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Aptamer-mediated universal enzyme assay based on target-triggered DNA polymerase activity

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ABSTRACT

We herein describe an innovative method for a universal fluorescence turn-on enzyme assay, which relies on the target enzyme-triggered DNA polymerase activity. In the first target recognition step, the target enzyme is designed to destabilize detection probe derived from an aptamer specific to DNA polymerase containing the overhang sequence and the complementary blocker DNA, which consequently leads to the recovery of DNA polymerase activity inhibited by the detection probe. This target-triggered polymerase activity is monitored in the second signal transduction step based on primer extension reaction coupled with TaqMan probe. Utilizing this design principle, we have successfully detected the activities of two model enzymes, exonuclease I and uracil DNA glycosylase with high sensitivity and selectivity. Since this strategy is composed of separated target recognition and signal transduction modules, it could be universally employed for the sensitive determination of numerous different target enzymes by simply redesigning the overhang sequence of detection probe, while keeping TaqMan probe-based signal transduction module as a universal signaling tool.

1. Introduction

Enzymes are deeply involved in a wide variety of cellular processes such as DNA replication, recombination, and repair, and indispensable tools in the field of biotechnology and bioengineering including polymerase chain reaction, site-directed mutagenesis, molecular cloning, and DNA sequencing (Hutter and Maysinger, 2013; Jang et al., 2014; Leung et al., 2013a; Qing et al., 2013). In addition, enzymes with the altered levels of activities have been utilized as an indicator for a number of pathological conditions (McCaffrey et al., 1981; Napirei et al., 2000; Park et al., 2014; Paz-Elizur et al., 2003; Xu et al., 2008; Zhu et al., 2014) and recognized as a novel family of pharmacological targets for the treatment of tumors (Liu et al., 2010; Xu et al., 2007). For example, the inactivation of a major human 3′→5′ exonuclease named TREX1 that is vital for controlling autoimmunity was reported to induce the impaired apoptosis and autoimmune-like inflammatory diseases (Leung et al., 2011; Richards et al., 2007; Yang et al., 2007). Therefore, it has been an important goal to develop a reliable method for enzyme assay in order to understand the biological processes and networks.(Acker and Auld, 2014).

The conventional methods to determine enzyme activities include gel electrophoresis coupled with radioactive labeling (Nimonkar et al., 2008), high-performance liquid chromatography (McLaughlin et al., 1987), and enzyme-linked immunosorbent assay (Jeltsch et al., 1993). Since all of these procedures possess several disadvantages including the requirement of sophisticated instrumentation, technical expertize, and long analysis times, alternative fluorescence-based approaches have attracted an intense research interest to overcome these limitations, which leads to the development of a number of new methods to determine the enzyme activities.(Dai and Kool, 2011).

Early work in this area relies on molecular beacon probe labeled with the fluorophore and quencher at both ends. This detection probe whose fluorescence is restored in response to the target enzymes has been applied to monitor various catalytic processes such as DNA cleavage, ligation, transcription, and phosphorylation (Li et al., 2014; Li et al., 2007; Liu et al., 2013; Wang et al., 2009; Wu et al., 2014). Recently, fluorophore-labeled DNA probes complexed with different kinds of nanomaterials, such as carbon nanoparticles (Wang et al., 2012), carbon nanotubes (Liu et al., 2011), conjugated polymers (Feng et al., 2007), gold nanoparticles (Deng et al., 2012; Kim et al., 2007), and graphene oxide (Jang et al., 2013; Zhang and Kong, 2013; Zhou et al., 2013), that function as effective quenchers in place of organic quenchers, have been employed to construct advanced methods

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for enzyme assay. Although the above-mentioned techniques based on fluorescence resonance energy transfer allow the sensitive and selective determination of enzyme activities, new detection probes labeled with fluorophore or quencher should be designed to detect other enzyme activities, which increase the total assay cost. Therefore, it is quite challenging to develop the cost-effective method that enables the analysis of a wide range of target enzymes.

