# Development of a single-plasmid system for screening site-specific DNA methylases

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Targeted methylases are engineered fusion proteins that catalyze sequence-specific methylation – a useful tool to research DNA methylation and edit epigenomes. To accelerate the development of targeted methylases, we created and validated a biobrick assay, and enzymatically active fusion proteins for use as a positive control. Our modular single-plasmid system allows methylase fusions to be easily cloned, expressed, and tested via inexpensive digestion and gel electrophoresis. Our biobrick enables quickly measuring the existence and extent of targeted methylation. It additionally includes validated primer-binding sites for methylation-sensitive bisulfite sequencing, and our E. coli chassis effectively eliminated noise associated with methylation studies. The rapid and affordable workflow enables the quick iterations of the design-build-test cycle necessary for protein engineering.

## Introduction

In the past, DNA methylases, the enzymes responsible for DNA methylation, have been fused to zinc fingers to confer site-specificity for manipulating methylation patterns in bacteria, mammalian cells, and ex vivo (3-5). However, these zinc finger fusion proteins are not fully optimized in terms of methylation efficacy and site-specificity, and many researchers have written that nonspecific methylation should be studied further (3, 5). Given the higher specificity and lower cost of TALE and dCas9 as alternatives to zinc fingers for genome editing, we expect researchers will soon begin the lengthy and expensive development process for optimized targeted methylases using these newer DNA binding domains (2).

Synthetic biology is characterized by the application of engineering principles such as abstraction, modularity and standardization to biological problems. Here, we propose a modular single-plasmid design, with a noiseless restriction-based assay, as an affordable and accelerated method for screening libraries of targeted methylases. Additionally, we demonstrate the results of using our assay for preliminary analysis of a novel fusion protein based on the TALE binding domain. Our assay is available as a biobrick (BBa_K1128001), and it follows a similar workflow as previously developed multi-plasmid systems for developing site-specific DNA methylases (9).

## Materials and Methods

Plasmid Assembly. The following experiments used our Biobrick backbone (BBa_K1128001) and inserts available from the Registry of Biological Parts and Addgene. The backbone is a modified pET-26b(+) plasmid, an IPTG-inducible T7 expression vector including the kanamycin resistance gene and a low-copy pBR322 origin of replication. It was purchased from Novagen and modified to include the Biobrick prefix and suffix for Assembly Standard 10 cloning, an sgRNA expression cassette for use with dCas9 (including a constitutive promoter and T7 terminator) downstream of the original expression cassette, and a "target site" with the recognition sequences for the TALE1 and Zif268 DNA binding domains. The gene for the prokaryotic DNA CpG methylase M.SssI with a 5' linker was inserted downstream of the T7 promoter.
Depending on the experiment, either Zif268 or TALE1 was cloned into the vector upstream of the methylase by restriction digest and ligation. To express M.SssI alone, the linker sequence was removed and a start codon was added by PCR. Zinc Finger 268, was purchased from Addgene (plasmid 12612), to which it had been donated by the Scot Wolfe lab (1). TALE 1 was purchased from Addgene (plasmid 27969),to which it had been donated by the Feng Zhang lab (2).

Bacterial Strains: Escherichia coli T7 Express [fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10] was obtained from New England Biolabs and used throughout the studies because of the T7 polymerase expression system and methylation sensitive restriction enzyme knockouts. NEB5-alpha E. coli from New England Biolabs were used as expression negative controls because they lack an inducible T7 expression system.

In vitro methylation: Purified M.SssI from NEB was incubated with plasmid DNA and S-adenosylmethionine (SAM) according to the manufacturer's protocol.

In vivo methylation: The plasmid was transformed into T7 Express competent cells, induced with varying concentrations of IPTG (0.1mM to 2mM) for varying amounts of time (2 to 24 hours).

