



Continuous monitoring of restriction endonuclease cleavage activity by universal molecular beacon light quenching coupled with real-time polymerase chain reaction

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ABSTRACT

We describe a method for sensitive monitoring of restriction endonuclease kinetics and activity by use of a universal molecular beacon (U-MB) coupled with real-time polymerase chain reaction (PCR). The method is used to monitor the progress of DNA cleavage in a sealed reaction tube and offers more accurate and high-throughput detection. The template has a universal tail hybridized with the U-MB and the remaining sequence is complementary to one of the restriction endonuclease digestion products. The U-MB is replaced by the extension of digested product and the fluorescence quenches. With this concept, one universal fluorescence probe can be used in different enzyme analytical systems. In the work described here, homogenous assays were performed with the restriction endonucleases *AluI*, *EcoRI*, *XhoI*, and *SacI* at smoothly controlled temperature. Cleavage efficiencies were determined, and the potential applications of this method were discussed. Furthermore, the *AluI* and *EcoRI* cleavage reactions were monitored online at varying substrate concentrations at the molecular level, and K_m , V_{max} , and K_{cat} values were calculated. The results suggest that U-MB monitoring of restriction endonuclease assays based on real-time PCR will be very useful for high-throughput, sensitive, and precise assays for enzyme activity screening and evolutionary biotechnology analysis.

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Restriction endonucleases are the enzymes that hydrolyze phosphodiester linkages in the nucleic acid backbone. They recognize palindromic sequences, usually 4–8 bp in length [1,2], and cleave the two strands of DNA within the recognition site to give a 5'-phosphate and a 3'-OH end [3]. These enzymes are essential in recombinant DNA procedures for molecular cloning and analysis of nucleic acids [4,5]. In addition, they are used extensively to find restriction fragment length polymorphisms in allelic genomic regions [6]. Understanding the fundamental characteristics of restriction enzymes will facilitate the more efficient use of these tools, and provide important information on protein–nucleic acid interactions in general. Kinetic studies on enzymes are important tools in studying biological interactions at the molecular level. To assay the cleavage efficiency of restriction enzymes, several traditional methods have been used, such as gel electrophoresis, filter binding, and high-performance liquid chromatography [7–9]. However, these methods are discontinuous, time consuming, costly, and laborious. Furthermore, they require radiolabeling for sensitive detection. A continuous UV assay based on hyperchromic effects has been reported, but it is limited to a narrow substrate

concentration range [10]. Enzyme-linked immunosorbent assay (ELISA)¹ has been used in restriction endonuclease detection, but it is also a discontinuous method [11]. In combination with advances in genetic engineering and structure determination, more sensitive and precise techniques have been developed to characterize the kinetics of enzyme activity. A promising approach is the fluorescence assay for monitoring DNA cleavage [12,13], which is based on fluorescence resonance energy transfer [14–16]. Dual-color fluorescence cross-correlation spectrometry and molecular beacons are quite successful for continuous monitoring. In most of these techniques, the substrates are tagged with two fluorescent dyes. Enzyme kinetics is determined by monitoring the change in fluorescence signal that occurs when the dyes are physically separated from each other as a result of cleavage. Different kinds of fluorescent probes are used, and it is costly to design corresponding probes or substrates tagged with two fluorescent dyes for analysis of different enzymes. Furthermore, most of these techniques are carried out in unsealed vessels, so the detection temperature cannot be controlled easily. Therefore, it was necessary to develop a convenient and universal high-throughput method for restriction endonuclease analysis.

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¹ Abbreviations used: ELISA, enzyme-linked immunosorbent assay; U-MB, universal molecular beacon; UT, universal tailed; PCR, polymerase chain reaction.

Molecular beacons are hairpin-shaped oligonucleotide probes that have wide application in detecting nucleic acids in solution [17,18]. In our previous work, we introduced the concept of the universal molecular beacon (U-MB) probe and successfully used it to detect breast cancer point mutations [19] and hepatitis B virus DNA [20]. An advantage of U-MB is that one probe can be applied universally in different nucleic acid and protein–nucleic acid interaction systems. In the work reported here, we attempted to establish an effective approach to monitoring restriction endonuclease cleavage activity in real time based on the U-MB. The mechanism underlying this method is shown schematically in Fig. 1. The detailed structure of the U-MB has been described previously [19]. The universal tailed (UT) template contains two parts: the universal tail is combined with the U-MB, and the remaining part is complementary to one of the cleaved products of the substrate. In the presence of the UT template, the U-MB hybridizes with the tail and emits fluorescence (Fig. 1a). When the substrate is cleaved by the restriction endonuclease and produces blunt or sticky ends (Fig. 1b), one of the products in each system (A—sticky end system, B—blunt end system) is too short to remain double-stranded, and one strand (oligo A1 or B1) combines with the UT template as a primer and extends to replace the U-MB with a Klenow fragment (DNA polymerase). Consequently, the fluorescence quenches (Fig. 1c). This constitutes the basis for analysis of the cleavage reaction. For different enzymes, the substrates and sequence of the UT template change, but the U-MB is the same. The detection process is carried out in a real-time PCR, which has the advantages of smooth temperature control, sealed tubes, and high throughput. The method was applied to continuous monitoring of the activity of *AluI*, *EcoRI*, *XhoI*, and *SacI*. The real-time PCR results for *EcoRI*, *XhoI*, and *SacI* were confirmed by HPLC. We also determined the kinetic constants of *AluI* and *EcoRI* from the initial cleavage rates.