Toward this goal, we have devised a new fluorescence turn-on enzyme assay based on our recent finding that DNA aptamer containing overhang sequence inactivates DNA polymerase by its hybridization with complementary target DNA, which was applied for the detection of target DNAs (Park et al., 2015b). The target enzymes are designed to destabilize detection probe derived from an aptamer specific to DNA polymerase containing the overhang sequence and the complementary blocker by reducing the duplex stability within the overhang region and recover the DNA polymerase activity inhibited by the detection probe, which is finally used to identify target enzymes in the signal transduction step. Unlike the general biosensing approaches that contain two elements (a biological recognition part that interacts with the target and a transduction part that converts the recognition event into measurable signal) in spatial proximity,(Hutter and Maysinger, 2013) we have separated these two elements and linked them by employing DNA aptamer that regulates DNA polymerase activity (Dang and Jayasena, 1996; Kolpashchikov and Stojanovic, 2005; Lin and Jayasena, 1997; Park et al., 2015a, 2016a,b). Therefore, this strategy could be universally applied to determine the activities of numerous different enzymes. The new technique based on this design principle was successfully applied to determine the activities of two target enzymes, exonuclease I and uracil DNA glycosylase (Table S1). The practical diagnostic capability of this system was also demonstrated by its use to detect UDG in cells and human blood serum.

2. Materials and methods

2.1. Materials

All DNA oligonucleotides used in the present study were synthesized from Genotech Co. (Daejeon, Korea) and purified by desalting, except for template DNA (purified by PAGE) and TaqMan probe (purified by HPLC). The sequences of oligonucleotides are listed in Table S2. Uracil DNA Glycosylase (UDG), human alkyladenine DNA glycosylase (hAAG), human 8-oxoguanine DNA glycosylase 1 (hOGG1), formamidopyrimidine-DNA glycosylase (Fpg), BamHI, Ribonuclease H, Exonuclease I (Exo I), Exonuclease III, Lambda exonuclease, uracil DNA glycoslyase inhibitor (UGI) and *Thermus aquaticus* DNA polymerase (Taq DNA polymerase) whose concentration is equivalent to 550 nM were purchased from New England Biolabs Inc. (Beverly, MA, USA). Ultrapure DNase/RNase-free distilled water was purchased from Bioneer® (Daejeon, Korea). All other chemicals were of analytical grade and used without further purification.

2.2. Exo I detection procedure

The reaction mixtures were separately prepared as part A and part B. Part A (total volume of 20 μL) composed of 500 μM dNTPs and 12 nM Exo I blocker (Fig. S1) in a 1X Taq 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) and incubated for 20 min. Exo I at varying concentrations or other enzymes, such as UDG, BamHI, Lambda exonuclease, Exonuclease III, and Ribonuclease H were then added to the solution and incubated at 37 °C for 30 min. After the solution was heated at 80 °C for 20 min to inactivate the enzyme, 400 nM Exo I detection probe was added to the solution, which was then slowly cooled to 25 °C and incubated for 20 min. Taq DNA polymerase (11 nM) was next added and incubated at 25 °C for 20 min. Part B (total volume of 20 μL) composed of 600 nM template, 600 nM primer and 500 nM TaqMan probe in a 1X Taq reaction buffer was heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C/s) and incubated for 60 min. Part A and B were mixed, followed by the fluorescence measurement on a C1000™ thermal cycler (Bio-Rad, CA, USA). The fluorescence signal from TaqMan probe during the primer extension reaction was monitored at every 2 min at 25 °C.

