



# Real-time monitoring of double-stranded DNA cleavage using molecular beacons

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## ABSTRACT

Traditional methods to assay enzymatic cleavage of DNA are discontinuous, time-consuming and laborious. Here, we report a new approach for real-time monitoring of double-stranded DNA cleavage by restriction endonuclease based on nucleic acid ligation using molecular beacon. Upon cleavage of DNA, the cleavage product can be ligated by DNA ligase, which results in a fluorescence enhancement of the molecular beacon. This method permits real-time monitoring of DNA cleavage and makes it easy to characterize the activity of restriction endonuclease and to study the cleavage reaction kinetics.

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## 1. Introduction

The DNA cleavage reactions catalyzed by restriction endonucleases are important in cellular processes, such as DNA replication, recombination and repair [1]. They also play significant roles in a variety of biotechnologies such as cloning, genetic analysis and genotyping. To assay the cleavage efficiency of these enzymes, several traditional methods such as gel electrophoresis, filter binding, high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) [2–5] are commonly used. All these methods, however, are discontinuous, time-consuming, laborious and usually require isotope labeling. There have been many recent efforts for the development of fluorescence assays which are based on fluorescence resonance energy transfer (FRET) for DNA cleavage [6–13]. These assays are continuous and real-time. However, up to now all of them have been designed only for doubly labeled DNA substrates with fluorophores, might interfere with the kinetic behavior of the restriction endonucleases. Furthermore, the potential for achieving high sensitivity through FRET has not been fully realized. Therefore, there is a great demand for continuous, convenient, sensitive assays for these reactions.

In this paper, a novel method is described for real-time monitoring of double-stranded DNA cleavage by using molecule beacons

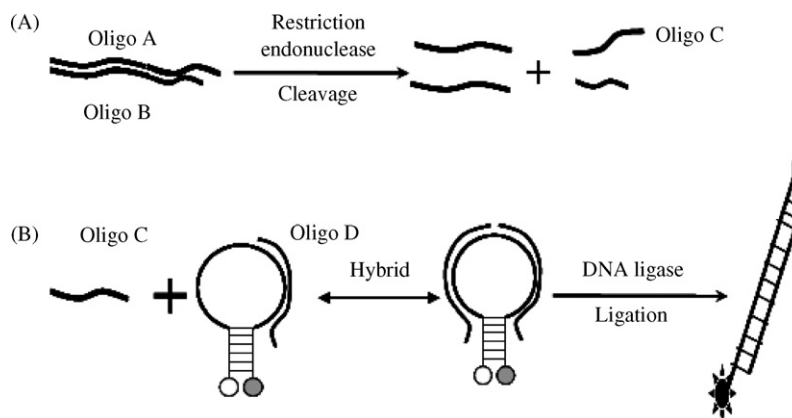
(MBs). Since they were first reported in 1996 [14], MBs have become a class of probes widely used in chemistry, biology, biotechnology, and medical sciences for biomolecular recognition, due in part to their high sensitivity and excellent specificity [15,16]. MB is not only a useful tool for DNA/RNA studies, but also a promising probe for monitoring DNA–protein interactions [17–19]. Previously, we have developed a method for real-time monitoring of nucleic acid ligation and phosphorylation using MB [20–22]. In this work, we further extend this new strategy for real-time monitoring of double-stranded DNA cleavage by restriction endonuclease based on nucleic acid ligation. Here, Rsa I endonuclease that recognizes the GTAC sequence was chosen as an example. This approach is simple, rapid, sensitive, and without requirement for doubly labeled DNA substrates.

