Supporting Information

Materials and methods

1. Chemicals and oligonucleotides

Oligonucleotides at HPLC grade were purchased from Sangon Biotech Co. Ltd. (Shanghai, China) without further purification. Their concentrations were determined by the absorption coefficiency of each sample. Their sequences are listed in Tables S1~S3, where the oligonucleotides for forming the circular templates have a phosphoric acid group at the 5'-terminal. EcoRI was purchased from Takara Biotechnology Co. Ltd. (Dalian, China) and phi29 DNA polymerase was purchased from NEB (Ipswith, MA). SYB Green II was from Invitrogen (Waltham, MA). T4 DNA Ligase was purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China).

2. Circulation reaction

For circulation, the 5'-end of the circular template (CT) was phosphorylated. The 5'- and 3'-portions of the circular template (1 μM) were hybridized with 2 μM of ligation oligonucleotide (LO) in a head-to-tail fashion, and were covalently linked by T4 DNA ligase (175 U) at 16 °C for 60 min. Ligation buffer contains 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, and 0.1 mM ATP in 10 μl reaction systems. The ligation product was used directly for the next RE cleavage and RCA analyses.

3. RE cleavage reactions and RCA detections

0.5 pmol of circular template was hybridized with 2 pmol of complementary oligonucleotide to form a short duplex substrate of EcoRI. The cleavage reaction was carried out in 10 μl reaction buffer containing 50 mM Tris pH 7.5, $MgCl_2$ 10 mM, DTT 1 mM, and NaCl 100 mM. The cleavage was initiated by adding 7.5 U EcoRI. After incubation at 37 °C for 5, 10, 30, 60 minutes, the cleavage was stopped by heating at 90 °C for 10 min. Then, 0.25 pmol of the cleaved circular template were added into RCA reaction solution which was composed of 50 mM Tris, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT (pH 7.5), SYB Green II (1:10000), phi29 DNA polymerase 3 U in 100 μl solution. Fluorescence signals were recorded on Microplate Reader (Infinite M200, Tecan, USA) with excitation wavelength at 480 nm and emission wavelength at 524 nm. The fluorescence emission was monitored for over ~20 min at 37 ºC.

4. FRET assay

This approach is based on the fluorescence resonance energy transfer (FRET) principle which has been described previously [1]. Briefly, the substrate of EcoRI is composed of three oligonucleotides: template, F-ON, and Q-ON. F-ON is complementary partially to the template, and Q-ON is complementary partially to another region of the template. Three types of oligonucleotides are prepared at the same concentration of 50 nM in 100 μl buffer containing 50 mM Tris, 10 mM $MgCl_2$, 1 mM DTT, and 100 mM NaCl (pH 7.5). The template and F-ON were mixed first, and the fluorescence intensity was measured as F0. After adding Q-ON, stable EcoRI substrates containing the EcoRI recognition sequence were formed. In this complex, the quencher and the fluorophore were so close in space that only very low fluorescence signal was observed

(defined as F1). Once EcoRI was added into the substrate solution, it started to cleave the recognition sequence. The cleaved 3'-portion of F-ON was too short to bind with the template and then released, generating a fluorescence signal Ft (Fig. S4). The relative fluorescence intensity (RF) was calculated using formulation RF = (Ft-F1) / (F0-F1).

In this experiment, fluorescence signals were recorded at 37 ºC on Microplate Reader (Infinite M200, Tecan, USA) with excitation wavelength at 480 nm and emission wavelength at 524 nm. The signals were collected every 15 seconds with an integration time of 20 microseconds, and the fluorescence emission was monitored for over ~20 min at 37 ºC.

5. PAGE analysis

Circular template was hybridized with CO to form substrates of EcoRI. The cleavage reaction were performed in 10 μl reaction buffer containing 50 mM Tris pH 7.5, $MgCl_2$ 10 mM, DTT 1 mM, and NaCl 100 mM. Cleavage was started by adding 7.5 U EcoRI at 37 °C, and was stopped by adding loading buffer containing 0.1% SDS, 5% Glycerol, and 0.005% Bromophenol Blue. The cleaved products were analyzed with 18% denaturing PAGE and fast silver staining as described [2].