Experimental

Reagents

AluI (10 units/ μ l), *EcoRI* (20 units/ μ l), *XhoI* (20 units/ μ l), *SacI* (20 units/ μ l) and Klenow fragment (5 units/ μ l) were purchased from New England Biolabs (NEB, UK). All oligonucleotides were synthesized and purified by HPLC from IDT (Coralville, IA, USA) and AUGCT (China). The U-MB was synthesized and purified by HPLC (Sangon Co., China). Sequences of the substrates, cleavage sites, and UT templates are listed in Table 1. Restriction endonuclease recognition sequences are highlighted in boldface and the

different nucleotides flanking the recognition site are underlined. Sequences of UT templates hybridized with the digested products are underlined. The reaction buffers were: for the *AluI* system, 50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl₂, and 1 mM dithiothreitol (pH 7.9); for the *EcoRI* system, 50 mM NaCl, 100 mM Tris–HCl, 10 mM MgCl₂, and 0.025% Triton X-100 (pH 7.5); for the *XhoI* system, 10 mM BisTris propane–HCl, 10 mM MgCl₂, 1 mM dithiothreitol (pH 7.0) and 10 μ g/ml bovine serum albumin; and for the *SacI* system, 50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl₂, and 1 mM dithiothreitol (pH 7.9).

Real-time PCR cleavage assay

U-MB quenching assays for *AluI*, *EcoRI*, *XhoI*, and *SacI* were performed individually.

1. *AluI*

All cleavage reactions were carried out in 50- μ l sealed tubes with 1 \times *AluI* reaction buffer, using Stratagene Mx3000p real-time PCR (USA). The thermal program was 200 cycles at 37 $^{\circ}$ C for 5 s, and fluorescence was measured at the end of each cycle. Some experiments were carried out by adding a hot shot procedure at 55 $^{\circ}$ C for 5 s within the segment. The flanking sequence optimization of the substrate was carried out at a concentration of 1.5 units Klenow fragment, 2 units *AluI*, 160 nM substrate, 160 nM U-MB, and UT template hybrids. Reaction conditions for monitoring the DNA cleavage process catalyzed by 0.1–20 units *AluI* and all other concentrations are as previously described. Reaction mixtures for determination of K_m contained 20–320 nM substrate. For the no enzyme control and no template control, ddH₂O was used.

The extent of restriction enzyme cleavage was calculated from the fluorescence data with the equation [21]

$$[\text{DNA}]_c = [\text{DNA}]_i \times (F_t - F_0) / (F_{\infty} - F_0) / \alpha,$$

where $[\text{DNA}]_c$ is the concentration of cleaved DNA, F_t is the fluorescence intensity at time t , F_{∞} is the fluorescence intensity at the plateau, F_0 is the initial fluorescence intensity, $[\text{DNA}]_i$ is the initial concentration of DNA, and α is the final quenching rate of each cleavage reaction.

2. *EcoRI*

Different flanking sequence optimization and real-time 3-bp flanking base cleavage reactions were carried out in 50 μ l 1 \times *EcoRI* reaction buffer, and the real-time PCR thermal procedure was the same as for *AluI*. Determination of K_m used reactions on 30–640 nM substrate, 5 units *EcoRI*, 1.5 units Klenow fragment, 160 nM U-MB, and UT template hybrids.

3. *XhoI* and *SacI*

All cleavage reactions were carried out in 50 μ l 1 \times reaction buffer and the real-time procedure was the same as for *AluI*. DNA cleavage reactions for *XhoI* or *SacI* contained 0.5 to 10 units of each.

Endonuclease assay and HPLC analysis

The sticky-end cleavage enzymes *EcoRI*, *XhoI*, and *SacI* (40 units) digested the respective substrates (concentration = 10 μ M) at 37 $^{\circ}$ C for 12 h, and then the mixtures were heated at 80 $^{\circ}$ C for 2 h to inactivate the enzymes. Digested products were purified with a Syringe Driven Filter Unit (Milllex-GV 0.22- μ m, Millipore USA). The products were then separated on a C18 column (4.6 \times 150 mm) at 1 ml/min, running a 30-min gradient from 7 to 20% acetonitrile for the *EcoRI* system, and a 25-min gradient from 10 to 17% acetonitrile for the *XhoI* and *SacI* systems, in 0.1 M triethylammonium acetate, pH 7.0, using an Agilent 1100 high-performance liquid chromatograph with a variable-wavelength UV detector at 260 nm.

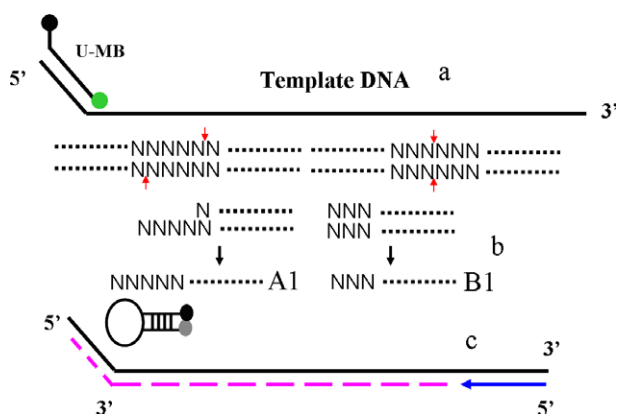


Fig. 1. Schematic of the U-MB light quenching assay for continuous monitoring of the cleavage of restriction endonuclease. (a) U-MB and UT template hybrids. (b) Two types of restriction endonuclease cleave DNA, producing blunt-end and sticky-end products. (c) The cleavage products dissociate and hybridize with the UT template, then extend to replace the U-MB, which induces fluorescence quenching.

