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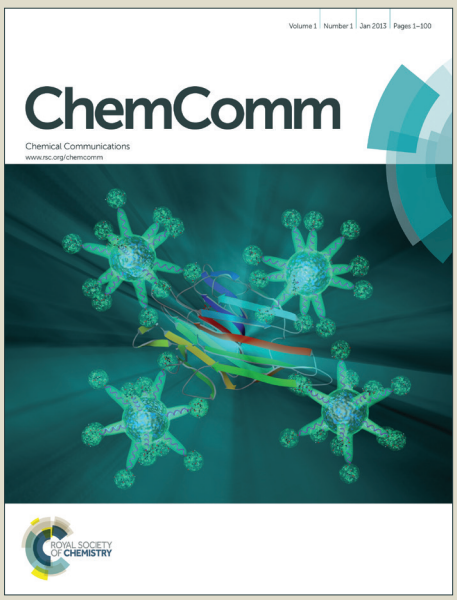

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ARTICLE TYPE

A RCA-based assay for analyzing individual strand break in DNA heteroduplexes cleavage by restriction endonucleases

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We have developed a rapid and high-throughput assay based on rolling circle amplification, to distinguish individual strand cleavage of DNA duplex by restriction endonucleases. As an illustration, we analyzed nicking activity of Nb.BbvCI and uneven cleavage of LNA modified DNA by EcoRI. This assay has potential for analyzing protein-DNA interactions.

Rolling circle amplification (RCA) is a natural DNA amplification process[1]. In RCA reaction, a circular single-stranded DNA template and a primer form a duplex, phi29 DNA polymerase extends the primer to synthesize a long DNA product containing hundreds and thousands of repeats whose sequence are complementary to the circular template. Since RCA process has the advantages of easy, rapid and isothermal reaction, it has been used in many bioassays, primarily in detecting DNA, RNA and other molecules[2]. To enhance its potential applications for other biological fields, we developed a RCA-based approach to expand its utility in studying the interactions of DNA with proteins, especially restriction endonuclease (RE). Nucleotide analogue-induced asymmetric cleavage of RE is important in precisely revealing RE's binding and catalytic mechanism. Asymmetric cleavage analysis was also useful for asymmetric cleavage REs such as nicking endonuclease (NEase or nickase). We show here a RCA-based assay able to analyze single strand break in DNA duplex asymmetric cleavage by RE, thus we term it as single strand cleavage analysis based on RCA (SSCAR).

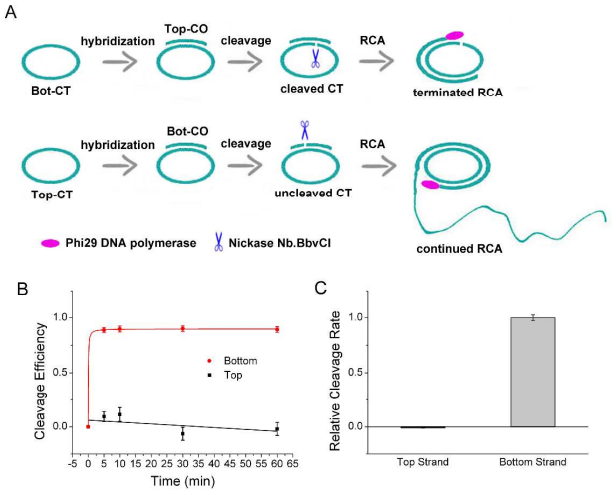

Fig. 1 (A) Schematic diagram of analyzing nicking activity by SSCAR. (B) Plots of cleavage efficiency of Bot-CT/Top-CO and Top-CT/Bot-CO substrates. (C) Relative cleavage rates of Bot-CT and Top-CT strands, respectively.

First, we have proved the concept of this approach by examining the single strand cleavage of DNA duplexes by nickase Nb.BbvCI. This nickase recognizes unpalindromic sequence of dsDNA. The top strand sequence is 5'-CCTCAGC-3', and the complementary sequence in bottom strand is 5'-GC↓TGAGG-3' which containing the cleavage site (indicated as arrow) [3]. We designed two experiments to distinguish the single strand cleavage accordingly. In the first situation, the bottom sequence of Nb.BbvCI was arranged on the circular template (named as Bot-CT strand) and the top sequence was on the complementary oligonucleotide (named as Top-CO strand) (Fig. 1A). Once the Bot-CT/Top-CO substrate was mixed with Nb.BbvCI, the circular template was cleaved, and phi29 DNA polymerase was unable to continue the RCA reaction (Fig. 1B). The cleavage rate monitored by RCA were linearly related to nickase concentration (Fig. S1). In contrast, when the top sequence was designed on the circular template, the nickase made a cut on the complementary strand and the circular template allowed the RCA reaction to be continued. The nicking efficiencies and the relative cleavage rates were then calculated (Fig. 1C). These results clearly proved the feasibility of this RCA-based approach to distinguish the single strand cleavage.