2.3. UDG detection procedure

The reaction mixtures were separately prepared as part A and part B. Part A (total volume of 20 μL) composed of 500 μM dNTPs, 400 nM UDG detection probe and 20 nM UDG blocker (Fig. S4) in a 1X Taq reaction buffer was heated at 90 °C for 5 min, cooled slowly to 37 °C (0.1 °C/s) and incubated for 20 min. UDG at varying concentrations or other enzymes such as hAAG, hOGG1, Fpg, BamHI, Exo I, and Lambda exonuclease were then added to the solution and incubated at 37 °C for 30 min. After the resulting solution was slowly cooled to 25 °C (0.1 °C/s) and incubated for 5 min, Taq DNA polymerase (11 nM) was then added and incubated at 25 °C for 20 min. Part B (total volume of 20 μL) composed of 600 nM template, 600 nM primer and 500 nM TaqMan probe in a 1X Taq reaction buffer was heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C/s) and incubated for 60 min. Part A and B were mixed, followed by the fluorescence measurement on a C1000™ thermal cycler (Bio-Rad, CA, USA). The fluorescence signal from TaqMan probe during the primer extension reaction was monitored at every 2 min at 25 °C. In UDG inhibition assay, UGI at varying concentrations were applied before the addition of UDG and all the procedures were the same as the UDG detection procedure. For the detection of UDG activities in human serum, UDG at different concentrations was spiked into the diluted human serum (5%) and each sample was analyzed by following the above-mentioned UDG detection procedure (Lu et al., 2015).

2.4. Calculation of limit of detection (LOD)

The limit of detection (LOD) was determined based on the conventional definition: (3 × standard deviation of background signal)/(slope of linear calibration curve) (Bechlin et al., 2013; Mavrikou et al., 2016). The background signals were obtained from samples without target enzymes and samples with different enzyme concentrations were prepared through serial dilution (Table S3 and S4).

2.5. Polyacrylamide gel electrophoresis (PAGE)

Sample solutions (total volume of 40 μL) were prepared as described in Exo I or UDG detection procedure except that the different concentrations of blocker DNA (200 nM), UDG (20 U/mL), and Exo I (200 U/mL) were used in Part A and TaqMan probe was excluded in Part B. The product solutions obtained after the incubation at 25 °C for 180 min were mixed with 6X loading buffer (Bioneer®, Daejeon, Korea) and subjected to electrophoresis analysis on a 15% precast polyacrylamide gel (Bio-Rad, CA, USA). The analysis was carried out in 1X TBE (89 mM Tris, 89 mM Borate, and 2 mM EDTA, pH 8.3) at 120 V for 120 min. After SYBR gold (Invitrogen, CA, USA) staining, gels were scanned using a UV transilluminator.

2.6. Melting curve analysis

In the assay for Exo I, sample solutions (total volume of 40 μL) containing 100 nM Exo I blocker, 250 μM dNTPs, and 1X EvaGreen™ (Seoul, Korea) in a 1X Taq reaction buffer were heated at 90 °C for 5 min, cooled slowly to 37 °C (0.1 °C/s) and incubated for 20 min. Exo I (100 U/mL) was then added to the solution and incubated for 30 min. After the resulting solution was heated at 80 °C for 20 min to inactivate the enzyme, 200 nM Exo I detection probe was added to each solution, which was then slowly cooled to 25 °C and incubated for 20 min. The fluorescence signal was measured on a C1000™ thermal cycler (Bio-Rad, CA, USA) as the temperature increased from 25 °C to 55 °C with an increment of 0.5 °C. The first derivative plot [-d(RFU)/dT] was used to determine the melting temperature.

In the assay for UDG, sample solutions (total volume of 40 μL) containing 200 nM UDG detection probe, 100 nM UDG blocker, 250 μM dNTPs, and 1X EvaGreen™ (Seoul, Korea) in a 1X Taq reaction buffer were heated at 90 °C for 5 min, cooled slowly to 37 °C (0.1 °C/s) and incubated at 37 °C for 20 min. UDG (10 U/mL) was then added to the solution and incubated at 37 °C for 30 min. The resulting solution was slowly cooled to 25 °C and then incubated at 25 °C for 20 min. The fluorescence signal was then measured on a C1000™ thermal cycler (Bio-Rad, CA, USA) as the temperature was increased from 25 °C to 55 °C with an increment of 0.5 °C. The first derivative plot [-d(RFU)/dT] was used to determine the melting temperature (Park et al., 2010).

