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Liang Cui, Zhi Zhu*, Ninghang Lin, Huimin Zhang, Zhichao Guan and Chaoyong James Yang*

ARTICLE TYPE

T7 Exonuclease-assisted cyclic enzymatic amplification method (CEAM) was combined with rolling circle amplification (RCA) to develop a RCA-CEAM dual amplification method for ultrasensitive detection of microRNA with excellent selectivity.

MicroRNAs (miRNAs), which are processed from longer endogenous hairpin transcripts by the enzyme dicer, comprise a class of endogenous non-coding small RNA molecules (19-25 nucleotides). Several hundred miRNAs have been found to encode in the human genome and dozens of them have now shown to regulate a diverse variety of cellular processes, both in normal physiology and in disease. Thus, for fully understanding their functions and diagnosis of human diseases, it is of great significance to develop a sensitive and selective method for the detection of miRNAs.

In the past decades, a variety of effective miRNA detection methods have been reported, from initial northern blotting, microarray to many recently developed sensitive methods based on signal amplification strategies. For example, Jonstrup et al. developed a novel miRNA detection system by using miRNA as template to cyclize padlock probes and subsequently as primer for rolling circle amplification (RCA). In Jonstrup’s work, linearly amplified detection of miRNA was achieved with a detection limit of ~10 pM. While RCA is a simple, reliable and isothermal amplification method, the limited sensitivity provided by linear amplification cannot satisfy the requirement of miRNA detection. Therefore, a new signal amplification strategy is needed to further improve the sensitivity.

Cyclic enzymatic amplification method (CEAM) based on nuclease, in which one target leads to many cycles of target-dependent nuclease cleavage of reporter probes for output signal amplification, is a recently developed method for simple and convenient nucleic acid detection. Taking Exonuclease III (Exo III) as an example, Exo III catalyzes the stepwise removal of mononucleotides from the 3’-blunt or recessed terminus of duplex DNA. Relying on this unique property, Exo III-based CEAM has been widely used for optical (fluorescence, SPR, UV-Vis, etc.) and electrical signal amplification in detecting DNA, proteins and small molecules. While proven to be simple, sensitive, and reliable, CEAM suffers from several inherent limitations when used for miRNA analysis. For instance, as a linear amplification process, CEAM can only achieve detection limits in the pM range. Furthermore, limited by the intrinsic properties of the nuclease used, most of the previous CEAMs are not applicable to RNA targets.

In this work, by taking the merits of RCA and CEAM while offsetting their drawbacks, we developed a RCA-CEAM dual amplification method for ultrasensitive detection of miRNA with excellent selectivity. Meanwhile, the proposed method was further successfully applied to differentiate the let-7a expression levels of hepatoma cells and normal hepatocytes, demonstrating its potential application in early cancer diagnosis.

As shown in Fig. 1, the system contains RCA reaction substrates (circular template, phi29 DNA polymerase, dNTPs) as well as CEAM reaction substrates (exonuclease, report probe). In this work, in order to improve hybridization kinetics and reduce background signal, we used linear molecular beacon (LMB) that was specially designed for CEAM as the report probe. LMB, which has been described in detail in previous literature, is a linear oligonucleotide probe with a fluorophore and a quencher attached to the terminal and penultimate nucleotides, respectively. The circular template of RCA was designed embedding the complementary sequences of target miRNA and reporter LMB. In the first step, binding of target miRNA to the circular template permits replication of the circular template with the help of phi29 DNA polymerase. RCA is an isothermal nucleic acid replication process, which produces a long ssDNA with numerous copies of the complementary sequence of the original circular template. This long linear ssDNA product thus consists of many repeating units that are complementary to the LMB, and can hybridize with thousands of LMB molecules.

In the second step, CEAM is carried out. According to the previous reports, although Exo III has been widely used in CEAM for detection of DNA and other analytes, it has extra residual exonuclease activity on ssDNA, resulting in reduced sensitivity due to a high background signal. Thus, in this work, we chose T7 exonuclease (T7 Exo), which hydrolyzes mononucleotides from blunt or recessed 5’-termini of duplex DNA but cannot work on ssDNA. As shown in Fig. 1, since T7 Exo only cleaves the 5’ recessed DNA strand from the duplex, the LMB is digested and the fluorophore is separated from the quencher to emit fluorescence, and then each unit is released to bind another LMB and initiates a new cleavage process. Through
such a cyclic hybridization-hydrolysis process, each RCA product has many units and each unit can induce the cleavage of a large number of LMBs to liberate numerous fluorophores. As a result, the fluorescence intensity of the solution will increase as the cycling reaction proceeds, resulting in highly efficient fluorescent signal amplification. Furthermore, since the RCA employs short sequence miRNA as the primer, non-active long-chain pr

