

## **Supplementary materials**

Engineering the DNA cytosine-5 methyltransferase reaction for  
sequence-specific labeling of DNA

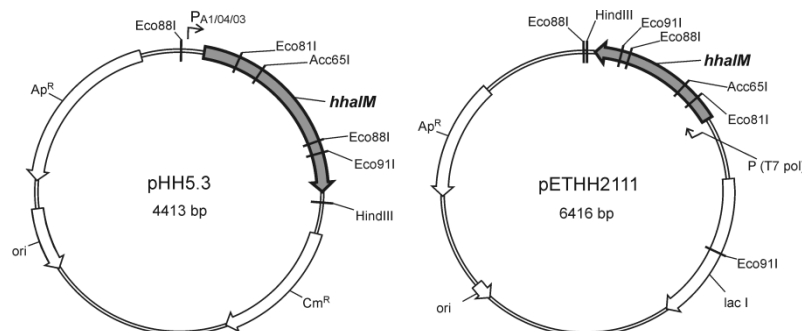
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and Saulius Klimašauskas**

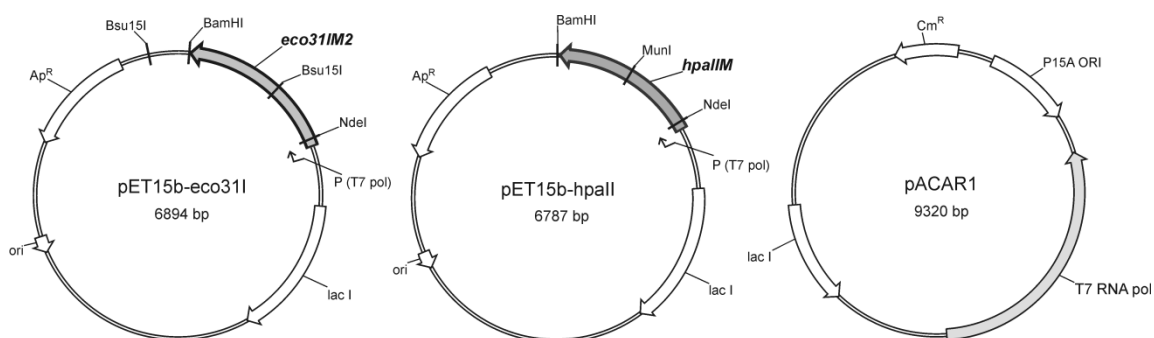
## Supplementary Methods

### Mutagenesis and purification of DNA methyltransferases

Plasmids containing *hhaIM* gene with single Q82A and N304A mutations had been previously produced as derivatives of M.HhaI expression vectors pHH5.3 and pETHH2111, respectively (9). Y254S mutation was introduced by the Kunkel method as described previously (15) using a mutagenic primer **II**. Full length *hhaIM* gene was restored by cloning R.Acc65I-R.Eco91I fragment into pHH5.3. M.HhaI variants containing two or three mutations were constructed by recombining appropriate fragments through unique R.Eco81I, R.Eco88I, R.Eco91I, R.Acc65I and R.HindIII sites. Protein expression was induced with 0.4 mM IPTG. WT HhaI and mutant MTases were purified as described previously (33). Briefly, MTases were selectively enriched by a high salt (0.4 M NaCl) back-extraction from the cell debris. Following extensive dialysis to remove bound endogenous AdoMet, MTases were purified by passing through a pre-column of Q-Sepharose followed by column chromatography on SP-Sepharose. All proteins appeared as sole bands (> 95%) in Coomassie-stained polyacrylamide gels. Protein concentrations were determined by active site titration with a fluorescent duplex oligonucleotide as described previously (33).



