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Enzymatic Amplification of DNA/RNA Hybrid Molecular Beacon Signaling in Nucleic Acid Detection

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Abstract

A rapid assay operable under isothermal or non-isothermal conditions is described wherein the sensitivity of a typical molecular beacon (MB) system is improved by utilizing thermostable RNase H to enzymatically cleave an MB comprised of a DNA stem and RNA loop (R/D-MB). Upon hybridization of the R/D-MB to target DNA, there was a modest increase in fluorescence intensity (~5.7x above background) due to an opening of the probe and concomitant reduction in the Förster resonance energy transfer efficiency. Addition of thermostable RNase H resulted in the cleavage of the RNA loop which eliminated energy transfer. The cleavage step also released bound target DNA, enabling it to bind to another R/D-MB probe and rendering the approach a cyclic amplification scheme. Full processing of R/D-MBs maximized the fluorescence signal to the fullest extent possible (12.9x above background), resulting in a ~2–2.8 fold increase in the signal-to-noise ratio observed isothermally at 50 °C following the addition of RNase H. The probe was also used to monitor real-time PCR reactions by measuring enhancement of donor fluorescence upon R/D-MB binding to amplified pUC19 template dilutions. Hence, the R/D-MB-RNase H scheme can be applied to a broad range of nucleic acid amplification methods.

Keywords

isothermal; non-isothermal; molecular beacon; thermostable RNase H; nucleic acids; cyclic amplification; Förster resonance energy transfer (FRET); real-time PCR

INTRODUCTION

Since their advent in 1996 [1–2], molecular beacons (MB) have become a dominant method of biomolecular recognition [3] due to their high selectivity, sensitivity, and stability. MB's are used to quantitatively detect DNA and RNA through the use of Förster Resonance Energy Transfer (FRET) [4], a distance-dependent nonradiative energy transfer phenomenon that can occur between a “donor” and “acceptor” fluorophore that exhibit spectral overlap. FRET is typically employed in the MB design by taking a stem-loop DNA oligomer and attaching a fluorescent donor and a nonfluorescent acceptor (the “quencher”) to the 3' and

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5' ends, respectively. The resulting tight proximity between the FRET pair inherent in the stem configuration produces a highly efficient FRET and concomitantly low donor fluorescence background (F_{close}) in the absence of probe targets [5]. When target oligomers recognize and bind to the complementary loop region of the MB to form a double strand, the stem is forced apart which parts the fluorophore from the quencher and leads to a reduction in FRET and concomitant increase in donor fluorescence intensity (F_{open}) [5]. Using this mechanism, MB's are able to have a high signal-to-noise (S:N) ratio when applied in immobilized platforms and especially solution based assays. Design improvements leading to higher S:N ratios have led MBs to surpass their originally envisioned application in monitoring PCR reactions [1–2]. As detailed by Wang *et al.* [5], they are now used in protein assays, enzyme monitoring, cellular mRNA detection, biosensors, molecular computing, and as aptamers.

Even though the MB's particular use of FRET has produced S:N ratios that are superior to what is seen from alternatives, the sensitivity of conventional MBs is nonetheless inadequate for detecting oligomeric targets found in low abundance. This limits their use to applications involving highly abundant or stimulated gene products [5]; it is thus desirable to improve their sensitivity as dictated by the S:N ratio calculated from [6]:

$$\frac{F_{\text{open}} - F_{\text{buffer}}}{F_{\text{close}} - F_{\text{buffer}}} \quad (1)$$

where F_{open} is the donor fluorescence when MB is bound to its target, F_{close} is the donor fluorescence in the absence of target, and F_{buffer} is the background fluorescence in the absence of probe. It is seen from Eqn. 1 that the S:N ratio of a particular MB design may be attenuated by: 1) incomplete initial quenching in the absence of targets, which increases F_{close} ; 2) incomplete abolishment of FRET once a target is bound, which decreases F_{open} ; and 3) a lack of the type of processive cycling seen in the Catacleave probe [7–8] or the SNP assay of the Liu lab [34] which would enable one target molecule to react with multiple MBs and reduce the concentration of targets needed to achieve a particular F_{open} . Most attempts at improving the S:N ratio have focused on improving the MB design to either reduce F_{close} by enhancing the initial quenching efficiency or increase F_{open} by enhancing the performance of the donor [5]. It is evident from the literature that targeting F_{close} has proven more successful to date based on fold enhancements of the S:N ratio.

The quenching efficiency seen in MB designs has been improved from a basal range of 85–97% [5] to higher values using a variety of design enhancements. Gold surfaces [9–10] and gold nanoparticles [11–12] have been employed as quenchers paired with fluorescent dyes like Rhodamine 6G and Fluorescein (FAM) [11]. Specifically, the average quenching efficiency for a Gold/Rhodamine 6G and Gold/Fluorescein pairing was found to be 99.5% and 98.7% with corresponding S:N ratios of 182 and 76, respectively [11]. Superquenching has also proven effective at reducing F_{close} and has involved the use of a multiple-quencher assembly paired with one fluorophore. A seminal study by Yang *et al.* showed that using a three quencher assembly instead of a single quencher significantly increased the S:N ratio of their MB design from 14 to 320 [13]. Besides the improvement of quenching efficiency, another strategy that has proven successful at reducing F_{close} is the use of a chemiluminescent detection approach with a greatly reduced background signal [14–15]. Chemiluminescent MBs have been able to achieve lower detection limits in the femto to attomolar range, which is ~4 orders of magnitude more sensitive than typical fluorescent MB probes [14]. Thus focusing design modifications on improving F_{close} has led to several orders of magnitude improvement in the MB S:N ratio.

