

## Molecular beacons for isothermal fluorescence enhancement by the cleavage of RNase HII from *Chlamydia pneumoniae*

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### Abstract

This article describes a new assay for isothermal enhancement of fluorescence intensity. The assay is based on the cleavage of duplexes formed by the chimeric DNA–rN<sub>1</sub>–DNA molecular beacon (cMB) and target DNA with *Chlamydia pneumoniae* RNase HII (CpRNase HII). The loop sequence of the cMB, which was designed according to the target sequence, contains a single ribonucleotide. The combination of CpRNase HII cleavage and cMB (RHMB) permitted a 90-fold increase in fluorescence intensity change compared with the hybridization reaction in the presence of the same amount of target DNA. These results indicate that the RHMB assay can enhance the fluorescence signal in real-time monitoring of the target DNA.

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**Keywords:** RNase H; Molecular beacons; *Chlamydia pneumoniae*; Isothermal fluorescence enhancement

A molecular beacon is a nucleic acid probe that has a fluorophore at the 5' end and a quencher at the 3' end [1]. In general, the fluorophore of a molecular beacon is quenched by close contact with the quencher. The cleavage of the loop leads to a separation of the fluorophore and the quencher from each other. Therefore, a fluorescence signal is emitted. However, because a molecular beacon initially is designed for thermal cycling-based amplification technology, such as PCR, applying the molecular beacon in an isothermal reaction is a challenge.

In most real-time detection systems, including *Taqman* and the molecular beacon, the fluorescent signal is proportional to the amount of target sequence [1–3]. Enhancing the fluorescent signal in the presence of constant amounts of target DNA is a challenge. An artificial DNA nicking system based on restriction enzymes has been developed to amplify the fluorescence signal with the constant target [4]. However, a special DNA recognition sequence of nick-

ase must be included in target DNA, and this limits the use of the system. In this article, we describe a new method to isothermally amplify the fluorescence signal by the cleavage of molecular beacons with RNase HII from *Chlamydia pneumoniae* (CpRNase HII)<sup>1</sup> in the presence of constant target DNA.

RNase H is an enzyme found in a variety of organisms, ranging from viruses to mammalian cells, and can recognize and cleave the RNA strand of the RNA/DNA duplex [5–7]. Based on differences in the amino acid sequences, RNase H is classified into two major families, type 1 and type 2 RNase H, which are evolutionarily unrelated [6,7]. CpRNase HII, which is type 2 RNase H, has been expressed and characterized [8]. Our further investigation demonstrated that CpRNase HII can cleave DNA–rN<sub>1</sub>–DNA/DNA duplexes [9].

<sup>1</sup> Abbreviations used: CpRNase HII, RNase HII from *Chlamydia pneumoniae*; cMB, chimeric DNA–rN<sub>1</sub>–DNA molecular beacon; RHMB, CpRNase HII cleavage and cMB; ssDNA, single-stranded DNA; T<sub>m</sub>, melting temperature; RCA, rolling circle amplification.

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In the current work, CpRNase HII was used to cleave the chimeric DNA-rN<sub>1</sub>-DNA molecular beacon (cMB) in the presence of target DNA. Based on this observation, a novel CpRNase HII cleavage and cMB (RHMB) assay was set up to detect nucleic acid targets with the increased fluorescence intensity. We demonstrate that multiple cMBs can be cleaved by CpRNase HII on a single target molecule under isothermal conditions.

## Materials and methods

### Preparation of cMB and oligonucleotides used for RHMB assay

cMBs, which were labeled with FAM at the 5' end and with Dabcyl at the 3' end, were the oligonucleotides containing one ribonucleotide at the middle of the sequences (Table 1). cMBs and corresponding oligonucleotides (TaKaRa, China) are detailed in Table 1.

### Preparation of ssDNA samples for RHMB assay

The *gapA* gene, which had a length of 439 bp, was prepared by PCR amplification from *Escherichia coli* genomic DNA. The forward primer was 5'-GCTAACCT-GAAATGGGACGA-3', and the reverse primer was 5'-GTCAGTTTGGCATTTCAGTTC-3', in which the forward primers were phosphorylated at the 5' end by T4-polynucleotide kinase. PCR was performed in a 25- $\mu$ l volume containing 20 ng *E. coli* genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, and

2.5 U *Taq* DNA polymerase. The PCR protocol consisted of a 5-min pre-PCR heating step at 95 °C, followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 54 °C for 60 s, and extension at 72 °C for 60 s, with a final 5-min extension step at 72 °C. The PCR fragment was purified and incubated with  $\lambda$ -exonuclease (Epicentre) at 37 °C to obtain target single-stranded DNA (ssDNA).