Table 1
Sequences of the substrates and UT templates

UT template sequence and cleavage site	Cleavage hybrid
<i>AluI</i> (CT) ₁₀ CCGGGCTCACACCTC ↓ AGCT TCGA ↑	CGTCACTCACTCAGCTCACA GCAGTGAGTGAGTCGAGTGT
	CGTCACTCACTCAGCTCACACCTC GCAGTGAGTGAGTCGAGTGTGGAG
	CGTCACTCACTCAGCTCACACCTCAAGG GCAGTGAGTGAGTCGAGTGTGGAGTCC
<i>EcoRI</i> (CT) ₁₀ CCGGGCCGGAATT ↓ GAATTC CTTAAG ↑	CCGGAATTCCGG GGCCTTAAGGCC
	ACCGGAATTCCGGG TGGCCTTAAGGCC
<i>XhoI</i> (CT) ₁₀ CCGGGGCACCAC ↓ CTCGAG GAGCTC ↑	1. GTGGTGCTCGAGTGCGCC CACCACGAGCTCACGCGG
	2. GTGGTGGTGGTGTCTCGAGTGC CACCACGAGCTCACG
	3. GTGGTGCTCGAGTGC CACGAGCTCACG
	4. GTGGTGCTCGAGT CACGAGCTCA
<i>SacI</i> (CT) ₁₀ CCGGGAGCTCCGT ↓ GAGCTC CTCGAG ↑	1. GAATTCGAGCTCCGTCCA CTTAAGCTCGAGGCAGCT
	2. GAATTCGAGCTCCGT CTTAAGCTCGAGGCA
	3. TTCGAGCTCCGT AAGCTCGAGGCA
	4. TTCGAGCTCC AAGCTCGAGG

Note. The restriction endonuclease recognition sequences are highlighted in boldface and the different nucleotides flanking the recognition site are underlined. The sequences of UT templates hybridized with the digested products are underlined.

Results

Real-time monitoring of *AluI* cleavage activity with different lengths flanking the recognition site

AluI is a restriction enzyme that cleaves substrates into blunt-end products. We analyzed its cleavage activity on substrates of different flanking lengths using the U-MB real-time PCR technique.

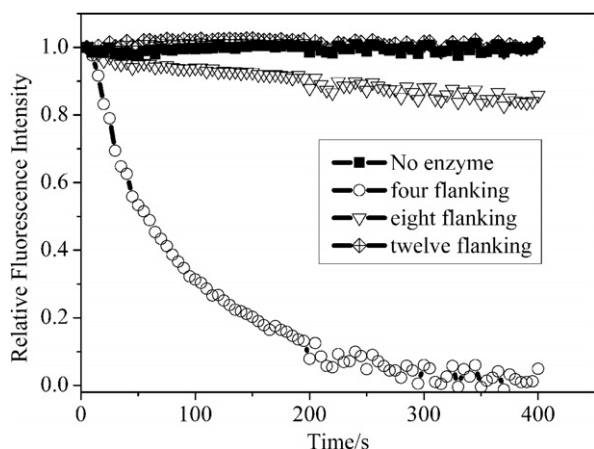


Fig. 2. Cleavage activity of *AluI* on the different sequences flanking the recognition site.

We analyzed 4-, 8-, and 12-bp lengths of sequences flanking the *AluI* substrate recognition site (Table 1). As shown in Fig. 2, the 4-bp flanking sequence showed the highest quenching efficiency at the beginning, the 8-bp flanking sequence had a slower fluorescence quenching rate, and the 12-bp flanking sequence exhibited no fluorescence change. We found that, for the detection of cleavage of the 12-bp flanking substrate, the fluorescence quenched with the addition of a 55 °C hot shot. From these results we concluded that the flanking sequence affected detection efficiency drastically, and that a short flanking sequence yielded high quenching efficiency. In the control system without restriction enzyme, no fluorescence change was observed.

Real-time monitoring of the cleavage activity of *EcoRI*, *XhoI*, and *SacI*

We further analyzed the cleavage efficiencies of three sticky-end cleavage enzymes, *EcoRI*, *XhoI*, and *SacI* (Table 1), with the established U-MB real-time PCR. As their cleavage products all have sticky ends, which may combine with each other by hydrogen bonding after digestion, the cleavage activity detection results are quite different from those for cleavage of blunt-end products by *AluI*.

In the *EcoRI* system, 3- and 4-bp flanking sequences were compared. The 3-bp flanking sequence showed high fluorescence quenching efficiency at 37 °C, but 4-bp flanking sequences exhibited relatively lower quenching efficiency. With the addition of a

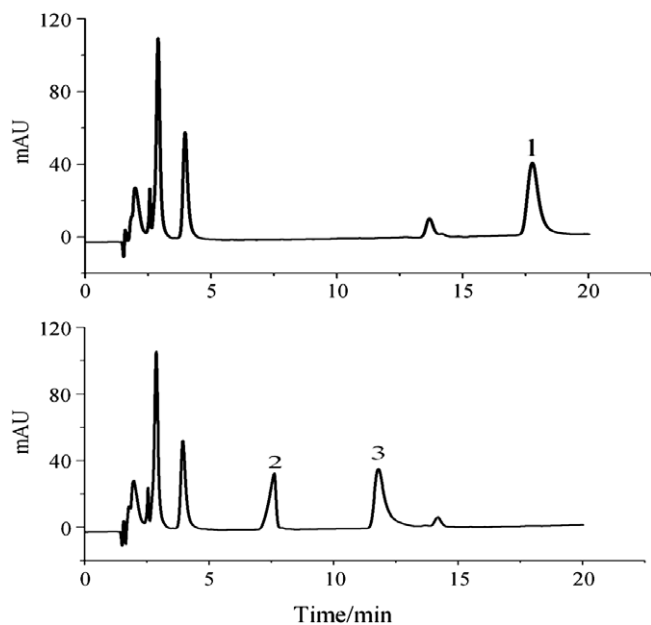


Fig. 3. HPLC analysis of *EcoRI* before and after catalyzed hydrolysis of the substrate double-stranded d(CCGGAATTCGG).

55 °C hot shot, the fluorescence quenching rate of the 4-bp system increased significantly. In the control system without restriction enzyme, no fluorescence change was observed.

In the *XhoI* system, four flanking sequences of different length (from 1 to 6 bp, see Table 1) were investigated. None of them exhibited a fluorescence quenching signal in the 37 °C thermal cycle. With the addition of a 55 °C hot shot, fluorescence quenched in the systems with 1- and 3-bp flanking sequences, but not in the 6-bp flanking sequence system. In the *SacI* system, also four flanking sequences of different length (6, 6–3, 3, and 1 bp) were tested. None of these gave a fluorescence quenching signal in the 37 °C thermal cycle; with the addition of a 55 °C hot shot, fluorescence quenched in the 3- and 6-bp flanking sequence systems, but not in the 1-bp flanking sequence system.