Methylation sensitive digestion protocol: 600 ng of purified DNA were digested with 1 ul of AvaI and 1ul of XbaI, 2 ul of NEBuffer 2.1, and nuclease free water to total 15 ul per reaction. The solution was incubated for one hour at 37oC. Gel electrophoresis was performed with 1.0% agarose gel in TAE buffer.
Additional methods and protocols: Other techniques we used are available for download at: http://2013.igem.org/Team:Penn/Protocols

Figure Legends

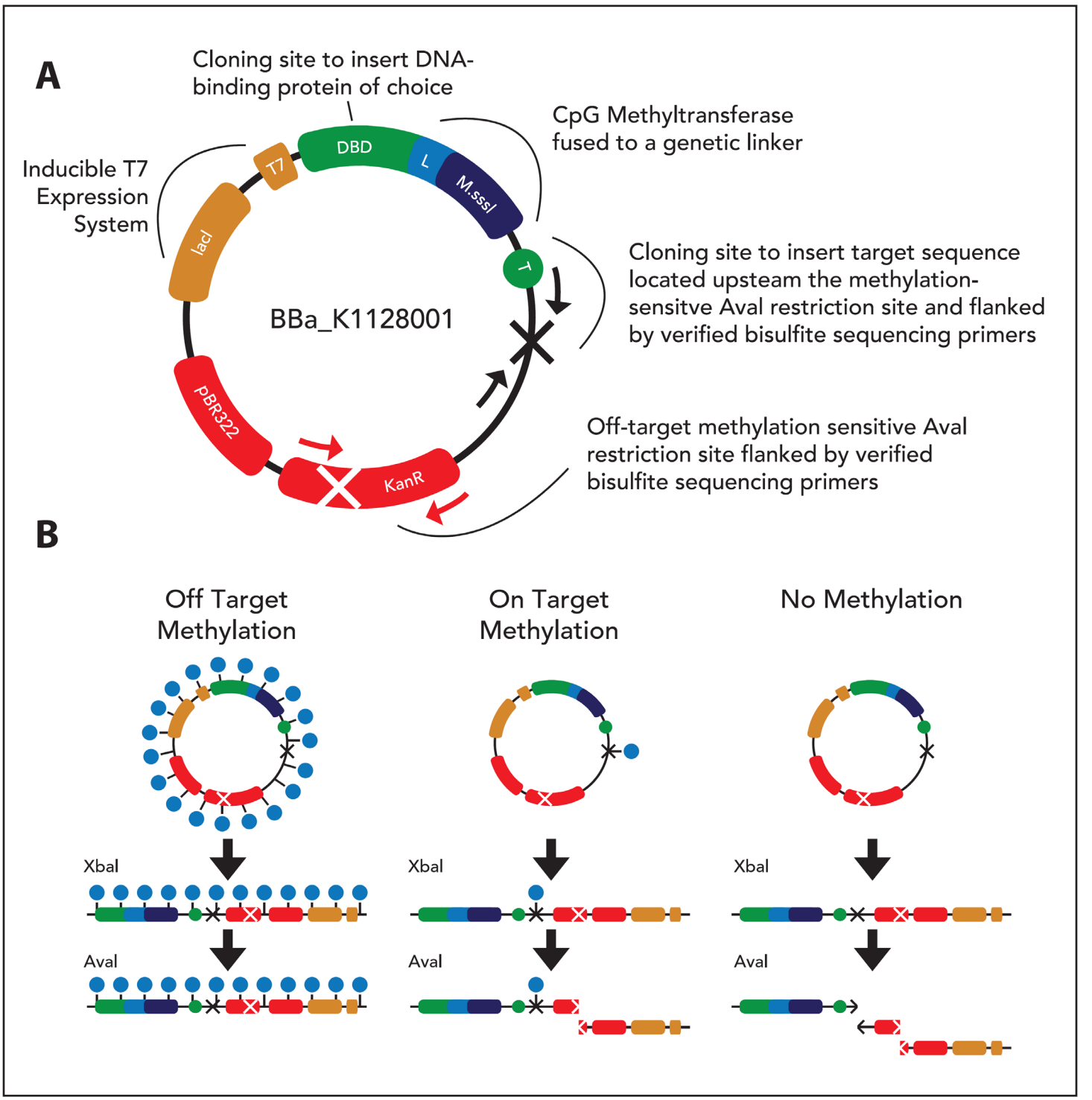


Figure 1. Design of a single-plasmid system for the construction, expression, and measurement of site-specific methylases. A) The single-plasmid system (BBa_K1128001) includes the necessary genetic parts to test site-specific methylases. The methylase, M.SssI with an up-stream linker sequence (BBa_K1128002), can be cloned in by standard biobrick assembly with EcoRI and PstI. B) A model showing how methylation sensitive restriction digest followed by gel electrophoresis distinguishes between methylation states.