Nowadays, the most commonly used methods for analyzing the RE (especially nickase) cleavage are electrophoresis-based [4]. In general, one strand of the duplex is either radioisotope-labeled or fluorophore-labeled prior to experiments. After incubation with REs, the cleaved products are analyzed using denaturing electrophoresis, and the cleavage process is examined by visualizing the labeled strands. These methods are laborious, time-consuming and low-throughput. Though methods based on fluorescence resonance energy transfer (FRET) have been also introduced into the endonuclease cleavage assay for their convenience, time-saving and high-throughput, they can only monitor the duplex DNA cleavage rather than individual strand cleavage of duplex [5]. Our RCA-based approach provides a rapid and convenient way to analyze the single strand cleavage of nickases. To our knowledge, analyzing the single strand cleavage of RE without electrophoresis and labeling has not been reported previously.

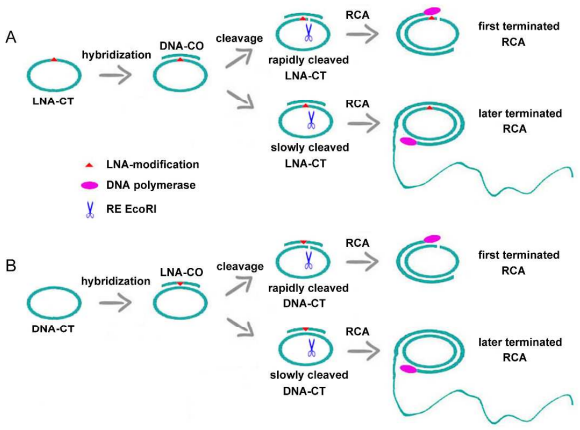

Fig. 2 Schematic diagram of analyzing LNA-induced asymmetric cleavage. (A) Analysis of CT cleavage affected by the LNA substitution on the circular template strand (LNA-CT). (B) Analysis of CT cleavage affected by the LNA substitution on the complementary strand (LNA-CO).

Next, we utilized this approach to explore the double strand cleavage by restriction endonuclease EcoRI. An EcoRI dimer recognizes a palindromic sequence G↓AATTC and cleaves both strands evenly. Both the circular strand and the complementary strand contain the EcoRI recognition sequence. As shown in Figs. S2A and S2B, at a fixed EcoRI concentration, the RCA signals were proportional to the CT concentration. When the substrate concentration was fixed, the RCA signals were adversely proportional to the EcoRI concentration (Figs. S2C and S2D). When both EcoRI and substrate concentrations were kept constant, the RCA fluorescence signals became steadily decreased as the cleavage reaction progressed and the relative cleavage rates were determined (Figs. S2E and S2F). Theses cleavage processes have been confirmed by gel electrophoresis (Fig. S2G).

Furthermore, we examined the ability of our RCA-based approach to distinguish the uneven cleavage. We intentionally created DNA heteroduplexes by substituting nucleotides on one strand of the EcoRI substrate duplex with locked nucleic acid (LNA). LNA is a nucleotide derivative characterized by a 2'-O, 4'-C-methylene-β-D-ribofuranosyl unit. This bicyclic ribosyl structure effectively locks the ribose in the *C3'-endo* sugar pucker conformation that is dominant in A-form DNA and RNA [6]. When substituting normal nucleotides, its *C3'-endo* sugar pucker conformation would disturb the local interactions between the EcoRI homodimer and the palindromic DNA sequence, and alter the original cleavage behavior.