Possible mechanism of EcoRI cleavage on LNA-modified substrates

The crystal structure of EcoRI-DNA complex has revealed 12 hydrogen bonds with purine bases and a variety of interactions with phosphate, as well as a set of van der Waals contacts between enzyme and substrate duplex [3]. The nucleotides adjacent to the recognition sequence also interact with EcoRI through phosphate backbones. The duplex DNA substrates are bended at three positions called neo-kinks: neo-1 kink in the middle of recognition sequence, and two neo-2 kinks at each end of the recognition sequence. Phosphate backbone interactions with enzyme are responsible for the formation of the distorted kinks. This includes three interactions called ‘key clamp contacts’, *i.e.* pNpGAApTTC [4]. EcoRI accesses the recognition sequence from the major groove widened by neo-1 kink.

It has been suggested that both direct readout by base-bonding and indirect readout by backbone-contact are significant for the specific recognition and cleavage by EcoRI, especially to those duplex-binding in a distorted way [5]. Therefore, exploring the backbone bonding is as important as base interaction. Many base analogues and phosphate analogues have been used to study the interaction of EcoRI-DNA [4, 6]. However, the interactions between sugar analogues with EcoRI have been much less explored. LNA has an ability to lock the ribosyl sugar to the *C3'-endo* pucker conformation which has been observed frequently in A-form DNA and RNA. LNA substitution will introduce an impact on the phosphate backbone. Thus, the local substitution might decrease the duplex flexibility and change the backbone bending.

During the EcoRI catalysis, three neo-kinks provide the most appropriate bending positions for enzyme cleavage. Thus, factors affecting these key bending sites will change its cleavage characteristics inevitably. In the case of LNA-CT modification analyses, all the LNA-substituted positions inhibit heavily the cleavage except T4 and T7. It should be noticed that T4 was located at neo-1 kink and T7 at neo-2 kink. When the bending by LNA is accord with the natural neokinks, the inhibition effects might be counteract to some extent. Four positions (T-1, G1, A2 and A3) at 5'-side of the recognition sequence cover almost all important interactions of both direct and indirect readout with EcoRI. Therefore, hydrogen bonds with three purine bases and three

phosphate contacts (T(-1)pG1, G1pA2 and A2pA3) assemble an elaborate network to coordinate the catalytic center. Many of the conserved residues are involved in the network, such as Arg200, Asp91, Glu111, Lys113 and Arg145. Thus, disturbance in this region is most likely to bring an intimate impact on the catalytic center and neo-kink formation. As a result, LNA substitutions at these positions generate strong resistance against the endonuclease cleavage. Since the T5 and C6 positions are distal to the cleavage site, the interaction with enzyme is relatively limited. It is speculated that the N terminal residues of α4 helix, Met137, Ala138 and Gly140 will absorb the distortion of T5 and C6 substitutions, and transmit through α4 helix to Arg145 and catalytic center. Consequently, LNA substitution at T5 and C6 positions might use α4 helix as a pathway to reduce the cleavage rate.

In the case of LNA-CO cleavage, the effects of LNA substitutions have to be transmitted to the complementary strand. This process can be realized through Watson-Crick base pairs and interaction between EcoRI dimer and DNA. LNA-substitutions at A3, T4, T5 and C6 positions reduced the cleavage rates significantly. Since they are complement to their opposite strand at cut site, thus the strong inhibitory effect is likely through base pairs to impress their backbone distortion on the 5'- half recognition sequence of the complementary strand. The G1 position, which forms hydrogen bonds of Arg200 and Arg203 from the opposite subunit of protein homodimer, may transmit the impact to the cleavage site of the complementary strand through this interaction. Surprisingly, A2 position modification remained most of the cleavage activity. Kurpiewski *et al.* have suggested a crosstalk ring which is formed by Glu144 and Arg145 side chains of both subunits of enzyme homodimer [7]. They crosslink the adenine bases of both strands, especially A2 position. This structure may provide a flexible and robust characteristic, which can resist the effect of A2 modification from the complementary strand.

