their lysates. Methyltransferases (MTases) are emerging as a key class of enzymes for the site-selective functionalization of biomolecules because of their versatility in terms of targets and functional groups they can accommodate.21–25 Most MTases are responsible for transferring a methyl group from the naturally occurring cofactor S-adenosyl-L-methionine (AdoMet), and have been identified with targets as diverse as small molecules, carbohydrates, proteins and nucleic acids. Most importantly, a wide range of MTases can accommodate larger groups in their binding pocket, so that by manipulating the chemical structure of AdoMet it is possible to hijack MTase machinery to introduce functional groups to biomolecules.21–28 Commonly, MTase-di-
rected ligation is used to introduce “clickable” groups that are used to label the biomolecules with fluorescent and other functional tags (Figure 1A).\textsuperscript{26-31} MTase-directed ligation is now finding application in imaging, and in genomic and metabolomic analysis.

**Figure 1** Schematic representation of methods for labeling of biomolecules. A) Current methodologies are based on the introduction, via one or multiple steps, of stable chemical linkers. Common challenges include site-selectivity, tolerance to functional groups or reversibility. B) Our method achieves site-selective MTase-directed *labeling* of DNA \textsuperscript{①} to then introduce chemical *tags* via azide-alkyne cycloaddition \textsuperscript{②}. This introduced functionality can then be removed (i.e. *un-tagged*) via exchange at the Schiff-base \textsuperscript{③}, to give an intermediate hydrazide-labeled DNA that can be *re-written* via Schiff-base formation \textsuperscript{④} to give the original functionality introduced via MTase-labeling. Alternatively, this hydrazide-labeled DNA can be functionalized via covalent chemistry to introduce a *permanent tag* if needed \textsuperscript{⑤}.

Invariably, chemical and enzymatic strategies to label biomolecules have been focused on developing stable linkages that rely on the covalent attachment of functional tags. However, this approach often results in the modification of the biomolecule with bulky functional groups that can compromise the chemical and physical properties of the targeted biomolecule. In recent years, efforts have been made to develop reversible chemistries for bioconjugation that let scientists remove the introduced chemical functionality once it has served its purpose.\textsuperscript{5,16,17} For instance, recent examples in the field of MTase-directed labeling have explored the use of disulfide linkers\textsuperscript{32} or of light-cleavable moieties,\textsuperscript{33,34} that can be cleaved under the right circumstances. Invariably however, current bioconjugation techniques lack the versatility to be re-written if needed and limit the study of these biomolecules to a single analytical process. This approach is at odds with the current trends in cell biology, where integrative approaches that combine multiple analytical methods are needed to provide a holistic understanding of cell behavior.\textsuperscript{35-38} This need is particularly true in single-cell analyses, where material is limited.

In order to address these limitations, here we present a new method for the functionalization of nucleic acids. The selectivity and robustness of MTase-directed labeling is combined with the reversibility of Schiff-bases and the efficiency of click chemistry to introduce a bifunctional chemical handle on DNA that allows us to perform multiple functionalizations (Figure 1B). To this end, we have developed a new class of *AdoMet* derivatives, containing an acyl hydrazone or oxime linker and a terminal azide at the sulfonium center. First, we demonstrate that representative MTases are able to accommodate these bifunctional cofactors to site-selectively label DNA, including oligonucleotide and plasmid DNA. Then, we identify that the acyl hydrazone-linker is amenable to cleavage via exchange with H\textsubscript{2}NOH-HCl, providing a route to *un-tag* the chemical functionality introduced. Furthermore, we demonstrate that the formed
hydrazide-labeled DNA can be re-written to recover the original chemical handle. Alternatively, this hydrazide-labeled DNA can be covalently functionalized using non-reversible chemistries to introduce permanent tags if required. The potential of the method to underpin new discoveries at the interface between chemistry and biology is demonstrated through the sequential labeling of DNA with two fluorescent dyes, and the development of a new protocol to sort fragments of DNA using magnetic beads. Labeled DNA fragments are sorted under mild conditions with high yields, remaining functional to be then amplified by real time polymerase chain reaction (real time PCR).

**Results & Discussion**

**Cofactor Synthesis – AdoMet Derivatives Carrying Schiff-Base Linkers And A Terminal Azide**

Our initial aim was to develop an AdoMet analog that carried a reactive and re-writable (or reversible) chemical handle. While several strategies are available to introduce reactive moieties to AdoMet derivatives, few of these are reversible, and none allow repeated functionalization of the target site. An ideal reactive linker should carry a chemical functionality orthogonal to common biological moieties (e.g. hydroxyl, amino and carboxyl). In nature, Schiff-bases are commonly used to this end and chemists have now exploited the versatility of the C=N bond to develop a plethora of applications for this dynamic chemistry. The stability of the C=N bond can be tailored as a function of the “amine” used, with hydrazides and alkoxyamines demonstrating the largest stability ranges. In particular, Dawson et al. have exploited this versatility to reversibly tag biotin onto proteins through an acyl hydrazone linker. The biotin-modified protein could be captured via affinity chromatography to then be released under mild conditions via competitive exchange. While this work clearly demonstrates the potential of Schiff-bases to reversibly label biomolecules, the site-selective modification of biomolecules with these chemistries still remains a challenge.

With these principles in mind, we prepared linkers 1a and 1b, carrying the desired hydrazide or alkoxyamine moieties protected as tert-butylcarbamates (see Section 3.1-3.7 in the Supporting Information for details). These linkers were then reacted with S-adenosyl-L-homocysteine (AdoHcy) under standard conditions to give Boc-protected AdoMet derivatives 2 (Scheme 1). Although MTases are not particularly affected by impurities in the cofactor mixture, the excess of linker 1 was removed via extraction with diethyl ether because the presence of this linker could result in non-specific alkylation of DNA in subsequent assays. The crude AdoMet derivatives 2 obtained this way were freeze-dried and immediately deprotected under acidic conditions. Purification of the hydrazide- and alkoxyamine-AdoMet derivatives 3 was performed by HPLC, to separate both diastereomers of each cofactor 3 (Figure S20 and Figure S23), a separation that was not possible at later stages. Our initial intention was to use these cofactors to introduce the reactive moiety in DNA but these deprotected AdoMet derivatives 3 slowly degraded (Figure S26-Figure S27), in particular following freeze-drying (Figure S28). For instance, HPLC analysis of cofactor 3b after freeze-drying revealed the presence of an additional peak at higher retention times (~31 min). MS analysis of this peak suggested a mass of 536.61 Da, very close to that of the alkoxy AdoMet derivative (537.62 Da). This difference in molecular mass, together with the increase in retention time, suggested degradation of 3b was also occurring via intramolecular nucleophilic attack of the terminal amine of the linker to the sulfonium center (Figure S28).
Scheme 1 – Synthesis of AdoMet derivatives 4. Conditions: i) HCO₂H, AcOH, 35 °C; ii) TFA; iii) 1.- TFA, 2.- 5, 20 mM ammonium acetate pH 5.5 water /MeOH. Full details can be found in the Supporting Information.

Degradation of AdoMet and derivatives is common but does not compromise their application. As just mentioned, MTases can promote transalkylation even with the complex mixture obtained upon degradation. Moreover, degradation is normally suppressed upon storage of these cofactors at low temperature and in a mildly acidic buffer (in our case 0.1% acetic acid 15-20 mM in cofactor). However, we decided to minimize the degradation observed via this intramolecular rearrangement (Figure S28). To this end, AdoMet derivatives 3 were reacted immediately after purification by HPLC with commercially available benzaldehyde 5 (Scheme 1). A slight excess of aldehyde 5 (1.2 equiv.) was employed to ensure full functionalization of the intermediate 3, and the obtained cofactors 4 freeze-dried and stored in 0.1% acetic acid solution without further purification. The cofactor analogues 4 that were formed now contained a reactive terminal azide, suitable for tagging via standard azide-alkyne cycloaddition and with the additional advantage of expanding the range of functional molecules that we had access to in the tagging step of our cycle (Figure 1). Additionally, cofactors 4 carried a Schiff-base linker that maintained the reversible functionality, giving us a route to remove and re-write chemical functionality. The chemical identity of these cofactors was confirmed via HRMS (Section 3.10 in Supporting Information). No degradation of AdoMet derivatives 4 was observed following this protocol (Figure S31, 4b as a representative example), and these cofactors could be used in the reversible labeling of DNA without further purification.

Writing Chemical Functionality – MTase-Directed Labeling

Following successful synthesis of AdoMet derivatives 4, a restriction assay was used to demonstrate that representative MTases could accommodate cofactors 4 to efficiently and site-selectively label DNA. In this assay, M.TaqI, an N6-adenine DNA MTase, was used to label pUC19 DNA, which has four recognition sites (TCGA) for this enzyme (Figure 2). Successful alkylation of DNA by M.TaqI results in protection of the plasmid from restriction digestion by R.TaqI, an endonuclease with the same target site as M.TaqI. Our intention was not only to demonstrate that cofactors 4 could be employed by a relevant MTase, but also that they could modify DNA with a complex topology (Figure 2B).
Figure 2 – MTase-directed labeling of plasmid DNA. (A) Schematic representation of restriction assay and (B) gel electrophoresis of pUC19 following enzymatic treatment with M.TaqI and/or R.TaqI in the presence and absence of AdoMet (375 μM) or AdoMet derivative 4b. In the absence of M.TaqI-mediated alkylation (lanes 4, 8 and 12), pUC19 is cut into fragments, of which the largest three can be identified by gel electrophoresis. M.TaqI-mediated alkylation with AdoMet (line 10) or derivative 4b (lanes 1-3 and 5-6) results in partial to full protection from restriction by R.TaqI, with mainly open circular and supercoiled plasmid DNA being observed by gel electrophoresis. Controls in the absence of AdoMet derivatives (lanes 11 and 12), in the absence of M.TaqI (lanes 4, 8 and 12) and in the absence of R.TaqI (lane 9) are included.