## 2. Experimental

### 2.1. Reagents and materials

The MB probe: 5'-TAMRA-CGATGCCGTGCTTGTAGTCCCGTCCATCG-DABCYL-3', oligonucleotide (Oligo) A: 5'-GACGAGAGGTA-CAAGACACG-3', Oligo B: 5'-CTTGTACCTCTCGTC-3' and Oligo D: 5'-GACGGGA-3' were synthesized by Dalian Takara Bio Inc. (Dalian, China), and the T4 DNA ligase was purchased from the same company. The Rsa I endonuclease was purchased from MBI Fermentas (Lithuania). The MgCl<sub>2</sub> and ATP were purchased from Amresco (Solon, OH). All other reagents were of analytical-reagent grade. Deionized water was obtained through a Nanopure InfinityTM

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**Fig. 1.** Schematic of real-time monitoring of DNA cleavage by restriction endonuclease using molecular beacon (A) DNA cleavage by restriction endonuclease; (B) the cleavage product hybridizes with the MB and can be ligated by DNA ligase, which restores the quenched fluorescence of the MB.

ultrapure water system (Barnstead/thermolyne Corp., Dubuque, IA).

## 2.2. Fluorescence measurement

All fluorescence measurements were carried out on an F2500 (Hitachi, Japan) with excitation at 521 nm and emission at 578 nm for TAMRA labeling at 5' end of MB as a fluorophore. Each experiment was carried out in a final volume of 100  $\mu$ L and incubated at 37  $^{\circ}$ C by an aqueous thermostat (Amersham) for about 8 min to a steady status before the addition of DNA ligase, after which the sample incubated until the fluorescence reached equilibrium again, then Rsa I endonuclease was introduced into the solution and stirred for 4 s, and the fluorescence intensity of sample was recorded synchronously.

## 2.3. Real-time monitoring of DNA cleavage

Two samples were prepared: sample A contained MB, Oligo A, Oligo B and Oligo D; sample B only contained MB. The concentrations of MB and oligos were 250 nM and 300 nM, respectively. Assays were carried out in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM  $MgCl_2$ , 1.0 mM DTT and 500  $\mu$ M ATP. In the real-time monitoring experiment of the DNA cleavage, the aforementioned two samples were incubated at 37  $^{\circ}$ C with the sequential addition of 1.4 units of DNA ligase and 40.0 units of Rsa I endonuclease.

## 2.4. Activity assay of Rsa I endonuclease

In the assay of Rsa I endonuclease activity experiments, samples were prepared containing 200 nM MB, 250 nM Oligo A, 250 nM Oligo B, 250 nM Oligo D and buffer (50 mM Tris-HCl (pH 8.0), 5 mM  $MgCl_2$ , 1.0 mM DTT, 500  $\mu$ M ATP). Before the addition of Rsa I endonuclease at various concentrations (1–320 units/ml), 1.4 units of DNA ligase was introduced into each sample. The cleavage velocities of samples were evaluated by analyzing the time courses respectively.

## 3. Results and discussion

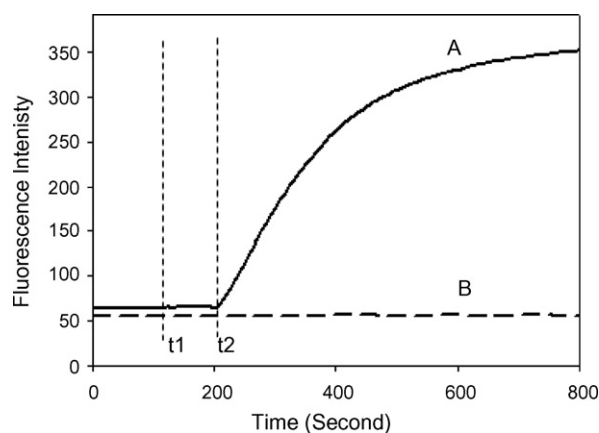
### 3.1. The experimental principle

The principle of this approach is illustrated in Fig. 1. The Oligo A and Oligo B are designed to hybridize with each other to form

