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Original article

Sensitive detection of DNA methyltransferase activity based on rolling circle amplification technology

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ABSTRACT

This work develops a fluorescence approach for sensitive detection of DNA methyltransferase activity based on endonuclease and rolling circle amplification (RCA) technique. In the presence of DNA adenine methylation (Dam) MTase, the methylation-responsive sequence of hairpin probe is methylated and cleaved by the methylation-sensitive restriction endonuclease Dpn I. The products cleaved by restriction endonuclease Dpn I then function as a signal primer to initiate RCA reaction by hybridizing with the circular DNA template. Each RCA product containing thousands of repeated sequences might hybridize with a large number of molecular beacons (detection probes), resulting in an enhanced fluorescence signal. In the absence of Dam MTase, neither methylation/cleavage nor RCA reaction can be initiated and no fluorescence signal is observed. The proposed method exhibits a dynamic range from 0.5 U/mL to 30 U/mL and a detection limit of 0.18 U/mL. This method can be used for the screening of antimicrobial drugs and has a great potential to be further applied in early clinical diagnosis.

1. Introduction

DNA methylation is regarded as a critical biochemical reaction which has been implicated in the regulation of a variety of biological processes across virtually every branch of the taxonomic tree [1–3]. DNA methylation is achieved by the catalysis of DNA methyltransferases (MTase), which specifically recognize short palindromic sequences and catalyze the transfer of a methyl group from several different donors to the target adenine or cytosine. DNA methylation is associated with the formation of heterochromatin and silencing of genes critical for the regulation of growth and proliferation [4,5]. This covalent modification of DNA functions as an important mediator of gene regulation and, together with covalent modifications of histone proteins, also forms the cornerstone for the burgeoning field of epigenetics. Cancer cells are characterized by hypomethylation throughout their genomes, plus hypermethylation of specific regions, which has been associated with transcriptional silencing [6,7]. Because of its key role in pathology and diagnostics, analysis of DNA methylation and MTase activities has increasingly received research interests. A few methods have been developed for the determination of DNA methylation and MTases activity, including polymerase chain reaction (PCR)-based techniques [8,9], light scattering techniques [10], colorimetric approaches [11–13], capillary electrophoresis [14], high-performance liquid chromatography (HPLC) [15], fluorescence [16–19], electrochemical [20,21], and chemiluminescence methods [22], *etc.* However, most of those methods have shortcomings, like being time-intensive, laborious treatment, radiolabeling substrate requirement, or immobilization of a target analyte to surfaces.

In recent years, isothermal amplification techniques, such as rolling circle amplification (RCA) [23], strand displacement amplification (SDA) [24], and nicking enzyme signal amplification (NESA) [25], have been frequently used for an enzyme assay due to their wide variety of choices and significant signal amplification. Among these amplification methods, the SDA [26] and NESA [27] methods have been used for the DNA MTase assay. RCA technology has proven very useful for highly sensitive detection of target nucleic acids and proteins [28,29]. In RCA-based assays, target quantification is achieved through the quantification of the RCA products. In this study, we adopted rolling circle amplification technique and molecular beacons to develop a sensitive and selective method to detect DNA methyltransferase activity. As shown as Scheme 1, we used DNA adenine methylation (Dam)

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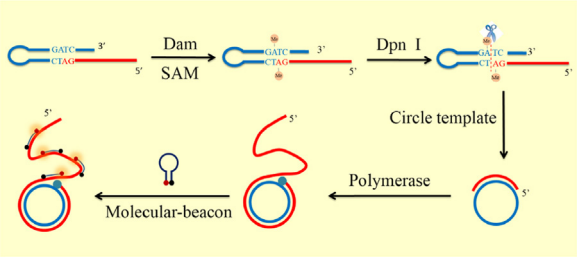

Scheme 1. Schematic diagram of DNA methylation detection based on the Dam MTase-restriction endonuclease Dpn I interaction system and RCA technology.

MTase and restriction endonuclease Dpn I endonuclease, which both had the same 5′-G-A-T-C-3′ recognition sequence. Dam MTase catalyzes the methylation of the sequence 5′-G-A-T-C-3′, and restriction endonuclease Dpn I cleaves the sequence 5′-G-Am-T-C-3′. The cleaved short oligonucleotide can hybridize with the circular DNA template and trigger RCA. Each RCA product containing a large number of repeated sequences might hybridize with a large number of molecular beacons (detection probes), resulting in an enhanced fluorescence signal. In contrast, in the absence of Dam MTase, the unmethylated DNA cannot be cleaved by the restriction endonuclease Dpn I. At the same time, Bst polymerase (large fragment) only can copy complementary sequences from the 3′ end of oligonucleotides, and the polymer reaction cannot be triggered. Therefore, no obvious change in the fluorescence intensity of the detection probe is observed. The unique ability of restriction endonuclease Dpn I to transduce DNA adenine methylation to sequence information was melded perfectly with the capabilities of RCA to amplify nucleic acid sequences.