The native M.TaqI substrate, AdoMet, was used as a positive control for these experiments. In the absence of cofactor, M.TaqI is unable to alkylate pUC19 and bands corresponding to the three biggest DNA fragments formed after digestion with R.TaqI were observed (Figure 2, lane 11). A similar effect is observed in the absence of M.TaqI (Figure 2, lane 12), demonstrating that neither isomer of AdoMet derivative 4b interferes with the ability of R.TaqI to digest plasmid DNA (Figure 2, lane 4 and 8). More importantly, in the presence of both diastereomers of 4b, M.TaqI was able to functionalize pUC19, although with different efficiencies, and with limited evidence of DNA digestion. In this case, only bands corresponding to open circular or supercoiled DNA were observed (Figure 2, lanes 1-3 and 5-7), similar to those observed when AdoMet was used (Figure 2, lane 10) or when no digestion was performed (Figure 2, lane 9). Dilution of the amount of cofactor used revealed that the second fraction had a higher activity. A similar effect was observed for the acyl hydrazide derivative 4a (Figure S3.2), demonstrating that both analogues had the potential to be employed for the labeling of biomolecules. The reactivity of both diastereomers was unexpected, as it is commonly assumed that MTases are stereoselective,27 and thus we considered that isomerization around the sulfonium center was occurring in the assay conditions. However, no isomerization was observed by HPLC following incubation at 50 °C of the second isomer of cofactor 4b (Figure S3.1). Thus, we can not rule out that the activity of M.TaqI with both isomers of 4b is due to a lack of specificity (for this stereocenter) of this MTase,22,50 isomerization in the presence of the MTase, or the presence of small amounts of the active stereoisomer.

Having demonstrated that M.TaqI was able to catalyze transalkylation of plasmid DNA with cofactors 4, we then decided to test whether other relevant DNA MTases could use these AdoMet derivatives as cofactors. M.MpeI is a cytosine-C5 MTase. Much like human DNMT1, this bacterial enzyme targets the CpG dinucleotide, whose methylation is involved in the mechanism of gene regulation in vertebrates.32,51,52 Thus, pUC19 was incubated with mutant M.MpeI (Q136A, N347A) and 4a, and then challenged with R.HaeI, a restriction enzyme that targets a subset of the CpG dinucleotides.53 To our delight, we also observed efficient transalkylation of plasmid DNA using this MTase (Figure S3.3), although only isomer II seemed to show significant activity with this enzyme.

Further evidence of the ability of MTases to alkylate DNA with AdoMet derivatives 4 was carried out using a 14-base pair (bp) oligonucleotide with one copy of the M.TaqI sequence (TCAΔ) in the center. MTase-directed labeling was achieved in the same way as the protection assay described above, however restriction digestion was not carried out. Our intention here was to monitor the MTase-directed labeling of the oligo DNA via HPLC, so that further details about the labeling process could be observed. Following
incubation of the oligo DNA with M.TaqI in the presence of AdoMet or AdoMet derivatives 4, samples were analyzed by HPLC and MS. HPLC analysis was performed above the melting temperature of the DNA so that both strands could be clearly identified in the chromatogram (Figure 3). A clear shift in the retention time was seen upon labeling with AdoMet (Figure S36), 4a or 4b (Figure 3, middle) when compared to the retention times of the unmodified DNA (Figure 3, top and Figure S35). This shift was observed for both peaks, demonstrating that M.TaqI was able to label both strands as a consequence of the palindromic nature of the sequence this MTase recognizes. Moreover, the shift was proportional to the size and nature of the linker transferred, with the AdoMet methylation resulting in a small shift in retention time (Figure S36) and the oxime derivate 4b giving the biggest shift (Figure 3). In these chromatograms, we could also see what we expected to be, and later confirmed, free hydrazide (Figure 3, left, peaks at ~ 13 min) and hydroxylamine (Figure 3, right, peaks at ~ 19 min). The presence of this small amount of un-tagged oligo DNA is likely due to hydrolysis under the HPLC conditions. Analysis of the individual peaks was carried out using MS, which confirmed labeling was successful and that only one linker per DNA chain had been conjugated. MS also confirmed the nature of the sidechain functionality introduced following incubation (Figure S35-Figure S44).

**Figure 3** - *Writing and un-tagging* chemical functionality on oligo DNA. Analytical HPLC chromatograms of oligo DNA (top) and oligo DNA following incubation with M.TaqI and 4 (middle), and with M.TaqI and 4, followed by incubation with 10 equiv. of H$_2$NOH-HCl in 10 mM ammonium acetate pH 4.0 (bottom). HPLC conditions: 0.1 M triethylammonium acetate buffer, pH 7.0 (A)/MeCN (B) gradient at 60 ºC. Under these conditions, oligo DNA melts and both strands of DNA can be observed independently.

**Un-Tagging** The Introduced Functionality – Competitive Exchange Of The Schiff-Base

Following successful labeling of the oligo DNA, these samples were then used to demonstrate the reversible nature of the Schiff-base introduced and its potential to be efficiently cleaved. Aliquots of labeled oligo DNA were incubated at 50 ºC for 1.5 h with 10 eq. of H$_2$NOH-HCl. This competing reagent was introduced to facilitate exchange with the Schiff-base and the pH of the samples was adjusted to pH 4.0.[4,5] HPLC analysis showed a clear shift in the retention time of the acyl hydrazone-labeled DNA following treatment with the competing reagent (Figure 3, left). Over 85% of the functional linker was cleaved under these conditions. The new peak shifted to lower retention time, as expected following the loss of the potentially hydrophobic aldehyde 5. MS analysis confirmed that these peaks (~13 min) corresponded to the hydrazide-labeled oligo DNA (Figure S46-Figure S47). Conversely, under these conditions the oxime-labeled DNA remained intact (Figure 3, right and Figure S48), consistent with the higher stability of this type of Schiff-base.[56]

Incubation of DNA with hydroxyl amine has been associated with the induction of mutations as a result of a nucleophilic attack to pyrimidine bases to give 6-hydroxylamino-5,6-dihydroxycytidine and N4-di-
Moreover, loss of purine bases has been reported at pH 4.59 To evaluate whether these side-reactions were being observed under the conditions used for cleavage of the Schiff-base, analysis of the HRMS for each of the oligo DNAs was performed. To our delight, no evidence of these side-reactions was observed. We believe the absence of DNA damage is the result of the low concentration of H2NOH·HCl used (typically 20 mM) and the short incubation times (typically 1.5 h).

**Re-Writing The Original Functionality Via Schiff-Base Formation**

The next stage was to demonstrate that the labeled DNA could be re-written to recover the original functionality introduced during the MTase-directed labeling with cofactor 4a (Figure 4A). To this end, the hydrazide-functionalized oligo DNA (Figure 4B) was incubated in the presence of an excess of aldehyde 5. As predicted, a clear shift in the retention time of the main peaks associated with oligo DNA was observed (Figure 4C). Comparison of this chromatogram to that obtained following incubation of oligo DNA with M.TaqI and cofactor 4a (Figure 4D), showed good overlap of the peaks associated with azide-functionalized DNA at ~ 6.2 and 6.4 min, and a similar ratio of this peak to that of the free hydrazide (at 3.8 and 4.0 min). The presence of a small amount of hydrazide-labeled oligo DNA is likely due to hydrolysis of the Schiff-base under the UPLC conditions. Three additional peaks were observed following re-writing with aldehyde 5, which overlapped with those observed when this aldehyde was incubated with H2NOH·HCl (Figure 4E). Analysis of the individual peaks was carried out by UPLC-MS which confirmed re-writing was successful that only one aldehyde had been conjugated, and the nature of the chemical functionality on the oligo DNA (Figure S49-Figure S50).

**Figure 4 – Re-writing oligo DNA.** (A) Schematic representation of un-tagging and re-writing of chemical functionality on oligo DNA using the AdoMet derivative 4a and aldehyde 5. Analytical UPLC chromatograms of oligo DNA following incubation with M.TaqI and 4a, followed by incubation with 10 equiv. of H2NOH·HCl in 10 mM ammonium acetate pH 4 (B), and oligo DNA from B followed by incubation with aldehyde 5 (C). Chromatogram of oligo DNA following incubation with M.TaqI and 4a (D) and of aldehyde 5 incubated with 10 equiv. of H2NOH·HCl in 10 mM ammonium acetate pH 4 (E) shown for comparison. UPLC conditions: 0.5 % triethyl amine, water/MeCN gradient at 60 °C. Under these conditions, oligo DNA melts and both strands of DNA can be observed independently.

**Write, Un-Tag and Permanent Tag.**

One common limitation of the current chemistries used for site-selective functionalization of biomolecules is their lack of versatility. Once a chemical moiety is introduced to, for instance, facilitate the purification of the biomolecule (e.g. biotin), these moieties remain attached to the targeted biomolecule. More importantly, these moieties can not be further functionalized under mild and straightforward conditions, to introduce new functionality (e.g. fluorophores, targeting ligands) often required for further research. Our strategy for MTase-directed labeling of DNA using AdoMet derivative 4a results in DNA that now carries a Schiff-base that can be efficiently cleaved. Removing the chemical functionality via exchange with H2NOH·HCl results in a hydrazide-labeled DNA that should be easily functionalized using...
standard covalent bioconjugation techniques. To this end, fragments of DNA generated by PCR, containing 17 CpG sites, were site-selectively labeled with M.Mpel. Labeling was followed by incubation with H₂NOH-HCl to remove the azide moiety and then reacted with a commercially available NHS-activated fluorophore ATTO 647N 7 to introduce a permanent fluorescent tag (Figure S34A). The reaction was monitored via gel electrophoresis (Figure S34B) and showed specific conjugation of ATTO 647N to hydrazide-labeled DNA. While no red-fluorescence was observed in the absence of ATTO 647N (Figure S34B, lanes 1, 3 and 5), this dye is positively charged and was able to non-specifically associate with the DNA in all samples (Figure S34B, lanes 2, 4 and 6). When we compared the intensities of the red and green channels (SYBR™ Green), to evaluate the degree of labeling with ATTO 647N per unit of DNA, we could observe that hydrazide-labeled PCR fragments were giving the highest ratio (Figure S34B, lanes 6), 4.8 times higher for the DNA than in the absence of the un-tag step (H₂NOH-HCl treatment, Figure S34B, lanes 4), and over 23 times higher than in the absence of MTase-directed labeling (Figure S34B, lanes 2).

