Biotechnology Journal
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Target-Activated DNA Polymerase Activity for Sensitive RNase H Activity Assay

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Herein, the ribonuclease H (RNase H) activity assay based on the target-activated DNA polymerase activity is described. In this method, a detection probe composed of two functional sequences, a binding site for DNA polymerase and a catalytic substrate for RNase H, serves as a key component. The detection probe, at its initial state, suppresses the DNA polymerase activity, but it becomes destabilized by RNase H, which specifically hydrolyzes RNA in RNA/DNA hybrid duplexes. As a result, DNA polymerase recovers its activity and initiates multiple primer extension reactions in a separate TaqMan probe-based signal transduction module, leading to a significantly enhanced fluorescence "turn-on" signal. This assay can detect RNase H activity as low as 0.016 U mL^{-1} under optimized conditions. Furthermore, its potential use for evaluating RNase H inhibitors, which have been considered potential therapeutic agents against acquired immune deficiency syndrome (AIDS), is successfully explored. In summary, this approach is quite promising for the sensitive and accurate determination of enzyme activity and inhibitor screening.

1. Introduction

Ribonuclease H (RNase H) is a ribonuclease that hydrolyzes the RNA strands of RNA/DNA hybrid duplexes through its endonucleolytic cleavage activities.[1,2] It has been found in almost all organisms from prokaryotes to eukaryotes, and its activity has been shown to be involved in crucial biological processes, including DNA replication, repair, and transcription.[3,4] Importantly, the RNase H activity of human immunodeficiency viruses (HIV)-1 reverse transcriptase (RT) is essential for the viral replication cycle, and it has been regarded as an attractive target for anti-HIV therapeutics.[5,6] Hence, it is desirable to develop an efficient method to monitor RNase H activity and its inhibition.

To date, various strategies for the RNase H activity assay have been developed, such as gel electrophoresis,[7] capillary electrophoresis,[8] high-performance liquid chromatography (HPLC),[9] acid-soluble release of RNA fragments,[10] colorimetry,[11,12] and fluorometry.[13,14] In particular, fluorometric methods have been extensively established due to their advantageous features of simplicity, reliability, and sensitivity.[15–22] The typical examples employing molecular beacons or G-quadruplexes rely on the fluorescence readouts resulting from RNase H-catalyzed reactions.[14,23,24] However, the signal response in these methods is not large enough for the sensitive determination of RNase H activity, which is attributed to the fact that a single target only promotes a single signaling event. In addition, these methods often require the direct modification of detection probes with fluorophore and quencher, which can interfere with the effective RNase H-catalyzed reactions, consequently raising issues such as poor sensitivity and reliability of the assays.[13,25,26]

In an attempt to address the issues in previous fluorometric methods, we developed a novel method for sensitive RNase H activity assays by designing a unique detection probe composed of a DNA polymerase-specific aptamer (DPA) and an RNA-containing strand (RS). The proposed method relies on our recent finding that a DNA aptamer, containing 5′ target-specific overhang sequence, can regulate the DNA polymerase activity in response to the target molecule.[27,28] In principle, RNase H, which degrades the RNA part of DNA/RNA hybrid duplexes, destabilizes the detection probe. Accordingly, DNA polymerase becomes activated and promotes multiple primer extension reactions on a separate TaqMan probe-based signaling probe, leading to a significant fluorescence enhancement. Utilizing the proposed system, we determined the RNase H activity with great performances and also screened RNase H inhibitors. Importantly, the developed system has separate detection and signaling probes, and thus it does not require the direct modification of the detection probe. In addition, the DNA polymerase that is activated by RNase H promotes multiple extension reactions, allowing for accurate and sensitive enzyme activity assays.