To further clarify the cleavage reaction, we carried out digestion reactions with *EcoRI* and *XhoI* (both 3-bp flanking systems), and separated the products by HPLC to analyze the cleavage efficiency of the enzymes. As illustrated in Fig. 3, the substrates hydrolyzed by *EcoRI* clearly generated two new products. In Fig. 3a, peak 1 was the substrate before digestion, and in Fig. 3b, peaks 2 and 3 represent the two new cleaved products. The results confirmed the high cleavage activity of *EcoRI*, which hydrolyzed the substrate completely in the cleavage reaction. In the case of *XhoI*, after digestion at 37 °C, two new peaks representing the cleaved products emerged, and the substrate peak area decreased to about half of the original amount, indicating the substrates were digested to a certain degree. The results for the *SacI* system were generally the same as those for the *XhoI* system. This proved that *XhoI* and *SacI* digested the substrate, but not with high cleavage efficiency.

Enzyme kinetics

Restriction endonucleases *AluI* and *EcoRI* catalyzed the cleavage of a phosphodiester bond in the palindromic recognition sequence of –AGCT and –GAATTC. The U-MB real-time PCR method allows the cleavage reaction to be monitored continuously in real time, so that it can be used to investigate DNA cleavage reactions, and is easily adapted to study reaction kinetics. Decreasing fluorescence indicates that the reaction is taking place and the DNA

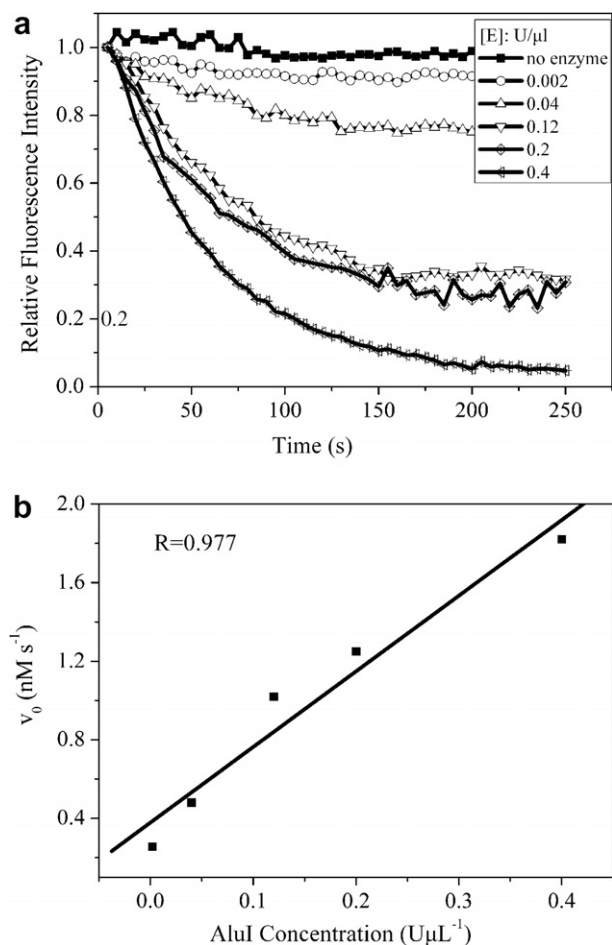


Fig. 4. (a) Time course of real-time PCR monitoring of DNA cleavage catalyzed by different amounts of *AluI*. (b) Initial cleavage velocity as a function of increasing *AluI* concentration.

digestion process can be monitored in real time. Enzyme cleavage reactions were carried out at varying enzyme concentrations to demonstrate the ability of the U-MB real-time PCR assay to sensitively quantify enzyme activity. *AluI* was chosen as an example. Fig. 4a shows the time curve for the cleavage of a fixed amount of substrate with concentrations of *AluI* ranging from 0.002 to 0.4 unit/μl. The initial digestion rate was measured from the first 20 s of the linear portion of the time curve. Initial cleavage rate and *AluI* concentration exhibited a good linear relationship (Fig. 4b). This linear relationship is consistent with the general model for restriction endonuclease reaction [22].

To further confirm the validity of the U-MB real-time PCR method, cleavage reactions were monitored over a wide range of substrate concentrations, from 20 to 320 nM in the *AluI* system and from 30 to 640 nM in the *EcoRI* system. The relationships between initial cleavage rate and substrate concentration are plotted in Figs. 5a and 6a. The initial cleavage rate V_0 was measured from the time curve. Plotting $1/V_0$ against $1/[S]$ ($[S]$ represents substrate concentration) yielded a straight line (Lineweaver–Burk plot; Figs. 5b and 6b). This confirms that these two enzymes obey the Michaelis–Menten equation, as already reported by others [8,11,23,24]. From the Lineweaver–Burk plot, the important kinetic parameters K_m (Michaelis–Menten constant), V_{max} (maximum initial velocity), and K_{cat} were all determined. For *AluI*, V_{max} , K_m , and K_{cat} were $6.25 \pm 0.45 \text{ nM s}^{-1}$, $186 \pm 14 \text{ nM}$, and $0.46 \pm 0.30 \text{ s}^{-1}$, respectively. For *EcoRI*, V_{max} , K_m and K_{cat} were $7.14 \pm 0.40 \text{ nM s}^{-1}$, $226 \pm 20 \text{ nM}$, and $0.22 \pm 0.20 \text{ s}^{-1}$, respectively.