In order to demonstrate the feasibility, we first systematically investigated the activity and specificity of T7 Exo. A polyacrylamide gel electrophoresis (PAGE) experiment was performed to test the activity of T7 Exo. Various concentrations of target cDNA were added to a constant concentration of TaqMan probe solution with T7 Exo. TaqMan probe was designed to be complementary to target cDNA and form dsDNA with a 5’ recessed terminus for T7 Exo digestion. As shown in lane 1 of Fig.2A, in the absence of target cDNA, there was no detectable hydrolysis of the TaqMan probe. When the target concentration was only 1% of the probe concentration (lane 2), the probe was partially digested. The gradual fading of the probe band was clearly observed with increasing target concentration from 10% (lane 3) to 100% (lane 4). The gel results confirmed that the target can trigger the cyclic hydrolysis of the probe. Furthermore, even a few target molecules could lead to the complete digestion of the probe, thereby establishing the feasibility of using T7 Exo for signal amplification.

To evaluate the ssDNA cleavage activity of T7 Exo, the fluorescence intensity of the TaqMan probe was monitored before and after binding to target cDNA in the presence of T7 Exo. As a control, the specificity of Exo III was also tested in the same way. In order to achieve a credible comparison, the TaqMan probes were designed to resect in both termini when hybridizing with targets, so they became the substrates of both T7 Exo and Exo III. As shown in Fig.2B, TaqMan probe bands are highlighted with the red rectangle. Lane 5 (probe only) and lane 6 (target only) are controls. (B) Time course study of T7 Exo and Exo III activity on ssDNA.

The performance of T7 Exo-assisted CEAM for detection of DNA was also investigated. The principle is similar to that for CEAM assisted by Exo III described in detail in previous reports. As shown in Fig.3A, with SybrGreen in the reaction system, after addition of 10 nM miRNA, the fluorescence intensity significantly increased, indicating a successful RCA reaction in the presence of miRNA. After the performances of RCA and CEAM were confirmed individually, we carried out the dual amplification method for the detection of miRNA. Theoretically, in the RCA step, in the presence of miRNA, the length of the RCA product would increase linearly with time, thus generating numerous repeating units that are complementary to LMB. In the CEAM step, a single unit from RCA could lead to the consumption of many LMBs and significant restoration of fluorescence. As a result, sensitive detection of miRNA can be achieved. As shown in Fig.3B, with increasing target concentration, a gradual increase in fluorescence intensity was observed. Fig.3C shows the relationship between fluorescence intensity change and target miRNA concentration. The fluorescence intensity change showed a good linear positive correlation towards target miRNA concentrations within the range from 25 fM to 1 pM, the apparent increase in fluorescence intensity was observed, showing that T7 Exo does not have any observable exo- nuclease activity against ssDNA at these experimental conditions. However, after addition of cDNA, a rise in fluorescence intensity was observed instantaneously, indicating a quick cleavage of the probes after hybridizing with cDNA. In contrast, even in the absence of target cDNA, a remarkable rise in fluorescence intensity was observed upon the addition of Exo III. This was likely due to the residual exo-nuclease activity of Exo III on ssDNA, resulting in a high background signal. Therefore, after addition of cDNA, there was no observable change in fluorescence intensity. The above results confirmed that T7 Exo showed better specificity on the dsDNA substrate than Exo III. The residual exo-nuclease activity on ssDNA of Exo III greatly compromised the detection sensitivity due to the high background signal. In contrast, under our experimental condition, no residual ssDNA cleavage activity was observed for T7 Exo, which makes it a desired candidate for designing T7 Exo-assisted CEAM to work with RCA.

The performance of T7 Exo-assisted CEAM for detection of miRNA cannot trigger the RCA reaction. Overall, by combining the two linear amplification methods, the dual amplification method may provide excellent sensitivity and selectivity for miRNA analysis.
employed the developed method to quantify the expression level of miRNA. The expression of let-7a in hepatoma cells and normal hepatocytes. With ultrahigh sensitivity and selectivity, the RCA-CEAM dual amplification method has great potential for miRNA discovery, functional studies, and clinical diagnoses relying on miRNA biomarkers.

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Notes and references

State Key Laboratory of Physical Chemistry of Solid Surfaces, Key Laboratory for Chemical Biology of Fujian Province, The MOE Key Laboratory of Spectrochemical Analysis & Instrumentation, Department of Chemical Biology, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, P. R. China; E-mail: cyyang@xmu.edu.cn, zhazhi@xmu.edu.cn

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