2. Experimental Section

2.1. Materials

All oligonucleotides used in the study were synthesized by Bioneer (Daejeon, South Korea) and purified by Bio-RP, except

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DOI: 10.1002/biot.201800645

the template and the TaqMan probe, which were purified by polyacrylamide gel electrophoresis (PAGE) and HPLC, respectively. The sequences of the oligonucleotides are listed in Table S1, Supporting Information. *Thermus aquaticus* (*T. aquaticus*) DNA polymerase (Taq DNA polymerase; 550 nM), uracil DNA glycosylase (UDG), lambda exonuclease (λ exo), exonuclease I (Exo I), T4 polynucleotide kinase (PNK), *Hin*dIII, and hOGG 1 were purchased from New England Biolabs Inc. (Beverly, MA, USA). *Escherichia coli* (*E. coli*) RNase H and SYBR Green I (10 000×) were purchased from Takara Biotechnology (Dalian, China) and Invitrogen (Carlsbad, CA, USA), respectively. Ethidium bromide (EtBr) and ellipticine were purchased from Bioneer (Daejeon, Korea) and Dingyan Chem Co. (Hangzhou, China), respectively. Ultrapure DNase/RNase-free distilled water purchased from Bioneer was used in all the experiments. All other chemicals were of analytical grade and used without further purification.

2.2. Procedure for RNase H Activity Assay

The reaction mixtures were separately prepared as part A and part B. Part A (total volume of 20 μL), consisting of 500 μM dNTPs, 400 nM DPA, and 400 nM RS in 1× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$), was heated at 90 °C for 5 min, cooled slowly to 37 °C (0.1 °C s^{-1}), and incubated for 20 min. RNase H at varying concentrations or other enzymes were added to part A and incubated at 37 °C for 30 min. Taq DNA polymerase (11 nM) was then added to the above solution and incubated at 25 °C for 20 min. At the same time, part B (total volume of 20 μL) consisting of 600 nM template, 600 nM primer, and 500 nM TaqMan probe in 1× reaction buffer was heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C s^{-1}), and incubated for 60 min. Parts A and B were then mixed, and the fluorescence signal from the TaqMan probe was immediately monitored at every 2 min at 25 °C during the primer extension reaction on a CFX Connect real-time polymerase chain reaction (RT-PCR) Detection System (Bio-Rad, Hercules, CA, USA).

2.3. Melting Curve Analysis

The reaction mixtures (total volume of 40 μL) containing 200 nM DPA, 200 nM RS, 250 μM dNTPs, and 1× SYBR Green I in 1× reaction buffer were heated at 90 °C for 5 min, cooled slowly to 37 °C (0.1 °C s^{-1}), and incubated for 20 min. RNase H (30 U mL^{-1}) was then added to the reaction mixtures and incubated at 37 °C for 30 min, followed by the cooling at 25 °C for 20 min. The fluorescence signal was measured on a CFX Connect RT-PCR Detection System (Bio-Rad) as the temperature increased from 25 °C to 75 °C with an increment of 0.5 °C. The first derivative plot (−d(RFU)/d*T*) was used to determine the melting temperature.

2.4. PAGE Analysis

The reaction products (total volume of 40 μL) were prepared according to Section 2.2 except that the TaqMan probe was excluded from part B and the primer extension reaction was carried out for 180 min. The reaction products were mixed with 6× loading buffer and resolved on 15% polyacrylamide gel. The electrophoresis was run in 1× TBE buffer for 180 min at 120 V with Mini-PROTEAN Tetra Cell Systems (Bio-Rad). After staining with EtBr, the gel image was obtained with the Gel Doc EZ Imager (Bio-Rad).

2.5. RNase H Inhibition Assay

All procedures were the same as those of the Section 2.2 except that EtBr or ellipticine at varying concentrations was added to part A and incubated at 37 °C for 10 min prior to the addition of RNase H.