The pUC19Eco31IRM plasmid was used as a template in PCR amplification of the *eco31IM2* gene. The *eco31IM2* gene was cloned into the expression plasmid vector pET15b (in R.NdeI and R.BamHI sites) to create pET15b-*eco31I*. This plasmid was used to express wild type protein, carrying N-terminal His6 affinity tag. N127A and Q233A mutations were introduced by megaprimer method using pET15b-*eco31I* as a template. In the first round of PCR one of the flanking primers (**III** for N127A substitution and **IV** for Q233A substitution) and the internal primer (**V** and **VI** respectively) were used to generate a megaprimer, which was purified and used with the other flanking primer (**IV** for N127A substitution and **III** for Q233A substitution) in the second round of PCR to generate the complete *eco31IM2* sequence with the desired mutation. The plasmids pET15b-*eco31I*N127A and pET15b-*eco31I*Q233A were constructed by cloning the modified *eco31IM2* gene into the pET15b through R.NdeI and R.BamHI sites. The plasmid pET15b-*eco31I*N127Q233A was constructed by recombining mutations from pET15b-*eco31I*N127A and pET15b-*eco31I*Q233A using the restriction endonuclease Bsu15I sites.



M.HpaII encoding plasmid pA-HpaII was used as a template in PCR amplification of *hpaIIM* gene. *hpaIIM* was cloned into the expression plasmid pET15b (in R.NdeI and R.BamHI sites) and plasmid pET15b-hpaII was constructed. This plasmid was used to express wild type protein, carrying N-terminal His6 affinity tag. Q104A and N335A mutations were introduced by megaprimer method using pET15b-hpaII as a template, two flanking primers **III** and **IV** and the internal mutagenic primers (**VII** for Q104A substitution and **VIII** for N335A). Mutagenesis was carried out as described above for M2.Eco31I. The plasmid pET15b-hpaIIQ104AN335A was constructed by recombining mutations from pET15b-hpaIIQ104A and pET15b-hpaIIN335A using R.MunI and R.BamHI sites.

*E.coli* ER2566 carrying a pET15b-borne MTase gene and a T7 RNA polymerase gene on a separate plasmid (pACAR1, G.Vilkaitis, unpublished data) was grown at 37°C in LB medium containing ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml) until cells reached OD<sub>600</sub> 0.8. Protein expression was induced by addition of IPTG to 1 mM concentration and cells were grown ~16 hours at 16°C. After that, cells were sonicated and the supernatant was applied onto IMAC HP column (GE Healthcare). Protein was eluted with an imidazole gradient from 10 mM to 500 mM, and pooled fractions were dialyzed against storage buffer (M2.Eco31I: 20 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.5, 100 mM KCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, 50% glycerol; M.HpaII: 20 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, 50% glycerol). All purification steps were carried out at 4°C. The molecular mass of purified enzymes was verified using MS analysis (single quadruple Hewlett-Packard 1100 series system).

## Supplementary reference

- Holz B, Klimašauskas S, Serva S, & Weinhold E (1998) *Nucleic Acids Res* **26**, 1076-1083.

**Supplementary Table S1. Synthetic DNA oligonucleotides used in this study.**

Duplex oligonucleotide	Sequence*	Remarks
<b>I</b>	5' -CATTAC <b>GCGC</b> CGGGTCTGGCTAT GTAAT <b>GCGC</b> GCCCAGGACCGATA-5'	M.HhaI substrate
<b>Primers for mutagenesis</b>		
<b>II</b>	AAAATGCCCCCGCCAGAAGCACTTAAGG	M.HhaI Y254S
<b>III</b>	TAATACGACTCACTATAGGG	flanking primers for megaprimer-based mutagenesis
<b>IV</b>	GGGTTATGCTAGTTATTGC	
<b>V</b>	TGGGGGCACCGCAGCACCGATTTG	M2.Eco31I N127A
<b>VI</b>	CCCCATGCGCGGGATTTTCT	M2.Eco31I Q233A
<b>VII</b>	GCAATAGAGAATGCCGCACAAGGAAA	M.HpaII Q104A
<b>VIII</b>	AAACAATTTGGTGCTTCAGTAGCA	M.HpaII N335A

\* Target site is shown boldface, mutated codons are italicized

**Supplementary Table S2. Apparent catalytic turnover rates ( $\text{min}^{-1}$ )\* of M.HhaI variants in reactions with AdoMet and its analogs (graphically presented in Figure 2c).**