**Sequential Functionalization With Complementary Fluorescent Dyes**

The final stage was to demonstrate the utility of our strategy and the potential to perform multiple functionalizations on the same sample of DNA. To this end, we decided to explore the consecutive labeling of short DNA fragments with two different fluorescent dyes. DNA fragments were first incubated with M.Mpel and cofactor 4a, to yield azide-functionalized DNA (Figure 5A, step ①). This functionalization resulted in a small shift in the migration time of the DNA on the gel (Figure 5B, step ①) but, as expected, no fluorescence was observed (Figure 5C, step ①). A further shift in the migration time was observed when the azide-functionalized DNA was reacted with TAMRA-DBCO 6 (Figure 5B, step ②) but, more importantly, emission from DNA-associated TAMRA fluorophore was clearly observed (Figure 5C, step ②). Removal of the TAMRA tag was achieved by incubation with an excess of H₂NOH-HCl. No fluorescence was observed from the resulting DNA fragments (Figure 5C, step ③) and a shift back to the original migration time was observed (Figure 5B, step ③), suggesting that this hydrazide linker had little impact on the physical properties of the DNA. Incubation of this hydrazide-labeled DNA with NHS-activated ATTO 647N 7 resulted in a new shift in migration time (Figure 5B, step ④) and the corresponding appearance of fluorescence, now visible under red illumination (Figure 5C, step ④).
**Figure 5 – Sequential Functionalization.** (A) Schematic representation of the sequential functionalization of DNA: DNA was first written via site-selective MTase-directed labeling using cofactor 4a ①. The obtained azide-functionalized DNA fragments were then reacted with TAMRA-DBCO tag ⑥ via azidealkyne cycloaddition ②. The introduced TAMRA was then removed via exchange with H₂NOH-HCl ③, to give hydrazide-labeled DNA fragments that were covalently functionalized with NHS-activated ATTO 647N ⑦ as a permanent tag ④. Functionalization was monitored using gel electrophoresis. Conditions: DNA concentration; 7 ng/µL, release buffer; 10 mM ammonium acetate, pH 6.8, 1 M NaCl, 0.01% SDS. DNA stained with GelRed®. Gel was visualized using a Bio-Rad Pharos FX (GelRed®: excitation, trans-UV; emission filter: 590/110 nm; TAMRA: excitation, epi-green illumination; emission filter: 602/50 nm). TAMRA channel was colored yellow and ATTO 647N ⑦ was colored red for visualization. (B) GelRed® channel and (C) Composite image of TAMRA and ATTO 647N channels. Full chemical structures of the fluorescent dyes are available in the Supporting Information.

**Application – DNA Sorting Via Reversible Capture And Further Amplification Via PCR**

Having demonstrated that each of the steps of our method were feasible, and that sequential functionalization of DNA was possible, we decided to test the potential of this method to underpin sequential manipulations of DNA that go beyond fluorescent tagging. A common application of MTase-directed labeling of DNA is the manipulation of DNA for sequencing. In this application, DNA carrying sequences of interest is normally sorted through site-selective labeling with affinity tags such as biotin, followed by capture with magnetic beads or via affinity chromatography. Release of the captured nucleic acid is commonly achieved using either denaturing conditions that disrupt the biotin-(strept)avidin binding. However, denaturing conditions for release can result in low percentages of labeled-DNA isolated (see below Figure 6B for an example). We anticipated that an alternative strategy could be employed using the AdoMet derivative 4a, but the presence of the acyl hydrazine linker would allow us to efficiently release the captured DNA under mild conditions. A similar concept has been employed for the development of photo-releasable and reducible linkers that avoid the use of denaturing conditions.

Our studies thus focused on the capture and release of the 203 bp DNA fragment containing 17 CpG sites targeted for alkylation by M.Mpelt. After labeling with cofactor 4a, the fragments were further modified by reaction with a bifunctional linker molecule, carrying biotin and dibenzocyclooctyne (DBCO) groups.
MTase-directed labeling was also performed with **AdoMet** derivative 8 that, while including an azide moiety for further functionalization using DBCO chemistry, did not contain the reversible acyl hydrazone linker. Capture experiments, using streptavidin-coated beads, were performed using a high salt tris buffer. We consistently captured in excess of 80% of the DNA (Figure 6C, Figure S51 and Figure S52), while almost no unlabeled DNA was adsorbed onto the magnetic bead under these conditions (Figure 6C, ■).

**Figure 6 – Capture and release of DNA using MTase-directed labeling and competitive exchange.**

(A) Schematic representation of functionalization of DNA fragments with a biotin tag using both MTase-directed labeling and subsequent DBCO conjugation. (B) Schematic representation of the capture and release of DNA fragments using the **AdoMet** derivative 4a. (C) Percentage of DNA remaining following capture with magnetic beads and (D) percentage DNA released from the magnetic beads following treatment with H$_2$NOH·HCl (cycles 1-3) and denaturing conditions (reflux in denaturing buffer, cycles 4 and 5). DNA was either unlabeled (■), incubated with M.Mpel and 4a (●), or incubated with M.Mpel and azide containing cofactor 8 (○). Conditions: DNA concentration; 7 ng/µL; capture buffer: 10 mM Tris, 1 M NaCl, pH 8.5; release buffer: 20 mM H$_2$NOH·HCl (22624 equiv. per labeling site), 10 mM ammonium acetate, pH 6.8, 1 M NaCl, 0.01% SDS; denaturing buffer. N = 3. Error bars indicate range. The amount of DNA was quantified using a Qubit fluorometer and normalized to the starting amount of DNA (C) or the amount of DNA captured (D).

Following capture, the DNA-coated beads were washed and then suspended in an ammonium acetate buffer solution with H$_2$NOH·HCl at 50 °C, to release the captured DNA via cleavage of the acyl hydrazone.
linker. Initial experiments with 3 equiv. of H$_2$NOH-HCl per CpG site on the DNA failed to release significant amounts of captured DNA (Figure S5.1). Increasing the amount of this competing agent significantly increased the amount of released DNA up to ~50% of the original DNA captured. We believe the need to increase the amount of H$_2$NOH-HCl is probably due to a combination of factors including moving from solution to solid phase, the relative high number and density of labeling sites present in the captured DNA, and the length of the DNA (203 bp in PCR fragments vs 14 bp for oligo DNA). The effect of pH on the release efficiency was also evaluated (Figure S5.2). While the optimal pH for acyl hydrazone exchange is around pH 4.5,54,55 the degree of ionization of the captured DNA would be higher at higher pH, minimizing non-specific interactions between the bead and the DNA. In our case, no significant effect of pH was observed on the amount of DNA released, with similar levels obtained for all three pHs tested (4.1, 4.9 and 7) (Figure S5.2). Further optimization of this protocol included reducing the concentration of DNA used during the capture experiments, with almost quantitative release obtained when 7 ng/µL instead of > 22 ng/µL were used (Figure 6D, ●).

To demonstrate that release was the result of the cleavage of the acyl hydrazone linker and not the result of non-specific interactions of the excess of H$_2$NOH-HCl, a sample of DNA was modified using M.Mpel and azide-containing AdoMet derivative 8. This cofactor lacks the required chemistry for reversible capture of the DNA (Figure 6B) but reacts in a similar fashion to 4a in the capture of MTase-labeled DNA (Figure 6A). In fact, similar levels of DNA capture were obtained using either of the cofactor analogues, 4a or 8. Incubating the captured DNA with an excess of H$_2$NOH-HCl gave no observable release of DNA modified with cofactor 8, even after four cycles of incubation (Figure 6D, ○). This lack of release was in sharp contrast to the almost quantitative release observed for DNA labeled with AdoMet derivative 4a (Figure 6D, ●). DNA labeled with AdoMet derivative 8 was only released from the magnetic beads using standard denaturing conditions (boiling in 0.1% SDS), although only 20% of the captured DNA was recovered, as opposed to the nearly quantitative release with cofactor 4a. Moreover, no additional release of DNA was observed from those beads used to capture the DNA modified with cofactor 4a (Figure 6D, ○).

Finally, and to show that the presence of the hydrazide linker did not affect the functionality of the released DNA, we tested the ability of a polymerase enzyme (Sso7d, Bio-Rad) to further amplify DNA that was captured and released from the beads. Polymerase chain reaction (PCR) is a common tool in molecular biology that underpins applications ranging from quantification of DNA to genetic fingerprinting and diagnosis.61,62 Amplification by PCR can be affected by the presence of bulky groups such as DBCO or biotin, and by DNA damage induced by incubation with hydroxyl amine.32,63,64 In our case, following capture and release, real time PCR (SsoAdvanced Universal SYBR™ Green Supermix, Biorad) showed an amplification curve that evolved in a similar fashion for both the modified and control DNA samples (Figure S5.3), indicating that the introduction of the acyl hydrazone-linker, and the incubation with 20 mM H$_2$NOH-HCl, had no impact on the ability of the DNA to be amplified via PCR. Although we can not rule out that the polymerase used in this assay can bypass damaged sites effectively, we anticipate side-reactions will be minimised in our conditions by the low concentration of hydroxyl amine used, the short incubation times used and running the experiments at pH 6.8 or above (Figure 6). Similar side-reactions could be expected upon prolonged storage of the un-tagged DNA through nucleophilic attack of the hydrazide moiety to cytosine residues, leading to inter- and intra-strand cross-links. However, we believe that this potential side-reaction can be minimized by immediately reacting the purified DNA fragments, following steps 4 or 5 in our reported methodology (Figure 1B), much like cofactors 4 were prepared to minimize the intramolecular nucleophilic attack observed for cofactors 3. Finally, the concentration determined using real time PCR (35 ± 11 ng/µL) was in good agreement with that obtained using a complementary, fluorescence-based measurement (Qubit fluorometer) of the eluted DNA, (concentration for the DNA 38 ng/µL). All together, these results demonstrate the potential of our approach, and of AdoMet derivative 4a to underpin the development of a mild and selective method to sort labeled DNA with high recovery efficiencies and functionality.
Conclusion

