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ARTICLE

Highly Sensitive Fluorescence Assay for Methyltransferase Activity by Exonuclease-aided Signal Amplification

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Abstract: DNA methylation, catalyzed by methyltransferases, plays critical roles in various biological processes in both prokaryotes and eukaryotes. Bacterial DNA adenine methyltransferases (DAM) are associated with bacterial pathogenesis and essential for bacterial virulence and viability. Since mammals do not methylate DNA at adenine, bacterial DAM is considered to be a great candidate target for developing new therapeutics for diseases. In the current study, we developed a simple, rapid and highly sensitive fluorescent method for the detection of DAM based on exonuclease-aided signal amplification. In the proposed strategy, a liberated Amplifier upon DAM methylation and Dpn I digestion of the substrate can hybridize with a reporter (FT) that contains a quencher (TRAMA) at the second base of the 3' end and a fluorophore (FAM) at the fifth base. Upon hybridization, exonuclease III degrades the reporter in the formed duplex DNA from 3' end successively, releasing the fluorophore from the quencher and resulting in an intensive appearance of fluorescent signal. The Amplifier will hybridize with another reporter and enter a new cycle, which therefore can amplify the signal and dramatically increase the detection sensitivity even with an extremely low amount of Amplifier. Using this strategy, the detection limit down to 0.0025 U/mL of DAM was achieved within a short assay time of 30 min. Furthermore, the assay was applied to evaluate endogenous DAM activity in *E. coli* cell at different growth stages as well as the effects of inhibitors on DAM activity. Given the attractive analytical performance, the sensing strategy may find many important applications in biomedical research and clinical diagnosis.

Introduction

DNA methylation plays a critical role in numerous biological processes, including embryogenesis, regulation of gene expression, genomic imprinting and X-chromosome inactivation.¹⁻³ The methyl group can be introduced into target cytosine or adenine in the specific DNA sequences by a group of enzymes known as DNA methyltransferases.⁴ Changes in DNA methyltransferase activities may lead to aberrant DNA methylation patterns that are associated with various types of diseases.⁵

Bacterial DNA adenine methyltransferase (DAM) are associated with bacterial pathogenesis and essential for bacterial virulence and viability.⁶⁻⁸ DAM can recognize GATC

sites in DNA and convert adenine into *N*⁶-methyladenine (*m*⁶A) with S-adenosylmethionine as the methyl donor. Methylation of adenine at N6 can protect bacterial DNA against cleavage by restriction enzymes.^{9, 10} Since adenine in DNA is not methylated and there is no reported DAM in mammals, hence, bacterial DAM could be a great candidate target for developing new therapeutics for diseases.^{9, 11} For this reason, sensitive detection of the activity of DAM and fast screening of their inhibitors have attracted intense interest in the past decades in both biochemical and clinical research.¹²⁻¹⁴

Traditional methods for DAM activity assay frequently employ the radioactive labeling^{15, 16} and immunoblot-based strategies¹⁵ coupled with high-performance liquid chromatography (HPLC) or gel electrophoresis detection. Although these methods are well-established, most of them either bring in contamination of radioactive materials or are labor-intensive. To overcome those drawbacks, some new methods have been developed for real-time and nonradioactive detection of DAM.¹⁷⁻²¹ For example, gold nanoparticle-based colorimetric assay,^{17, 20} methylation-responsive DNAzyme-based signal amplification colorimetric assay,¹⁹ and light scattering of carbon nanotubes based label-free assay²² have been developed to detect DNA

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methytransferases activity. The sensing platforms often suffer from tedious nanoparticle preparation and functionalization or relative low detection sensitivity. In addition, a variety of electrochemical and electrogenerated chemiluminescence (ECL) biosensors have been designed for the quantitative analysis of DAM activity. Despite the attractive sensitivity, their performances have been compromised by cumbersome multistep processes and long assay times.^{14, 23} Recently, isothermal amplification techniques, such as strand displacement amplification (SDA)¹⁸ and rolling circle amplification (RCA)²⁴ were developed for DNA methyltransferase assay. The strand displacement amplification-based colorimetric method allows simple visualization of DNA methyltransferase activity, but the detection sensitivity of the colorimetric method is limited. RCA method exhibits high sensitivity, while it involves multiple assay steps and normally requires relatively long assay time. Therefore, the development of rapid, simple and highly sensitive method for the DAM assay is still a demand.