2. Experimental

2.1. Materials

Oligonucleotides designed in this study were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China), and the sequences of all oligonucleotides are listed in Table 1. The underlined portion in the probe is the specific recognize sequence for Dam methyltransferase. The Dam MTase (*Escherichia coli*), restriction endonuclease Dpn I, Bst DNA polymerase (large fragment), S-adenosylmethionine (SAM), dNTP and the corresponding buffer solution were purchased from New England Biolabs Inc. (NEB, U.K.). The gentamycin sulfate was purchased from Bo Mei Biotechnology Co., Ltd. Other chemicals were of analytical grade and were used without further purification. The solutions in the experiments were prepared with ultrapure water (Milli-Q 18.2 MΩ cm, Millipore System Inc.). Several buffers were used in the present work: The methylation buffer contained 10 mmol/L Tris–HCl (pH 7.5 @ 25 °C), 15 mmol/L $MgCl_2$, 50 mmol/L NaCl, and 1 mmol/L DTT. The RCA-reaction buffer was composed of 20 mmol/L Tris–HCl (pH 8.8 @ 25 °C), 10 mmol/L KCl, 2 mmol/L $MgSO_4$, 10 mmol/L $(NH_4)_2SO_4$, and 0.1% Triton X-100.

Table 1
Sequences of oligonucleotides used.

Note	Sequence (5′-3′)
Hairpin probe	5′-GTT GGC GAA GAT CCC GCT TCT TTT GAA GCG GGA TCT TCT TTT-3′
Circular template	5′-p-CTT CGC CAA CTT GTT TCC TTT CCT TGA TAC TTT GAC CTT TCT TTC TTT CGA CTA AGC AGT TGT-3′
Detection probe	5′-(FAM)-AGC TAA TCC TTG ATA CTT TGA CTT AGC T-(DABCYL)-3′

2.2. Methylation assay by gel electrophoresis

The reaction mixtures (200 μL) consisted of a 200 nmol/L hairpin probe, 1× methylation buffer, 160 μmol/L SAM, 20 units of restriction endonuclease Dpn I, and 40 units of Dam MTase. In the gel electrophoresis assay, 5 μL samples were removed from the reaction solution after having been incubated at 37 °C for 90 min and then heated at 65 °C for 15 min to stop the reaction. Subsequently, the samples were put on a polyacrylamide gel (20% acrylamide, 19:1 acrylamide/bisacrylamide) to separate the cleaved products from the substrate. The electrophoresis was carried in 1× TBE (pH 8.0) at 100 V constant voltage for 3 h, and then the gel was scanned using the Gel Image Analysis System (Tanon 2500R, Shang Hai, China).

2.3. Apparatus and fluorescence measurements

Fluorescence spectra were measured using a Hitachi F-7000 fluorescence spectrometer (Hitachi, Japan) with a personal computer data processing unit. Excitation and emission slits were all set for 5.0 nm band-pass. The fluorophore of FAM was excited at 488 nm, and the emission spectra from 500 nm to 600 nm were collected. The fluorescence intensity at 521 nm was used to evaluate the performance of the proposed assay strategy. All measurements were carried out at room temperature unless stated otherwise.

2.4. RCA and Dam MTase detection

A Dam MTase assay buffer solution was prepared in an Eppendorf tube containing 1× methylation buffer, 1 μmol/L substrate oligonucleotide (namely Prime), 160 μmol/L SAM, 20 units of restriction endonuclease Dpn I, and a different activity of Dam MTase. The methylation was allowed to proceed at 37 °C for 90 min and then heated at 65 °C for 15 min to stop the reaction. Then, 20 μL of the resulting solution was added to 20 μL of RCA-reaction buffer containing 1× RCA-reaction buffer, 500 μmol/L dNTPs and 0.2 U/μL Bst DNA polymerase (large fragment). The polymerization reaction was carried out at 60 °C for 60 min. Subsequently, the resulting solution was incubated at 80 °C for 20 min to inactivate the Bst DNA polymerase (large fragment). After the resulting solution was cooled to ambient temperature, the detection probes were added and allowed to hybridize with the RCA products for about 40 min prior to the fluorescence measurement.

3. Results and discussion

3.1. Design and feasibility of RCA based Dam MTase assay

As mentioned above, in the presence of Dam MTase, the enzyme will catalyze the methylation reaction on the recognition sequence to yield the methylation duplex DNA 5′-G-Am-T-C-3′. Then, the cleavage reaction by restriction endonuclease Dpn I was initiated, and the cleaved short oligonucleotide hybridized with the circular DNA template and triggered rolling circular amplification. The fluorescence signal was acquired after RCA product sequences opened the molecular beacons.

To confirm our assay method, gel electrophoresis experiments were carried out. As shown in Fig. 1A, new bands appear in lane 3 when both Dam MTase and restriction endonuclease Dpn I are added in the test solution, suggesting that a methylation reaction has taken place, and the methylated DNA had been cleaved into