Here, we have presented a new versatile method to site-selectively write, remove and re-write functional tags on DNA. To this end, two new S-adenosyl-L-methionine (AdoMet) derivatives incorporating a bifunctional chemical handle, carrying both a Schiff-base linker and a terminal azide, were prepared. The ability of two relevant methyltransferases (MTases), including M.Mpel a bacterial analog of human DNMT1, to label DNA using these cofactors was demonstrated, and the versatility of this methodology was explored by labeling DNA across a range of sizes, including oligo and plasmid DNA. The combination of MTase-directed labeling and reversible acyl hydrazone chemistry allowed us to site-selectively label DNA with a bifunctional chemical handle, to enable tagging and un-tagging of chemical functionality. Each of the steps has been demonstrated using a range of analytical and bioanalytical tools. This combination of known chemistries has not been described before, and we have demonstrated the potential of this methodology to underpin new biological applications through the sequential labeling of DNA with two complementary fluorescent dyes, via writing → tagging → un-tagging and permanent tagging. Moreover, a new protocol was developed to sort labeled fragments of plasmid DNA, fragments that could be quantitatively recovered under mild conditions. The recovered DNA fragments retained the functionality of an unmodified DNA for PCR amplification, a critical step in molecular biology and the application of functional DNA.

We think the versatility of our method will have an impact beyond the research presented here. The combination of azide-alkyne cycloaddition and acyl hydrazone exchange should enable the introduction of a broad range of chemical moieties and functional tags. Also, a range of MTases that target other biomolecules (e.g. RNA, proteins) have been reported, thus making the presented method adaptable for bioconjugation to a broad range of targets. Moreover, the principles behind the presented method (i.e. write → tag → un-tag → re-write...) should be readily expanded to other methods for site-selective functionalization of these biomolecules, including other chemical and chemoenzymatic methods. Ultimately, these methodologies should enable sequential analyses on the same target biomolecule (e.g. imaging → capture → sequence), expanding the analytical methods available to understand the biological role of these biomolecules and our ability to exploit them. Our efforts to explore some of these directions will be reported in due course.

Supplementary information

Full experimental details can be obtained in the Supporting Information, including materials and methods, safety statement, structure of the reactive dyes used, and experimental details for the synthesis and characterization of cofactors and precursors, for MTase expression and purification, for gel electrophoresis, for oligonucleotide HPLC and UPLC-MS analysis, for PCR amplification, for PCR product labeling, and for DNA sorting.

**AUTHOR INFORMATION**

**Author Contributions**

A.W., E.J., R.K.N. and P.F.T. conceived and designed the experiments. A.W., S.C., K.U and E.J. synthesized and purified cofactors. A.E.R. and J.K. produced and confirmed the activity of the M.Taq1 and M.Mpel enzymes. A.W. and E.J. performed the experiments demonstrating re-writability. Q.S. performed real time PCR. A.W. performed all other experiments. P.F.T. and R.K.N. secured funding. A.W., E.J., R.K.N. and P.F.T. analyzed the data and wrote the paper, with all other authors contributing to the final version of the manuscript.

**Conflicts of Interest**

The authors declare the following competing financial interest(s): A.W., E.J., R.K.N. and P.F.T. are named on a patent application (GB1913598.7) related to this work.
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# Supporting Information

**Site-selective and Re-writable labeling of DNA through Enzymatic, Reversible and Click Chemistries.**

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## Table of Contents

1. Safety Statement .................................................................................................................. 2
2. General Materials and Methods ............................................................................................ 2
   2.1. Structure of Dyes ........................................................................................................... 2
   2.2. LC Analysis, Concentration and Yield Determination .................................................. 2
3. Synthesis of Cofactors .......................................................................................................... 3
   3.1. 8-Hydroxyoct-6-ynoic acid (10) .................................................................................. 3
   3.2. tert-Butyl 2-(8-hydroxyoct-6-ynoyl)hydrazine-1-carboxylate (11) ............................. 5
   3.3. tert-Butyl 2-(8-bromoocct-6-ynoyl)hydrazine-1-carboxylate (1a) .............................. 7
   3.4. 7-Bromo-hept-1-yne (12) ............................................................................................ 9
   3.5. 8-Bromoocct-2-yn-1-ol (13) ....................................................................................... 10
   3.6. tert-Butyl ((8-hydroxyoct-6-yn-1-yl)oxy)carbamate (14) ........................................... 12
   3.7. tert-Butyl ((8-bromoocct-6-yn-1-yl)oxy)carbamate (1b) ........................................... 14
3.8. Synthesis of 2a and 2b: General Procedure for the Coupling of Bromides 1 with AdoHcy .................................................................................................................. 15
3.9. Synthesis of Cofactors 3a and 3b: Cofactor Deprotection ................................................. 16
3.10. Synthesis of Cofactors 4a and 4b: Aldehyde Coupling .................................................. 23
4. M’Tase Expression and Purification ....................................................................................... 24
   4.1. M.TaqI ............................................................................................................................. 24
   4.2. M.Mpel .......................................................................................................................... 25
5. Gel Electrophoresis .............................................................................................................. 25
   5.1. General procedure ......................................................................................................... 25
6. Oligonucleotide LC .............................................................................................................. 28
   6.1. Enzymatic Labeling ....................................................................................................... 28
   6.2. Un-Tagging – Competitive Exchange of the Schiff-Base ............................................ 28
7. PCR Product: Amplification, Labeling, Capture and Release ........................................... 36
   7.1. PCR Amplification ......................................................................................................... 36
   7.2. PCR Product Labeling ................................................................................................. 36
   7.3. DNA Capture ............................................................................................................... 36
   7.4. General DNA Release .................................................................................................. 37
7.5. DNA Quantification Using Qubit Fluorometer ............................................................. 37
7.6. Real Time PCR Amplification of Purified DNA ............................................................ 38
1. Safety Statement

No unexpected safety hazards were encountered. Azides can be explosive upon contact. This hazard depends on the molecular mass of the azide and it is recommended that the number of N does not exceed the number of C, and that the overall number of C and O is at least three times bigger than the number of N. Enzymes can cause skin and eye irritation, and can be sensitizers. When handling enzymes, care should be taken to avoid inhalation of aerosols and contact with skin and eyes.

2. General Materials and Methods

Reagents were purchased from Sigma-Aldrich except 6-heptyn-1-ol, which was purchased from Fluorochem. N-Boc-hydroxylamine was purchased from Alfa Aesar. N-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-4-formylbenzamide (5) was purchased from BroadPharm. TAMRA-dibenzylcyclooctyne conjugate 6 was purchased from Click Chemistry Tools LLC. CutSmart® buffer, pUC19 plasmid, proteinase K, HaeII and TaqI (R.TaqI) were purchased from New England BioLabs (NEB). The DNA sequence for 14 bp oligo was 5’-GCCGCTCGATGCCG-5’, and was purchased from Integrated DNA technologies®. Flash chromatography was performed using Geduran Si 60 from Merck and TLC (F254) analysis was performed using 60 Å silica gel from VWR International. TLC plates were visualized by staining with potassium permanganate stain or UV absorption. NMR data was acquired on a Bruker Avance III operating at 300 or 400 MHz. MS spectra were obtained on a Xevo® G2-XS-ToF (Waters) and Synapt-G2-S from electrospray ionization (ESI) and time-of-flight (TOF) measurement in negative or positive ion mode. IR spectra were acquired on Agilent Technologies Cary 600 Series FTIR Spectrometer. UV-Vis absorbance measurements were performed on Shimadzu BioSpec-nano.

2.1. Structure of Dyes

Figure S1 Structure of the dyes used in this work.

2.2. LC Analysis, Concentration and Yield Determination

Analytical RP-HPLC for 3a,b and 4a,b was performed on Shimadzu LC-20 Prominance equipped with ACE 5 C18 (250 x 4.6 mm, flow rate 1 mL/min). Elution with 10 mM ammonium acetate pH 5.5/MeCN gradient: 3-30% MeCN over 20 minutes, 30-97% over 15 minutes hold 97% for 10 minutes. Preparative RP-HPLC was performed on Agilent Technologies 1260 Infinity equipped with ACE 5 C18 (250 x 21.2 mm, 100 Å, flow rate 10 mL/min). Elution with 10 mM ammonium acetate pH 5.5/MeCN gradient: 3-30% MeCN over 30 minutes, 30-97% over 20 minutes, hold 97% 5 minutes.

Analytical RP-HPLC for oligonucleotides was performed on Agilent Technologies 1260 Infinity equipped with Phenomenex Gemini® C18 (150 x 4.6 mm, 5 μm, 100 Å, flow rate 1 mL/min) at 60 °C. Elution with a 0.1 M triethyl amine acetate buffer, pH 7.0/MeCN gradient: gradient A: 5-18% MeCN over 25 min, to

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1 Bräse, S., Gil, C., Knepper, K. and Zimmermann, V. Angew. Chem., Int. Ed. 2005, 19, 5188-5240.
2 https://amfep.org/library/_files/amfep-guide-on-safe-handling-of-enzymes-updated-in-2013.pdf. Accessed 10/01/2019
100% 5 min, hold at 100% 10 min, lower to 5% for 5 min; gradient B: 5-31% MeCN over 50 min, to 100% 10 min, hold at 100% 5 min, lower to 5% for 10 min. Gradient A was used for unlabeled and alkylated oligonucleotides and gradient B was used for all remaining samples. The UV-detection was carried out at 260 nm.