In the current study, we present a simple, rapid, and highly sensitive strategy for the DAM activity assay by methylation-sensitive cleavage and exonuclease-aided signal amplification. As shown in Figure 1, a single-stranded DNA (FT) labelled with a quencher (TRAMA) at the second base of the 3' end and a donor fluorophore (FAM) at the fifth base was used as reporter. The fluorescence of the fluorophore was perfectly quenched by FRET because the TAMRA and FAM is quite close to each other (only 3 bases away). Another hairpin DNA (Probe) containing DAM recognition site of GATC was used as the substrate of DAM. Once the Probe was methylated by DAM, it could be recognized and cleaved by Dpn I, which allows the release of a single-stranded oligodeoxynucleotide (Amplifier) in the presence of exonuclease III (Exo III). The Amplifier can hybridize to the single-stranded FT reporter to form a double-stranded DNA with recessed 3'-terminus on the FT, which subsequently triggers the Exo III-aided Amplifier recycling reaction and therefore can significantly enhance the detection sensitivity of DAM. Using this method, we further examined DAM activity in different growth phases of *E. coli* cells and evaluated the inhibition of DAM by certain drugs. Given the attractive analytical characteristics, the sensing strategy could be used to evaluate DAM activity and screen suitable inhibitor drugs of DAM for disease(s) treatment in biomedical research and clinical diagnosis.

Results and discussion

Strategy rationale

The strategy for the sensitive assay of DAM activity is shown in Figure 1. A hairpin DNA (Probe) with a sequence of GATC in the stem region is designed as the substrate of DAM. The Probe contains Exo III-resistant 3' protruding terminus and can be recognized and cleaved by Dpn I once the adenine

in the GATC site is methylated by DAM. Then double-stranded DNA (dsDNA) with blunt 3' end is generated upon Dpn I cleavage, which further initiates the digestion by Exo III to degrade the shorter strand from the 3' terminus and liberates the longer strand (Amplifier). The Amplifier then hybridizes with a reporter FT that contains a quencher (TRAMA) at the second base of the 3' end and a fluorophore (FAM) at the fifth base. Upon hybridization, Exo III degrades the FT in the formed duplex DNA from 3' end successively, releasing the fluorophore from the quencher and resulting in an intensive appearance of fluorescent signal. The Amplifier will hybridize with another FT and enter a new cycle, which therefore can amplify the signal and dramatically increase the detection sensitivity even with an extremely low amount of Amplifier. Thus, an exonuclease-aided amplification of the fluorescent signal could be achieved through the cycling of the Amplifier to allow the sensitive assay of DAM.

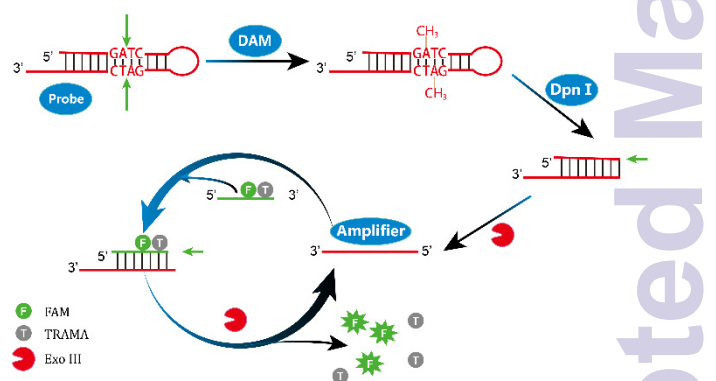


Figure 1. Schematic diagram of the assay for DAM activity based on exonuclease-aided signal amplification.

Investigation of feasibility of the DAM activity assay

Several experiments were carried out to evaluate the feasibility of the proposed strategy for DAM activity assay. First, we investigated the Exo III-aided signal amplification (Figure S1, Supporting Information). When different amounts of Amplifier were mixed with FT in the presence of Exo III, a gradual increase of the fluorescent signal was observed. And the obvious fluorescent signal can be seen even the concentration of Amplifier was as low as 0.01 nM, indicating the high detection sensitivity may be achieved.