The true mechanism for LNA substitutions could be more complicated and the proposed mechanisms need further confirmation. However, we can see that the effects of LNA substitution on both strands are universal. It seems that three neokinks might work as a whole, and aberration of a local site can disrupt the whole optimal conformation of enzyme-substrate complex. Studies on EcoRV have suggested that the interference arising from one site substitution is likely due to the disruption of the whole network of protein-DNA rather than a simple result of deleting one contact [5].

In addition, one of our previous studies showed that 2'-O-methyl nucleotide (2'-OMeN) and 2'-fluoro nucleotide (2'-FN) modifications at T-1 and T7 positions inhibited remarkably the cleavage of EcoRI. These modifications are different from LNA modification although they have the similar C3'-endo pucker conformation. It is speculated that the size, electronegativity and hydrophobicity of these moieties at the C2' position might create a complex interaction network.

Tables

Table S1. Oligonucleotides used for analyzing Nb.BbvCI cleavage

	Sequences
LO	5'-GTATGCGTATGTCTCCGTGC
Top-CT	5'-p-ATACGCATACCTGTCCTCAGCTGGCTAAAAGCACACGCACGGAGAC
Bot-CO	5'-TAGCCAGC↓TGAGGACAG
Bot-CT	5'-p-ATACGCATACCTGTGC↓TGAGGTGGCTAAAAGCACACGCACGGAGAC
Top-CO	5'-TAGCCACCTCAGCACAG

*The recognition sequence of Nb.BbvCI is underlined. The arrows indicate the cleavage site. LO stands for the ligation oligonucleotide used to circularize the liner strand.

Table S2. Oligonucleotides for studying cleavage on LNA-modified strand.

	Sequences
DNA-CT	5'-p-ATACGCATACCTGTGAATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-T(-1)	5'-p-ATACGCATACCTGtGAATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-G1	5'-p-ATACGCATACCTGTgAATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-A2	5'-p-ATACGCATACCTGTGaATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-A3	5'-p-ATACGCATACCTGTGAaTTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-T4	5'-p-ATACGCATACCTGTGAAtTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-T5	5'-p-ATACGCATACCTGTGAATtCTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-C6	5'-p-ATACGCATACCTGTGAATTcTGGCTAAAAGCACACGCACGGAGAC
LNA-CT-T7	5'-p-ATACGCATACCTGTGAATTCtGGCTAAAAGCACACGCACGGAGAC
DNA-CO	5'-TAGCCAGAATTCACAG
LO	5'-GTATGCGTATGTCTCCGTGC

*The recognition sequence of EcoRI is underlined. LNA nucleotides are in the lower case. LO stands for the ligation oligonucleotide used to circularize the liner strand.

Table S3. Oligonucleotides for studying cleavage on LNA-unmodified strand.

	Sequences
DNA-CT	5'-p-TGAGCACTAGGTCTCCGTGCGTGTGCTTTTAGCCAGAATTCACAG GTATGCGTATGTTTCCTAAGCTATCATTGGAC
DNA-CO	5'-ATACGCATACCTGTGAATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-T(-1)	5'-ATACGCATACCTGtGAATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-G1	5'-ATACGCATACCTGTgAATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-A2	5'-ATACGCATACCTGTGaATTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-A3	5'-ATACGCATACCTGTGAaTTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-T4	5'-ATACGCATACCTGTGAAtTCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-T5	5'-ATACGCATACCTGTGAATtCTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-C6	5'-ATACGCATACCTGTGAATTcTGGCTAAAAGCACACGCACGGAGAC
LNA-CO-T7	5 '-ATACGCATACCTGTGAATTCtGGCTAAAAGCACACGCACGGAGAC
LO	5'-CTAGTGCTCAGTCCAATGAT

*The recognition sequence of EcoRI is underlined. LNA nucleotides are in the lower case. LO stands for the ligation oligonucleotide used to circularize the liner strand.