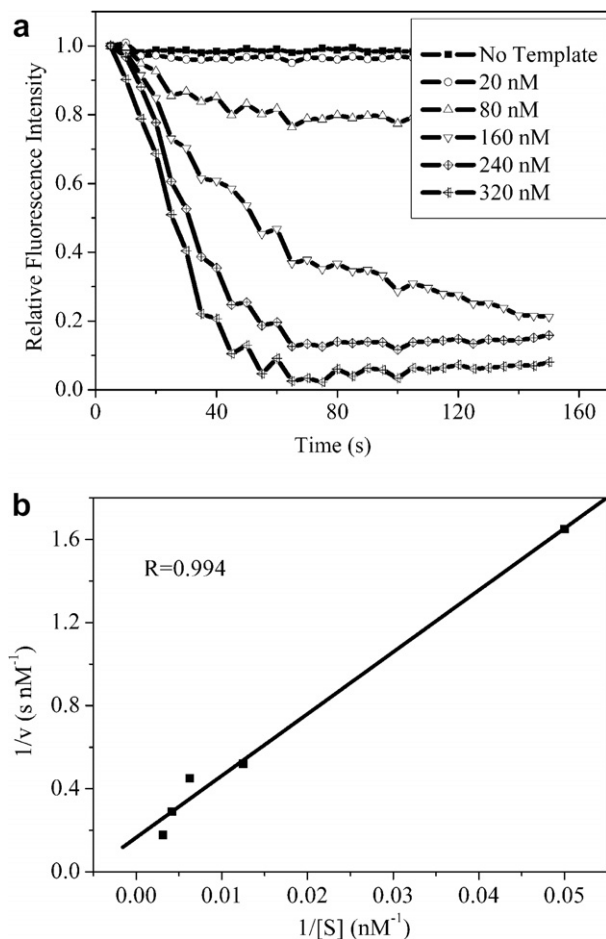


Fig. 5. (a) Time curves for the cleavage of substrate by *AluI*. The concentration of substrate was increased from 20 to 320 nM. (b) Lineweaver-Burk plot of cleavage of the substrate by *AluI*.

Discussion

Rate-limiting step of real-time PCR detection

In principle, the detection process comprises four steps: (1) cleavage of the substrate by the restriction endonuclease, (2) dissociation of the cleaved products, (3) hybridization of one product with the UT template, (4) extension to replace the U-MB with the Klenow fragment. The rate-limiting step of the overall process can be hydrolysis [21,25] or dissociation, or both, depending on the substrate sequences [26].

AluI displayed high cleavage efficiency on substrates with short flanking sequences, and we found that the rates of dissociation and hybridization of the products did not contribute much to the measured rates of cleavage because dissociation of the <6-mer sequence was relatively fast at 37 °C. When the flanking part was long, after digestion, the cleaved products could not separate at 37 °C to continue the replacement of the U-MB reaction. When a 55 °C hot shot was added, the products had enough energy to dissociate, and then the subsequent reactions could occur. Generally, the rate-limiting step in the hydrolysis reaction may be different for long DNA and shorter substrates. Product release is the rate-limiting step for the cleavage of long DNA [27], whereas phosphodiester bond hydrolysis is the rate-limiting step with shorter substrates [23]. Similarly observed in our technique, the cleavage reaction was the rate-limiting step for the shorter substrates, whereas product dissociation was the rate-limiting step for long

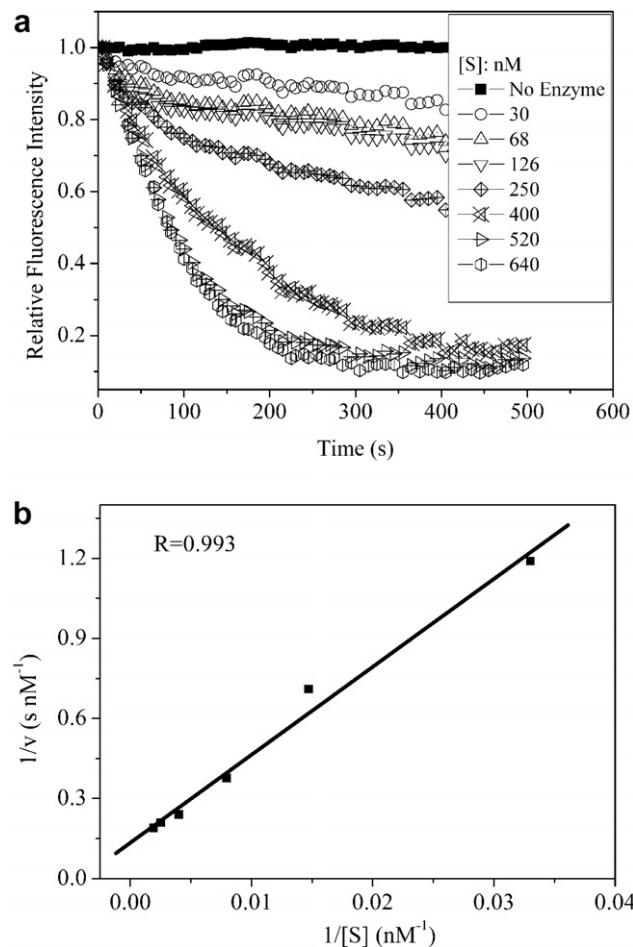


Fig. 6. (a) Time curves for cleavage of the substrate by *EcoRI*. The concentration of substrate was increased from 20 to 320 nM. (b) Lineweaver-Burk plot of the cleavage of substrate by *EcoRI*.

DNA, and the continuous cleavage by *AluI* of short substrates was successfully detected by real-time PCR at 37 °C.

In the four steps of detection, other than the substrate flanking sequence, the DNA polymerase reaction did not influence cleavage rates. Only in the system with the Klenow fragment did fluorescence not change. The fluorescence quenched and the quenching efficiency increased with increasing concentrations of restriction enzyme. Similar results were also reported by another group who used the primer extension reaction to monitor endonuclease activity [25]. From the analysis, the fluorescence quenching rate detected in this method was due mainly to the cleavage digestion DNA reaction, and flanking length was a critical factor of the rate-limiting step in cleavage activity analysis by U-MB quenching real-time PCR.

Flanking sequences affected restriction endonuclease cleavage activity and continuous real-time PCR detection

When the real-time PCR and HPLC results are combined, *EcoRI* and *XhoI* differed with respect to cleavage activity on the short synthetic DNA substrates. *EcoRI* has high cleavage activity on short flanking substrates [28], so its cleavage reaction can be successfully detected with the real-time PCR method. But when the flanking sequence was long, it was more difficult for sticky-end products to dissociate at 37 °C. When a hot shot procedure was added, the dissociation was accelerated and the cleavage products were detected.