Then, we used polyacrylamide gel electrophoresis to examine the cleavage of the methylated hairpin Probe by Dpn I. The results showed that the methylated Probe can be efficiently cleaved by Dpn I (Figure S2, lane 4, Supporting Information). While in the reaction mixture that lacks either DAM or Dpn I, Probe cannot be cleaved by Dpn I (Figure S2, lane 2 and 3, Supporting Information).

Subsequently, we evaluated the feasibility of the proposed DAM activity assay. As shown in Figure S3 (Supporting Information), a significant fluorescent signal was

observed when all the three enzymes of DAM, Dpn I and Exo III were present. However, in the absence of any one of the enzymes, no obvious fluorescent signal was observed, demonstrating the feasibility of this method for the assay of DAM activity. In addition, the fluorescent signals of the controls were extremely low, which could be attributed to the efficient quenching of the fluorophore (FAM) by quencher (TRAMA) due to their short distance. The low background can therefore further contribute to the high detection sensitivity of the assay.

Optimization of assay conditions for DAM activity

To obtain high signal amplification efficiency by Exo III-aided recycling, the core enzyme of Exo III in the proposed strategy was experimentally optimized. As shown in Figure S4, the relative fluorescent intensity was the strongest with using 0.5 U of Exo III. With the further increase of Exo III, the specificity of Exo III can decrease since the background became higher due to the non-specific degradation of the reporter FT. Thus, 0.5 U Exo III was used for the following experiments.

We then further optimized the Exo III incubation time. The result showed that relative fluorescent intensity reached a plateau within 10 min (Figure 2A). Thus, 10 min was used as Exo III incubation time for the following experiments. Finally, we optimized the DAM incubation time. The result showed that the fluorescent intensity reached a plateau when the incubation time was 20 min (Figure 2B). Therefore, 20 min of DAM incubation time was used for the subsequent experiments. Taken together, the total assay time was 30 min, which is more rapid than previously established methods for DAM assay (Table S1, Supporting Information).

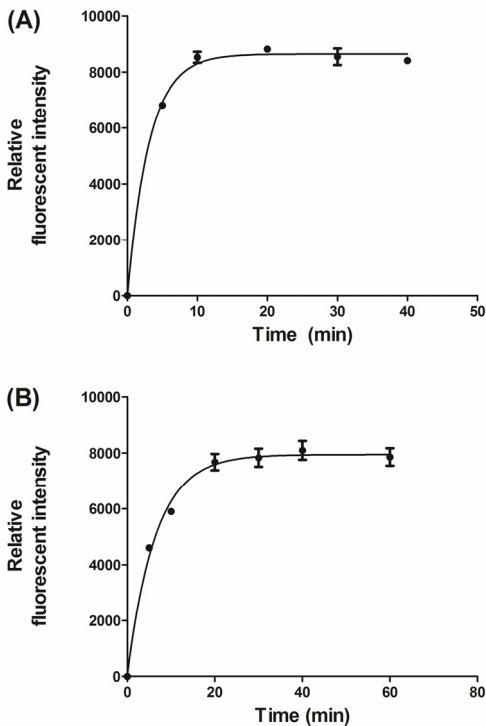


Figure 2. Optimization of the incubation time of Exo III (A) and DAM (B).

Assay of DAM activity

To investigate the analytical performance of the proposed strategy, we measured the DAM activity at various concentrations under the optimized conditions. By combining low-background reporter FT with high-efficiency Exo III-aided signal amplification, an ultra-sensitive detection was achieved and even as low as 0.0025 U/mL of DAM can be readily observed (Figure 3A), which is much lower than previously reported assay for DAM activity (Table S1, Supporting Information). The linear ranges of the assay for DAM activity were evaluated using DAM with the range from 0 to 50 U/mL. As shown in Figure 3B, a relatively good linearity was obtained across 3 orders of magnitude ($R^2=0.9885$).