A strategy along different lines to improving the S:N ratio of the MB has been the enhancement of F_{open} by increasing the maximal fluorescence intensity of a given fluorophore using techniques such as multiple fluorophore based systems [16–19], wavelength-shifting MBs [20], conjugated polymers [21], and quantum dot MB systems [22]. In particular, quantum dots and conjugated polymers have been shown to produce a much higher fluorescence intensity than that of a single small molecule organic fluorophore. The maximal fluorescence intensity of a single quantum dot can have the same magnitude as the combined emission from 20 individual organic fluorophores [22]. Due to their high quantum efficiency, conjugated polymers, such as the water-soluble polyelectrolyte Poly (phenylene ethylene) (PPE), have also been shown to be 6 times brighter than conventional organic dyes such as Cy3, Fluorescein, tetramethylrhodamine, and Alexa Fluor 488 [5]. Under the experimental conditions tested by Tan *et al.*, a single PPE chain was shown to be 20 and 6 times brighter than a Cy3 and Alexa Fluor 488, respectively [5]. However, it is evident that attempts to improve F_{open} by employing enhanced donors have only increased the S:N by one order of magnitude.

Another F_{open} -oriented improvement that could be made to general MB design is to ensure that once the MB has recognized and bound to its target, FRET is abolished by maximizing the distance between the fluorophore and quencher. According to Förster theory, FRET efficiency is inversely proportional to the sixth power of the distance between the centers of the fluorophore and quencher [23]. A sufficient increase in distance would drive the FRET efficiency to zero and ensure the maximum possible donor emission regardless of the fluorophore chosen. Current MB technologies can only separate the fluorophore/quencher pairing by a distance dictated by the number of bases in the complimentary loop region. If a bound loop could be selectively digested while leaving the target intact, this would not only maximize separation of the donor from the quencher, but also liberate the previously bound target and allow it to bind to another free probe. This would create an amplification cycle wherein each target single-stranded DNA (ssDNA) participates in multiple cleavage events until the entire MB population is processed [27]. A generalized implementation of this approach could be to render the entire loop of a MB as a ribonucleotide sequence complementary to target and use commercially available Thermostable RNase HI to selectively digest the loop.

Technically, previous studies performed by the Liu lab [24–25,34] have employed CpRNase HII [24–25] and TthRNase HII [34] to selectively nick MBs containing a single ribonucleotide in their the loop sequence (DNA-rN1-DNA MBs) when bound to target DNA with a perfectly matched sequence specifically nicked the MB loop portion of the target•MB helix, allowing the liberated target to bind to another free and unprocessed probe. This amounted to an ingenious assay for detecting single-nucleotide polymorphisms (SNPs) that takes advantage of CPT to amplify the signal when an SNP is present. Though the Liu lab cast the technique as originally envisioned with CpRNase HII as amenable to general oligonucleotide detection [24], there are some limitations with the approach that make it less effective for this purpose. The CpRNase HII based assay must be run isothermally at mesophilic temperatures and cannot be combined with approaches requiring thermal cycling, such as PCR or double-stranded DNA detection. Later, the addition of TthRNase HII [34] to the method made it compatible with thermal cycling, but the assay was not readily compatible with PCR due to enzyme-mediated inhibition of amplification, which is a serious drawback. Furthermore, the technique requires expression and purification of nickases that are not commercially available. Also, multiple studies have shown that the binding efficiency and kinetics that control RNase H mediated cleavage is better with four or more ribonucleotide bases as opposed to three or less [28–29].

We rationalized that a more generalized approach to marrying MBs and CPT for trace oligonucleotide detection would be to rely on the faster kinetics produced by the combination of a full RNA loop perfectly matched to target and Thermostable RNase HI, which is commercially available and requires at least 4 consecutive ribonucleotides for binding and digestion [8]. Thus the technique would require relying completely on stable DNA/RNA hybridization for target recognition and concomitant melting of the stem, which to our knowledge has never been done. Thus in this study, we developed and tested a generalized design for a hybrid MB compatible with CPT and PCR that contains a DNA stem and a long 21-bp RNA loop that is processed with Hybridase™ Thermostable RNase HI in nuclease free buffer. We showed through a melting curve analysis that the ssDNA target was recognized by and annealed to the R/D-MB loop to form a double-stranded helix in which the stem was broken apart and an initial fluorescence increase was obtained ($F_{\text{open-1}}$). After adding thermostable RNase HI enzyme, the RNA loop strand of the R/D-MB•target complex was specifically cleaved, producing an enhanced donor fluorescence signal ($F_{\text{open-2}}$) and leaving behind an intact, unbound ssDNA target as expected [26–27]. Testing a constant probe concentration against varying concentrations of ssDNA target in the presence of RNase H demonstrated accumulation of cleaved R/D-MB probes until a maximum F_{open} was achieved. Finally, application of the R/D-MB probe in monitoring a PCR was demonstrated. The combined experiments show that complete reliance on RNA to DNA hybridization works very well in MBs, producing a broadly applicable scheme that has superior S:N ratios and kinetics when combined with thermostable RNase HI.