We prepared two groups of heteroduplexes. The first group includes eight circular template strands each containing single LNA substitution at different positions (named as LNA-CTs) and one complementary DNA strand (named as DNA-CO) (Fig. 2A and Table S2). The LNA-substituted positions include six nucleotides of the EcoRI recognition sequence GAATTC, one nucleotide flanking the 5'-site (T(-1)) and one nucleotide flanking the 3'-site (T7) of the EcoRI recognition sequence. In this design, we are able to monitor how the LNA substitutions affect the EcoRI cleavage on the modified strand. In contrast, the second group includes one DNA circular template strand (DNA-CT) and eight LNA-substituted CO strands (LNA-COs) (Fig. 2B and Table S3). This design will allow us to test how the LNA substitutions affect the EcoRI cleavage on the unmodified strand.

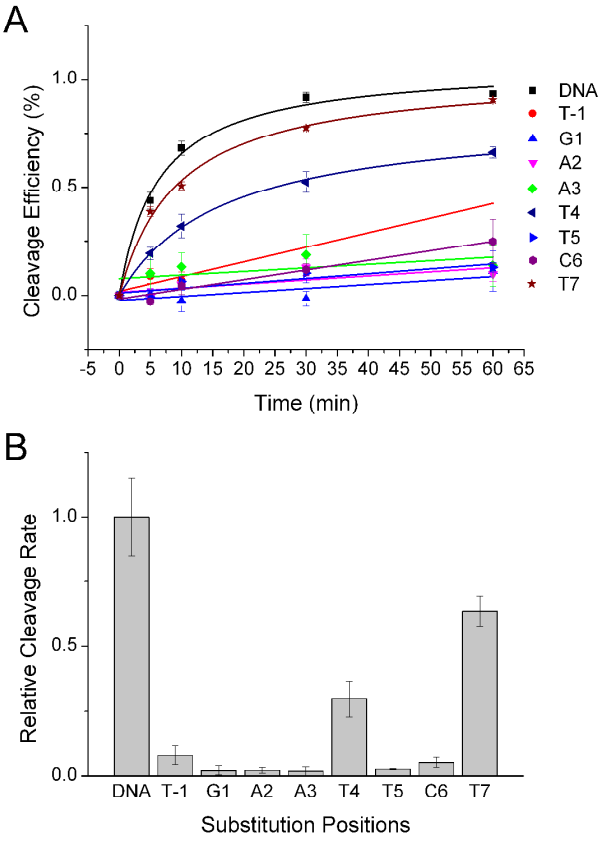

Fig. 3 EcoRI cleavage on CT-modified substrate. (A) Plots of cleavage efficiency of LNA-CTs at different modified positions. The un-substituted 'DNA' is used as control. (B) Relative cleavage rates of the LNA-CTs in comparison with that of control.

Fig. 3 shows the enzymatic cleavage of eight LNA-CT/DNA-CO substrate heteroduplexes by EcoRI and the calculated relative cleavage efficiencies. The EcoRI cleavage is significantly inhibited by LNA substitutions at T(-1), G1, A2, A3, T5 and C6 positions (by > 95%), while the cleavage is repressed by LNA substitutions at T4 and T7 positions only by 70% and 25%, respectively, reflecting the effects of LNA substitutions on enzymatic cleavage. When LNA substitutions are made on the complementary strand (LNA-CO), EcoRI cleaves the circular template strand differently. The EcoRI cleavage is strongly inhibited by the LNA substitution at G1, A3, T4, T5 and C6 positions. The enzymatic cleavage is slightly repressed by the LNA substitution at A2 position, and is not affected at all when LNA substitutions are made at the T(-1) and T7 positions (Fig. 4). The cleavages of LNA-CT/DNA-CO heteroduplexes and DNA-CT/LNA-CO heteroduplexes are verified by gel electrophoresis (Fig. S3). These results demonstrate clearly that LNA-substitutions on one strand can influence the enzymatic cleavage on the modified strand quite differently from that on the unmodified strand.