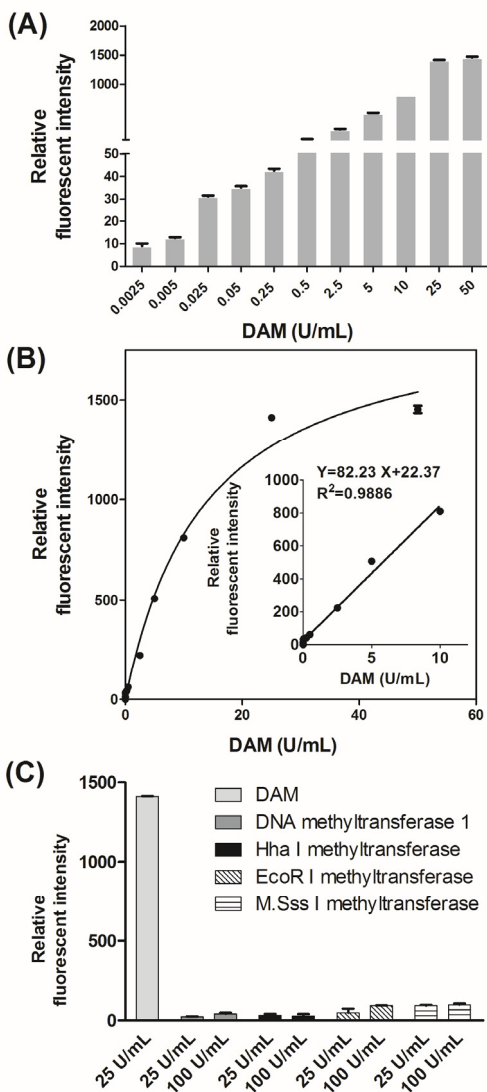


Figure 3. (A) Relative fluorescent signal intensity in the presence of different concentrations of DAM. (B) Linear curve of the assay for DAM activity based on exonuclease-aided signal amplification. (C) Selectivity of the assay for DAM.

We further evaluated the selectivity of this assay for DAM activity. In this respect, DNA methyltransferase 1, Hha I methyltransferase, EcoR I methyltransferase, M.Sss I methyltransferase, were selected as interference methyltransferases to assess the selectivity of this method. The results showed that only DAM can induce a remarkable fluorescence enhancement, while the other four methyltransferases have no obvious fluorescence change, even when adding 4-fold more of the methyltransferases (Figure 3C). These results demonstrated that the amplification strategy for DAM activity exhibited good selectivity originating from the high specific sequence recognition between DAM and the hairpin substrate.

Generally, the strategy we developed here is simple, highly sensitive and can be accomplished within 30 min. As far as we know, the assay time of this method is relatively short compared with most of the established approaches that typically ranged from 60 min to 420 min. Also the detection limit of our assay is 0.0025 U/mL, which is better than most of the previously developed approaches. A detailed comparison of these assays for DAM activity is listed in Table S1 (Supporting Information).

Inhibition of DAM activity

DAM inhibition is attractive because humans lack this particular enzyme. Therefore, DAM represents an excellent candidate for antibiotic development. In our research, we examined the inhibition effects of gentamycin (broad-spectrum antibiotic) and 5-fluorouracil (an anticancer drug) on DAM activity using our developed strategy. Previous reports suggest that gentamycin and 5-fluorouracil had no influence on the activity of Dpn I and Exo III when the concentration was lower than 100 μM .^{25, 26} Therefore, the effect of gentamycin and 5-fluorouracil should go through the inhibition of DAM. As shown in Figure 4, gentamycin and 5-fluorouracil displayed dose-dependent inhibition for DAM. The calculated IC_{50} for gentamycin and 5-fluorouracil were 11.3 μM and 8.82 μM , respectively, which were at the same level as previous reports.^{14, 19, 23, 27} These results demonstrated that the proposed DAM activity assay can be successfully applied in DAM inhibitor screening and is potentially a useful tool for antibiotic drug discovery.