MATERIALS AND METHODS

Materials

Hybridase™ thermostable RNase H was from Epicentre Technologies and RNase H reaction buffer (75 mM KCl, 50 mM Tris-HCl @ pH 8.3, 3 mM MgCl₂, and 10 mM dithiothreitol (DTT)) was from New England Biolabs. All PCR related agents were purchased from Invitrogen, including Native Taq DNA polymerase, 10 mM dNTP mixture, 10 μM forward (5′-TGTGGAATTGTGAGCGGATAAC-3′) and reverse (5′-CCTCTTCGCTATTACGCCAG-3′) primers, and a 16 reaction GeneTailor™ Site-Directed Mutagenesis System. Plasmid purification was done using the Qiagen Plasmid Mini Purification Kit. Stock (100 μM) ssDNA target and R/D-MB solutions were synthesized by Sigma and Biosearch Technologies, respectively.

R/D-MB Probe Design

The R/D-MB probe used in this study was designed using the software Beacon Designer from Premier Biosoft International (<http://www.premierbiosoft.com>). The sequences of the R/D-MB and ssDNA target were designed as followed:

Molecular Beacon Probe: FAM-5′-CTCGCG-CCGCAGAUUAUAGUGGACGCC-
CGCGAG-3′ - BHQ1

Target ssDNA Sequence: AAAAA-GGCGTCGACTATAATCTGCGG-AAAAA

The DNA stem region of the R/D-MB probe is underlined and the RNA loop region is italicized. Attached at the 5′ end of the DNA stem is the donor fluorophore, Fluorescein, while attached at the 3′ end is a Black Hole Quencher 1. The complimentary ssDNA target sequence is shown in plain typeface and the two end-flanking regions are italicized. A melting temperature (T_m) of 72 °C was predicted for the design, as well as an annealing temperature (T_a) of 64 °C for half-maximal annealing between the RNA loop region and ssDNA target. A StepOne™ Real-Time PCR system was set to monitor and record fluorescence as a function of temperature using ramps from 25 to 95 °C at 0.3 degrees per second.

Fluorescence Measurements

All fluorescence measurements involving melting curve analysis, target recognition and binding, RNase H cleavage, and monitoring of real-time PCR reactions were performed using a StepOne™ Real-Time PCR system from Applied Biosystems. All real-time fluorescence data was monitored and recorded using StepOne Software v2. Recorded fluorescence intensities were means calculated by averaging fluorescence intensity values from three replicates per sample type.

RNase H Cleavage Reactions and Quantifying the S:N Ratio

RNase H cleavage reactions were carried out in a 1x RNase H reaction buffer with 50 nM of R/D-MB probe, 0.1–200 nM of ssDNA target, and 2.5 U of thermostable RNase HI in a total reaction volume of 20 µL. R/D-MB probe and target ssDNA were first incubated at 95 °C for 2 min, followed by the addition of RNase H and monitoring of fluorescence intensity over a 100 min timeframe at a set reaction temperature using the StepOne™ Real-Time PCR system. Fluorescence excitation light was passed through an optical filter suitable for FAM.

To determine the sensitivity of the assay at a set reaction temperature, samples were carefully prepared that could be used to quantify the S:N ratio as given in Equation 1. For each experimental run, a control sample was made containing only 1x RNase H buffer made up in nuclease free water (Bioexpress) and used to measure F_{buffer} . F_{close} was measured from samples containing only R/D-MB probe in an RNase H buffer and nuclease free water. F_{open} was measured by creating two different kinds of samples, each in 1x RNase H buffer: R/D-MB probe + target ssDNA and R/D-MB probe + target ssDNA + RNase H. Two different sets of S:N ratios were calculated, one representing $F_{\text{open-1}}$ (R/D-MB + target ssDNA), and the other $F_{\text{open-2}}$ (R/D-MB + target ssDNA + RNase H) as shown in Fig. 1.

R/D-MB/RNase H Concentration Dependence Experiments

Different concentrations of ssDNA target were incubated with 50 nM R/D-MB probe in the presence or absence 2.5 U of thermostable RNase HI in 1x RNase H reaction buffer made up in nuclease free water for a total sample volume of 20 µL. The 50 nM R/D-MB probe concentration was selected after running several preliminary experiments to determine the lowest probe concentration that could be used to achieve a strong fluorescence signal with a constant ssDNA target concentration (data not shown). S:N ratios at each target ssDNA concentration tested was calculated according to Eqn. 1 by comparing the fluorescence intensities of samples containing either R/D-MB + non-target ssDNA, R/D-MB + target ssDNA, and R/D-MB + target ssDNA + RNase H. All fluorescence measurements were recorded using the 48 well-plate StepOne™ Real-Time PCR system over a 100 min timeframe.

Real-Time PCR Reactions

Prior to running any PCR reactions, site-directed mutagenesis was used (*via* the GeneTailor™ Site-Directed Mutagenesis kit) to insert the ssDNA target sequence complimentary to the RNA loop of the R/D-MB into a pUC19 plasmid. After the sequence insertion, the plasmid was transformed into DH5α™-T1^R *E. coli* competent cells, and plasmid purification was carried out using a Qiagen Plasmid Mini Purification Kit. A 1% agarose gel was run to show that the target sequence (239 bp) was indeed inserted into the plasmid (data not shown).