XhoI exhibited low cleavage activity at 37 °C. We believe the flanking sequence is the key factor affecting the cleavage efficiency of the restriction endonuclease reaction and real-time PCR detection in the case of *XhoI*. In the 1-bp flanking system, the synthetic substrate was too short for the enzyme to combine and digest. Low cleavage activity on very short flanking sequences (<3-mer) of *XhoI* was also reported in other studies [28]. Interaction between the restriction endonuclease and the DNA substrate is crucial for cleavage [29]. Evidence suggests that the discrimination efficiency of restriction endonucleases on a substrate changes with different sequences flanking the recognition site [30–33]. When the flanking part was short, some enzymes had very low combining activity on the sequence, and the cleavage reaction did not take place. Also, the location of the recognition sites in the DNA molecule is crucial for DNA cleavage. On linear DNA with two recognition sites close to one end, both flanked by the same sequences, the inner site is cleaved preferentially to the outer site; when this DNA is circularized, the two sites are cleaved equally [34]. So the bias of some restriction enzymes toward low activity is due to the linear short substrate, and *XhoI* exhibited low activity on short substrates. For the 3- and 6-bp flanking systems of *XhoI*, the cleavage products were too long to dissociate at 37 °C and remained double-stranded because of the sticky-end hydrogen bonding. So the fluorescence quenching signals were not detected until the hot shot step was added.

The activity of many restriction endonucleases depends on the sequence flanking the recognition site, and the hydrolysis rates differ greatly with various flanking sequences. So we conclude that a longer flanking sequence is likely to result in good cleavage activity of the restriction endonuclease, but at the same time, it reduces the dissociation activity. Once the cleavage activity of the endonuclease on the substrate is confirmed, a shorter flanking sequence makes the real-time PCR method more sensitive. This U-MB real-time PCR technique is suitable for monitoring the digestion of synthetic substrates by a restriction endonuclease with high cleavage activity.

Kinetic analysis

The method described in the *AluI* and *EcoRI* kinetic studies demonstrated the utility of U-MB real-time PCR to assess kinetic parameters of the enzymatic DNA cleavage reaction. A restriction enzyme concentration detectable at 0.002 unit/ μ l and substrates down to the nanomolar range demonstrated the high sensitivity of this technique. Monitoring enzyme cleavage activity by dual-color fluorescence cross-correlation spectroscopy yields great detection results and indicates a linear range of more than one magnitude [35]. With our method, the linear range of *AluI* concentration was more than two orders of magnitude (0.002–0.4 unit/ μ l), which is comparable to that reported by Wang [25].

EcoRI kinetics has been investigated a lot before. The K_m value depends sensitively on the size of the substrate [8,35]. K_m is usually higher with short synthetic DNA oligomers (80–230 nM, in one case up to 7 μ M) [36,37] than with plasmid substrates (1–10 nM) [38–41]. Compared with the reported values, the K_m (226 \pm 20 nM) obtained by our method is comparable to the results for synthetic oligomers. And the K_{cat} (0.22 \pm 0.20 s⁻¹) is also in the range that has been analyzed before [42]. This confirms that our method is reliable and has the potential to become a model system for restriction enzyme activity detection.

Temperature influence

In the restriction enzyme cleavage reaction, some digestion occurred rapidly in the first 40 s. The time required to mix enzyme and DNA, seal the tube(s), and start the experiment in the reverse

transcription PCR machine was found to be about 15 s. To determine if any of the substrate was cleaved before the reaction actually started, we performed a comparison experiment. We measured the restriction enzyme activity at room temperature (about 25 °C), and the results indicated nearly no change in fluorescence in the 10 thermal cycles at 25 °C (about 50 s). But with and increase in time, fluorescence did exhibit a little decrease. This means that *EcoRI* has low activity at 25 °C. So, before the detection starts at 37 °C, the influence of mixing time on the cleavage reaction at room temperature is negligible, but operating time should be kept as short as possible. Also, the activity of *EcoRI*, after being heated at 60 °C for 5 min, showed little change in comparison with normal *EcoRI*. In our experiment, the hot shot was shorter than 5 min, so the effect on enzyme activity was insignificant. However, higher temperatures (65 °C) and longer heating times (>20 min) would destroy the enzyme activity.

Potential applications

Cleavage efficiency close to the termini of DNA fragments is helpful in selecting enzymes most likely to cleave the end of a DNA fragment, choosing the order of addition of two restriction endonucleases for a double digestion, or designing PCR experiments in which the synthesized DNA fragment is to be subsequently digested with a restriction endonuclease. With our method, all reactions were restriction endonuclease cleavages on close-end recognition sites. For example, the *EcoRI* 3-bp flanking system showed high cleavage activity on the close-end recognition site, whereas the *XhoI* 3-bp flanking system exhibited low digestion efficiency, so it would be much better to design the recognition site for *EcoRI*, rather than *XhoI*, at the termini in designing substrate sequences. This technique not only clarifies the difference in cleavage efficiency of restriction endonucleases on recognition sites close to the end of the substrate, but also provides an easy way to test the varying requirements that restriction enzymes have for the number of bases flanking the recognition site by digesting a series of short double-stranded DNA substrates.

Flanking sequences are likely to influence the structure and dynamics of the DNA, both of which are important in the recognition process. So, enhancing the potential for analysis of cleavage activity of restriction enzymes among different flanking substrates is important for elucidating the molecular basis for enzyme selectivity. In this technique, it is only necessary to design different flanking sequences in the substrate to detect cleavage activity in a simple process. And the results permit comparison of restriction enzyme activities on substrates. So, this method can be used for rapid, preliminary, high-throughput selection of restriction endonuclease activity.