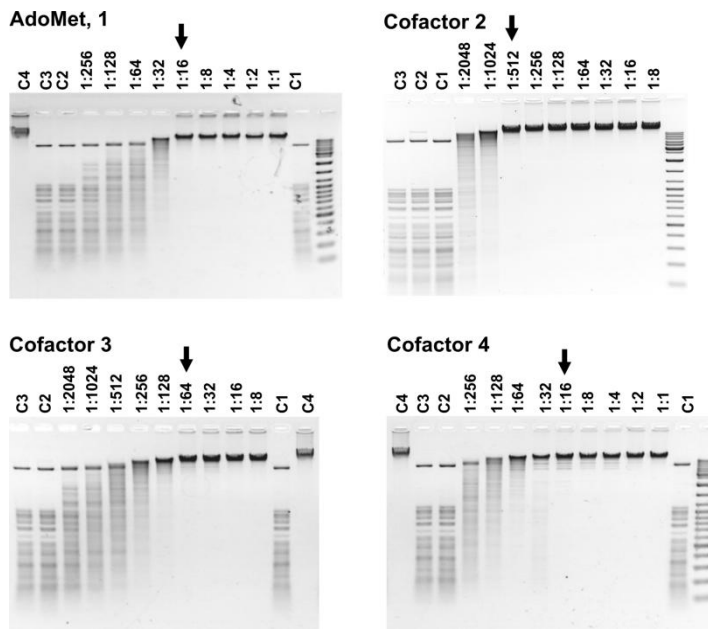
M.HhaI variant	1, AdoMet	cofactor 2	cofactor 3	cofactor 4
WT	2.133	0.017	0.002	0.002
Q82A	0.133	0.067	0.002	0.002
Y254S	2.133	0.267	0.067	0.002
N304A	0.533	4.267	2.133	0.004
Q82A/Y254S	0.533	0.533	0.067	0.002
Q82A/N304A	0.267	8.533	2.133	0.133
Y254S/N304A	0.533	4.267	2.133	0.267
Q82A/Y254S/N304A	0.267	8.533	1.067	0.267

\* Errors are within one two-fold dilution (+100/-50% or  $\pm 0.3 \log_{10}$  units)

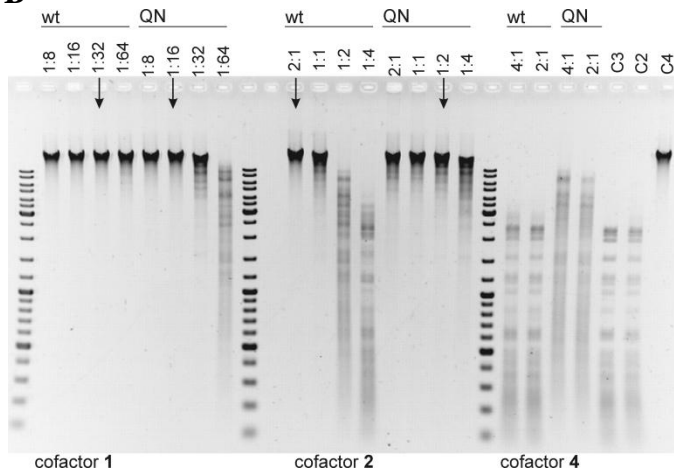
**Supplementary Table S3. Apparent catalytic turnover rates ( $\text{min}^{-1}$ )\* of M.HpaII and M2.Eco31I variants in reactions with AdoMet and its analogs (graphically presented in Figure 6c).**

MTase variant	1, AdoMet	cofactor 2	cofactor 4
M.HpaII WT	0.1333	0.0021	0.0000
M.HpaII Q104A/N335A	0.0667	0.0167	0.0003
M2.Eco31I WT	0.2667	0.0083	0.0003
M2.Eco31I N127A	0.0042	0.0167	0.0042
M2.Eco31I N127A/Q233A	0.0167	0.0667	0.0167