Real-time PCR reactions were carried out in 1x RNase H buffer using 2.5 U of Native Taq DNA polymerase, 200 nM R/D-MB probe, 250 nM of dNTP mixture, 200 nM of ssDNA primer (reverse and forward), 2.5 U of thermostable RNase H, and 1 µL of diluted pUC19 template in a total reaction volume of 50 µL. The PCR protocol followed consisted of 1

cycle of 95 °C for 3 min, followed by a 40-cycle amplification scheme wherein each cycle consisted of a 95 °C incubation step for 30 sec, followed by a 50 °C step for 45 s, and a 72 °C step for 30 s. The protocol was completed with a final extension step at 72 °C for 8 min. Real-time data was collected during the 50 °C annealing stage. Triplicates were run for each serial dilution of template and a “no template” control was used to test for the presence of contamination.

Results

Thermal Denaturation Profiles

To verify the integrity of the R/D-MB probe design, the melting temperature of the R/D-MB probe stem and the temperature for half-maximal annealing between the RNA loop region of the R/D-MB and the complimentary ssDNA target sequence were measured and compared with predicted values. Melting curves were determined by measuring the fluorescence of the 200 nM R/D-MB as a function of temperature in the absence and presence of 200 nM ssDNA target and are shown in Fig. 2. Comparison between the thermal denaturation profiles of the R/D-MB + target or R/D-MB probe and the values predicted for T_a or T_m , respectively, confirmed that our design produced a properly functioning R/D-MB having a T_a of approximately 64 °C and T_m of 72 °C.

Isothermal Temperature Dependence of R/D-MB Performance

Having established that the R/D-MB probe showed the expected thermally dependent behavior, the next experimental step in testing the proposed scheme (Fig. 1) was to confirm the enhancements of F_{open} expected from nuclease-mediated loop cleavage and cyclic amplification. HybridaseTM thermostable RNase HI was chosen for this purpose as it is able to digest the RNA portion of a RNA-DNA hybrid, without affecting ssDNA or unhybridized RNA, over a broad temperature range that extends as high as 95 °C. Furthermore, its optimal activity is 3-fold higher than that of *E. coli* RNase H. Though thermostable RNase HI is optimally active above 65°C, since the T_m and T_a of the R/D-MB probe is 72 °C and 64 °C, respectively, reaction temperatures lower than T_a were chosen for isothermal characterization. Thus to determine the optimum reaction temperature which would maximize the S:N ratio as stated in Eqn. 1, isothermal RNase H cleavage reactions were carried out at 45, 50, 55, and 60 °C. No temperatures below 45 °C were tested, as using temperatures > 45 °C ensured the prevention of non-specific binding.

Shown in Fig. 3A are the results from a representative set of isothermal experiments from which we determined the RNase-H-dependent enhancements of the S:N ratio seen at a specific temperature (in this case, 50 °C). The fluorescence background (F_{close}) was first measured from samples containing R/D-MB only or R/D-MB plus non-target ssDNA, both of which gave fluorescence intensities ~7× higher than control samples of buffer alone. In the presence of ssDNA target, donor fluorescence intensity increased by another ~5.7× to a level representing F_{open-1} . When thermostable RNase H was added to a R/D-MB + target ssDNA sample, an additional ~2.2× increase in fluorescence intensity (or a ~12.9× increase over the intensity of samples containing R/D-MB + non-target) was observed that corresponds to F_{open-2} . The fact that the trace for the test sample containing R/D-MB probe + target + RNase H reached saturation suggests that all of the R/D-MB probes were processed by the cyclical binding of ssDNA target and RNA-loop cleavage by RNase H. Hence at 50 °C with the concentrations tested, cleavage of all R/D-MB probes was nearly complete within 10 min after the reaction had started.

Final fluorescence intensities were determined according to this scheme at the isothermal reaction temperatures of 45, 50, 55, and 60 °C, and then these intensities were used to

calculate the ratios of S:N according to Eqn. 1 (Fig. 3B). Two ratios were computed, one for $F_{\text{open-1}}$ and the other for $F_{\text{open-2}}$. Over the temperatures tested, the highest S:N ratios were obtained at 45°C (S:N = 19.9 and 9.7 for $F_{\text{open-2}}$ and $F_{\text{open-1}}$, respectively), followed by 50°C and 55°C (S:N = ~14–15 and ~6.4), and lastly 60 °C (S:N = 7.9 and 3.5). The reason for the higher S:N ratios at 45°C compared to the next highest temperature at 50 °C (~1.4× difference) was that at lower temperatures and in the absence of ssDNA targets, there is a higher probability that an individual R/D-MB within the whole population of probes will be found with an annealed stem (see red trace in Fig. 2). This would result in a higher overall quenching efficiency averaged over the probe population and thus a lower F_{close} . Comparing the S:N ratios based on $F_{\text{open-2}}$ with the S:N ratios based on $F_{\text{open-1}}$, it was evident that the addition of RNase H increased the overall S:N ratio of the assay by ~2x at each temperature setting. Based on these results, the optimal isothermal temperature setting for our R/D-MB + RNase H reaction system is likely 45 °C due to the lower F_{close} value in comparison to the other settings. However, subsequent experiments were conducted at 50 °C because it was expected that the higher temperature would be more effective when applying the R/D-MB in PCR applications. Nevertheless, these results demonstrated our rationale that the combination of the full RNA loop of the R/D-MB with thermostable RNase HI cleavage reactions could provide an F_{open} oriented enhancement to the S:N ratio of a conventional MB system, and that this enhancement is applicable over a broad temperature range and obtainable over a useful time scale.