2.7. Cell culture and preparation of cell-free extracts

Human lung epithelial cancer (A549) cells purchased from the Korean Cell Link Bank (Seoul, South Korea) were cultured in RPMI-1640 media (Thermo Scientific, USA) supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. For the preparation of cell-free extracts, cells were collected using trypsin-EDTA (Thermo Scientific, USA) and washed three times with PBS. Cells (5×10^6) were then lysed by PRO-PREP™ protein extraction solution kit (Intron Biotechnology, South Korea) according to the manufacturer's instructions. The concentration of isolated total protein was quantified by Bradford assay.

3. Results and discussion

3.1. Overall detection procedure for a universal enzyme assay

The novel fluorescence turn-on strategy for a universal enzyme assay relies on DNA aptamer that binds to Taq DNA polymerase with high affinity and efficiently inhibits DNA polymerase activity. (Dang and Jayasena, 1996) As illustrated in Fig. 1, detection probe derived from an aptamer specific to DNA polymerase containing the overhang sequence and the complementary blocker DNA effectively binds to and inactivates DNA polymerase (Park et al., 2015b). However, target enzyme removes blocker DNA from detection probe and prevents its inhibition against DNA polymerase (1, Fig. 1). The activated DNA polymerase promotes a primer extension reaction in the separate signal transduction module based on TaqMan probe, a DNA probe modified with a fluorophore (FAM) at one end and a quencher (BHQ1) at the other end (2, Fig. 1). Since TaqMan probe that is initially quenched gives a highly enhanced fluorescence signal after being cleaved by 5′→3′ exonuclease activity of DNA polymerase during a primer extension reaction, DNA polymerase activated by target enzymes would produce a significantly higher fluorescence signal than that in the absence of target enzymes. The difference of fluorescence signal could be used to determine target enzyme activity. Since target recognition and signal transduction steps are systematically separated, numerous different enzymes that are involved in DNA repair processes could be universally assayed by simply redesigning the overhang sequence of detection probe and complementary blocker DNA, while retaining the same signal transduction module (Fig. 1).

3.2. Assay of Exo I activity

In our first example, we applied our strategy to determine the activity of exonuclease I (Exo I) that specifically catalyzes the removal of nucleotides from single-stranded DNA in the 3′ to 5′ direction (Song et al., 2014; Zhang and Kong, 2013). In the procedure, the blocker DNA denoted as Exo I blocker is first treated with Exo I and then incubated with Exo I detection probe whose overhang sequence is complementary to Exo I blocker (1, Fig. 2). Exo I cleaves Exo I blocker into deoxyribonucleoside 5′-monophosphates (5′ dNMPs) and 5′-terminal dinucleotide (5′ NpN-OH 3′),

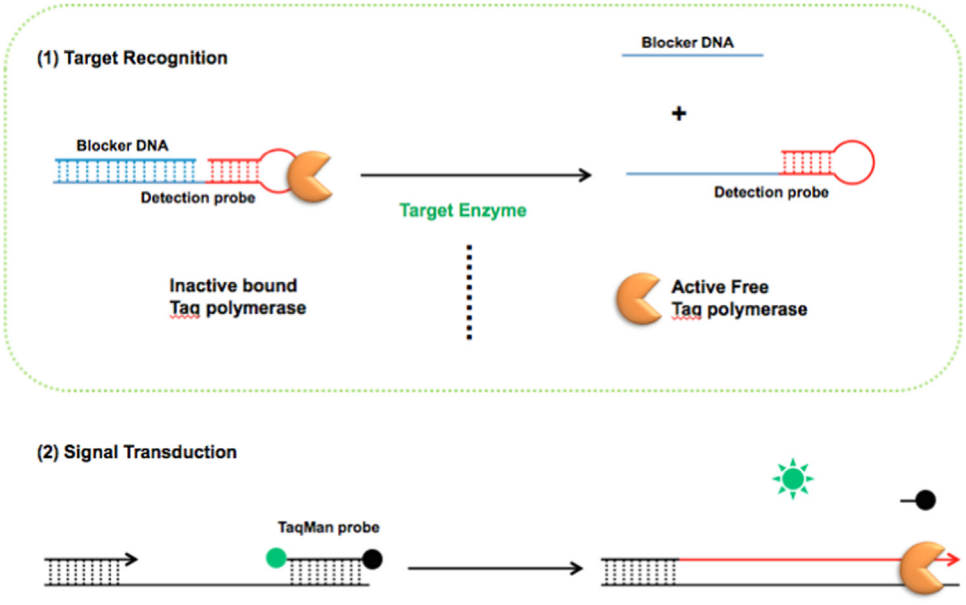

Fig. 1. Schematic illustration of a universal, fluorescence turn-on enzyme assay based on target-triggered polymerase activity. This sensing system is composed of separated target recognition and signal transduction steps, which allows the universal assay of many different target enzymes.