## Results

The completed plasmid (BBa_K1128001) includes all the genetic parts necessary to both express a site-specific methylase and report its activity in terms of site-specificity and methylation efficacy (Figure 1A). By methylating the plasmid in vitro with purified M.SssI as well as by methylating the plasmid in vivo by expressing M.SssI with the T7 promoter, we verified the digestion band pattern appeared as expected (Figure 2A). To verify the plasmid could report analog levels of methylation, it was methylated in vitro for varying degrees of time, and a linear relationship between time methylated and the band's normalized intensity level after quantitative image analysis was observed (Figure 2B).

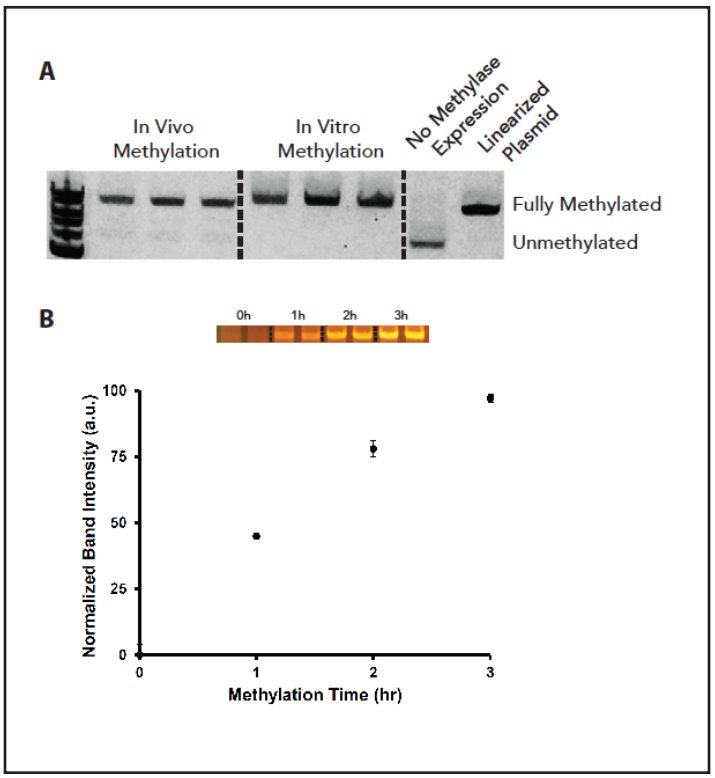


Figure 2. Detection of methylation in vitro and in vivo with the single-plasmid system. A) M.SssI was cloned and expressed in T7 Express cells in vivo, and M.SssI was incubated with the plasmid in vitro. As a negative control, the same plasmid was transformed into NEB 5 alpha competent E. coli.These groups were digested with AvaI and XbaI. As a positive control for the fully methylated band, the plasmid was linearized by digestion with only XbaI, mimicking complete protection against AvaI digestion. B) The plasmid was methylated in vitro for increasing amounts of time with M.SssI. The bands shown and quantified with imageJ are the bands corresponding to full methylation of the plasmid.

Then, the zinc finger-M.SssI fusion was expressed from two plasmids, which were the same except for the presence or lack of the zinc finger's nine base pair binding site upstream of the "target" AvaI cut site. After a 10-hour induction, only the fully methylated band was visible, regardless of the presence or lack of the zinc finger's binding site (Figure 3). We then constructed the first TALE-methylase fusion protein, to our knowledge. After a 4-hour induction, the two most visible bands corresponded to no methylation and non-specific methylation. Less visible were the highest band corresponding to full methylation and the lowest band corresponding to site-specific methylation (Figure 4). This experiment was repeated for 0, 2, 6, and 24 hour inductions with 0, .1, 1, and 2 μM IPTG. Greater induction time and IPTG concentration corresponded to greater intensity for the full methylation band (not shown).

