



## Chimeric RNA–DNA molecular beacon assay for ribonuclease H activity

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Current methods to detect and assay ribonuclease H (RNase H) activity are indirect and time-consuming. Here we introduce a direct and sensitive method, based on the fluorescence quenching mechanism of molecular beacons, to assay RNA cleavage in RNA:DNA hybrids. An RNA–DNA chimeric beacon assay for RNase H enzymatic activity was developed. The substrate is a single-stranded RNA–DNA chimeric oligonucleotide labeled with a 5'-fluorescein and a 3'-DABCYL. The fluorophore (fluorescein) of the probe is held in close proximity to the quencher (DABCYL) by the RNA:DNA stem-loop structure. When the RNA sequence of the RNA:DNA hybrid stem is cleaved, the fluorophore is separated from the quencher and fluorescence can be detected as a function of time. Chimeric beacons with different stem lengths and sequences have been surveyed for this assay with *E. coli* RNase H. We found that the beacon kinetic parameters are in qualitative agreement with previously reported values using more cumbersome assays. This method permits real-time detection of RNase H activity and a convenient approach to RNase H kinetic and mechanistic study.

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### INTRODUCTION

RNase H specifically hydrolyzes the RNA strand of an RNA:DNA duplex through an endonucleolytic mechanism (for review, see <sup>1</sup>). The enzyme is important in several cellular processes including DNA replication, DNA repair and transcription.<sup>1</sup> RNase H activity has been demonstrated for a large and growing family of proteins. Enzymes with RNase H activities have been identified in many sources, including *E. coli*,<sup>2,3</sup> yeast,<sup>4,5</sup> trypanosomes,<sup>6</sup> calf thymus,<sup>7,8</sup> and other cells.<sup>9–12</sup> In addition to the cellular RNase H enzymes, RNase H activity has been found associated with retroviral reverse transcriptase.<sup>13,14</sup> RNase H has also found wide application in molecular biology and biotechnology, e.g. removal of mRNA from mRNA:cDNA duplex and specific cleavage in RNA with synthetic DNA. In antisense

therapy, RNase H appears to play a critical role in the antisense effects of oligodeoxynucleotides.<sup>1,15</sup> It also displays decreased specificity when DNA oligomers form mismatched duplexes to untargeted mRNAs that are also substrates for RNase H.<sup>16,17</sup> The three-dimensional structure of the *E. coli* RNase H has been determined, and suggests a possible mechanism of substrate recognition and cleavage.<sup>18,19</sup> RNase H has been found to be a processive endonuclease. First, it hydrolyzes endonucleolytically at a ribonucleotide of the RNA:DNA duplex, then processively performs 3'-5' exonucleolytic cleavage.<sup>20–22</sup> Digestion with this enzyme produces 5'-phosphate and 3'-hydroxyl termini.<sup>23</sup> The complete digestion of poly(rA):poly(dT) with *E. coli* RNase H yields oligoribonucleotides with varying chain lengths, ranging from monomers to hexamers.<sup>21,24</sup> *E. coli* RNase H appears to require 4–6 base pairs of RNA:DNA

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hybrids as substrates to bind and cleave effectively.<sup>21,25</sup> In order to understand more about RNA hydrolysis by RNase H, it is necessary to develop a real-time, sensitive and non-isotope labeled method to study these enzymes. To assay the enzyme activities and evaluate the kinetic parameters, a number of traditional methods have been used, such as the acid soluble release of RNA fragment,<sup>26</sup> gel electrophoresis<sup>27</sup> and HPLC.<sup>28</sup> Acid soluble and gel electrophoresis techniques require radioisotope-labeled substrates, and micromolar concentration of substrate is needed for the HPLC method. All of these methods are indirect, time-consuming, and laborious. The assay we describe here is a real-time method with a fluorescence enhancement of up to 40-fold. The large fluorescence increase was achieved by taking advantage of the fluorescence quenching mechanism inherent to molecular beacons.