3. Results and Discussion

3.1. Detection Principle of the RNase H Activity Assay

The basic principle of a new strategy to determine the RNase H activity is illustrated in **Figure 1**. In the assay, the detection probe that serves as a substrate for RNase H is formed by the hybridization of the DPA and RS (5′-DNA–RNA–DNA-3′; RS). In the absence of RNase H, the detection probe remains intact and binds to the DNA polymerase, inhibiting its activity. On the other hand, the presence of RNase H destabilizes the detection probe by catalyzing the hydrolysis reactions on its RNA part and thus the DNA polymerase with 5′ → 3′ exonuclease activity becomes activated. As a result, multiple primer extension reactions are initiated by active DNA polymerase in a separate signal transduction module composed of primer/template complexes in conjunction with a TaqMan probe labeled with a fluorophore (FAM) and a quencher (BHQ1) at each end. Importantly, DNA polymerase that completes one cycle of extension reaction goes to another primer/template complex and initiates another extension reaction. In this manner, the DNA polymerase activated by RNase H induces multiple cycles of extension reactions, leading to a significant fluorescence enhancement.

3.2. RNase H-Catalyzed Destabilization of the Detection Probe

First, we performed a melting curve analysis to confirm the destabilization of the detection probe by the specific catalytic reaction of RNase H. As shown in Figure S1, Supporting Information, the peak at 57 °C corresponding to the melting temperature of the detection probe (DPA + RS) disappeared upon the addition of RNase H (curves 1, 2, and 3). On the other hand, the control detection probe (DPA + DS) in which the RNA part of RS is replaced with DNA (DS) exhibited the same peak at 62 °C corresponding to the melting temperature of the control detection probe (DPA + DS) regardless of the presence of RNase H (curves 4 and 5), indicating that the detection probe is destabilized by the specific catalytic reaction of RNase H.

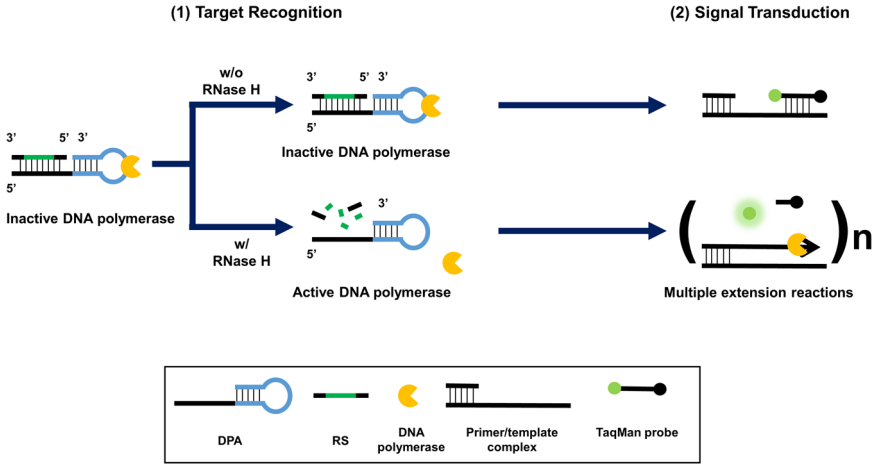

Figure 1. Schematic illustration (not drawn to scale) of the RNase H activity assay based on the target-activated DNA polymerase activity.

3.3. Detection Feasibility of the RNase H Activity Assay

Then, we validated the feasibility of the proposed method by measuring the time-dependent fluorescence intensities during the primer extension reaction under different conditions (**Figure 2**). As expected, the DPA alone did not inhibit the DNA polymerase, but DPA stabilized by RS to form the detection probe and bound to and suppressed the DNA polymerase, as evidenced by the negligible fluorescence increase (1 and 2 in Figure 2A,B). In contrast, upon the addition of RNase H, the detection probe became destabilized, as confirmed by melting curve analysis (Figure S1, Supporting Information), and no longer inhibited the DNA polymerase, leading to the significantly enhanced fluorescence signal (3 in Figure 2A,B). In addition, it was also confirmed that the control detection probe (DPA + DS) even in the presence of RNase H, suppresses DNA polymerase activity, consequently leading to the negligible fluorescence enhancement (4 and 5 in Figure 2A,B). To further support the above fluorescence results, the PAGE analysis that confirms the generation of primer extension product was carried out. As shown in the inset of Figure 2B, an intense band for the extension product appeared in lanes 1 and 3, which correspond to the samples where the significant fluorescence enhancement took place. However, in other cases where the fluorescence increase was negligible (lanes 2, 4, and 5), only a band for the primer/template complex was observed. Overall, these results are consistent with the above fluorescence results, ensuring that the DNA polymerase is activated by the specific catalytic reaction of RNase H that destabilizes the detection probe.