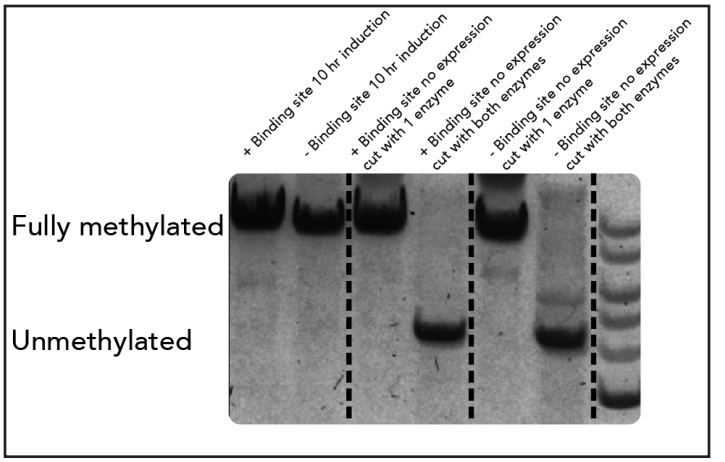


Figure 3. Zinc finger-M.SssI does not show site-specific methylation. The plasmid was modified to make a negative control with the zinc finger's binding site removed. Lanes 3 and 4 are controls to show the zinc finger-M.SssI plasmid digests as expected when linearized and when expression is not induced. Lanes 5 and 6 repeat those controls for the plasmid with the binding site removed. Lanes 1 and 2 show the two plasmids after 10 hours of induced expression of zinc finger-M.SssI and digestion with XbaI and AvaI.

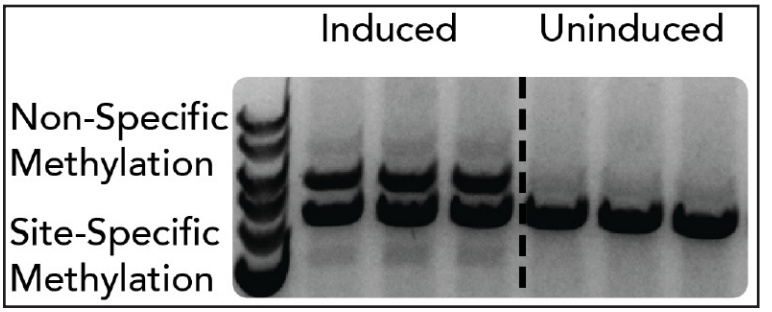


Figure 4. TALE-methylase shows non-specific methylation and lower enzymatic activity. The TALE-methylase was induced and expressed for 4 hours or not induced, then digested with XbaI and AvaI.

## Discussion

The modular plasmid proved to be an effective system to rapidly generate data on targeted methylases. Including the different DNA binding domains, presence or absence of the target binding site, and varied induction

parameters, we were able to produce quantifiable data for over 100 conditions in 3 weeks, including time for cloning. Site-specific methylation is reported by this assay only if greater than 10% of the plasmids are methylated at only the target AvaI site, based on the limits of visibility with our gel electrophoresis set up and loading 600 ng of DNA. In the future, this system could be used to accelerate and lower costs of library screening as researchers optimize promising TALE-methylases or Cas-methylases. Optimization could include varying linker lengths, performing directed evolution, or creating a split-reconstitution system with two DNA binding domains fused to subunits of the methylase. This latter approach could overcome the off-target methylation inherent to any bifunctional system with an active methylase effector (7). In many cases, the ultimate application of targeted methylation will be in mammalian systems, but the E. coli chassis is effective for library screening because the lack of endogenous CpG methylase makes the assay noiseless.

Recently, researchers produced other tools for engineering other aspects of the epigenome, including: TALE-histone modifiers and TALE-DNA demethylases (8, 10, 11). Our new biobrick could be the enabling tool that allows researcher to complete a new toolkit for investigating the role of epigenetics in development and disease by direct manipulation of the primary epigenetic patterns.

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Author Contributions
JT and DC conceived the project idea and designed the BioBricks in consultation with STG, JSM, and BC. JT, DC, BK, MC, and DF designed and performed all experiments. All authors contributed to data analysis and writing the manuscript. STG, MSM, AV, and BYC supervised all aspects of the projects.

Notes
The authors declare no competing financial interest.

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