### RHMB assay

Various oligonucleotides or ssDNA were incubated with cMBs (Table 1) to generate the cMB/Oligo or cMB/ssDNA substrates of CpRNase HII. CpRNase HII was prepared according to the reported procedure [8]. The CpRNase HII reaction solution (100  $\mu$ l) contained 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM  $\beta$ -ME, 10  $\mu$ g/ml bovine serum albumin, 2 U ribonuclease inhibitor (TaKaRa, China), different amounts of substrates, and CpRNase HII (100 ng,  $\sim 1.8 \times 10^{-3}$  U) [8]. The CpRNase HII reaction mixture was incubated at 37 or 33 °C in a Fluoroskan Ascent FL or Fluoroskan Ascent Type 374 (Thermo Labsystems, Finland), and fluorescence was monitored at time points ranging from 3 to 60 min. The excitation was set at 485 nm, and the emission was set at 527 nm. The fluorescence intensity changes were obtained according to the formula  $\Delta F = F_t - F_0$ , where  $F_t$  is the fluorescence intensity of the reaction mixture after the CpRNase HII cleavage or hybridization reaction and  $F_0$  represents fluorescence intensity at the start of the reaction.

Table 1  
Oligonucleotides used for CpRNase HII cleavage reaction

Oligonucleotide	Nucleotide sequence (5'-3')
cMB-ra	(FAM)-CGCGATGCTGCAGGAA <b>a</b> TCGATATCAAATCGCG-(Dabcyl)
Oligo-T(21)	<b>TTGATATCGATTTCCTGCAGC</b>
Oligo-T(19)	<b>TGATATCGATTTCCTGCAG</b>
Oligo-T(17)	<b>GATATCGATTTCCTGCA</b>
Oligo-T(15)	<b>ATATCGATTTCCTGC</b>
Oligo-T(13)	<b>TATCGATTTCCTG</b>
Oligo-T(11)	<b>ATCGATTTCCT</b>
cMB-ru	(FAM)-CGCGATTGGTGAGGGC <b>u</b> GGGTGGATGGATCGCG-(Dabcyl)
Oligo-A(21)	<b>CCATCCACCCAGCCCTCACCA</b>
Oligo-A(19)	<b>CATCCACCCAGCCCTCACC</b>
Oligo-A(17)	<b>ATCCACCCAGCCCTCAC</b>
Oligo-A(15)	<b>TCCACCCAGCCCTCA</b>
Oligo-A(13)	<b>CCACCCAGCCCTC</b>
Oligo-A(11)	<b>CACCCAGCCCT</b>
cMB-rc	(FAM)-CGCGATT <b>AA</b> ACTGCAC <b>c</b> ACCA <b>ACTA</b> ATATCGCG-(Dabcyl)
Oligo-G(21)	<b>ATTAGTTGGTGGTGCAGTTTA</b>
Oligo-G(19)	<b>TTAGTTGGTGGTGCAGTTT</b>
Oligo-G(17)	<b>TAGTTGGTGGTGCAGTT</b>
Oligo-G(15)	<b>AGTTGGTGGTGCAGT</b>
Oligo-G(13)	<b>GTTGGTGGTGCAG</b>
Oligo-G(11)	<b>TTGGTGGTGCAG</b>

Note. Deoxyribonucleotides are shown by uppercase letters, and ribonucleotides are in bold and shown by lowercase letters. The italic portions of the cMB-rc sequence indicate the random sequences that are not complementary to the 439-nt ssDNA.

## Results

### Determination of optimal length of complementary sequences between cMBs and target DNA

The effect of oligonucleotide size and sequence dependence on the CpRNase HII cleavage was conducted to determine the appropriate length of complementary sequences between cMBs and target DNA. cMBs were hybridized with complementary oligonucleotides (Table 1) to form the substrates of CpRNase HII. The cleavage of CpRNase HII on cMB/Oligo had been performed at 37 or 33 °C. Different fluorescence intensity changes were obtained (Fig. 1). For cMB-ra/Oligo-T duplexes, Oligo-T(15) was the most desirable oligonucleotide for the highest fluorescence intensity changes at 37 °C. Lower fluorescence intensity changes were obtained at 37 °C for the oligonucleotides whose melting temperature ( $T_m$ ) was higher or lower than that of Oligo-T(15). However, Oligo-T(13), whose  $T_m$  was lower than that of Oligo-T(15), was the most desirable oligonucleotide at 33 °C.