3.4. Analytical Performances of the RNase H Activity Assay

Next, we optimized the reaction conditions for the efficient determination of the RNase H activity. The results of the experiments, in which the concentration of RS and the RNA length within the detection probe was varied, show that 200 nM

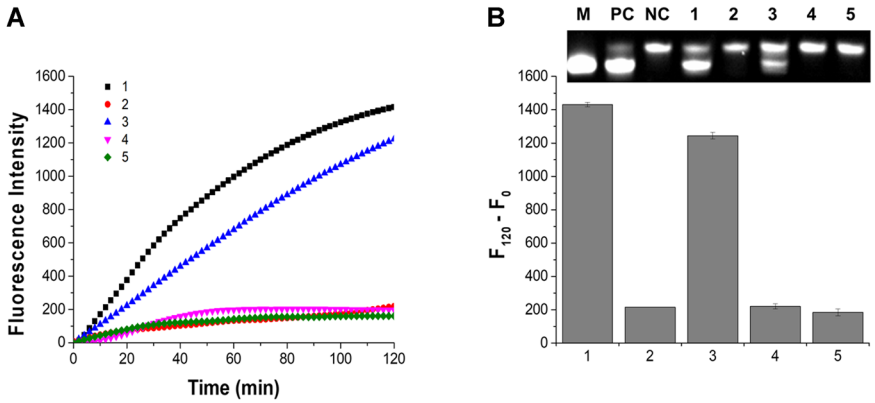

Figure 2. Feasibility of the RNase H activity assay. A) Time-dependent fluorescence intensities from the TaqMan probe during the primer extension reaction, and B) fluorescence signal changes ($F_{120} - F_0$), where F_{120} and F_0 are the fluorescence intensities measured at 120 min and 0 min during the primer extension reaction, respectively. Inset in (B): the PAGE image of the primer extension reaction products. M, template/complement; PC, Template + primer + DNA polymerase; NC, Template + primer; 1, DPA; 2, DPA + RS; 3, DPA + RS + RNase H; 4, DPA + DS; and 5, DPA + DS + RNase H. PC was included in the samples from 1 to 5. The final concentrations of DPA, RS, DS, DNA polymerase, and RNase H are 200 nM, 200 nM, 200 nM, 5.5 nM, and 30 U mL^{-1}, respectively.

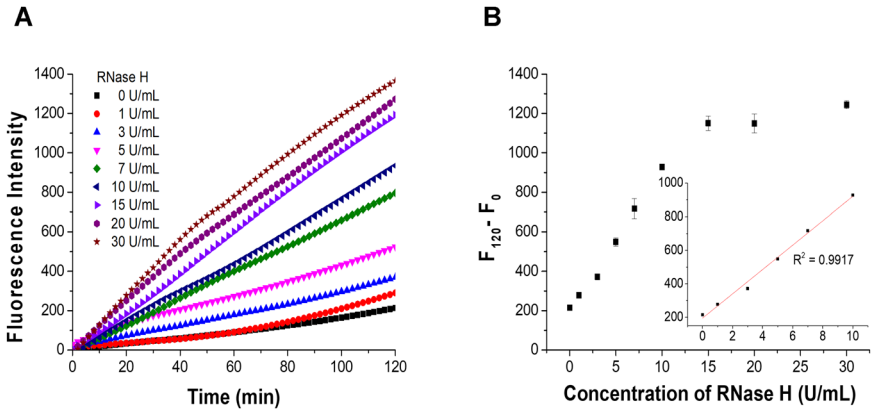

Figure 3. Sensitivity of the RNase H activity assay. A) Time-dependent fluorescence intensities from the TaqMan probe during the primer extension reaction and B) fluorescence signal changes ($F_{120} - F_0$) in the presence of varying concentrations of RNase H, where F_{120} and F_0 are the fluorescence intensities measured at 120 min and 0 min during the primer extension reaction, respectively. Inset in (B): linear relationship between ($F_{120} - F_0$) and RNase H concentration (0–10 U mL^{-1}). The final concentrations of DPA, RS, and DNA polymerase are 200 nM, 200 nM, and 5.5 nM, respectively.