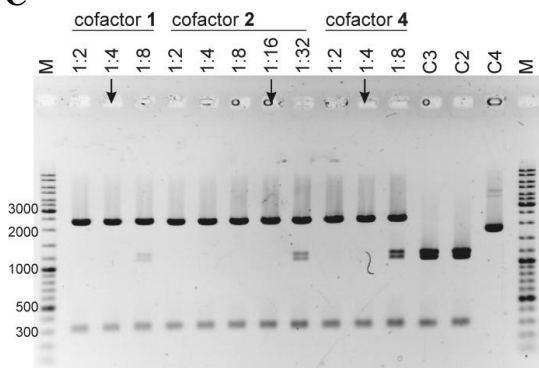
\* Errors are within one two-fold dilution (+100/-50% or  $\pm 0.3 \log_{10}$  units)

**A**

Engineered M.HhaI (variant Q82A/Y254S/N304A) was incubated with lambda DNA and cofactor **1**, **2** or **4** (300  $\mu$ M) at 37°C for 1 h at a series of dilutions (molar ratios of MTase to its target sites are as indicated) and then treated with R.Hin6I endonuclease. Lanes representing highest MTase dilutions that render full protection of target sites in DNA are marked with arrows. Control reactions: C1 - no MTase, C2 - no cofactor, C3 - no MTase, no cofactor, C4 - untreated  $\lambda$  DNA (see Materials and Methods for details).

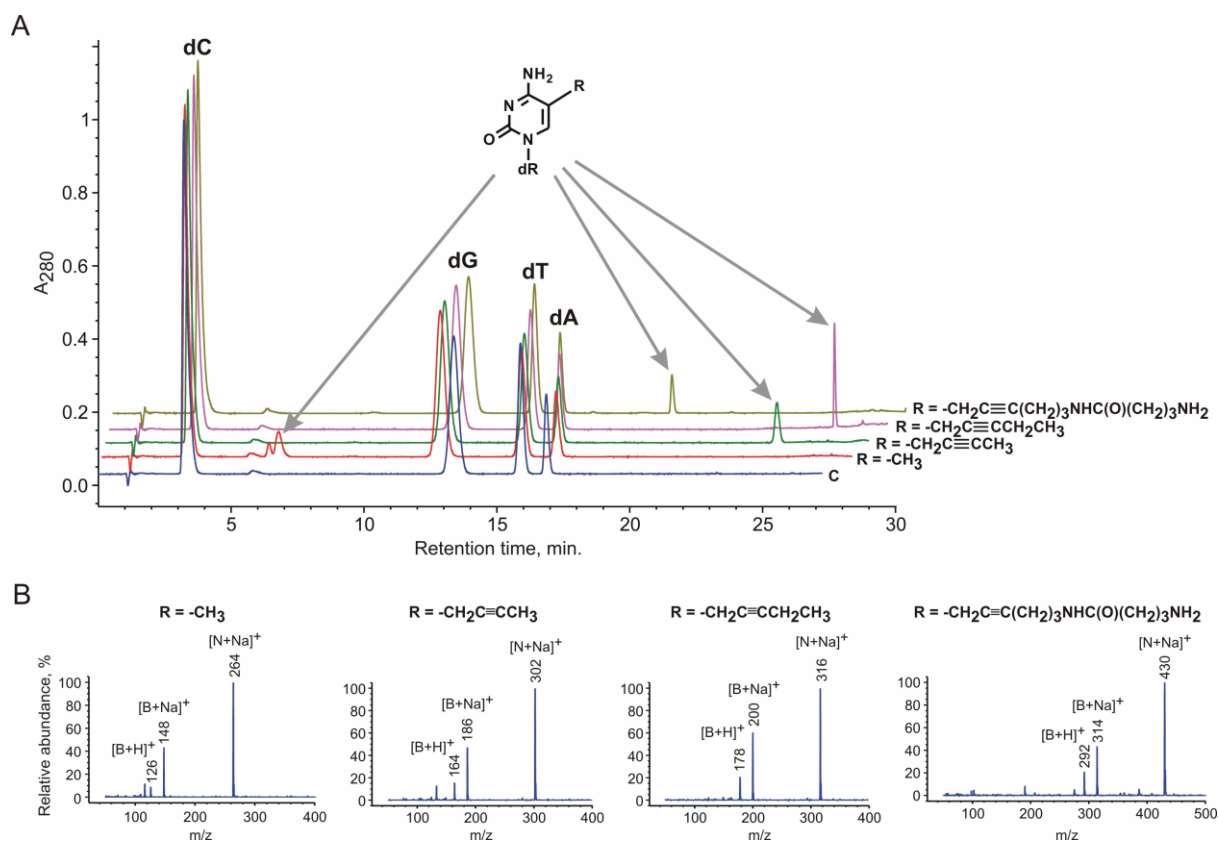
**B**

Two-fold serial dilutions of M.HpaII wild type (wt) or its variant Q104A/N335A (QN) were incubated with lambda DNA and cofactor **1**, **2** or **4** for 4 h at 37°C, and then treated with R.HpaII endonuclease. Other details as above.