Figures

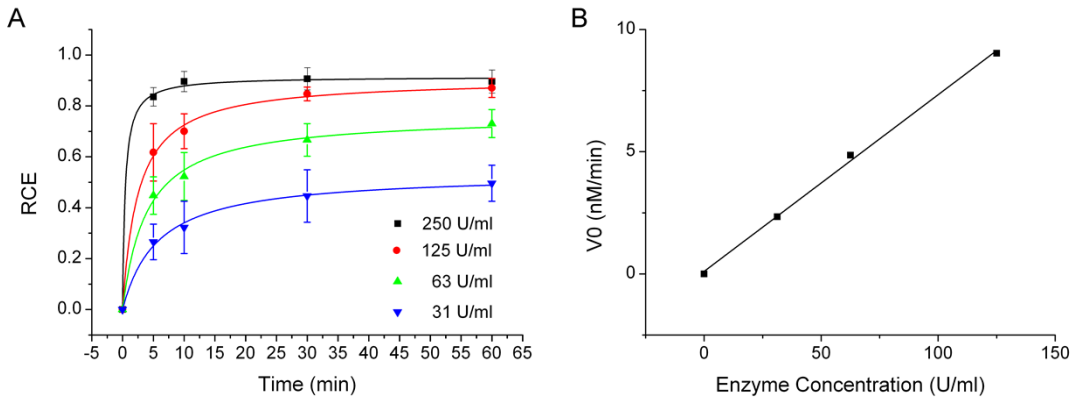
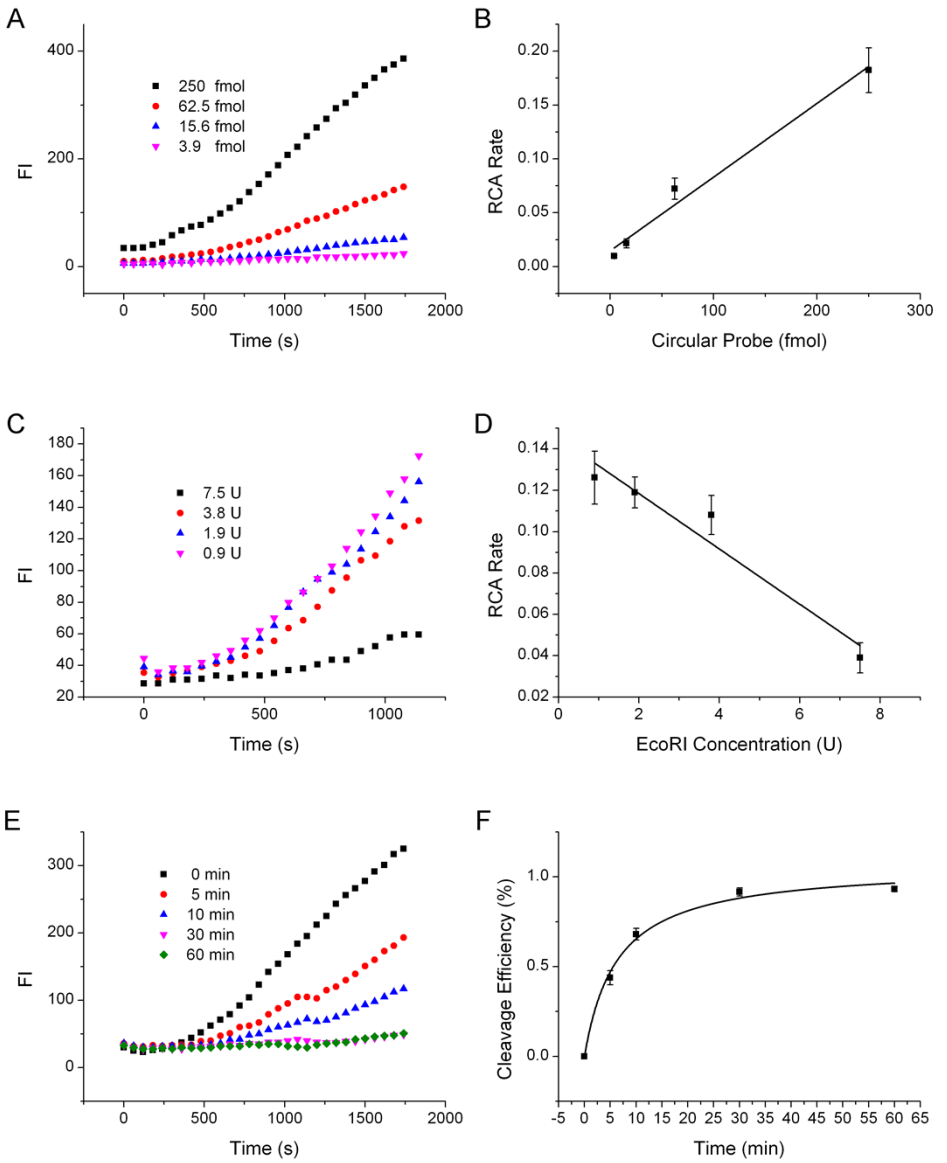