of RS and 6 bp of RNA part lead to the lowest background signal and the highest signal-to-noise ratio (S/N), respectively (Figure S2, Supporting Information). Under the optimized conditions, we checked the detection sensitivity of the proposed system by measuring the time-dependent fluorescence intensities as a function of the RNase H concentration (**Figure 3**). As shown in Figure 3B, the fluorescence signal changes ($F_{120} - F_0$) increased concentrations up to 15 U mL^{-1} and reached a plateau at concentrations >15 U mL^{-1}. An excellent linear relationship ($R^2 = 0.9917$) was established in the range from 0 U mL^{-1} to 10 U mL^{-1}, and the limit of detection (LOD) was calculated to be 0.016 U mL^{-1}, using the equation of $3\sigma/S$, where σ is the standard deviation at the blank and S is the slope of the linear relationship, respectively (inset in Figure 3B). Importantly, the LOD obtained by the proposed approach was comparable to or lower than those of previous fluorometric methods (**Table 1**). In addition, it should be noted that this strategy does not require the direct modification of fluorophore and quencher on the RNase H substrate and thus overcomes the drawbacks in the previous methods.

To demonstrate the detection selectivity of this method, the extents of fluorescence enhancement induced by other enzymes including UDG, λ exo, Exo I, PNK, *Hin*dIII, and hOGG 1 in addition to RNase H were also assessed. The results in **Figure 4** show that the significant fluorescence enhancement only takes place in the presence of RNase H, while other enzymes induce the weak fluorescence increase comparable to that of blank, confirming that the devised method is highly selective to RNase H.

3.5. RNase H Inhibition Assay

Finally, we evaluated the capability of the developed strategy to screen candidate RNase H inhibitors that have been considered as the promising therapeutic drugs to treat intractable diseases such as AIDS.[29] As model inhibitors for RNase H, we selected ellipticine and EtBr, which were confirmed to have no direct effect on the DNA polymerase activity (Figure S3, Supporting Information) and measured the fluorescence responses of our system in the presence of RNase H inhibitors at different concentrations. The results in **Figure 5** show that the relative activity of RNase H, which is defined as the ratio of fluorescence signal change ($F_{120} - F_0$) in the presence of an inhibitor to that in the absence of an inhibitor, decreases with increasing concentrations of ellipticine (black) or EtBr (red), respectively. In addition, the IC_{50} values of model inhibitors, the concentration of inhibitor reducing the enzyme activity to 50%, were calculated to be 2.36 μM and 4.94 μM, respectively, which are in

Table 1. Comparison of the developed method with previous fluorometric ones.

Key materials/methods	LOD [U mL^{-1}]	Linear range [U mL^{-1}]	Limitations	References
Fluorophore- and quencher-labeled molecular beacon	0.5	1–80	• Direct modification of the RNase H substrate • A single signaling event	Liu et al.[26]
Dual-pyrene-labeled molecular beacon	5	—	• Direct modification of the RNase H substrate • A single signaling event	Chen et al.[14]
G-quadruplex/NMM complex	0.2	0–4	• A single signaling event	Hu et al.[23]
FAM-labeled molecular beacon substrate and graphene oxide	0.005	0.1–1	• Direct modification of the RNase H substrate • A single signaling event	Zhao et al.[25]
DNAzyme-assisted cascade reaction	0.01	0.02–20	—	Wang et al.[18]
Target activated DNA polymerase activity	0.016	0–10	—	This work