**C**

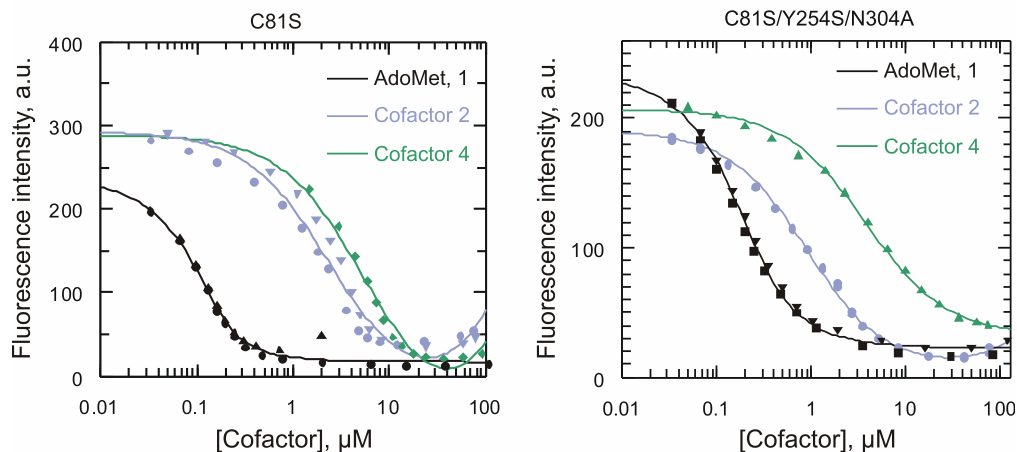
Two-fold serial dilutions of engineered M2.Eco31I variant N127A/Q233A were incubated with plasmid pUC19 DNA and cofactor **1**, **2** or **4** for 4 h at 37°C, and then treated with R.PvuII (to produce linear DNA fragments) and R.Eco31I. Protection of a unique Eco31I site leads to the disappearance of the 1130 bp and 1234 bp fragments.