Fig. S1 Nb.BbvCI cleavage behavior monitored by RCA. (A) Nb.BbvCI relative cleavage efficiency with respect to the Nb.BbvCI concentration. (B) The calculated cleavage initial velocities of Nb.BbvCI as a function of enzyme concentration.


A
FI
Time (s)
250 fmol
62.5 fmol
15.6 fmol
3.9 fmol
B
RCA Rate
Circular Probe (fmol)
C
FI
Time (s)
7.5 U
3.8 U
1.9 U
0.9 U
D
RCA Rate
EcoRI Concentration (U)
E
FI
Time (s)
0 min
5 min
10 min
30 min
60 min
F
Cleavage Efficiency (%)
Time (min)

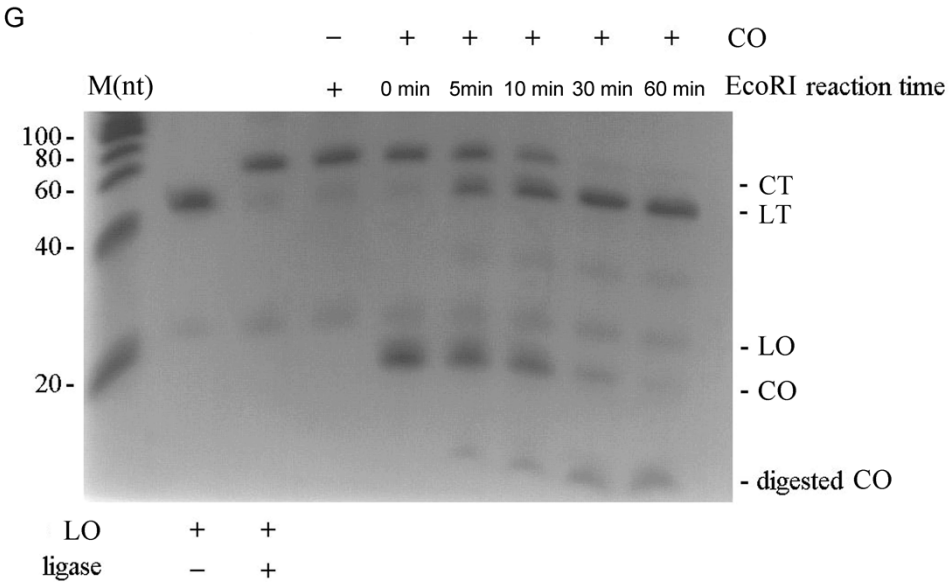

G
M(nt)
EcoRI reaction time
0 min
5min
10 min
30 min
60 min
CO
100
80
60
40
20
CT
LT
LO
CO
digested CO
LO
ligase