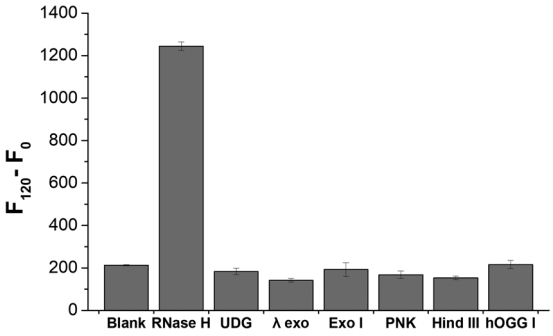

Figure 4. Selectivity of the RNase H activity assay. Fluorescence signal changes ($F_{120} - F_0$) in the presence of RNase H (30 U mL^{-1}) and other enzymes (50 U mL^{-1}), where F_{120} and F_0 are the fluorescence intensities measured at 120 min and 0 min during the primer extension reaction, respectively. The final concentrations of DPA, RS, and DNA polymerase are 200 nM, 200 nM, and 5.5 nM, respectively.

good agreement with those from previous reports.[17,23,30] These results clearly support that the present strategy is capable of screening potential chemicals that inhibit the RNase H activity.

4. Conclusion

In summary, we have developed a novel method for sensitive RNase H activity assays based on the RNase H-responsive activation of DNA polymerase. Importantly, this strategy is composed of two separate steps, target recognition and signal transduction, and thus the direct modification of fluorophore and quencher in the target-specific probe is not required. In addition, multiple cycles of primer extension reaction induced by active DNA polymerase enable the sensitive determination of RNase H, which is demonstrated by the low LOD. Finally, the practical utility of this assay to screen the inhibitors was demonstrated by testing the model drug, ellipticine, and EtBr.

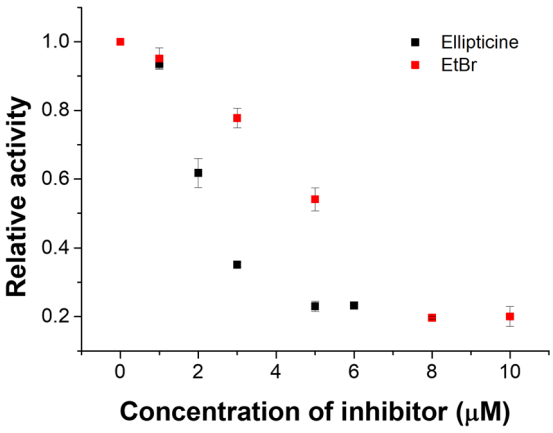

Figure 5. RNase H inhibition assay to screen the candidate RNase H inhibitors. Relative activities of RNase H were defined as the ratio of fluorescence signal change ($F_{120} - F_0$) in the presence of inhibitors to that in the absence of inhibitors (ellipticine: black and EtBR: red), where F_{120} and F_0 are the fluorescence intensities measured at 120 min and 0 min during the primer extension reaction, respectively. The final concentrations of DPA, RS, DNA polymerase, and RNase H are 200 nM, 200 nM, 5.5 nM, and 30 U mL^{-1}, respectively.

With these outstanding features, we expect that the developed system would be readily adapted to the assays for other enzyme activities at low-cost by simply redesigning the detection probe specific to the target enzymes while retaining the same TaqMan probe-based signaling probe.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Financial support to this study was provided by the Mid-career Researcher Support Program (No. 2018R1A2A1A05022355) and Bio-Synergy Research Project (No. 2015M3A9C4070484) of the National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning (MSIP) of Korea. Financial support was also provided by the Center for BioNano Health-Guard funded by the MSIP of Korea as Global Frontier Project (Grant H-GUARD_2013M3A6B2078964).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

aptamer, DNA polymerase, enzyme activity assay, fluorescent biosensor, RNase H

Received: October 18, 2018
Revised: December 11, 2018
Published online: May 20, 2019

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