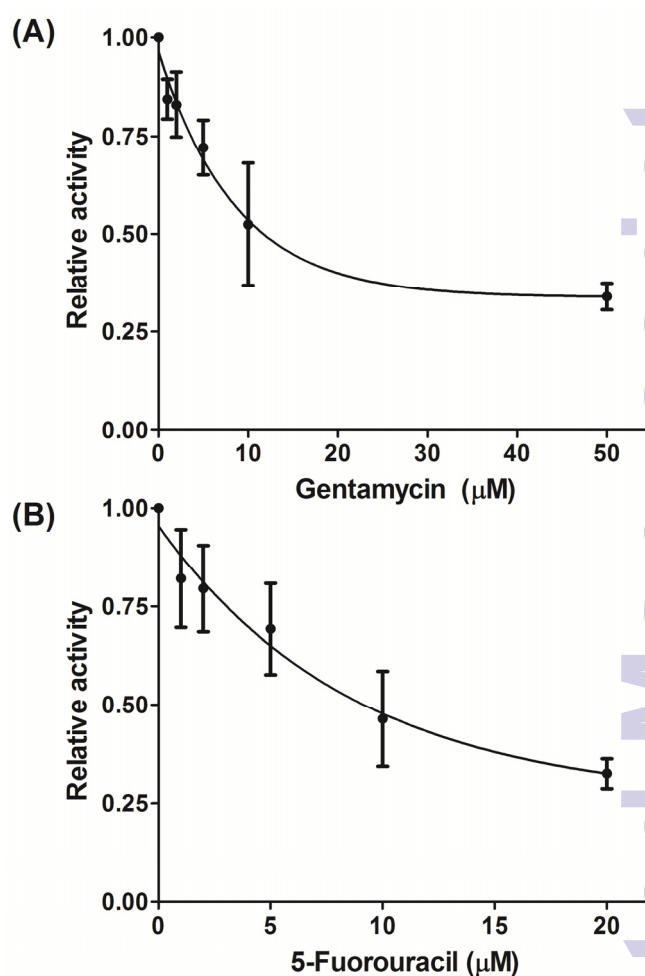


Figure 4. Inhibition of DAM activity by gentamycin (A) and 5-fluorouracil (B).

Assay of DAM activity in *E. coli* cells

The developed assay was then applied to examine the endogenous DAM activity in *E. coli* cells. As shown in Figure 5A, the relative fluorescent intensity showed a dose-dependent increase of the total proteins of *E. coli* cells. As for the calculation of DAM activity in *E. coli* cell lysate, we first determined the total protein concentration using Bradford Protein Assay. Then certain volume of *E. coli* cell lysate was added into the reaction buffer and DAM activity (U/mL) was determined by the calibration curve in Figure 3B. Therefore DAM activity (U/mg) can be obtained with knowing the DAM activity (U) and the total protein amount (mg) in the certain volume of *E. coli* cell lysate.