**Figure S1. Multiple turnover analysis of alkyltransferase activity using DNA protection assays**

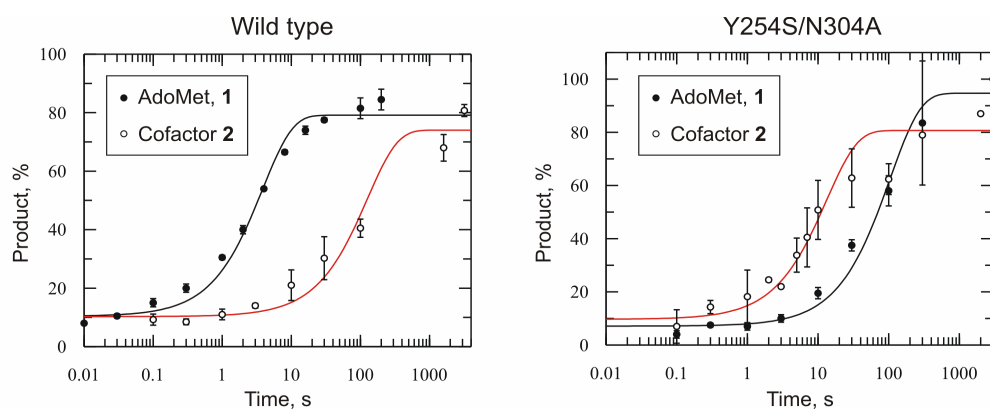


**Figure S2. Analysis of transalkylation products formed in duplex oligodeoxynucleotides.**

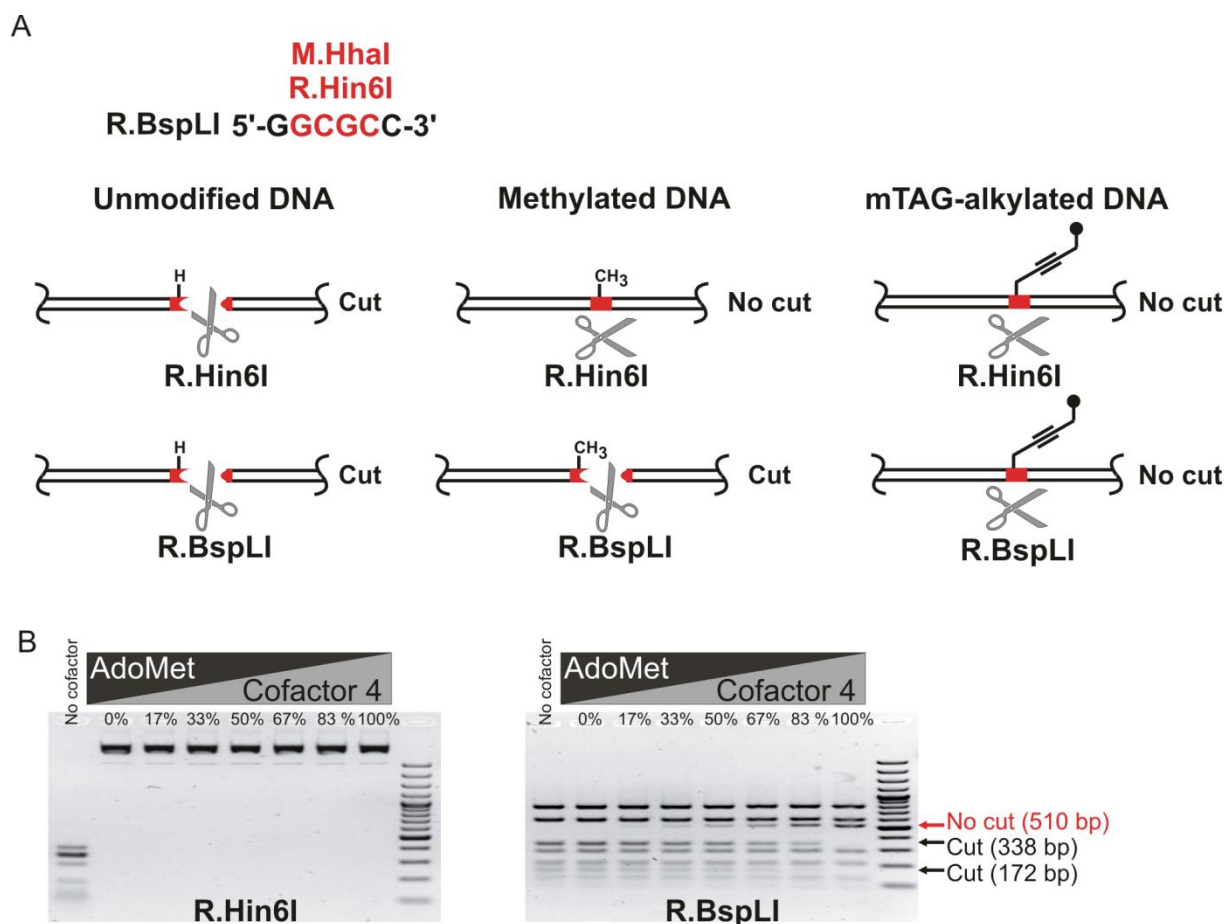
(A) HPLC analysis of enzymatically fragmented duplex oligodeoxynucleotides (dR = 2'-deoxyribose) obtained after modification with M.HhaI (Q82A/Y254S/N304A variant) in the presence of AdoMet or its analogs; (B) ESI-MS analysis of the modified nucleosides obtained with AdoMet or cofactor analogs **2–4** and M.HhaI. N denotes 2'-deoxynucleoside; B, nucleobase.