Molecular beacon is a term that has been given to single-stranded DNA that can form a stem-loop secondary structure with a fluorophore and quencher at either end.<sup>29</sup> When the stem-loop is in the closed conformation, fluorescence is quenched due to close proximity of the fluorophore and quencher. Hybridization to a complementary nucleotide sequence or hydrolysis of one or both of the stems causes an increase in fluorescence due to separation of the fluorophore and the quencher.<sup>29</sup> DNA molecular beacon assays have been described for single-stranded specific DNases, the restriction endonuclease *Bam*HI and small non-enzyme DNA cleavage agents.<sup>30–32</sup>

Here we describe the preparation of RNA/DNA chimeric molecular beacon, which contains a single-stranded RNA–DNA chimeric oligonucleotide labeled with a 5'-fluorescein as fluorophore and a 3'-DABCYL as quencher (Fig. 1). The fluorophore of the probe is held in proximity to the quencher by the stem-loop structure. When the RNA sequence of the RNA:DNA hybrid stem is cleaved, the fluorescence of the fluorophore is manifested. In order to find the optimal chimeric beacon, beacons with different stem size, sequence, and orientation were tested against *E. coli* RNase H. The loop was kept at 5 nucleotides because it is the most stable size.

## MATERIALS AND METHODS

### Materials

DNA phosphoramidites and DABCYL-CPG were obtained from Glen Research. The 2'-silyl protected RNA phosphoramidites were obtained from Annovis (Crucchem). Fluorescein phosphoramidite was

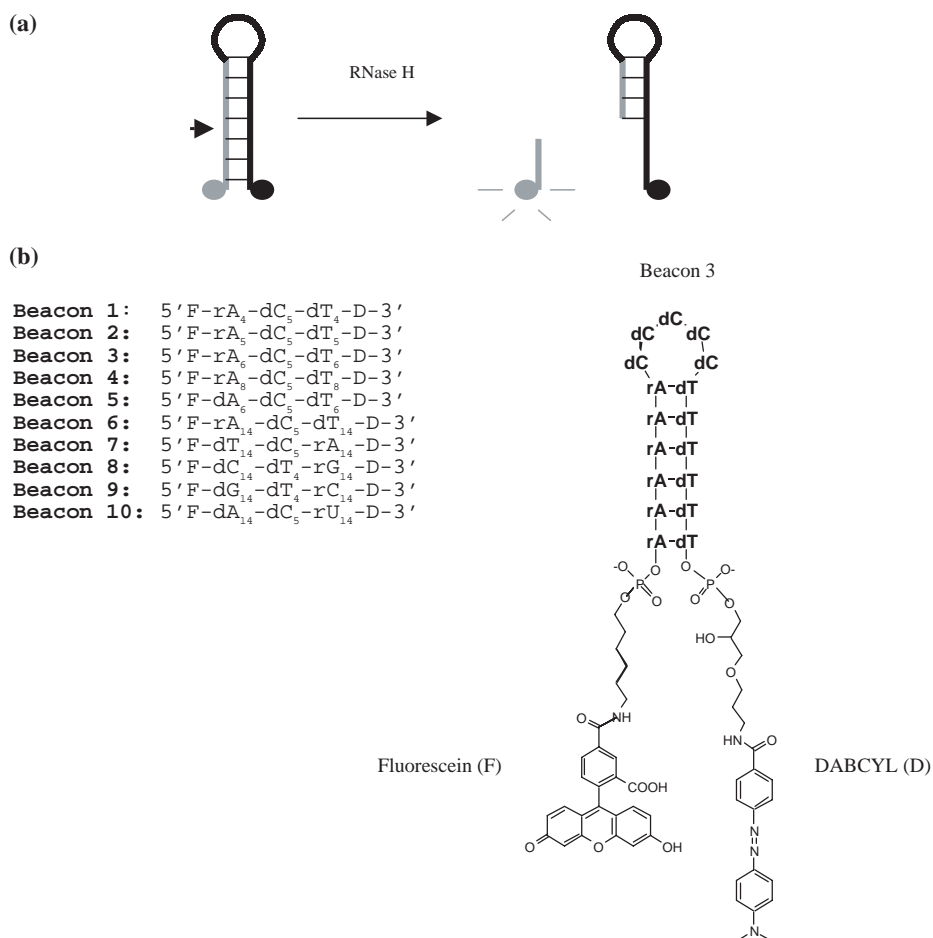
purchased from Biosearch Technologies, Inc. *E. coli* RNase H with enzyme activity of 5 units/ $\mu$ l was purchased from United States Biochemical (USB), where one unit of RNase H catalyzes the hydrolysis of 1 nmole of RNA in radiolabeled poly(rA):poly(dT) to acid-soluble material in 20 min at 37°C. The enzyme specific activity is 419,972.4 units per mg of *E. coli* RNase H.