Moreover, this method provided different temperature segments during the reaction process. From the view of the hot shot segment, application of this method could widen to encompass longer flanking sequences in substrate optimization analysis, and also could be a convenient technique to analyze enzyme activities at different temperatures, especially in comparative investigation of the cleavage activity of some artificial enzymes at temperatures other than 37 °C. So, smooth control of the temperature system assists analysis of the cleavage reaction to a wider scope.

Because enzyme activity and kinetic parameters may change significantly under different cleavage reaction conditions, the detection results from different probing systems are often not comparable for restriction enzymes. In our method, it is only necessary to change the substrates and the corresponding sequences of the UT template. Such a universal detection model enables relatively stable and uniform reaction conditions for analysis of restriction enzyme activity, and this is particularly advantageous in distinguishing the activity of the same enzyme on different substrates and/or at

different temperatures. This also offers the potential for high-throughput screening of inhibitors or cofactors of these enzymes.

Similar to other assays for monitoring DNA cleavage based on fluorescence resonance energy transfer, the method described here is applicable only under multiple turnover conditions and may be problematic if the enzyme first nicks DNA and dissociates from it before cleaving the second strand. It is more suitable for those restriction enzymes that have high cleavage efficiency on short substrates and those homodimer restriction enzymes that cleave the two strands of the substrate simultaneously so that the cleaved products may undergo the subsequent reactions smoothly.

Conclusion

For the first time, the U-MB quenching assay has been used to monitor the progress of nucleic acid cleavage reactions in sealed tubes using real-time PCR, resulting in accurate kinetic quantification of restriction endonucleases and realization of high-throughput applications. This simple assay with high sensitivity and a smooth temperature control procedure is widely useful in characterizing new restriction enzymes and their cleavage reactions. It is also a fast and effective way to screen restriction enzyme cleavage activity on substrates with different flanking sequences. This assay has the potential for high-throughput analysis of other modifying enzymes and searching for their inhibitors or cofactors. The method not only provides a yes-or-no decision on cleavage activity of the enzyme on the substrate, but also yields accurate cleavage activity kinetic data. In this study, restriction endonuclease kinetics was analyzed at high sensitivity, the detection limit was low, and the assay time was short. It provides a very useful method for high-throughput monitoring of endonuclease activity in real time.