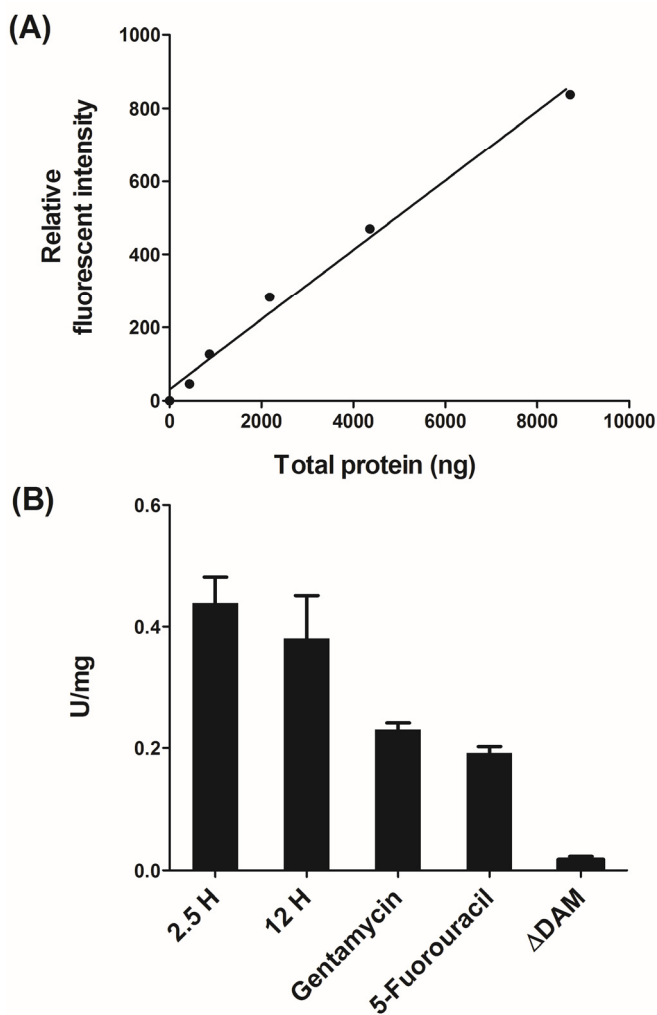


Figure 5. (A) Measurement of the activity of DAM in *E. coli* cells. (B) The activity of DAM at different growth phases of *E. coli* cells and in DAM negative (Δ DAM) *E. coli* cells, and the inhibition of DAM activity by gentamycin (20 μ M) and 5-fluorouracil (20 μ M).

We then compared the DAM activity in different growth stages of *E. coli* cells. The exponential growth stage of *E. coli* occurred between 1.5 and 3 h, and the growth rate slowed down and then entered the stationary growth stage after 12 h. The result showed that the activity of DAM was slightly higher in exponential growth stage than in stationary growth stage (no significant difference, $p = 0.482$, Figure 5B), with the mean DAM activity being 0.44 U/mg at exponential growth stage and 0.38 U/mg at stationary growth stage. This phenomenon may attribute to the higher level of DAM in *E. coli* cells at exponential growth stage, which is required for the fast growth of *E. coli* cells.^{28, 29}

In addition, we examined the inhibition of DAM from *E. coli* cell lysate by gentamycin and 5-fluorouracil. The results showed that DAM activity can be efficiently inhibited by gentamycin and 5-fluorouracil (Figure 5B). Furthermore, no obvious DAM activity was observed in DAM-negative (Δ DAM) *E. coli* of JM110 (Figure 5B). These results suggested that the

obtained signal is derived from DAM activity, not from the non-specific cleavage of the Probe. Combining these experiment results, the strategy we developed can be readily applied to evaluate DAM activity in complex biological samples.

Experimental

Reagents

DAM, DNA methyltransferase 1, Hha I methyltransferase, EcoR I methyltransferase, M.Sss I methyltransferase and Dpn I were purchased from New England Biolabs (Ipswich, MA, USA). Exo III was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). All oligodeoxynucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences of these ONDs are listed in Table 1. Yeast extract, peptone, and agar were purchased from Becton Dickinson Medical Devices Co., Ltd. (Shanghai, China). The cell lysis buffer RIPA and Bradford Protein Assay Kit were provided by Beyotime Institute of Biotechnology (Shanghai, China). The water used throughout the study was purified by a Milli-Q apparatus (Millipore, Bedford, MA). Other chemicals of analytical grade were purchased from Sigma-Aldrich (Beijing, China).

Table 1. Sequences of ONDs.

ONDs	Sequences *
Hairpin probe	5'- <u>TATACGCACCGTCCTACGATCCGTTTTTCG</u> <u>GATCGTAGGACGG</u> <u>TGCGTATAAATG</u> -3'
FT	5'-TATACGCACCG/i6FAMdT/CC/iTAMdT/A-3'
Amplifier	5'-TCGTAGGACGGTGCGTATAAATG-3'
3'-FAM labeled probe	5'- <u>TATACGCACCGTCCTACGATCCGTTTTTCG</u> <u>GATCGTAGGACGG</u> <u>TGCGTATAAATG</u> -FAM-3'

*The bases underlined can form stem of the hairpin substrate. Highlight in red are the recognition sites of GATC for DAM and Dpn I. Highlight in italic are the liberated ssODN (Amplifier) after Dpn I digestion.

Culture of bacterial cells

The GW5100 (DAM positive) and JM110 (DAM negative) *E. coli* cells were cultured according to our previous method.³⁰ Briefly, a colony was inoculated into 5 mL of liquid medium (5 g/L yeast extract, 10 g/L Trypton, 10 g/L NaCl) and incubated at 37 °C in a shaker (250 rpm) for 12 h. Then, 50 μ L of the cell suspension was added into 5 mL of medium and incubated for 2.5 h or 12 h. Subsequently, the cell suspension was centrifuged at 5,000 rpm to obtain cell pellet followed by rinsing with Milli-Q water twice. The resulting *E. coli* cells were lysed using RIPA lysis buffer. The protein contents were determined using Bradford Protein Assay Kit according to manufacturer's recommended protocol.