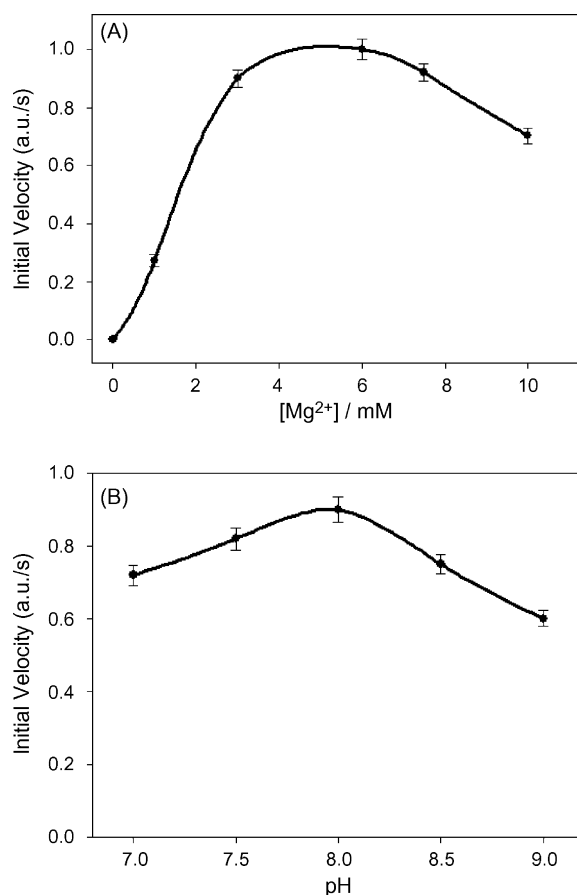
a DNA duplex containing the Rsa I endonuclease recognition site. Upon cleavage, the resulting Oligo C which is complementary with 3' half part of MB's loop is expected to dissociate readily from the duplex. The Oligo C together with Oligo D, can hybridize with the MB's loop to form a ligatable nick and ligated by DNA ligase, resulting in the complete opening of MB and leading to a full fluorescence restoration. Thus, the DNA cleavage reaction by Rsa I endonuclease can be monitored in real-time based on this principle.

### 3.2. Real-time monitoring of double-stranded DNA cleavage

The fluorescence intensity of these samples was monitored and the time courses were plotted in Fig. 2. As illustrated in Fig. 2, after the addition of DNA ligase and Rsa I endonuclease, there was no fluorescence change in curve B (corresponding to sample B). This result implied that MB's conformation was not affected by DNA ligase and Rsa I endonuclease. The only factor that could affect the conformation of MB altering its fluorescence was the presence of the target DNA hybridizing with MB. In sample A represented by curve A, after the addition of DNA ligase, the fluorescence did not change. After the addition of Rsa I endonuclease, the fluorescence was enhanced rapidly, implying MB's hybridization with the target DNA. According to the mechanism demonstrated in Fig. 1, the DNA cleavage reaction took place, and the newly produced DNA could "open" the MB by hybridizing with the loop



**Fig. 2.** Monitoring of the Rsa I endonuclease activity in real-time. The curves A and B represent the time courses of samples A and B, respectively. At time  $t_1$ , DNA ligase was added into the samples; while at time  $t_2$ , Rsa I endonuclease was added.



**Fig. 3.** Effect of  $Mg^{2+}$  and pH. Assays were carried out at  $37^{\circ}C$  in a final volume of  $100\ \mu L$ . The amounts of DNA ligase and Rsa I endonuclease were 1.4 units and 40.0 units, respectively. The experiments were performed in triplicate.

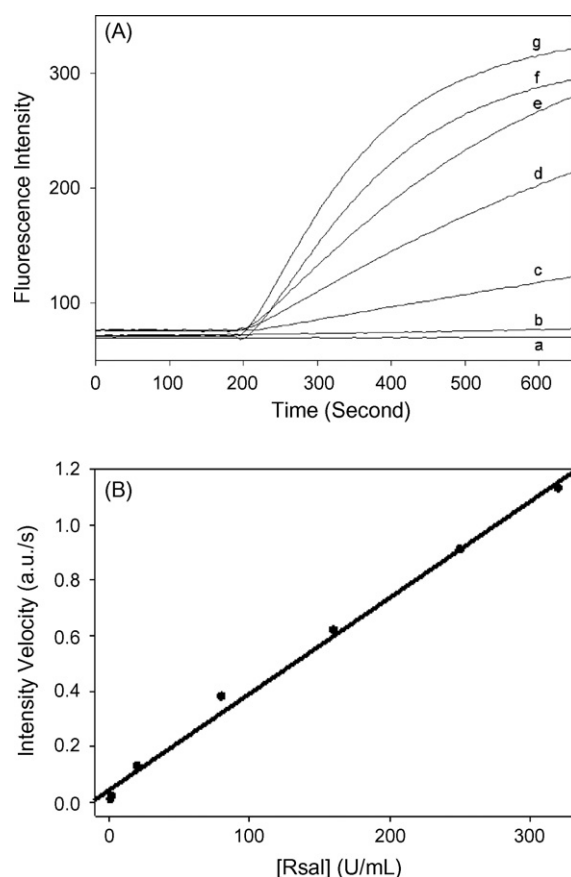
of MB. Based upon the above data, DNA cleavage reactions by Rsa I endonuclease can be monitored in real-time by this novel approach.