Analytical RP-UPLC-MS for oligonucleotides was performed on Waters Ltd. Xevo-G2-XS Tof equipped with ACQUITY UPLC® Oligonucleotide BEH C18 (50 x 2.1 mm, 1.7 μm, 130 Å, flow rate 0.2 mL/min) at 60 °C. Elution with a 0.5 % triethyl amine water/MeCN gradient: gradient: 0-100% MeCN over 20 min, hold at 100% 5 min, lower to 0% for 5 min. The UV-detection was carried with a diode array and the trace at 260 nm represented. MS detection was done on negative mode.

The concentration and yields for 3 and 4 were determined by UV absorbance measurements at 260 nm, performed in 0.1 % acetic acid. For the calculation, molar extinction coefficient was used: $\varepsilon_{260} = 15,400 \text{ dm}^3\text{mol}^{-1}\text{cm}^{-1}$.

3. Synthesis of Cofactors

3.1. 8-Hydroxyoct-6-ynoic acid (10)

A solution of 6-heptynoic acid (2 g, 15.87 mmol) was made in dry THF (42 mL) under argon, to this, HMPA (34.9 mmol, 6.13 mL) was added and the solution was cooled to -78 °C. To this, n-BuLi (1.6 M in hexanes, 34.9 mmol, 21.8 mL) was added dropwise whilst maintaining the temperature below -60 °C. The solution was then warmed to -40 °C and stirred for 1 h. After 1 h, paraformaldehyde (1.47 g, 47.6 mmol) was added via powder funnel under an argon flow. The reaction mixture was then warmed to 45 °C for 4 h. After reaction, the mixture was quenched with 1 M HCl to pH 4-5 and extracted with EtOAc. Organic layer was then dried over Na$_2$SO$_4$ and the EtOAc was removed by rotary evaporation giving the crude product. Purification was completed using flash column chromatography (silica gel, Hex:EtOAc, 6:4): Yield = 68%; $R_f = 0.27$ (Hex:EtOAc, 6:4); $^1$H-NMR (300 MHz, DMSO-$d_6$) $\delta$ 12.03 (s, 1H), 5.03 (s, 1H), 4.02 (d, $J = 2.6$ Hz, 2H), 2.29 – 2.14 (m, 4H), 1.63 – 1.50 (m, 2H), 1.50 – 1.39 (m, 2H); $^{13}$C-NMR (101 MHz, DMSO) $\delta$ 174.3, 83.8, 80.5, 49.1, 33.1, 27.6, 23.7, 23.7, 17.7; IR (cm$^{-1}$) $\nu$ max: 3003, 1700, 1411, 1218, 1133, 1002; HRMS-ESI(-): calcd for C$_8$H$_{11}$O$_3$: 155.0708, found: 155.0713 [M-H].
Figure S2. $^1$H-NMR spectrum of 8-hydroxyoct-6-ynoic acid (10)

Figure S3. $^{13}$C-NMR spectrum of 8-hydroxyoct-6-ynoic acid (10)
3.2. tert-Butyl 2-(8-hydroxyoct-6-ynoyl)hydrazine-1-carboxylate (11)

8-hydroxyoct-6-ynoic acid (10) (1.35 g, 8.65 mmol) and tert-butyl carbazate (1.4 g, 10.38 mmol) were dissolved in 2:1 THF:H₂O (13.5:6.75 mL). To this, EDC·HCl (1.87 g, 9.52 mmol) was added slowly over 15 minutes. The mixture was left to stir for 3 h and then extracted with EtOAc. The organic layer was washed with 0.1 M HCl, water and brine and the organic layer was collected, dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure yielding the product as a white solid: Yield = 63%; Rf = 0.14 (Hex:EtOAc 1:1) ³H-NMR (400 MHz, DMSO-d₆) δ 9.47 (s, 1H), 8.66 (s, 1H), 5.04 (t, J = 5.9 Hz, 1H), 4.02 (dt, J = 5.9, 2.2 Hz, 2H), 2.19 (tt, J = 7.1, 2.2 Hz, 2H), 2.06 (t, J = 7.2 Hz, 2H), 1.58 (p, J = 7.3 Hz, 2H), 1.50 – 1.32 (m, 12H); ¹³C-NMR (101 MHz, DMSO) δ 172.0, 155.6, 84.3, 80.9, 79.4, 49.6, 33.0, 28.5, 28.1, 24.7, 18.2; IR (cm⁻¹) ν max: 3300, 1668, 1370, 1245, 1157; HRMS-ESI(+): calcd for C₁₃H₂₂N₂O₄Na: 293.1477, found: 293.1477 [M+Na].

Figure S4: Infrared spectrum of 8-hydroxyoct-6-ynoic acid (10)
Figure S5 $^1$H-NMR spectrum of tert-butyl 2-(8-hydroxyoct-6-ynoyl)hydrazine-1-carboxylate (11)

Figure S6 $^{13}$C-NMR spectrum of tert-butyl 2-(8-hydroxyoct-6-ynoyl)hydrazine-1-carboxylate (11)
3.3. tert-Butyl 2-(8-hydroxyoct-6-ynoyl)hydrazine-1-carboxylate (1a)

A solution of tert-butyl 2-(8-hydroxyoct-6-ynoyl)hydrazine-1-carboxylate (11) (300 mg, 1.11 mmol) was made in dry DCM (3.33 mL) and cooled on ice. Triphenylphosphine (437 mg, 1.67 mmol) was added and left to dissolve, once dissolved tetrabromomethane (552 mg, 1.67 mmol) was added slowly. The reaction was then brought to room temperature and left to stir for 1 h. After reaction, the solvent was removed under reduced pressure and the crude mixture was purified by flash column chromatography (Hex:EtOAc, 7:3): Yield = 55%; Rf = 0.15 (Hex:EtOAc 7:3); ¹H-NMR (300 MHz, DMSO-d₆) δ 9.48 (s, 1H), 8.67 (s, 1H), 4.21 (t, J = 2.3 Hz, 2H), 2.27 (tt, J = 6.9, 3.4 Hz, 2H), 2.06 (t, J = 7.4 Hz, 2H), 1.65 – 1.31 (m, 13H); ¹³C-NMR (101 MHz, DMSO) δ 171.4, 155.2, 87.7, 78.9, 76.3, 54.9, 39.5, 32.5, 28.0, 27.3, 24.1, 17.9, 17.2; HRMS-ESI(+): calcd for C₁₃H₂₇N₂O₃NaBr: 355.0633, found: 355.0634 [M+Na].
Figure S8 $^1$H-NMR spectrum of tert-butyl 2-(8-bromooct-6-ynoyl)hydrazine-1-carboxylate (1a)

Figure S9 $^{13}$C-NMR spectrum of tert-butyl 2-(8-bromooct-6-ynoyl)hydrazine-1-carboxylate (1a)
3.4. 7-Bromo-hept-1-yne (12)

A solution of 6-heptyn-1-ol (5 g, 44.6 mmol) was made in dry DCM (60 mL) and cooled on ice. To this, triphenylphosphine (17.6 g, 67 mmol) was added, upon complete dissolution, tetrabromomethane (22.2 g, 67 mmol) was added slowly. The reaction mixture was brought to room temperature and stirred for 1 h. After completion, the solvent was removed under reduced pressure. Hexane was added to the crude forming a white suspension. The hexane fraction was filtered, collected and then the solvent was removed. An oily residue remained which was purified by flash column chromatography with hexane: Yield = 91%, Rf = 0.45 (hexane); $^1$H-NMR (300 MHz, DMSO-$d_6$) $\delta$ 3.53 (t, $J$ = 6.7 Hz, 2H), 2.75 (t, $J$ = 2.7 Hz, 1H), 2.23 – 2.10 (m, 2H), 1.89 – 1.74 (m, 2H), 1.50 – 1.43 (m, 4H); $^{13}$C-NMR (101 MHz, DMSO) $\delta$ 84.3, 71.3, 35.0, 31.7, 27.0, 27.7, 17.6; IR (cm$^{-1}$) $\nu$ max: 3290, 2918, 1437, 1120, 635, 540.

Figure S10 $^1$H-NMR spectrum of 7-bromo-hept-1-yne (12)
3.5. 8-Bromoocct-2-yn-1-ol (13)

A solution of 7-bromohept-1-yne (12) (20.56 mmol, 3600 mg) was made in Dry THF (12.3 mL) and cooled to -78 °C under Argon. To this, a solution of n-BuLi in hexanes (1.6 M, 13 mL) was added dropwise, whilst maintaining the temperature below -60 °C. The reaction mixture was then warmed to 0 °C in an ice bath at which point paraformaldehyde (1718 mg, 55.5 mmol) was added under a flow of argon and stirred for 30 minutes. The mixture was then warmed to room temperature and left to stir, while the temperature was maintained below 30 °C until the exothermic reaction had stopped. The mixture was
then heated to 45 °C for 2 h. Once complete the reaction was extracted with ether and sat. NH₄Cl. The organic layer was collected, dried over anhydrous sodium sulfate and the solvents were removed under reduced pressure to yield the crude product as an oil. Purification was completed by flash column chromatography (Hex: EtOAc 9:1). The product was then collected as a colorless oil: Yield = 55%; Rf = 0.15 (Hex: EtOAc 9:1); 1H-NMR (300 MHz, DMSO- d₆) δ 5.04 (t, J = 5.7 Hz, 1H), 4.03 (dt, J = 5.5, 2.1 Hz, 2H), 3.54 (t, J = 6.7 Hz, 2H), 2.20 (m, 2H), 1.88 – 1.75 (m, 2H), 1.52 – 1.40 (m, 4H); 13C-NMR (101 MHz, CDCl₃) δ 86.1, 78.8, 77.2, 51.5, 33.7, 32.4, 27.8, 27.5, 18.7.