Fluorescence monitoring procedure

All Fluorescence measurements were performed on a spectrofluorometer (Hitachi Ltd., F-4600, Japan) at 25 °C. The excitation and emission slit widths were 5 nm and 10 nm, respectively. The excitation was made at 494 nm with a recording emission ranging from 505 to 550 nm. The highest fluorescent emission intensity was recorded at 518 nm.

Assay of DAM activity

All of these standard solutions were prepared under 4 °C and stored at –20 °C. The reaction mixture (200 µL) consisted of 2.5 nM hairpin Probe, 50 nM FT, 160 µM SAM (S-adenosylmethionine), 5 U of Dpn I endonuclease and various amounts of DAM. The reaction buffer contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM KCl, and 1 mM DTT. The experiment was performed at 37 °C for 20 min. After that, 0.5 U of Exo III was added, and the mixture was incubated at 37 °C for 10 min. To achieve the best performance, the incubation time of DAM and Exo III was optimized.

The synthesized oligodeoxynucleotides of Amplifier was used to demonstrate the feasibility of the Exo III-aided signal amplification. The reaction mixture (200 µL) consisted of 50 nM FT, 0.5 U of Exo III and various amounts of Amplifier. The reaction buffer contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM KCl, and 1 mM DTT. The experiment was performed at 37 °C.

As for the assay of *in-vivo* DAM activity of *E. coli* cells, the RIPA-cell lysate (0.5 µL–10 µL) was directly added into the reaction mixture, and the subsequent analysis was identical to the assay of standard DAM.

Influence of inhibitors on DAM activity

DAM is closely related to cancer and bacterial virulence, so inhibition of DAM activity holds promise for the development of anti-cancer drugs and antibiotic agents. To further examine the inhibitors screening ability of the proposed assay, the influence of drugs on DAM activity was investigated by using gentamycin (broad-spectrum antibiotic) and 5-fluorouracil (anti-cancer drugs) as two model inhibitors. First, inhibitors (gentamycin or 5-fluorouracil) were added at various concentrations with hairpin Probe (2.5 nM) in 200 µL reaction buffer (20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM KCl, and 1 mM DTT). Then 50 nM FT, 160 µM SAM, 5 U of Dpn I endonuclease and 1 U of DAM were added, and the mixture was incubated at 37 °C for 20 min followed by additional incubation for 10 min after adding Exo III.

Conclusions

In the current study, we developed a simple, rapid, and highly sensitive fluorescent assay for the detection of DAM activity by methylation-sensitive cleavage and exonuclease-aided signal amplification. By combining low-background reporter with high-efficiency Exo III-

aided signal amplification, a very low detection limit of DAM (0.0025 U/mL) was achieved and the whole assay can be accomplished within 30 min. The application of the assay for the detection of DAM activity in *E. coli* cells further demonstrated that the proposed strategy showed a great potential for the detection of DAM activity in complex biological samples. Given the attractive analytical performance, the sensing strategy also can be applied to screen inhibitors of DAM as well as in biomedical research and clinical diagnosis.

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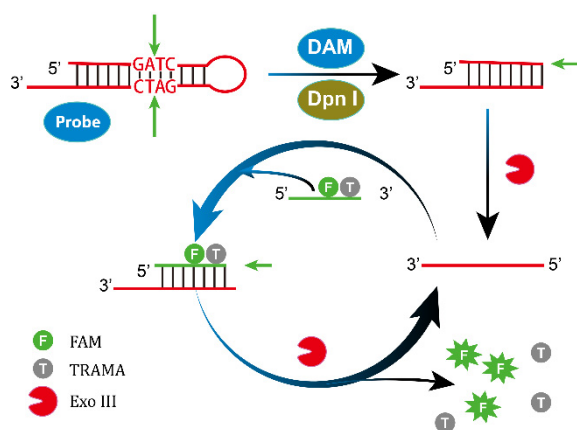
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A highly sensitive fluorescence assay for DNA adenine methyltransferase activity was developed using exonuclease-aided signal amplification.