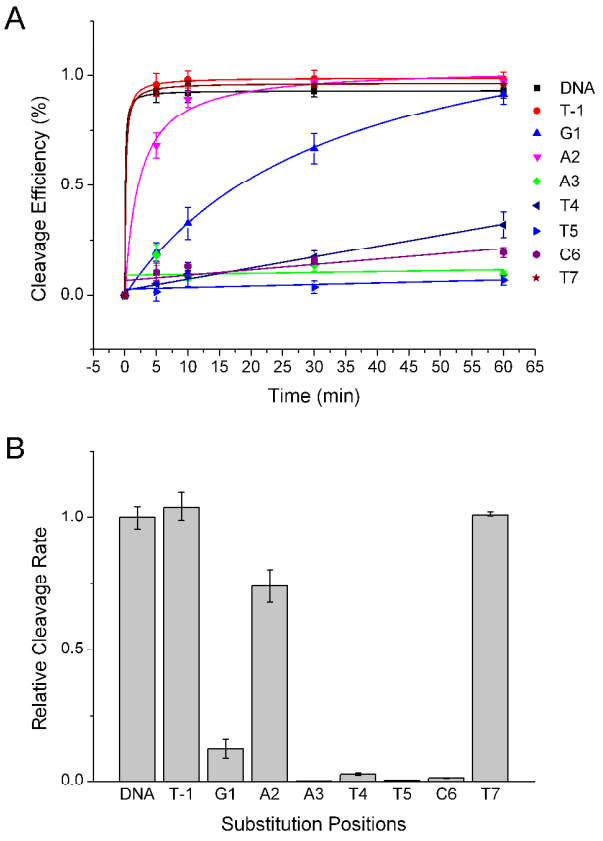

Fig. 4 EcoRI cleavage on CO-modified substrate. (A) Plots of cleavage efficiency of CT affected by LNA-CO at different modified positions. The un-substituted 'DNA' is used as control. (B) Relative cleavage rates of the LNA-COs in comparison with that of control.

FRET-based assays for enzyme cleavage have been widely used in many studies for their convenience and sensitivity[5d, 7]. However, analyzing each individual strand break of RE cleavage by FRET is challenging, To verify the utility of this RCA-based approach, we also utilized the FRET-based enzymatic analytic assay. The substrate of this assay consists of three oligonucleotides: a template (T-ON), one labeled with fluorophore at 5'-end (F-ON) and one labeled with a quencher at 3'-end (Q-ON). When forming a stable substrate, there is no fluorescence signal due to the effective energy transfer from the fluorophore to the quencher. Enzymatic cleavage will cut the substrate into several fragments which are too short to stay together as a stable duplex. Disassociation causes the separation of the fluorephore and the quencher and results in an increase of fluorescence signals (Figs. S4A and S4B). However, this assay is unable to distinguish which strand is cleaved fast. Thus, when the FRET-based results show no fluorescence signal, it implies simply that the cleavages on both strands are strongly inhibited or extremely slow. In contrast, when the fluorescence signal is observable, the fluorescence signal reflects the rapidly cleaved fragment if the duplex is cleaved unevenly. As a result, the FRET-based assay always measures the fast cleavage strand. Thus, when making comparisons, the cleavage efficiencies of FRET-based assay (C_{FRET}) will be always equal to the fast cleavage of the RCA-based approach (C_{LNA-CT} or C_{DNA-CT}). Fig. S4C illustrates this comparison which shows a high consistence between the FRET-based assay and the RCA-based approach.

Restriction endonucleases have been extensively utilized as powerful tools in genetic engineering and synthetic biology [8]. In addition, they also represent proper models for investigating the protein-DNA interactions, since their structures determined by X-ray crystallographic and NMR spectroscopic methods have disclosed many specific interactions (hydrogen bond formation, ionic attractions and coordinate interactions). These interactions play substantially important roles in recognition and cleavage of DNA duplexes by endonucleases [9]. Understanding these molecular interactions can advance the design of biologically engineered antibodies, vaccines, enzymes and functional proteins for many applications. Mapping the cleavage patterns by using nucleotide substitutions for particular nucleotides offers an alternative biochemical strategy to probe the detailed interactions [10]. The different cleavage patterns of DNA-enzyme complexes reflect the disturbed interactions between the amino acid residues of RE and their recognized DNA sequence. Since substitution will cause different effects on both the modified and the unmodified strand cleavage of duplex, distinguishing each individual strand break is extremely important in these studies. For a long time, the only choice is denaturing electrophoresis. Here, we demonstrate the feasibility of the electrophoresis-free and RCA-based approach to analyze the single strand cleavage by nickases and asymmetric cleavage by restriction endonucleases. This approach could be developed into a universal, easy, fast, convenient and high-throughput method to investigate molecular interactions of DNA-protein complexes.

We developed a novel RE cleavage assay based on RCA. This assay has the ability of analyzing individual strand cleavage of DNA duplex by REs. It has potential to be used in uneven cleavage of REs.

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Notes and references

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‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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