### Synthesis of DNA/RNA chimeric molecular beacons

All of the molecular beacons (Fig. 1) were synthesized on an Expedite 8909 synthesizer with a modified program. The modified program combines the standard 1  $\mu$ mol DNA synthesis protocol and 1  $\mu$ mol RNA synthesis protocol from the manufacturer. Upper case letters (A, C, G, and T) were used for DNA cycles and lower case letters (a, c, g, and u) for the RNA cycles. By using this modified protocol, a chimeric DNA:RNA oligonucleotide can be synthesized without switching the protocol. The oligonucleotides were deprotected with concentrated ammonia/ethanol mixture (3:1) at 50°C for 18 h. The samples were then lyophilized to dryness. The 2'-silyl groups of the RNA nucleotides were removed by adding 250  $\mu$ l of anhydrous triethylammonium trihydrogen fluoride/N-methylpyrrolidinone/triethylamine (10:15:7.5).<sup>33</sup> The solution was heated at 65°C for 1.5 h with occasional stirring. The RNA/DNA chimeric molecular beacon was then precipitated by adding 50  $\mu$ l of 3 M sodium acetate and 1 ml of n-butanol, followed by centrifugation at 12,000 g for 20 min. The orange pellet was washed with ethanol and dried for 10 min in a Speed-Vac<sup>®</sup>. The beacons then were purified by HPLC (BioCAD Sprint with oligo R3 column, 4.6 $\times$ 150 mm). A 10–25% gradient of 0.1 M triethylamine acetate-acetonitrile was run over 35 min with 3 ml/min flow rate. The product peak was collected. After lyophilization, the beacon was precipitated using 3 M sodium acetate and ethanol. Gel capillary electrophoresis (Beckman MDQ) was used to check the purity of the beacons. All the molecular beacons were over 90% pure, with no evidence of fluorescein-only labeled oligonucleotide.

### Analytical instruments

Fluorescence measurements were carried out on Perkin Elmer MPF4 and LS-50B spectrofluorometers. MPF-4 is equipped with a digital data collecting system. The assays were either done at room temperature (23°C), or at 37°C using a jacketed



**Fig. 1.** Schematic drawings of the cleavage of chimeric molecular beacon by RNase H and chimeric beacon. (a) The stem-loop structure brings the fluorophore (●) and the quencher (●) in close proximity. In this conformation, the fluorescence is quenched. Upon hydrolysis of the RNA strand (—) of the RNA:DNA hybrid stem by RNase H, the fluorophore is liberated from the beacon, resulting in a large increase in fluorescence. (b) Sequences of the beacons are shown. Beacons have 5'-fluorescein (F) as fluorophore and 3'-DABCYL (D) as quencher. Chimeric molecular beacons consist of an RNA:DNA stem (4–14 bp) and a DNA-loop (4–5 nt). The RNA:DNA hybrid stem is the substrate of RNase H. Beacon 5 contains only DNA as a control. The structure of beacon 3 is shown in detail.

cuvette holder and a circulation pump. All samples were analyzed with excitation at 490 nm and emission at 520 nm.