**Figure S3. Cofactor binding to binary protein-DNA complex.** Left panel shows cofactor binding to C81S mutant and cognate DNA complex. Right panel shows cofactor binding to triple C81S/Y254S/N304A mutant and cognate DNA complex. Binding was monitored by following Trp41 fluorescence decrease upon increasing concentrations of native cofactor AdoMet or its analogs **2** and **4**.

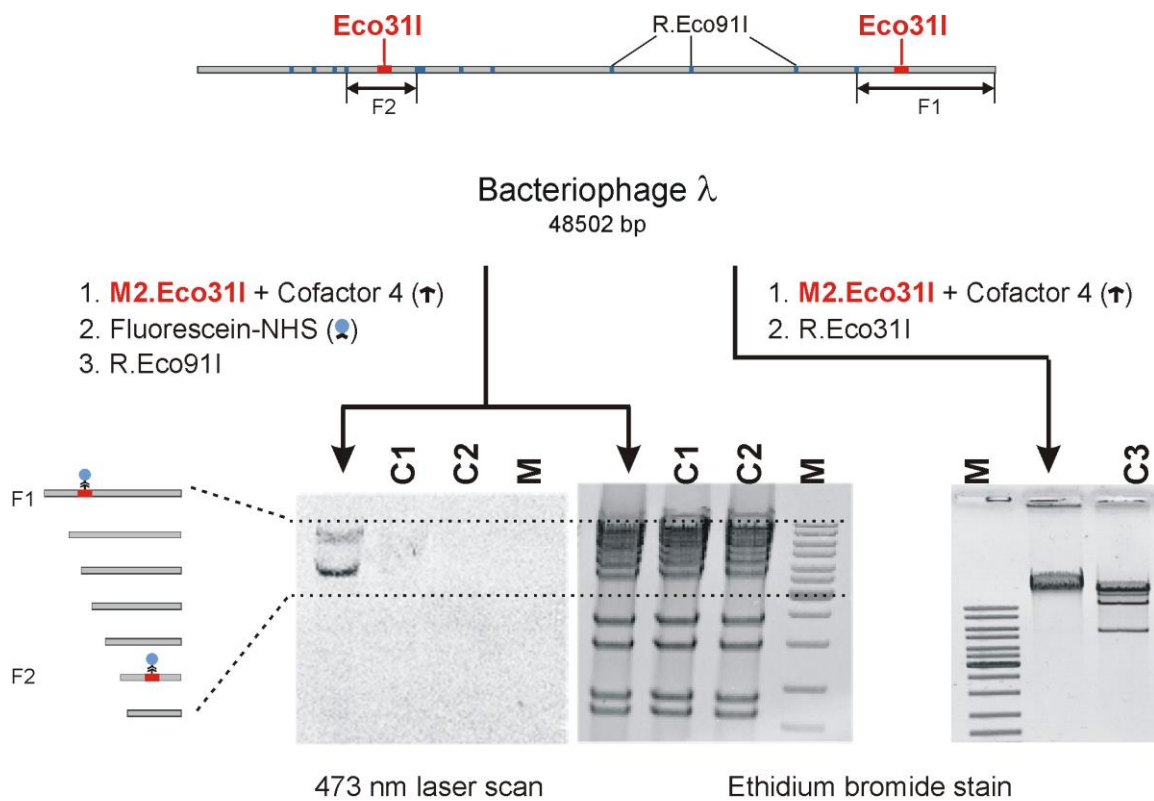


**Figure S4. Product formation under single turnover reaction conditions.** DNA modification by WT and Y254S/N304A mutants of M.HhaI in the presence of natural cofactor and its analog **2** was followed as described in Methods. Reaction rates  $k_{chem}$  were obtained by fitting data to a single exponential equation.