ssDNA Target Concentration Dependence of R/D-MB Performance

Having characterized the temperature dependence of the R/D-MB at one set of concentrations, it was important to examine the dependence of probe performance on the concentration of target ssDNA. Representative traces of donor fluorescence intensity versus time (min) are plotted in Fig. 4A. Fig. 4B shows the corresponding S:N ratios based on $F_{\text{open-1}}$ (determined from samples with R/D-MB + target) or $F_{\text{open-2}}$ (determined from samples with R/D-MB + target + RNase H) at a 100 min incubation time. Both Figs. 4A and 4B illustrate the effects of the relative concentration of target ssDNA on probe performance. At target ssDNA concentrations of 20 nM and greater, all R/D-MB probes were mostly processed by RNase H enzymatic activity within a 40 min timeframe as indicated by the fluorescence amplitude reaching saturation at ~30,000 arbitrary units. When the target ssDNA was approximately equimolar or in excess to the probe (i.e. 40 nM) in samples containing RNase H, saturation was reached within 20 min. The 200 nM target ssDNA condition reached saturation within less than 1 min, suggesting that the kinetics of RNase H binding and cleavage are very rapid at the 50 nM substrate level. Thus at lower concentrations of ssDNA target, the rate of the cleavage reaction may be limited by the target-to-probe binding equilibrium and lower effective substrate (i.e. target•probe) concentrations. However, even at a concentration 500× more dilute than that of R/D-MD, a detectable change in fluorescence intensity was observed for 0.1 nM ssDNA target in the presence of RNase H. It was noted that signals from the lowest concentrations of ssDNA target tested (i.e. 0.1 nM and 1 nM) in the presence of RNase H did not reach saturation within the 100 min timeframe, but it was evident that saturation would have been reached given a longer observation time.

Depicted in Fig. 4B is the [target ssDNA] dependence after a 100 min sample incubation time of RNase H mediated enhancement of the S:N ratio. Note that the x-axis was deliberately left unscaled because [probe•target], the true substrate of RNase H, was not determined. Enhancement was quantified by comparing the S:N ratio based on $F_{\text{open-1}}$ (from samples incubated without RNase H) with the S:N ratio based on $F_{\text{open-2}}$ (from samples matched in concentration of ssDNA target and containing RNase H). Though the concentration of the true substrate of RNase H (i.e. probe•target) was not determined, the

S:N ratio based on $F_{\text{open-2}}$ increased in relation to increasing target ssDNA concentration until 5 nM ssDNA target was reached. At data points ≥ 5 nM the S:N ratio based on $F_{\text{open-2}}$ saturated at ~ 20 , suggesting that this was the maximum ratio possible at 100 min of reaction time due to the cleavage-induced abolishment of FRET within every probe in the sample population. Similarly, the S:N ratio based on $F_{\text{open-1}}$ steadily increased with higher target ssDNA concentration. One can see that the increase in $F_{\text{open-1}}$ started to decrease relative to the increase in [ssDNA] starting at the 100 nM data point. An RNase H mediated enhancement of the S:N ratio by $\sim 2\times$ of was observed when [target ssDNA] ≥ 20 nM or when the molar ratio of target to probe was less than 2:5. This is due to the fact that as more R/D-MB was bound by ssDNA target, the more $F_{\text{open-1}}$ approached the $F_{\text{open-2}}$ determined at the same target ssDNA concentration. This made sense, as the relatively longer 21-bp RNA loop should facilitate good separation between donor fluorophore and quencher when the RNA loop of a R/D-MB is hybridized with a ssDNA target. Nevertheless, the fact that the saturation point associated with $F_{\text{open-2}}$ was $\sim 1.2\times$ the highest point associated with the maximum $F_{\text{open-1}}$ suggests that the signal is appreciably enhanced by the complete abolishment of FRET. Thus RNase-H-mediated enhancement of S:N is expected to improve the detection of target ssDNA, and especially at concentrations much lower ($\sim 10^{-1}\times$) than that of the R/D-MB due to cyclic processing.