Figure S13 1H-NMR spectrum of 8-bromooct-2-yn-1-ol (13)
3.6. tert-Butyl ((8-hydroxyoct-6-yn-1-yl)oxy)carbamate (14)

To a solution of N-Boc Hydroxylamine (890 mg, 6.55 mmol) in DMF (4.3 mL), 8-bromooct-2-yn-1-ol (13) (1200 mg, 5.85 mmol) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (1000 mg, 6.55 mmol) were added. The solution was stirred at 50 °C for 20 h. Once complete, the reaction was extracted with DCM and 15% citric acid solution. The organic phases were dried over anhydrous sodium sulfate, collected and the solvent was removed under reduced pressure. A colorless oil was collected as the crude product. This was further purified by flash column chromatography (Hex: EtOAc 8:2). The product was collected as a colorless oil: Yield = 73 %; Rf = 0.27 (Hex: EtOAc 8:2); $^1$H-NMR (300 MHz, DMSO-$d_6$) $\delta$ 9.91 (s, 1H), 5.03 (t, $J$ = 5.9 Hz, 1H), 4.02 (dt, $J$ = 5.9, 2.2 Hz, 2H), 3.66 (t, $J$ = 6.2 Hz, 2H), 2.17 (tt, $J$ = 6.7, 1.7 Hz, 2H), 1.40 (m, 15H); $^{13}$C-NMR (101 MHz, DMSO) $\delta$ 156.1, 84.0, 80.4, 79.4, 75.1, 49.1, 28.1, 28.0, 27.1, 24.8, 17.9; MS: m/z [M+H] = 258.2.
Figure S15 $^1$H-NMR spectrum of tert-butyl ((8-hydroxyoct-6-yn-1-yl)oxy)carbamate (14)

Figure S16 $^{13}$C-NMR spectrum of tert-butyl ((8-hydroxyoct-6-yn-1-yl)oxy)carbamate (14)
3.7. **tert-Butyl ((8-bromooct-6-yn-1-yl)oxy)carbamate (1b)**

A solution of tert-butyl((8-hydroxyoct-6-yn-1-yl)oxy)carbamate (14) (1 g, 3.89 mmol) was made in dry DCM (5.2 mL) and cooled on ice. To this, triphenylphosphine (1.53 g, 67 mmol) was added. Upon complete dissolution tetrabromomethane (1.94 g, 67 mmol) was added slowly. The reaction mixture was brought to room temperature and allowed to stir for 1 h. After completion, the solvent was removed under reduced pressure. Purification was completed using flash column chromatography (Hex:EtOAc 8:2): Yield = 67%; Rf 0.52 (Hex:EtOAc, 8:2); \(^1\)H-NMR (300 MHz, DMSO-d\(_6\)) δ 9.90 (s, 1H), 4.21 (t, \(J = 2.4\) Hz, 2H), 3.66 (t, \(J = 6.2\) Hz, 2H), 2.25 (tt, \(J = 6.9, 2.4\) Hz, 2H), 1.40 (m, 15H); \(^13\)C-NMR (101 MHz, DMSO) δ 156.0, 87.8, 79.3, 76.2, 75.0, 39.5, 28.0, 27.6, 27.0, 24.7, 18.0, 17.2; IR (cm\(^{-1}\)) ν max: 1712, 607; MS: m/z [M+Na] = 342.35/344.35, [M-BuOH] = 246.38/248.38.

Figure S17: \(^1\)H-NMR spectrum of tert-butyl ((8-bromooct-6-yn-1-yl)oxy)carbamate (1b)
3.8. Synthesis of 2a and 2b: General Procedure for the Coupling of Bromides 1 with AdoHcy

A solution of S-adenosyl-l-homocysteine (AdoHcy) (15 mg, 0.04 mmol) was made in a 1:1 mixture of formic and acetic acid (300 µL). Linker 1 (1.2 mmol, 30 equiv.) was then added dropwise, on ice. The reaction mixture was warmed to 35 ºC and left to stir overnight. After overnight stirring, the reaction mixture was extracted with diethyl ether and the aqueous layer was collected and dried by lyophilization: MS: m/z [M+H] = 638, HRMS-ESI(+): calcld for C_{23}H_{34}N_7O_6S: 536.2291, found: 536.2302 [M+H-Homocysteine] (2a), [M+H] = 624 (2b). Compounds 2 were immediately used in the next step without further purification.
3.9. Synthesis of Cofactors 3a and 3b: Cofactor Deprotection

The crude product (2a or 2b) was dissolved in TFA (400 µL) and left to stir for 2 h at room temperature. After reaction, the acid was removed under a flow of argon. The crude reaction mixture was then dissolved in water (2 mL) and purified by Prep-HPLC. Retention times: 3a isomer I = 17.51 min, isomer II = 18.73 min, 3b isomer I = 25.47 min, isomer II = 28.24 min: MS: m/z [M+H] = 538, HRMS-ESI(+): calcd for C_{18}H_{26}N_{7}O_{4}S: 436.1767, found: 436.1768 [M+H-Homocysteine] (3a), [M+H] = 524 (3b). Compounds 3 were immediately used in the next step without further characterization.

Figure S20 Preparative-HPLC chromatogram of cofactor 3a. Conditions for analytical HPLC: 3-30% over 30 minutes, 30-97% over 20 minutes, hold 97% 5 minutes. 20 mM ammonium acetate pH 5.5. Flow rate 10 mL/min
Figure S21 Mass spectrum of the peak at 17 min from Figure S20. 538.0: M + H⁺, 550.0: M + Na⁺.
Figure S22 Mass spectrum of the peak at 18.5 min from Figure S20. 537.9: $M + H^+$, 549.9: $M + Na^+$. 
Figure S23 Preparative-HPLC chromatogram of cofactor 3b. Conditions for analytical HPLC: 3-30% over 30 minutes, 30-97% over 20 minutes, hold 97% 5 minutes. 20 mM ammonium acetate pH 5.5. Flow rate 10 mL/min.
Figure S24 Mass spectrum of the peak at 24.5 min from Figure S23. 525.0: M + H⁺.
Figure S25 Mass spectrum of the peak at 26 min from Figure S23. 525.0: M + H⁺.
Figure S26 Consecutive analytical HPLC chromatograms of cofactor 3a after incubation at 37 °C (Isomer I) following isolation via preparative HPLC. Conditions for analytical HPLC: 3-30% over 20 minutes, 30-97% over 15 minutes, hold 97% for 10 minutes. 10 mM ammonium acetate pH 5.5. Flow rate 1 mL/min. 14 min = 3a (Isomer I), 33 min = 3a (Isomer I) – Homoserine lactone.

Figure S27 Consecutive analytical HPLC chromatograms of AdoMet derivative 3a after incubation at 37 °C (Isomer II) following isolation via preparative HPLC. Conditions for analytical HPLC: 3-30% over 20 minutes, 30-97% over 15 minutes, hold 97% for 10 minutes. 10 mM ammonium acetate pH 5.5. Flow rate 1 mL/min. 16.5 min = 3a (Isomer I), 33 min = 3a (Isomer I) – Homoserine lactone.
Figure S28 Analytical HPLC chromatogram of AdoMet derivative 3b following isolation via preparative HPLC and freeze-drying, and proposed degradation pathways. Conditions for analytical HPLC: 3-30% over 20 minutes, 30-97% over 15 minutes, hold 97% for 10 minutes. 10 mM ammonium acetate pH 5.5. Flow rate 1 mL/min.

3.10. Synthesis of Cofactors 4a and 4b: Aldehyde Coupling

Crude deprotected cofactors 3 were mixed, directly after collection from preparative HPLC, with aldehyde 5 (1.2 equiv.) and the mixture rolled for 30 min at room temperature. The reaction mixture was then dried by lyophilization, and then resuspended in 100 µL 0.1% Acetic Acid and stored at -20 °C without further purification. MS: m/z [M+H] = 869 (4a), [M+H] = 856 (4b); HRMS-ESI(+): calcd for C38H54N11O10S: 856.3776, found: 856.3780 [M+H]+ (4a). Calcd for C38H53N12O10S: 869.3728, found: 869.3729 [M+H]+ (4b). HPLC retention times: 33.9 min (4a), 31.9 (4b iso 1), 32.0 (4b iso 2).

Figure S29 Analytical HPLC chromatogram of cofactor 4a (Isomer I) after freeze-drying. Conditions for analytical HPLC: 0.1 mL/min 3-100% over 60 min. 0.1% formic acid water methanol gradient. Excess aldehyde 5 at 40 min.

3 Acetone should be avoided during lyophilization, as exchange with the acyl hydrazone/oxime moiety can be observed.
Figure S30 Analytical HPLC chromatogram of cofactor 4a (Isomer II) after freeze-drying. Conditions for analytical HPLC: 0.1 mL/min 3-100% over 60 min. 0.1% formic acid water methanol gradient. Excess aldehyde 5 at 40 min.

Figure S31 Consecutive analytical HPLC chromatograms of AdoMet derivative 4b after incubation at 50 °C (Isomer II). Conditions for analytical HPLC: 3-30% over 20 minutes, 30-97% over 15 minutes, hold 97% for 10 minutes. 10 mM ammonium acetate pH 5.5. Flow rate 1 mL/min. 13 min = Adenine, 31 min = 4b (Isomer II), 38 min = 3a (Isomer I) – Homoserine lactone.