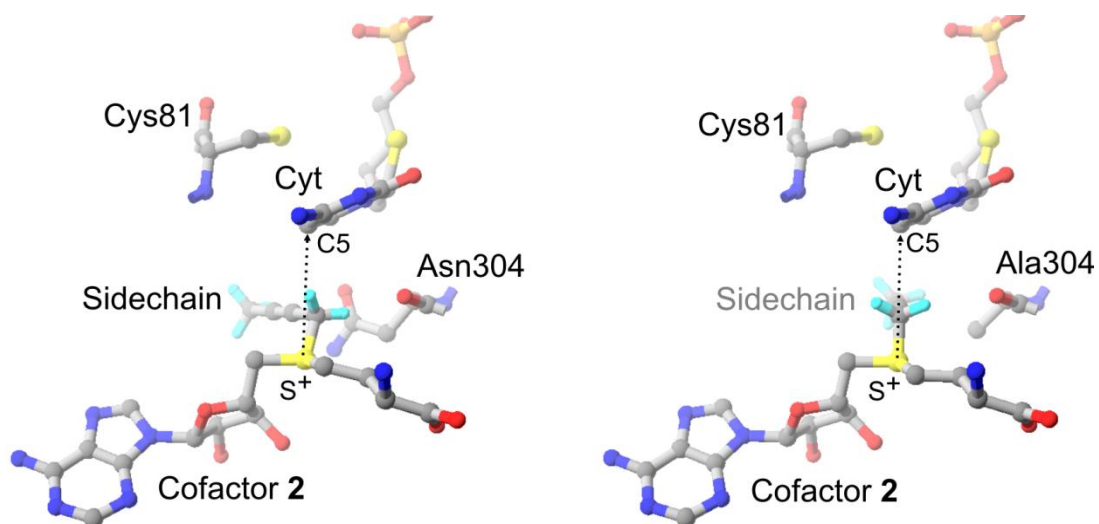
**Figure S5. mTAG alkylation of DNA in the presence of AdoMet.** pUC19 plasmid DNA was modified with M.HhaI (Q82A/Y254S/N304A mutant) and a mixture of AdoMet and cofactor **4** supplied in different molar fractions as indicated (total cofactor 300  $\mu$ M, 0.5  $\mu$ M DNA target sites, 0.25  $\mu$ M M.HhaI, 4 h at 37°C) (A) Detection of mTAG alkylation at a unique overlapping GGCGCC site using R.Hin6I (GCGC-specific) and R.BspLI (GGNNCC-specific) restriction endonucleases. Protection from R.Hin6I hydrolysis indicates that DNA is fully modified (methylated or alkylated), whereas R.BspLI is insensitive to methylation but is inhibited only by alkyl groups deposited at an overlapping target site. The GGCGCC site is located in a 510 bp fragment which is cleaved into 338 bp and 172 bp fragments by R.BspLI. (B) Analysis of mTAG-alkylated pUC19 DNA shows full immunity of the modified DNAs to R.Hin6I (left) and partial to complete immunity to R.BspLI (right) in line with increasing amounts of cofactor **4** (shown as % of AdoMet + cofactor **4**) present in the reaction (see also **Figure 4** in the main article).





**Figure S6. Sequence-specific labeling of bacteriophage lambda DNA at two internal sites.**

DNA was amino-modified with an engineered variant of the M2.Eco31I MTase (recognition sequence GGTCTC) in the presence of Cofactor 4 and then treated with a fluorescein-N-hydroxy-succinimide ester (fluorescein-NHS). Labeled DNA was cleaved with R.Eco91I endonuclease to produce fourteen fragments (largest are illustrated on the left) and analyzed by agarose gel electrophoresis. Fluorescein imaging using a 473 nm laser scanner displays only the two fragments (F1 and F2) that contain labeled target GGTCTC sites. C1 and C2 - controls with no MTase or no cofactor added, respectively; M - DNA size marker GeneRuler™ DNA Ladder Mix.



**Figure S7. Conformational flexibility of bound cofactor analog in M.HhaI.** A wider solvent channel in the cofactor pocket of the mutant M.HhaI (right) permits rotation of the butynyl side chain of cofactor **2** into the newly created space to position the transferable carbon atom into a more favorable pre-catalytic conformation in which one of the two hydrogen atoms assumes a less obstructive position for an in-line attack of the C5 nucleophile. This conformation is impossible in the WT MTase due to sterical constraints from the Asn304 residue. Model is based on ternary complex structure of HhaI methyltransferase with AdoMet and DNA containing 4'-thio-2'-deoxycytidine (PDB ID: 6MHT)