### Chimeric molecular beacon assay for RNase H

Assays were carried out in 50  $\mu$ l of USB RNase H buffer (20 mM TRIS-HCl, pH 7.5, 20 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 0.1 mM DTT), containing 20 or 50 nM molecular beacon and various amounts of RNase H. An increase in fluorescence emission at 520 nm, upon excitation at 490 nm, indicates the progress of beacon hydrolysis. The maximum fluorescence emission was determined by incubating the beacons with excess RNase H or DNase I. For determination of the Michaelis-Menten kinetic parameters, the molecular beacon concentration was

varied from 25 to 500 nM, in the range of the previously reported  $K_m$  values for RNase H.<sup>19,21</sup> Initial rate measurements were obtained in the first 30 s, with an enzyme concentration of 3 nM (25 units/ml). All experiments were repeated 2–3 times. Data in Table 1 were obtained by performing curve fitting to the Michaelis-Menten equation using IGOR Pro version  $\pi$  (WaveMetrics, Inc., Lake Oswego, OR, USA).

## RESULTS AND DISCUSSION

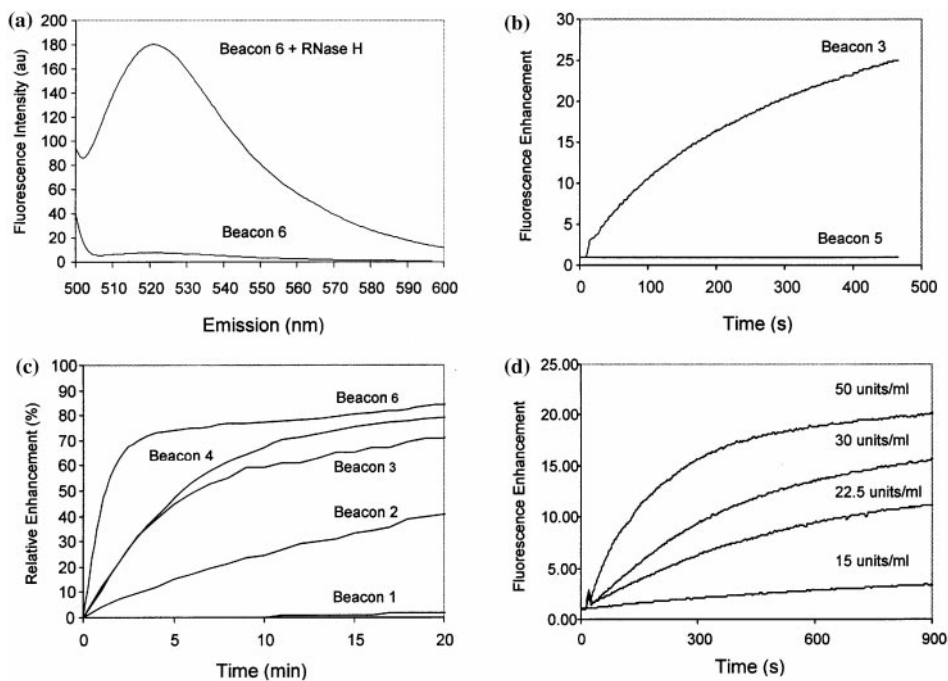
### Design and construction of chimeric molecular beacon

Fluorescence self-quenching efficiency of a molecular beacon depends on both the stem size

**Table 1.** The kinetic parameters of chimeric molecular beacons for *E. coli* RNase H

| Substrate | $K_m$ (nM)   | $k_{cat}$ ( $s^{-1}$ ) | $V_{max}$ ( $nM \cdot s^{-1}$ ) | $k_{cat}/K_m$<br>( $10^6 M^{-1} \cdot s^{-1}$ ) |
|-----------|--------------|------------------------|---------------------------------|---|
| Beacon 6  | $16 \pm 13$  | $0.6 \pm 0.4$          | $1.8 \pm 1.2$                   | $3.8 \pm 3.9$                                   |
| Beacon 7  | $80 \pm 60$  | $0.1 \pm 0.03$         | $0.4 \pm 0.1$                   | $2 \pm 1$                                       |
| Beacon 8  | $80 \pm 30$  | $0.05 \pm 0.01$        | $0.1 \pm 0.02$                  | $0.6 \pm 0.3$                                   |
| Beacon 9  | $60 \pm 30$  | $0.02 \pm 0.003$       | $0.06 \pm 0.01$                 | $0.3 \pm 0.2$                                   |
| Beacon 10 | $130 \pm 10$ | $0.03 \pm 0.001$       | $0.1 \pm 0.004$                 | $0.2 \pm 0.02$                                  |

Data were obtained in 20 nM TRIS-HCl buffer, pH 7.5, containing 20 nM KCl, 10 nM MgCl<sub>2</sub>, 0.1 nM EDTA, and 0.1 nM DTT at 37°C.