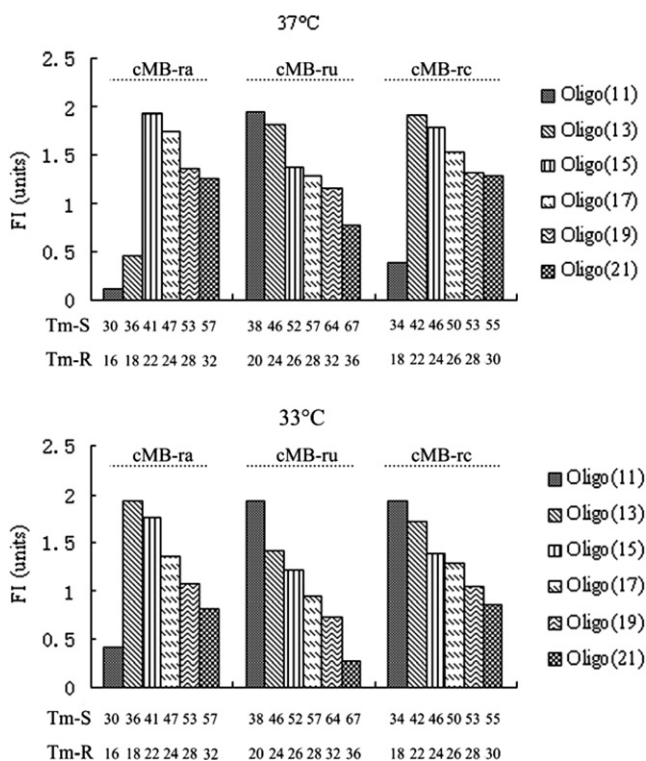


Fig. 1. Cleavage of CpRNase HII on the substrates formed by cMBs and different lengths of oligonucleotides. Here 100 nM cMBs was cleaved by 100 ng CpRNase HII in the presence of 10 nM corresponding oligonucleotides. The reactions were carried out at 37 or 33 °C for 30 min and measured with a Fluoroskan Ascent FL or Fluoroskan Ascent Type 374. FI (units) represents the fluorescence intensity of the mixture. Tm-S denotes the melting temperature of oligonucleotides used in the cleavage reaction. Tm-R denotes the melting temperature of oligonucleotides that are complementary to the cleaved cMBs after the cleavage reaction. Both Tm-S and Tm-R were generated by an oligonucleotide properties calculator in 50 mM NaCl [15]. Data represent the averages of three independent experiments with standard errors less than 5%.

The analogous results were obtained for cMB-ru/Oligo-A and cMB-rc/Oligo-G duplexes. From these results, we can confirm that the optimal length of complementary sequence between cMBs and target DNA is related to the  $T_m$  of the complementary sequence and the reaction temperature. This phenomenon could be interpreted as follows. The cMB/Oligo duplex is cleaved by CpRNase HII to form a nicked duplex, which is thermolabile at the reaction temperature and would be dissociated to let the target free. The free target would hybridize to another cMB and initiate another CpRNase HII cleavage reaction. For the duplexes with a high  $T_m$ , the cleavage products are more thermostable, leading to the difficulty of starting another cycle of CpRNase HII-catalyzed reactions. Consequently, after several cycles of cleavage, the accumulated fluorescence intensities of cMB-ra/Oligo-T(15), cMB-ru/Oligo-A(11), and cMB-rc/Oligo-G(13) duplexes are higher than those of other duplexes at 37 °C. The reason why cMB-ra/Oligo-T(11), cMB-ra/Oligo-T(13), and cMB-rc/Oligo-G(11) cannot be cleaved effectively at 37 °C may be that Oligo-T(11), Oligo-T(13), and Oligo-G(11) have a  $T_m$  lower than 37 °C, and so it does not benefit them to form the stable substrate of CpRNase HII with the complementary cMBs. Thus, the appropriate length of complementary sequences between cMBs and target DNA was determined according to their  $T_m$  at the constant reaction temperature.