Since the prior conclusions about S:N ratios were drawn from data tabulated at 100 minutes; this begs the question of whether the trends observed would still hold true at shorter incubation times. Fig. 4C provides the answer by tabulating S:N ratios as a function of [target ssDNA] based on $F_{\text{open-2}}$ at incubation times of 10, 20, 30, 45, and 60 minutes. For comparison, S:N ratios based on $F_{\text{open-1}}$ were tabulated at 10 and 60 min. The data is depicted as a series of plots for clarity. As in Fig. 4B, the x-axis was deliberately left unscaled because [probe•target] was not determined. It can be seen that at 10 min, RNase H mediated cleavage of R/D-MBs enhanced the S:N ratio at [target ssDNA] ≥ 1 nM, but at [target ssDNA] < 1 nM actually reduced the S:N ratio. This was likely due to the fact that at [target ssDNA] = 0.1 nM the concentration of probe•target must logically be ≤ 0.1 nM, resulting in a slow rate of cleavage. However, at 20 min the S:N ratio was enhanced at every [target ssDNA] tested, and at 30 min enhancement was generally even greater. For lower target ssDNA concentrations (i.e. 1 and 5 nM), RNase H mediated enhancement was as high as $\sim 2.8\times$. It is also seen that as incubation times increase going from 10 min and up to 60 min, the S:N decreases with [target ssDNA] ≥ 20 nM. This can be explained with the slight increase in fluorescence signal associated with the R/D-MB only sample (F_{close}) (Fig 4A) with time. This was also observed with the R/D-MB + non-target sample. This slight increase in F_{close} with time would increase the denominator for the S:N calculation as shown in Eqn. 1, and thus would decrease the S:N with time. This is also why S:N ratios as high as ~ 30 are observed at shorter incubation times, vs. the maximum of ~ 20 seen at 100 min in Fig. 4B. The phenomenon was likely due to the choice of 50 °C [5] as an assay temperature, as our traces at 45 °C did not show any increase in fluorescence with time (data not shown). Overall, the F_{open} -oriented enhancements to the S:N ratio offered by the R/D-MB + RNase H design approach requires a trade-off between better signal and longer incubation times, especially at lower concentrations of target ssDNA.

Since it was evident that this trade-off stems from the rate of the concentration dependence of the rate of the RNase H cleavage reaction, mono-exponentials were fit to our data on the [target ssDNA] dependence of R/D-MB fluorescence intensity in the presence of RNase H. Nonlinear regression of the monoexponentials to the data produced excellent fits (R^2 0.997), implying that even though [probe•target] was unknown, [probe•target] increased as [target ssDNA] was increased, as would be expected. Fig. 4D shows approximated first-order rate constants at the target ssDNA concentrations tested, and it is seen that the rate constants increased as the target ssDNA concentration increased. Unfortunately, it was not

possible to fit the data sets to the Michaelis Menten model because [probe•target] was unknown. Future experiments could be conducted to determine the equilibrium concentration of probe•target based on protocols from Behlke *et al.* [30–31], and Yang *et al.* [32]. However, the data presented in Fig. 4D is still useful as a “standard curve” for predicting the original concentration of target ssDNA based on the rate of fluorescence increase. Overall, our experiments characterizing the ssDNA target concentration dependence of R/D-MB + RNase H performance further demonstrated our rationale that the full RNA loop approach offers a broadly applicable F_{open} -oriented enhancement of S:N ratio.

Real-Time PCR Experiments

It was important to test the R/D-MB in the context of a PCR reaction, since real-time PCR has been accepted as the standard method for nucleic acid detection and quantification [33]. Presently, sequence-specific probes used in real-time PCR applications include Taqman, MBs, and scorpion primers. Of these nucleic acid detection probes, real-time PCR studies done by Wang *et al.* [33] have shown the MBs produced the highest S:N ratios and are the most sensitive out of the common nucleic acid probes used. However, one serious drawback of MBs during PCR is that only one MB probe can hybridize to a single amplified DNA sequence during a given PCR cycle. This limits the sensitivity of the scheme for nucleic acid detection because the progressive accumulation of fluorescence signal is completely dependent on amplification of target ssDNA [8]; but in a CPT (cyclic probe technology) approach, the cyclic nature of target binding, probe cleavage, and target release facilitates the accumulation of fluorescence intensity based on the activity of RNase H. Thus we rationalized that the R/D-MB approach could be applied as an application of CPT in PCR.

To test this rationale and establish the performance of the R/D-MB + thermostable RNase HI system during a typical PCR, we performed real-time PCR experiments with a pUC19 plasmid containing a 239-bp insert that served as the DNA template in the presence of 200 nM R/D-MB and 2.5 U thermostable RNase H in a 50 μ L PCR buffer solution. Since PCR is not an isothermal process, it is necessary to introduce a detection phase within the amplification cycle. This was done by including a 45 sec R/D-MB probe cleavage step at 50°C [8]. This step serves as a precaution to allow the RNA loop of the R/D-MB to hybridize with its ssDNA target, while also giving appropriate time for RNase H to cleave its substrate consisting of probe•target. Using an initial template concentration of 155 pM, serial dilutions were made ranging from a dilution factor of 10 $\times$ to 10⁵ $\times$ more dilute. The results plotted in Fig. 5A shows the normalized fluorescence intensities based on RNase H mediated cleavage of the different concentrations of diluted template DNA that were tested versus PCR cycle number. The threshold cycle C_t , which is defined as the PCR cycle at which the initial rise in exponential fluorescence begins for a given template dilution, was identified as the point when the level of fluorescence started to rise above the normalized value of 0.3. C_t values for each template dilution were determined accordingly and plotted against the log of the template dilution as shown in Fig. 5B. The expected linear relationship between template dilution and threshold cycle was obtained, and from this plot it is a simple matter to determine the initial concentration of an unknown sample once the C_t value is known. Thus the R/D-MB + RNase H scheme performs robustly over the thermal cycling approach that PCR represents, and thus could likely be applied to other assays based on thermal cycling, such as the detection of double stranded DNA.