**Fig. 2.** Chimeric molecular beacon assay for *E. coli* RNase H. Cleavage by *E. coli* RNase H and DNase I were carried out in a buffer consisting of 20 nM Tris-HCl, pH 7.5, 20 nM KCl, 10 nM MgCl<sub>2</sub>, 0.1 nM EDTA, and 0.1 nM DTT. All time course assays used  $\lambda_{ex} = 490$  nm and  $\lambda_{em} = 520$  nm. (a) Fluorescence emission intensity of beacon 6 and its cleavage products as a function of wavelength upon excitation at 490 nm. [Beacon 6] = 20 nM; [RNase H] = 3 nM; 30 min at room temperature. (b) Specificity of chimeric molecular beacons for RNase H. Chimeric beacon 3 (50 nM) or control beacon 5 (50 nM) were hydrolyzed by 100 units/ml of *E. coli* RNase H at 37°C. 100% cleavage of the beacons was achieved by adding 400 units/ml of DNase I. (c) The effects of chimeric beacon stem length on *E. coli* RNase H activity. 50 nM of chimeric beacon 1, 2, 3, 4 or 6 with 400 units/ml of *E. coli* RNase H at 37°C. 100% cleavage of the beacons was achieved by adding 400 units/ml of DNase I. (d) Time curves of cleavage of chimeric beacon 6 by *E. coli* RNase H at different enzyme concentrations at 37°C. Reaction conditions were: [Beacon 6] = 50 nM; [RNase H] = 15 units/ml, 22.5 units/ml, 30 units/ml, or 50 units/ml; 1 unit of *E. coli* RNase H =  $1.35 \times 10^{-3}$  mol.

and the distance between the fluorophore and quencher. We prepared a set of chimeric molecular beacons containing 4–14 bp RNA:DNA stem with sequence: poly(rA):poly(dT), poly(rC):poly(dG), poly(rG):poly(dC), and poly(rU):poly(dA). The loop of these probes consisted of a dT<sub>4</sub> or dC<sub>5</sub> loop to ensure nonhybridizing interactions with the stem sequence. The substrate sequences adopted a stable stem-loop structure at 37°C and 1 M Na<sup>+</sup> using the DNA folding program, MFOLD (Michael Zuker, Rensselaer Polytechnic Institute,

<http://bioinfo.math.rpi.edu/~mfold>). The 5'-fluorophore of the beacons was fluorescein, whereas the 3'-quencher was DABCYL (Fig. 1). In a typical molecular beacon, the quenching efficiency of this pair is about 99.9%.<sup>34</sup> The chimeric beacons were synthesized with phosphoramidite chemistry using a modified protocol in one step. The purified beacons had a fluorescence signal enhancement of up to 40-fold after the beacons were completely hydrolyzed by nuclease (see Fig. 2a).



the maximal velocity of the reaction. This could be due to the loop and/or end modification (i.e. fluorescein or DABCYL) interfering in the process of RNase H binding to the beacon. The results indicate that *E. coli* RNase H slightly prefers the beacon with rA<sub>14</sub> at the 5'-end rather than at the 3'-end.

## CONCLUSIONS

An assay for RNase H activity has been developed using chimeric beacons. The chimeric molecular beacon assay for RNase H is highly sensitive with fluorescence enhancement of up to 40-fold. This method permits real time detection of RNase H activity and makes it easy to study the enzymatic kinetics. *E. coli* RNase H cleaves a chimeric beacon containing a poly-rA sequence in the 5' arm with the highest efficiency. We found that *E. coli* RNase H enzyme activity is sequence dependent. The sequence preference of RNase H for beacons with ribonucleotides in the 3' end is: rA<sub>14</sub> ≈ rG<sub>14</sub> > rU<sub>14</sub> ≈ rC<sub>14</sub>. These molecular beacons will be used to probe the cellular level of RNase H.

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