### Enhancement of fluorescence intensity with RHMB assay

We used different amounts of cMB-ra to detect 10 nM Oligo-T(15) by the CpRNase HII cleavage reaction or by the hybridization reaction. As shown in Table 2, the fluorescence intensity changes increased with the increasing cMB-ra by the CpRNase HII cleavage reaction. However, for the hybridization reaction, the fluorescence intensity changes exhibited 10-fold excessive cMB-ra. At the same amount of cMB-ra and target DNA, the fluorescence intensity changes produced by the CpRNase HII cleavage reaction were much greater than those produced by the hybridization reaction. Furthermore, in the presence of 100-fold excess of cMB-ra, the fluorescence intensity change of cleavage increased 90-fold compared with that

Table 2  
Fluorescence intensity changes in the presence of different folds of cMBs

		cMB-ra (nM) <sup>a</sup>			
		10	100	500	1000
$\Delta F^b$	Cleavage <sup>c</sup>	0.242	2.136	11.228	21.739
	Hybridization <sup>d</sup>	0.078	0.239	0.243	0.241

Note. Data represent the averages of three independent experiments with standard errors less than 5%.

<sup>a</sup> Indicates the concentration of cMB-ra in the reaction mixture.

<sup>b</sup> Indicates the fluorescence intensity changes of the reaction mixture.

<sup>c</sup> Different concentrations of cMB-ra were mixed with 10 nM Oligo-T(15) to be cleaved by 1000 ng CpRNase HII at 37 °C for 60 min.

<sup>d</sup> Different concentrations of cMB-ra were hybridized with 10 nM Oligo-T(21) at 37 °C for 60 min.

of hybridization. These data demonstrate that multiple cMBs can be cleaved on one target molecule by the CpRNase HII cleavage reaction.

The fluorescent signal generated by the cleavage of 1000 nM cMB-ra in the presence of different concentrations of Oligo-T(15) and the signal generated by the hybridization of 1000 nM cMB-ra with 32 nM Oligo-T(21) are illustrated in Fig. 2. In the presence of 16 or 32 nM target DNA, the fluorescence intensity of the mixture generated by cleavage rose rapidly at first and then asymptotically approached a plateau with a large number of fluorescence intensities being obtained. However, the fluorescence intensity of the mixture generated by the hybridization increased a little. The fluorescence intensity was low with 1 or 2 nM target DNA by the cleavage of CpRNase HII, and its time dependence became linear.

#### Application of ssDNA as target in RHMB assay

We extended this RHMB assay by applying 439-nt ssDNA, which was obtained by digestion of the PCR fragment of *E. coli gapA*. The cMB-rc used in the reaction has 13 bases complementary to 439-nt ssDNA. From the results in Fig. 3, we can deduce that the fluorescence enhancement can also be applicable to the long ssDNA with the RHMB assay.

#### Specificity of RHMB assay

To investigate the specificity of the CpRNase HII-catalyzed reactions, three different concentrations of 439-nt ssDNA, which were obtained by digestion of the PCR fragment of *E. coli gapA*, were detected in the presence or absence of 5  $\mu$ g excess total calf thymus DNA. There were no significant fluorescent changes caused by excess calf thymus DNA (Fig. 4). These results indicate that the RHMB assay has high specificity. Therefore, the RHMB assay can

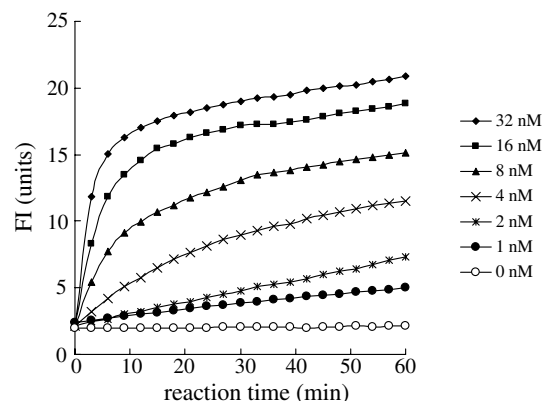


Fig. 3. Time course of cleavage reaction of cMB-rc with long ssDNA. Here 1000 nM cMB-rc was cleaved by 1000 ng CpRNase HII at 37 °C for 60 min in the presence of different concentrations of 439-nt ssDNA. The concentrations of 439-nt ssDNA are denoted by numbers beside the curves. FI (units) represents the fluorescence intensity of the reaction mixture.

be used to enhance the fluorescence signal of a target in a complex DNA mixture.