DISCUSSION

From the standpoint of Eqn. 1, our study has introduced a novel F_{open} oriented approach to enhancing the S:N ratio of a conventional MB system by including a full RNA loop in the MB design that is complimentary to a desired target ssDNA sequence and is intended to be

cleaved by Hybridase™ thermostable RNase HI. This combines the inherently low F_{close} of an MB, due to tight localization between the fluorophore and quencher, with all the advantages of CPT to surpass some of the F_{open} oriented weaknesses affecting conventional MB systems. It was demonstrated in this study that complete reliance on RNA-DNA hybridization chemistry worked in well in the MB scheme (Fig. 2) at greater than mesophilic temperatures (Fig. 3). With adequate incubation time, fluorophore-quencher FRET could be completely abolished (Fig. 4B) and all the R/D-MB probes could be processed (Figs. 4B, 4C). The accumulation of fluorescence signal in the system presented was thus a direct result of multiple cycles of R/D-MB hybridization to ssDNA target followed by thermostable RNase HI mediated cleavage of the RNA loop of the bound probe. As with any CPT assay, this method allows just a single ssDNA target to bind to multiple R/D-MBs until all the R/D-MBs are cleaved by RNase H. Most significantly, this F_{open} oriented approach to improving the sensitivity of a conventional MB offers amplification that is fully compatible with PCR, and is additional to and should be compatible with many of the other previously reported F_{open} - and F_{close} -oriented approaches to enhancing conventional MB sensitivity.

In comparison to one of its closest relatives found in the literature, the CataCleave probe [7–8], the R/D-MB + thermostable RNase H system offers several advantages due to its nature as a MB. For example, hybridization of the CataCleave probe to its target sequence does not lead to any change in donor fluorescence emission, meaning that detection requires that RNase H mediated cleavage is applied. As demonstrated in Fig. 4, the R/D-MB-RNase H system can be applied as a conventional MB without addition of RNase H if desired, with good performance still obtained. It may also be seen that RNase H mediated enhancement of the S:N ratios seen in our study is greater than that obtained by Harvey *et al.* with the CataCleave approach. Even with 200 nM CataCleave probe at the highest [target ssDNA] tested, Harvey *et al.* obtained an S:N ratio of ~6, whereas S:N ratios as high as ~30 were seen in our study (Fig. 4C). Presumably, this is due to a higher initial fluorescence background (F_{close}) associated with the CataCleave probe than compared to the R/D-MB. In the CataCleave probe design the fluorophore/quencher pairing are separated by four ribonucleotides, which results in a greater distance and concomitantly reduced FRET than that facilitated by the stem configuration of a conventional MB probe.

As mentioned in the introduction, other studies by Liu *et al.* [24–25,34] incorporated single ribonucleotides into the loop portion of their MB design with the intention that RNase H nickases would nick the hybridized probes in what amounts to a very useful assay for the detection of single nucleotide polymorphisms (SNPs) [34]. One major difference between the approach presented in this study and that of the Liu lab is that the SNP probe relies principally on DNA-DNA hybridization for target recognition and stem melting. Though the SNP probe scheme was presented as potentially generally applicable to trace oligonucleotide detection, initial studies based on the use of CpRNase HII [24–25] produced designs only applicable to isothermal and mesophilic temperature settings. The S:N ratios seen in those studies were also lower than was demonstrated here; for example, a maximum S:N ratio of ~14 may be calculated based on the background fluorescence from probe without target present vs. the fluorescence intensity observed when cleavage of 1000 nM probe, facilitated by 32 nM target, saturates the system [24]. This maximum S:N ratio of ~14, which is less than half of what was observed in our study with much less probe consumed, is what would be expected if the SNP probe was applied generally. Also, due to the lower temperature settings required by the assay, it was likely more susceptible to non-specific binding. Though the assay was improved in a later study by using TthRNase HII from *T. thermophilus* instead of CpRNase HII [34], it was still incompatible with PCR apparently due to RNase H mediated inhibition of the amplification reaction. As can be seen from Fig. 5, our full-RNA loop probe is able to function very well in the PCR environment.

Another expectation for our design was that the use of a full RNA loop and thermostable RNase HI would lead to a more rapid assay time. We rationalized that using an entire loop comprised of RNA would ensure maximal enzyme binding and cleavage efficiency. From our traces on R/D-MB + ssDNA samples (Fig. 3 and 4), probe-target hybridization appears to be rapid, suggesting that the rate limiting factor is the concentration of probe•target. Compared to the TthRNase HII based SNP assay from the Liu lab [34], our assay is approximately twice as fast. One can see in Fig. 4C from our study that in our assay a S:N of 20 was achieved for 0.1 probe:target within 20 minutes. Using Fig. 2 from Liu et al. 2010 to obtain an estimate of F_{close} , Fig. 3A shows that it took 40 minutes for the SNP assay to achieve a S:N ratio of 20. Hence the full RNA loop R/D-MB + thermostable RNaseHI assay presented in this study is indeed able to achieve higher S:N ratios more rapidly.