4. MTase Expression and Purification

4.1. M.TaqI

*Escherichia coli* T7 Express Competent (High Efficiency) (C2566) cells were transformed with pET28a::M.TaqI and allowed to recover in 495 µL SOC, 250 rpm and 37°C, for 30 minutes to 1 h. 150 µL of recovered cells were spread onto a sterile LB-agar (Melford) plate supplemented with kanamycin to a final concentration of 50 µg/mL and grown overnight at 37 °C. A colony was picked and used to inoculate 10 mL LB media (Melford) supplemented with kanamycin final concentration of 50 µg/mL and left shaking overnight at 37 °C 250 rpm. 5 mL of overnight culture was used to inoculate 2x 400 mL of LB media (Melford), supplemented with kanamycin to a final concentration of 50 µg/mL and left shaking 180 rpm at 37 °C until an OD600 reading of 0.4-0.6 was reached. IPTG was added to a final concentration 0.5 mM and left shaking at 20 °C, 180 rpm for 16 h. Cells were harvested by centrifugation (20 minutes at 3993 xg, 4 °C). The supernatant was discarded, and the pelleted cells were resuspended in 20 mL of 1% PBS supplemented with EDTA-free Protease Inhibitor Cocktail (Sigma) (pH 7.4). Resuspended cells
were then disrupted using an emulsiflex at a pressure of 12K psi and passed through the funnel repeatedly for a total of 5x. The resulting cell lysate was then centrifuged at 13500 rpm, 4 °C for 14 minutes. The supernatant was collected, and further EDTA-free Protease Inhibitor Cocktail tablets added. The supernatant was then passed through a 0.22 µM Sartorius syringe filter. 1 mL of Ni-NTA agarose beads were centrifuged at 500 xg for 5 minutes. The supernatant was removed, 1 mL of 1% PBS was added and the beads were washed by inverting several times followed by centrifuged at 500 xg for 5 minutes and removal of the PBS (repeated 5x). A final 1 mL of 1% PBS was added to the beads to create a 50% slurry, 50 µL of which was mixed per 1 mL of cell lysate and left rotating end over end for 1 h at 4 °C. Biorad EconoColumns were washed with 1x PBS and the sample was added to the column. Next, 25 mL of wash buffer (PBS, 1M NaCl, 20 mM imidazole) followed by 5 mL elution buffer (PBS, 200 mM NaCl, 250 mM imidazole) and left for 10 minutes, then eluted. An Amicon Ultra 0.5 mL 10 KDa kit was used to exchange the buffer following the manufacturer’s instructions. Storage buffer was made up of PBS, 5 mM EDTA and 5 mM β-mercaptoethanol. Protein presence was checked using SDS-PAGE and stored in 50% glycerol at -20 °C.

4.2. M.Mpe1

E. coli T7 Express Competent (High Efficiency) (C2566) cells were transformed with pET28a::mutated mpeIM (M.Mpe1) and allowed to recover in 200 µL of LB (Melford) at 37 °C, 200 rpm for 1 h. 50 µL of recovered cells were spread onto a sterile LB-agar (Melford) plate supplemented with kanamycin to a final concentration of 50 µg/mL and grown overnight at 37 °C. A colony was selected and used to inoculate 25 mL of sterile LB (Melford) supplemented with kanamycin (50 µg/mL), grown overnight at 37 °C, 200 rpm. From this, 20 mL was added to a 5L flask containing 2L of LB (Melford) supplemented with kanamycin (50 µg/mL) and grown at 37 °C, 180 rpm until an OD600 reading of 0.4-0.8 was reached. The temperature was then reduced to 28 °C and the culture was supplemented with IPTG to a final concentration of 0.5 mM and incubated for 10 h at 180 rpm. The culture was then split into 2x 1L centrifuge containers and harvested by centrifugation (20 minutes at 3993 x g, 4 °C). Cell pellets were washed in 50 mL of pre-chilled phosphate buffered saline (PBS) and re-harvested (20 minutes at 3993 x g, 4 °C). Cell pellets were then resuspended in 20mM Tris-HCl, 40mM imidazole, 0.5mM TCEP, EDTA-free Protease Inhibitor Cocktail (Sigma) (pH 7.4) and 1mg/mL of DNAase I and cells were lysed using an Emulsiflex-C3 (Avastin). Cell lysate was cleared via ultracentrifugation (30 min at 48,384 x g, 4 °C) and the supernatant collected and filtered (0.45µM filter). A 5 mL HisTrap HP column (GE healthcare) was equilibrated with 25x column volumes (CV) of equilibration buffer (20mM Tris-HCl, 40mM imidazole, 0.5mM TCEP, EDTA-free Protease Inhibitor Cocktail (Sigma)). The supernatant was added to the column and allowed to circulate overnight at 4 °C. A HisTrap HP column was then loaded onto an AKTA and the column was washed with 30x CV of equilibration buffer followed by 30x CV of high salt wash buffer (20mM Tris-HCl, 40mM imidazole, 0.5mM TCEP, 500mM NaCl and EDTA-free Protease Inhibitor Cocktail (Sigma)). M.Mpe1 was then eluted into 20mM Tris-HCl, 0.5mM TCEP, 500mM NaCl, EDTA-free Protease Inhibitor Cocktail (Sigma) using a 0-500 mM gradient of imidazole, collected into 2 mL fractions. Fractions containing the most protein were pooled, and buffer exchanged into 10 mM Tris-HCl, 0.5 mM TCEP, 1 mM EDTA (pH 7.5) and 50% glycerol. A final protein concentration of 1.7 mg/mL was achieved.

5. Gel Electrophoresis

5.1. General procedure

On ice, a master mix was created by mixing 79.5 µL molecular grade water, 10 µL (10x NEB CutSmart buffer) and 3 µL pUC19 (1000 ng/µL). 2x 9.25 µL was taken for tubes 4, 8 (restriction controls) and 0.25 µL of each cofactor isomer and 0.5 µL water was added. To the remaining master mix 4 µL M.TaqI (0.3 mg/mL) was added and mixed. The master mix was then split into 2x 19.5 µL (1,5) and 4x 10 µL and labeled (2,3,6,7). A 2x serial dilution was made by adding 0.5 µL 4a to the final concentration 300 µM to tubes 1/5 and mixed. 10 µL was then taken from tubes 1/5 and added to 2/6 and continued until tubes 6/7 discarding the final 10 µL. Additional controls for this experiment were also set up (Table 1). All samples were incubated at 50 °C for 1 h before adding 0.5 µL restriction enzyme (R.TaqI) to all tubes...
Samples were again incubated for 1 h at 50 °C. 0.5 µL proteinase K was added to all tubes and incubated at 50 °C for 1 h before being run on 1% agarose gel to analyze. A 2-Log DNA Ladder (0.1–10.0 kb) (NEB) was used in all the gels. Relevant bands have been labeled.

Table 1: Controls set up for all protection assays

|                     | AdoMet control (9/10) | No cofactor (11) | No MTase (12) |
|---------------------|-----------------------|------------------|---------------|
| 10x NEB CutSmart    | 2 µL                  | 1 µL             | 1 µL          |
| pUC19 (1000 ng/µL)  | 1 µL                  | 0.5 µL           | 0.5 µL        |
| AdoMet (3.5 mM)     | 0.5 µL                | -                | -             |
| M.TaqI              | 0.125 µL              | 0.125 µL         | n/a           |
| water               | 16.5 µL               | 8.5 µL           | 8.5 µL        |

Figure S32 Gel electrophoresis of pUC19 following enzymatic treatment with M.TaqI and/or R.TaqI in the presence and absence of AdoMet or AdoMet derivative 4a.
Figure S33: Gel electrophoresis of pUC19 following enzymatic treatment with M.MpeI and/or R.HaeII in the presence and absence of AdoMet or AdoMet derivative 4a.

Figure S34: (A) Schematic representation of the further functionalization with NHS-activated ATTO 647N 7. (B) Gel electrophoresis of pUC19 fragments. DNA was either unlabeled (lanes 1 and 2), incubated with M.TaqI and 4a (lanes 3 and 4), or incubated with M.MpeI and 4a followed by incubation with H₂NOH-HCl in 10 mM ammonium acetate pH 4 (lanes 5 and 6). The NHS ester of ATTO 647N 7 is shown as a control (lane 7). Conditions: DNA concentration: 7 ng/µL, un-tag buffer; 10 mM ammonium acetate, pH 6.8, 1 M NaCl, 0.01% SDS. DNA stained with SYBR™ Green. Gel was visualized using a ChemiDoc MP Imager (Bio-Rad) (SYBR™ Green: excitation, trans-UV; emission filter, 590/110 nm; ATTO 647N 7: excitation, epi-red illumination; emission filter: 700/50 nm). SYBR™ Green channel was colored green and ATTO 647N 7 was colored red for visualization. Full chemical structures of the fluorescent dyes are available in Figure S1.
6. Oligonucleotide LC

6.1. Enzymatic Labeling

For each sample a solution of oligo (120 µL, 10 µM), buffer (40 µL, 10x NEB CutSmart buffer), M.TaqI (45 µL), water (189 µL) and cofactor (6 µL, 20 mM) was made. Samples were incubated at 50 °C for 1.5 h. After incubation, proteinase K (2.5 µL) was added and the samples were incubated at 50 °C for a further 1 h. The samples were then purified using the Qiagen Qiaquick nucleotide clean up kit and eluted into 50 µL water and their concentration was measured by Shimadzu BioSpec-nano. Samples not to be un-tagged were taken and stored in the fridge until LC analysis.

6.2. Un-Tagging – Competitive Exchange of the Schiff-Base

To the labeled DNA, a solution of H₂NOH-HCl in water (10 µL, 10 equiv. per site) was added. The pH of the solution was then adjusted using 100 mM ammonium acetate buffer (pH 4.0, 7 µL). The samples were then incubated at 50 °C for 1.5 h and then stored in the fridge until analysis.

Table 2: Mass spec data collected of the fractions collected during HPLC for each oligonucleotide strand.

|                  | Strand 1 | Strand 2 |
|------------------|----------|----------|
|                  | 5'-CGGGGAGCTACGGC-3' | 3'-GCCGCTCGATGCGG-5' |
| Unlabeled        | Calculated: 4289.8 | Observed: N/A |
|                  | Calculated: 4240.8 | Observed: N/A |
| Methylated       | Calculated: 4304.8 | Observed: 4304.28 |
|                  | Calculated: 4255.8 | Observed: 4255.26 |
| Acyl Hydrazone   | Calculated: 4773.8 | Observed: 4773.36 |
|                  | Calculated: 4724.8 | Observed: 4724.76 |
| Oxime            | Calculated: 4760.8 | Observed: 4760.32 |
|                  | Calculated: 4711.8 | Observed: 4711.32 |
| Hydrazide        | Calculated: 4441.8 | Observed: 4441.8 |
|                  | Calculated: 4392.8 | Observed: 4392 |

Figure S35 Analytical-HPLC chromatogram of 14 bp oligo DNA. Conditions: 0.1 M TEAA buffer against acetonitrile. 5-18% MeCN over 25 minutes, 18-100% over 5 minutes, hold 100% 10 minutes, drop to 5% and hold 5 minutes, 60 °C.
Figure S36 Analytical-HPLC chromatogram of 14 bp oligo DNA incubated in the presence of M.TaqI and AdoMet. Conditions: 0.1 M TEAA buffer against acetonitrile. 5-18% MeCN over 25 minutes, 18-100% over 5 minutes, hold 100% 10 minutes, drop to 5% and hold 5 minutes, 60 °C.