#### Discussion

Our study has introduced a novel RHMB assay to enhance the fluorescence signal by the specific cleavage of CpRNase HII on cMBs. Compared with the conventional molecular beacon assay method and DNA microassay technology, where a fluorescent signal is predominantly proportional to the amount of target sequence [1–3,10], the RHMB assay amplifies detection signals from reacted molecular beacons rather than the targets. The fluorescent signal can be accumulated through multiple cycles of beacon target hybridization and CpRNase HII cleavage reaction. In this way, a copy of the DNA target supports multiple copies of cMB cleavage by CpRNase HII.

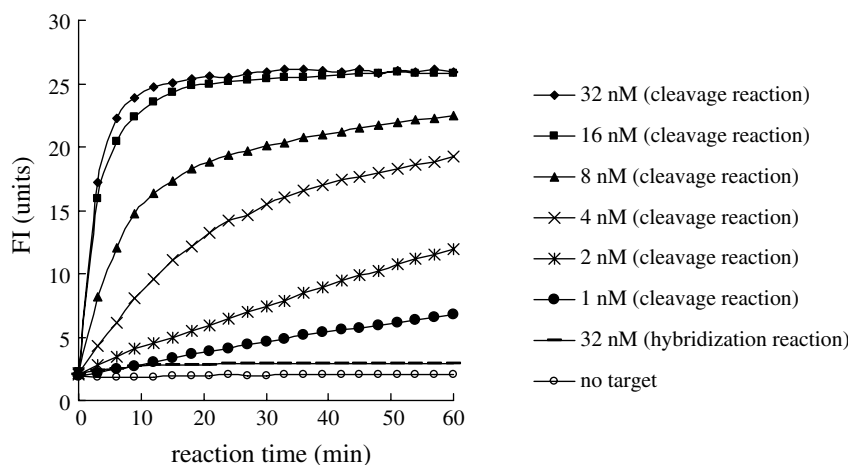


Fig. 2. Time course of cleavage or hybridization reaction with cMB-ra. Here 1000 nM cMB-ra was cleaved by 1000 ng CpRNase HII at 37 °C for 60 min in the presence of different concentrations of Oligo-T(15), and 1000 nM cMB-ra was hybridized with 32 nM Oligo-T(21) at 37 °C for 60 min. The concentrations of Oligo-T(15) are denoted by numbers beside the curves. FI (units) represents the fluorescence intensity of the reaction mixture.

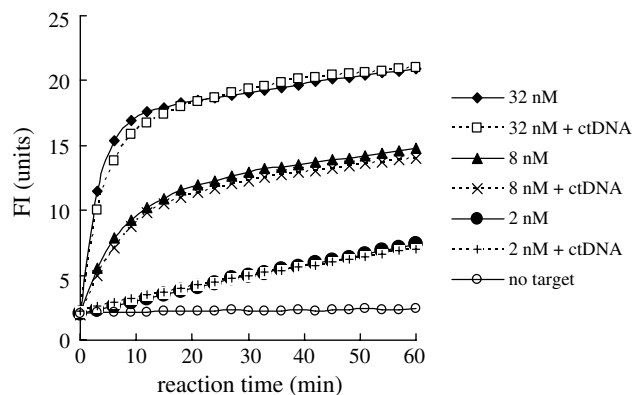


Fig. 4. Time course of cleavage of cMB-rc in the presence or absence of calf thymus DNA. Here 1000 nM cMB-rc was cleaved by 1000 ng CpRNase HII at 37 °C for 60 min in the presence or absence of 5 µg calf thymus DNA. The concentrations of 439-nt ssDNA are denoted by numbers beside the curves. ctDNA represents calf thymus DNA. FI (units) represents the fluorescence intensity of the cleaved mixture.

RHMB amplifies the fluorescence signal with high efficiency under isothermal conditions without significant influence of the copresence of nontarget DNA. The mismatches of DNA–rN<sub>1</sub>–DNA/DNA duplexes negatively affect the cleavage of CpRNase HII [9]. Therefore, the RHMB assay is highly specific for the target sequence, and this is beneficial for alleviating the general problem of backgrounds associated with constant temperature detection.

RHMB assay is simple and can be carried out under isothermal conditions. The reaction system consists of only two components: cMB and CpRNase HII. The RHMB assay may contribute to the real-time monitoring of the rolling circle amplification (RCA) and other reactions [11–14]. In the future, advances in molecular technology resulting in higher sensitivity will further improve the performance of this RHMB assay.

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