### 3.3. Optimization of the experimental conditions

To obtain the optimum conditions for this assay, the effects of  $Mg^{2+}$  and pH have been investigated as they are important factors influencing enzyme activity. As shown in Fig. 3A, the initial velocity of DNA cleavage reached its maximum at an  $Mg^{2+}$  concentration of 5.0 mM and then declined when the concentration of  $Mg^{2+}$  increased. So, 5.0 mM was chosen in our experiments hereafter. According to Fig. 3B, the initial velocity of DNA cleavage increased at pH 7.0–8.0, reached its maximum at pH 8.0, and then declined at pH 8.0–9.0. Therefore, pH 8.0 was selected for further study. Thus, the method developed here is convenient and accurate in detecting the effects of various catalytic conditions on DNA cleavage reactions. It will enable a better understanding of the enzymatic reactions performed under various conditions.

### 3.4. Assay of Rsa I endonuclease

Applied with these optimum conditions (pH 8.0 and 5.0 mM  $Mg^{2+}$ ), a series of reactions using various concentrations of Rsa I endonuclease have been carried out. The time courses are shown in Fig. 4A. The result manifests that, as Rsa I endonuclease concentration was increased, the fluorescence enhancement



**Fig. 4.** (A) Time courses of real-time monitoring of DNA cleavage process catalyzed by various Rsa I endonuclease concentrations: (a) 1 unit/ml; (b) 2 units/ml; (c) 20 units/ml; (d) 80 units/ml; (e) 160 units/ml; (f) 250 units/ml; (g) 320 units/ml. (B) Relationship between initial reaction velocity and concentration of Rsa I endonuclease. Assays were carried out at  $37^{\circ}C$  in a final volume of  $100\ \mu L$  under optimized conditions (50 mM Tris-HCl (pH 8.0), 5.0 mM  $MgCl_2$ , 1.0 mM DTT and 500  $\mu M$  ATP).

rate was increased. Within the enzyme concentration ranging from 1.0 unit/ml to 320 units/ml, a good linear correlation ( $r^2 = 0.9932$ ) between initial velocity of fluorescence enhancement and Rsa I endonuclease concentration was observed (Fig. 4B). The detection limit was 1.0 unit/ml. The assay avoids any indirect and time-consuming problems arising from stopping the reaction, such as in gel electrophoresis methods. And real-time monitoring avoids any subsequent detection and analysis step, so it shortens the detection time for each sample. So the real-time assay we have developed here is convenient, quick and sensitive, and has a wide dynamic range, which makes it a useful method to analyze the Rsa I endonuclease activity.

## 4. Conclusion

In conclusion, we have developed a novel, sensitive, continuous fluorescence assay method to monitor the cleavage of double-stranded DNA by restriction endonuclease based on nucleic acid ligation using MB. This assay used MB DNA probes to perform the signal transduction. Under optimized conditions, Rsa I endonuclease analysis could be realized within 5 min with a detection limit down to 1.0 unit/ml without the need for labeling of DNA substrates. This method permits real-time monitoring of DNA cleavage and makes it easy to characterize the activity of endonucleases and to study the cleavage reaction kinetics.

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