Figure S37. Mass spectrum of the peak at 10.53 min from Figure S36 (methylated 14 bp oligo strand 1, M – 3H)
Figure S38. Mass spectrum of the peak at 11.64 min from Figure S36 (methylated 14 bp oligo strand 2, M - 3H).

Figure S39 Analytical-HPLC chromatogram of 14 bp oligo DNA incubated in the presence of M.TaqI and 4a. Conditions: 0.1 M TEAA 5-36% over 50 minutes, 60°C.
Figure S40. Mass spectrum of the peak at 27 min from Figure S39 (14 bp oligo strand 1 incubated with M.TaqI and 4a. 4773.36: M – H; 4795.28: M + Na – H).

Figure S41. Mass spectrum of the peak at 28 min from Figure S39 (14 bp oligo strand 2 incubated with M.TaqI and 4a. 4724.76: M – H; 4746.96: M + Na – H).
Figure S42. Analytical-HPLC chromatogram of 14 bp oligo DNA incubated in the presence of M.TaqI and 4b. Conditions: 0.1 M TEAA buffer against acetonitrile. 5-18% MeCN over 25 minutes, 18-100% over 5 minutes, hold 100% 10 minutes, drop to 5% and hold 5 minutes, 60 °C.

Figure S43. Mass spectrum of the peak at 36 min from Figure S42 (14 bp oligo strand 1 incubated with M.TaqI and 4b. 4760.32: M – H; 4795.28: M + Na – H; 4795.28: M + K – H; 4819.20: M + Na + K – 2H).
Figure S44. Mass spectrum of the peak at 36.8 min from Figure S42 (14 bp oligo strand 2 incubated with M. TaqI and 4b. 4710.32: M – H; 4732.28: M + Na – H; 4750.28: M + K – H).

Figure S45 Analytical-HPLC chromatogram of 14 bp oligo DNA incubated in the presence of M. TaqI and 4a, followed by treatment with H$_2$NOH·HCl (10 equiv) in a pH 4 10 mM ammonium acetate buffer. Conditions: 0.1 M TEAA buffer against acetonitrile. 5-18% MeCN over 25 minutes, 18-100% over 5 minutes, hold 100% 10 minutes, drop to 5% and hold 5 minutes, 60 °C.
Figure S46. Mass spectrum of the peak at 12.7 min from Figure S45 (14 bp oligo strand 1 incubated with M.TaqI and 4b, followed by treatment with $\text{H}_2\text{NOH-HCl}$. 1502.96: M – 3H).

Figure S47. Mass spectrum of the peak at 14 min from Figure S45 (14 bp oligo strand 2 incubated with M.TaqI and 4b, followed by treatment with $\text{H}_2\text{NOH-HCl}$. 1463.28: M – 3H; 1471.94: M + Na – 4H).
Figure S4B Analytical-HPLC chromatogram of 14 bp oligo DNA incubated in the presence of M.TaqI and 4b, followed by treatment with H$_2$NOH-HCl (10 equiv.) in a pH 4 10 mM ammonium acetate buffer. Conditions: 0.1 M TEAA buffer against acetonitrile. 5-18% MeCN over 25 minutes, 18-100% over 5 minutes, hold 100% 10 minutes, drop to 5% and hold 5 minutes, 60 °C.

Figure S49 MS spectra of the 14 bp oligo DNA, strand 1, after re-witting with aldehyde 5 (1192.51: M – 4H; 1590.35: M – 3H; 1598.01: M + Na – 4H).
7. PCR Product: Amplification, Labeling, Capture and Release

7.1. PCR Amplification

The PCR product was prepared by amplification of a section of the plasmid pUC19 using the corresponding primers (Forward primer: 5'-GCC AGG AAC CGT AAA AAG-3' and Reverse primer: 5'-AGA AAG GCG GAC AGG TAT-3'). The DNA fragment amplified was 203 bp with 31 CpG sites. For amplification, a mastermix of buffer (200 µL, NEB 2x high-fidelity mastermix), forward primer (4 µL, 100 µM), reverse primer (4 µL, 100 µM), pUC19 (2 µL, 10 ng/µL) and water (190 µL) was prepared. The mastermix was then aliquoted into 50 µL portions. Amplification was completed using the program: 98 °C for 45 s, 40 cycles 98 °C for 15 s, 62 °C for 45 s, and 72 °C for 60 s. After amplification, the DNA was purified on silica column (Sigma-Aldrich PCR clean up kit) and eluted into water.

7.2. PCR Product Labeling

Once collected, the DNA fragment was labeled using the CpG specific DNA methyltransferase M.Mpel. For labeling, samples were made up of: DNA (66 µL, 40 ng/µL), buffer (10 µL, 10x NEB CutSmart buffer), cofactor (3 µL, 15 mM), M.Mpel (5 µL), and water (16 µL). Samples were incubated at 37 °C for 1 h after which proteinase K (2 µL) was added and incubated at 50 °C for 1 h. Finally, a solution of diazo-DBCO-Biotin (2 µL, 50 mM, Jena Bioscience) was added and incubated for a further 1 h at 37 °C. After labeling, the DNA was purified using a Qiagen purification kit and the DNA was eluted into a high salt Tris buffer (10 mM Tris, 1 M NaCl, pH 8.5, Tris A) ready for capture.

7.3. DNA Capture

After labeling, the DNA was captured onto streptavidin-coated magnetic beads (Dynabeads myone C1). For capture, 10 µL of the bead stock was washed 2x with 70 µL Tris A and then all supernatant was removed. The bead pellet was then resuspended in the DNA solution previously eluted (500 ng, 70 µL, Tris A). This solution was shaken at room temperature for 15 minutes. After capture, the bead mixture was placed on a magnetic rack and the DNA concentration of the eluent was measured. The eluent was then placed into a fresh bead pellet to capture the remaining DNA. This process was repeated 3x until the majority of DNA was captured.
After capture, the bead pellets were resuspended in water and washed 2x. The beads were then finally placed within the relevant buffer ready for release.

### 7.4. General DNA Release

To release captured DNA (500 ng, 200 bp) the bead pellets were suspended in ammonium acetate buffer (63 µL, 11.1 mM, 1 M NaCl, 0.01% SDS, var. pH) and to this, a solution of H$_2$NOH.HCl (7 µL, var. equiv.) was added. The bead solution was then shaken at 50 °C for 1 h. After shaking, the solution was placed on a magnet, the supernatant was removed and the DNA concentration was measured. This process was repeated until all DNA had been released.

![Graph](image1.png)

**Figure S51 Left:** Percentage of DNA remaining in solution following capture of DNA. Traces have been labeled with the corresponding release experiment. **Right:** Percentage of DNA released from the magnetic beads following treatment 3 (●), 30 (○) or 300 (■) equiv. of H$_2$NOH-HCl in acetate buffer at pH 4.9. In all cases, DNA (2500 ng, 19.9 pmol) was incubated with M.Mpel and 4a and then purified prior to capture. Capture was then performed in all cases using approx. 1 µg DNA (9 pmol, 22 ng/µL). n = 1. The amount of DNA was quantified using a Qubit fluorometer and normalized to the starting amount of DNA (Left) or the amount of DNA captured (Right).

![Graph](image2.png)

**Figure S52 Left:** Percentage of DNA remaining in solution following capture for these experiments. Traces have been labeled with the corresponding release experiment. **Right:** Percentage DNA released from the magnetic beads following treatment with H$_2$NOH-HCl (300 equiv.) in acetate buffer at pH 4.1 (■), 4.9 (●) or 7.4 (○). In all cases, DNA (2500 ng, 19.9 pmol) was incubated with M.Mpel and 4a and then purified prior to capture. Capture was then performed in all cases using approx. 1 µg DNA (8 pmol, 22 ng/µL). n = 1. The amount of DNA was quantified using a Qubit fluorometer and normalized to the starting amount of DNA (Left) or the amount of DNA captured (Right).

### 7.5. DNA Quantification Using Qubit Fluorometer

DNA quantification was performed using the Qubit® 3.0 Fluorometer. A broad-range (BR) assays kit was used according to the instructions provided by the supplier.
7.6. Real Time PCR Amplification of Purified DNA

DNA following capture and release (Figure 6) was prepared by PCR amplification of a known DNA fragment (332 bp) from the Lambda bacteriophage genome (NEB). Real time PCR was performed using the SsoAdvanced™ Universal SYBR™ Green Supermix (Bio-Rad) with appropriate primers (forward primer: 5’- GTG GTG AAA GGG CAG AGC A -3’ and reverse primer: 5’- AGG GCC AGA TGC TCA ATG C -3’). The amplification program was set as: 98 °C for 45 s, 40 cycles 98 °C for 15 s, 68 °C for 45 s, and 72 °C for 60 s. The captured/released DNA was diluted 500 times, prior to running the qPCR experiment.

![Real-time PCR](image)

Figure S53 Real-time PCR if pUC19 fragments following capture and release experiment (Figure 6): (Left) A standard curve is generated using samples with known concentrations, from left to right, at 1000 pg/µL, 100 pg/µL, 10 pg/µL and 1 pg/µL. (Right) Amplification curves for the captured/released DNA, where concentrations in the PCR reaction were calculated to be 83 pg/µL, 79 pg/µL and 45 pg/µL. These correspond to released DNA concentrations of 41.5 ng/µL, 39.5 ng/µL and 22.5 ng/µL respectively.