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References

- [1] A. Pingoud, A. Jeltsch, Structure and function of type II restriction endonucleases, *Nucleic Acids Res.* 29 (2001) 3705–3727.
- [2] A.S. Bhagwat, Restriction enzymes: properties and use, *Methods Enzymol.* 216 (1992) 199–224.
- [3] A. Pingoud, A. Jeltsch, Recognition and cleavage of DNA by type II restriction endonuclease, *Eur. J. Biochem.* 246 (1997) 1–22.
- [4] A. Guttman, Z. Ronai, C. Barta, Y.M. Hou, M. Sasvari-Szekely, X. Wang, S.P. Briggs, Membrane-mediated ultrafast restriction digestion and subsequent rapid gel microchip electrophoresis of DNA, *Electrophoresis* 23 (2002) 1524–1530.
- [5] R.J. Roberts, Restriction enzymes and their isoschizomers, *Nucleic Acids Res.* 18 (1990) 2331–2365.
- [6] D. Botstein, R.L. White, M. Skolnick, R.W. Davis, Construction of a genetic linkage map in man using restriction fragment length polymorphisms, *Am. J. Hum. Genet.* 32 (1980) 314–331.
- [7] S. Halford, A. Goodall, Modes of DNA cleavage by the *EcoRV* restriction endonuclease, *Biochemistry* 27 (1988) 1771–1777.
- [8] L.W. McLaughlin, F. Benseler, E. Graeser, N. Piel, S. Scholtissek, Effects of functional group changes in the *EcoRI* recognition site on the cleavage reaction catalyzed by the endonuclease, *Biochemistry* 26 (1987) 7238–7245.
- [9] J. Alves, T. Ruter, R. Geiger, A. Fliess, G. Maass, A. Pingoud, Changing the hydrogen-bonding potential in the DNA binding site of *EcoRI* by site-directed mutagenesis drastically reduces the enzymic activity, not, however, the preference of this restriction endonuclease for cleavage within the site –GAATTC–, *Biochemistry* 28 (1989) 2678–2684.
- [10] T.R. Waters, B.A. Connolly, Continuous spectrophotometric assay for restriction endonucleases using synthetic oligodeoxynucleotides and based on the hyperchromic effect, *Anal. Biochem.* 204 (1992) 204–209.
- [11] A. Jeltsch, A. Fritz, J. Alves, H. Wolfes, A. Pingoud, A fast and accurate enzyme-linked immunosorbent assay for the determination of the DNA cleavage activity of restriction endonucleases, *Anal. Biochem.* 213 (1993) 234–240.
- [12] S.P. Lee, M.K. Han, Fluorescence assay for DNA cleavage, *Methods Enzymol.* 278 (1997) 343–363.
- [13] E.S. Roddy, M. Price, A.G. Ewing, Continuous monitoring of a restriction enzyme digest of DNA on a microchip with automated capillary sample introduction, *Anal. Chem.* 75 (2003) 3704–3711.
- [14] S.S. Ghosh, P.S. Eis, K. Blumeyer, K. Fearon, D.P. Millar, Real-time kinetics of restriction endonuclease cleavage monitored by fluorescence resonance energy transfer, *Nucleic Acids Res.* 22 (1994) 3155–3159.
- [15] K. Eisenschmidt, T. Lanio, A. Jeltsch, A. Pingoud, A fluorimetric assay for on-line detection of DNA cleavage by restriction endonucleases, *J. Biotechnol.* 96 (2002) 185–191.
- [16] J.J. Li, R. Geyer, W.H. Tan, Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA, *Nucleic Acids Res.* 28 (2000) e52.
- [17] S. Tyagi, F.R. Kramer, Molecular beacons: probes that fluoresce upon hybridization, *Nat. Biotechnol.* 14 (1996) 303–308.
- [18] A.S. Piatek, S. Tyagi, A.C. Pol, L.P. Miller, F.R. Kramer, D. Alland, Molecular beacon sequence analysis for detecting drug resistant in *Mycobacterium tuberculosis*, *Nat. Biotechnol.* 16 (1998) 359–363.
- [19] X.M. Li, Y. Huang, Y. Guan, M.P. Zhao, Y.Z. Li, Universal molecular beacon-based tracer system for real-time polymerase chain reaction, *Anal. Chem.* 78 (2006) 7886–7890.
- [20] X.M. Li, Y. Huang, C. Song, M.P. Zhao, Y.Z. Li, Several concerns about the primer design in the universal molecular beacon real-time PCR assay and its application in HBV DNA detection, *Anal. Bioanal. Chem.* 388 (2007) 979–985.
- [21] S.P. Lee, D. Porter, J.G. Chirikjian, J.R. Knutson, M.K. Han, A fluorometric assay for DNA cleavage reactions characterized with *BamHI* restriction endonuclease, *Anal. Biochem.* 220 (1994) 377–383.
- [22] A.L. Lehninger, D.L. Nelson, M.M. Cox, Principles of Biochemistry, Worth, New York, 1993.
- [23] C.A. Brennan, M.D. Van Cleve, R.I. Gumport, The effects of base analogue substitutions on the cleavage by the *EcoRI* restriction endonuclease of octadeoxyribonucleotides containing modified *EcoRI* recognition sequences, *J. Biol. Chem.* 261 (1986) 7270–7278.
- [24] J. Alves, C. Urbanke, A. Fliess, G. Maass, A. Pingoud, Fluorescence stopped-flow kinetics of the cleavage of synthetic oligodeoxynucleotides by the *EcoRI* restriction endonuclease, *Biochemistry* 28 (1989) 7879–7888.
- [25] C.B. Ma, Z.W. Tang, K.M. Wang, W.H. Tan, X.H. Yang, W. Li, Z.H. Li, X.Y. Lv, Real-time monitoring of restriction endonuclease activity using molecular beacon, *Anal. Biochem.* 363 (2007) 294–296.
- [26] D. Mark, C. Van, I.G. Richard, Influence of enzyme–substrate contacts located outside the *EcoRI* recognition site on cleavage of duplex oligodeoxyribonucleotide substrates by *EcoRI* endonuclease, *Biochemistry* 31 (1992) 334–339.
- [27] B. Terry, W. Jack, P. Modrich, Mechanism of specific site location and DNA cleavage by *EcoRI* endonuclease, *Gene Amplif. Anal.* 5 (1987) 103–118.
- [28] Cleavage close to the end of DNA fragments (oligonucleotides), New England Biolabs technical reference (2005–2006).
- [29] D.R. Lesser, M.R. Kurpiewski, L. Jen-Jacobson, The energetic basis of specificity in the *EcoRI* endonuclease–DNA interaction, *Science* 250 (1990) 776–786.
- [30] C. Wenz, M. Hahn, A. Pingoud, Engineering of variants of the restriction endonuclease *EcoRV* that depend in their cleavage activity on the flexibility of sequences flanking the recognition site, *Biochemistry* 37 (1998) 2234–2242.
- [31] C. Wenz, A. Jeltsch, A. Pingoud, Probing the indirect readout of the restriction enzyme *EcoRV*, *J. Biol. Chem.* 271 (1996) 5565–5573.
- [32] C.C. Yang, D. Topal, Nonidentical DNA-binding sites of endonuclease *NaeI* recognize different families of sequences flanking the recognition site, *Biochemistry* 31 (1992) 9657–9664.
- [33] J.D. Taylor, S.E. Halford, The activity of the *EcoRV* restriction endonuclease is influenced by flanking DNA sequences both inside and outside the DNA–protein complex, *Biochemistry* 31 (1992) 90–97.
- [34] B.J. Terry, W.E. Jack, P. Modrich, Facilitated diffusion during catalysis by *EcoRI* endonuclease: nonspecific interactions in *EcoRI* catalysis, *J. Biol. Chem.* 260 (1985) 13130–13137.
- [35] U. Ketting, A. Koltermann, P. Schwill, M. Eigen, Real-time enzyme kinetics monitored by dual-color fluorescence cross-correlation spectroscopy, *Proc. Natl. Acad. Sci. USA* 95 (1998) 1416–1420.
- [36] T.M. Nordlund, S. Andersson, L. Nilsson, R. Rigler, A. Graslund, L.W. McLaughlin, Structure and dynamics of a fluorescent DNA oligomer containing the *EcoRI* recognition sequence: fluorescence, molecular dynamics, and NMR studies, *Biochemistry* 28 (1989) 9095–9103.
- [37] P.J. Greene, M. Gupta, H.W. Boyer, W.E. Brown, J.M. Rosenberg, Sequence analysis of the DNA encoding the *EcoRI* endonuclease and methylase, *J. Biol. Chem.* 256 (1981) 2143–2153.
- [38] S.E. Halford, N.P. Johnson, J. Grinstead, The reactions of the *EcoRI* and other restriction endonucleases, *Biochem. J.* 179 (1979) 353–365.
- [39] S.E. Halford, N.P. Johnson, J. Grinstead, The *EcoRI* restriction endonuclease with bacteriophage lambda DNA: kinetic studies, *Biochem. J.* 191 (1980) 581–592.
- [40] S.E. Halford, N.P. Johnson, The *EcoRI* restriction endonuclease covalently closed DNA and ethidium bromide, *Biochem. J.* 199 (1981) 767–777.
- [41] W.E. Jack, B.J. Terry, P. Modrich, Involvement of outside DNA sequences in the major kinetic path by which *EcoRI* endonuclease locates and leaves its recognition sequence, *Proc. Natl. Acad. Sci. USA* 79 (1982) 4010–4014.
- [42] D.J. Wright, W.E. Jack, P. Modrich, The kinetic mechanism of *EcoRI* endonuclease, *J. Biol. Chem.* 274 (1999) 31896–31902.