In summary, our R/D-MB + thermostable RNase H system amplifies the fluorescence signal under both isothermal and non-isothermal conditions as illustrated by our basic RNase H cleavage and real-time PCR experiments. This allows our method to be compatible with a variety of experimental protocols. As demonstrated by our isothermal experiments, the S:N ratio of a typical MB system was enhanced by as high as $\sim 2.8\times$ by thermostable RNase H mediated cleavage, and S:N ratios as high as ~ 30 (with a 10 min incubation) were observed. It was shown that the approach is useful over a variety of time scales. It was also demonstrated that the assay works under thermal cycling conditions, and a PCR was successfully monitored using the full RNA loop R/D-MB + thermostable RNase H scheme. Since the approach constitutes an MB design, it is compatible with all previously reported design enhancements reported for improving MB performance, *e.g.* the use of superquenchers, better fluorophores, *etc.* For example, if the ~ 22.9 fold reduction in F_{close} seen in Yang et al. [13] was obtained with our system, one could expect the maximum $F_{open,1}$ from Fig. 4B to be increased from ~ 16.5 to ~ 377 , and the maximum $F_{open,2}$ to increase from ~ 20 to ~ 457 . Thus other MB enhancements could drastically improve the S:N ratio of our design. Another compatible enhancement would be to create a longer stem that is part of the target-binding sequence [31], which could produce higher hybridization rates while also improving selectivity. Likewise, using the R/D-MB + thermostable RNase HI approach presented in this study is expected to enhance the performance of most existing MB designs.

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Figure 1.

This schematic shows the three step process of how FRET changes occur within the DNA/ RNA MB-(R/D-MB) hybrid. First (1) the R/D-MB is in a closed state where FRET is completely quenched. (2) This is then followed by a target oligomer binding to the RNA loop portion and thus parting the fluorophore/quencher some initial distance apart and creating an initial fluorescence signal. Lastly, (3) thermostable RNase H cleaves the RNA loop portion of the RNA:DNA hybrid, causing the stem to completely break apart and permanently separating the fluorophore and quencher apart, thereby completely abolishing FRET. The RNase H cleavage step also leaves behind intact, unbound target ssDNA, allowing it to bind to its next R/D-MB complement and creating a cyclic processing scheme.

Figure 2.

Shown are the thermal denaturation profiles of the designed R/D-MB probe (red circles) and probe in the presence of target ssDNA (blue squares). The half maximal annealing temperature T_a of 64 °C predicted by the Beacon Designer software from Premier Biosoft International is depicted as a solid blue line that extends from the x-axis to the melting curve. The temperature T_m of 72 °C at which R/D-MB stem was predicted to melt is shown with a red extended line. It can be seen that there is a good correspondence between the values predicted for T_a and T_m and the values indicated by the melting curves.

Figure 3.

Temperature dependence of thermostable RNase H reactions with R/D-MB probe. (A) Representative traces of fluorescence intensity from the R/D-MB probe (500 nM) in the absence or presence of target ssDNA (500 nM), either with or without RNase H enzyme (2.5 units) at 50°C. (B) S:N ratios (blue scale on right hand axis) of R/D-MB + target ssDNA and R/D-MB + target ssDNA + RNase H at 45°C, 50°C, 55°C, 60 °C and their corresponding fluorescence (red scale) intensities along with R/D-MB + non-target. The S:N ratio of the R/D-MB + target ssDNA + RNase H samples was ~2–2.4 times higher than that of the R/D-MB + target ssDNA samples at each temperature setting.

Figure 4.

Target ssDNA concentration dependence of the performance of the R/D-MB probe + thermostable RNase H detection scheme. (A) Raw data, showing the fluorescence intensity curves as a function of target ssDNA concentration (0.1–200 nM) from samples containing 50 nM R/D-MB and either the absence or presence of RNase H (2.5 U). Also shown are buffer, probe-only, and probe + non-target ssDNA controls. (B) Fluorescence intensities at 100 min of incubation time from samples containing 50 nM R/D-MB + variable [target ssDNA] and either the absence (orange bars) or presence (teal bars) of thermostable RNase H. Associated S:N ratios were determined based on F_{open-1} for the samples without RNase H present (blue open circles) and based on F_{open-2} for samples containing RNase H (blue squares). Note that the x-axis was deliberately left unscaled because $[probe \cdot target]$, the true substrate of RNase H, was not determined. (C) S:N ratios associated with F_{open-2} (*i.e.* samples containing RNase H) at different incubation times are plotted as a function of target ssDNA concentration. S:N ratios associated with F_{open-1} determined at 10 and 60 min are included for comparison. Note that points on the x-axis were deliberately left unscaled, since the true substrate of the RNase H reaction is $probe \cdot target$, the concentration of which was not determined. (D) Rate constants from monoexponential fits ($R^2 = 0.997$ for each trace) describing the first-order kinetics of R/D-MB probe binding and cleavage by RNase H are plotted against target ssDNA concentration. As with panel C, the x-axis was left unscaled since the concentration of the true substrate of the RNase H reaction, $probe \cdot target$, was not determined.

Figure 5.

Monitoring of real-time PCR experiments using the R/D-MB + thermostable RNase H scheme. (A) PCR template serial dilutions were performed at 10^{-1} (green squares), 10^{-3} (blue triangles), 10^{-4} (purple circles), and 10^{-5} (black diamonds). Dilutions of the pUC19 template were amplified in the presence of 200 nM R/D-MB and 2.5 U of thermostable RNase H in a 50 μ L PCR buffer solution. (B) Plot of C_t values versus fold dilution of pUC19 template (115 pM starting concentration). From this plot, it is simple to determine the initial concentration of an unknown pUC19 template sample once the C_t value is known.