Fig. S2 EcoRI cleavage behavior monitored by RCA. (A) Fluorescence signal changes of RCA with respect to the circular template concentration when the EcoRI concentration was fixed. (B) The linearity of the RCA rate with respect to the circular template concentration. (C) Fluorescence signal changes of RCA after digestion with respect to the EcoRI concentrations when the circular template concentration was fixed. (D) The adverse relationship of the RCA rate with respect to the EcoRI concentration. (E) Fluorescence signal changes of RCA reaction at different reaction time when both circular template and EcoRI concentrations were kept constant. (F) Relationship between the cleavage efficiency with respect to the cleavage time. (G) Denaturing PAGE analysis of single strand cleavage. Lane 2~3: Circular template is circularized by ligase when complement to LO. Lane 4: Without complementary oligonucleotide (CO), circular template is unable to be cleaved by EcoRI even after 60 min reaction. Lane 5~9: in the presence of CO, circular template is gradually cleaved by EcoRI at 0, 5, 10, 30 and 60 min.

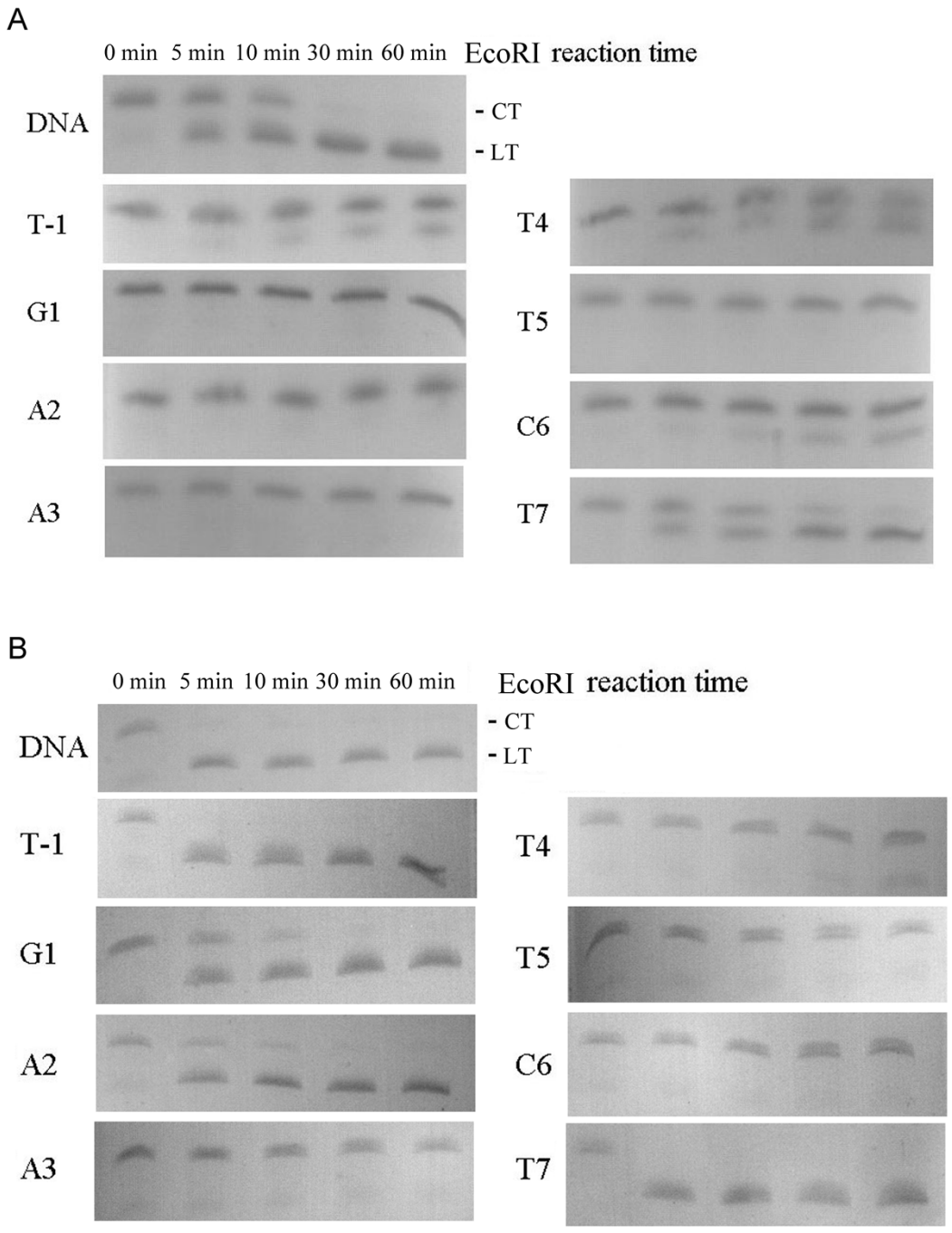


Fig. S3 (A) Denaturing PAGE analysis of the cleavage of LNA-modified circular templates (LNA-CT at different modification positions) by EcoRI. DNA duplex was used as control. EcoRI reaction time is 0, 5, 10, 30 and 60 min, respectively. (B) Denaturing PAGE analysis of the cleavage of DNA circular template (DNA-CT) affected by LNA-modified complementary strand (LNA-CO at different modification positions). DNA duplex was used as control. EcoRI reaction time is 0, 5, 10, 30 and 60 min, respectively.

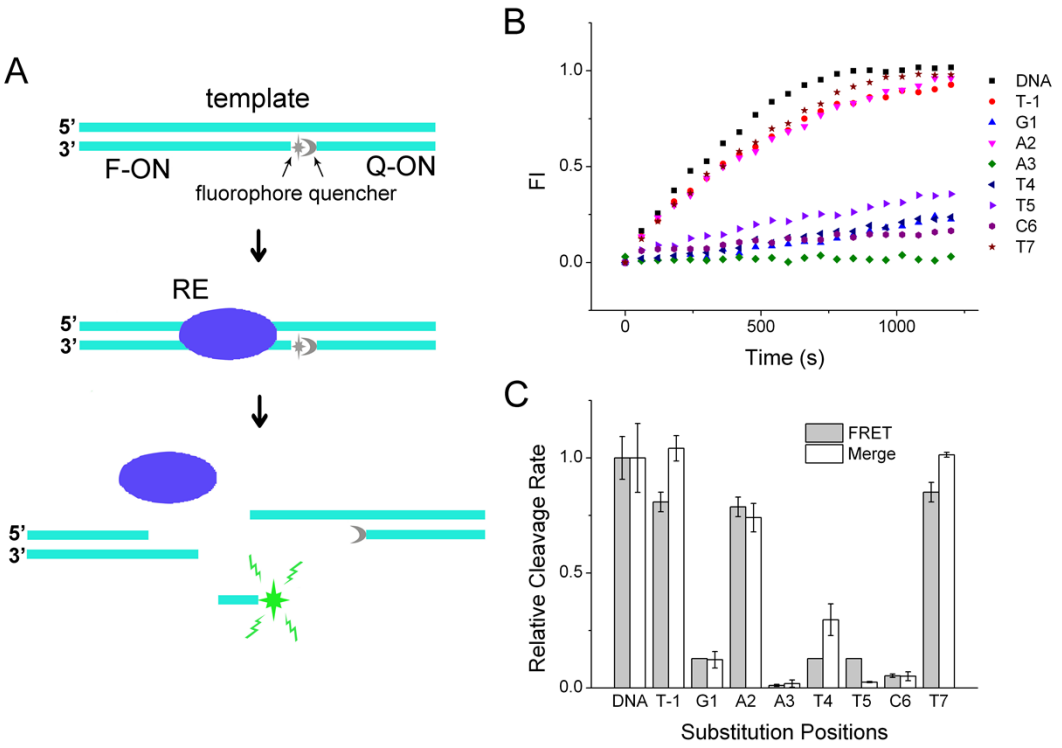


Fig. S4 (A) Schematic diagram showing the FRET assay for analyzing restriction endonuclease cleavage. (B) Fluorescence signal changes of the LNA modified template strand at different modification positions. (C) Comparison of the relative EcoRI cleavage rates determined by the RCA-based analysis and the FRET-base assay. The cleavage rates showed the effect of LNA substitutions at different positions.

References

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