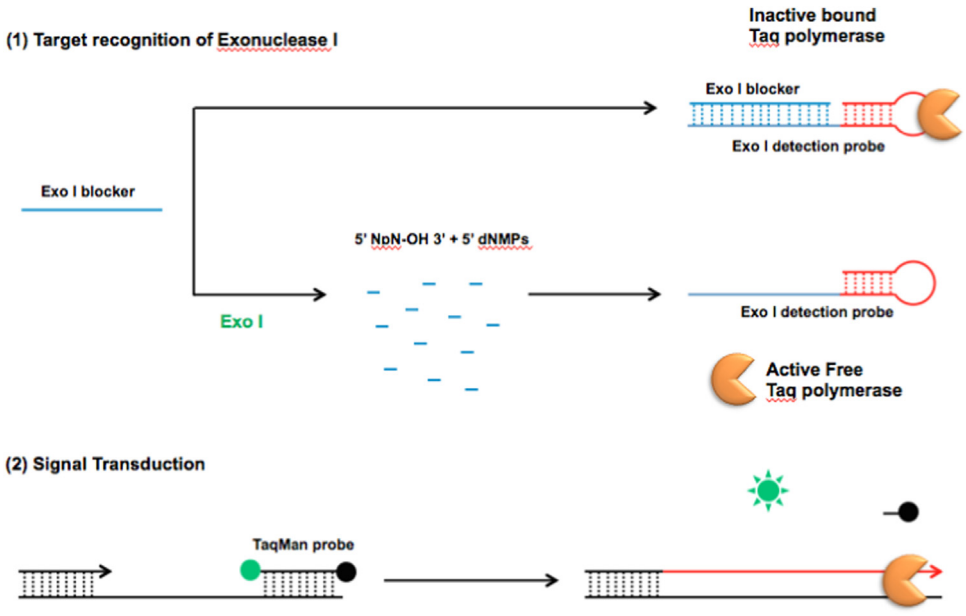

Fig. 2. Schematic illustration for Exo I assay based on target-triggered polymerase activity.

which are not able to stabilize Exo I detection probe, thereby preventing its inhibition against DNA polymerase. The activated DNA polymerase would produce the significantly increased fluorescence signal by promoting the multiple extension reactions in the separated signal transduction step, which is used to determine the activity of Exo I (2, Fig. 2).

To demonstrate the feasibility of this strategy, we prepared the samples in different conditions and measured the fluorescence signals produced from TaqMan probe during the primer extension reaction. As expected, Exo I detection probe alone in the absence of Exo I blocker was not able to inhibit DNA polymerase activity and free DNA polymerase produced a highly enhanced fluorescence signal (1, Fig. 3). On the other hand, the presence of Exo I blocker stabilized Exo I detection probe to be capable of binding to and inhibiting DNA polymerase, consequently leading to quite reduced fluorescence signal (2, Fig. 3 and Fig. S1). Most importantly, the inactivated DNA polymerase was totally restored to produce a highly enhanced fluorescence signal when Exo I blocker was treated by the target enzyme, Exo I that cleaves Exo I blocker into 5′ dNMPs and 5′ NpN-OH 3′ (3, Fig. 3). These observations were further confirmed by electrophoretic analysis of the extension products. As presented in Fig. S2, DNA polymerase became activated and the extension product appeared in the upper position only in the case where Exo I blocker was treated by Exo I (3, Fig. S2).

To verify that the observed fluorescence enhancement is induced by the specific catalytic activity of Exo I, a control Exo I blocker whose terminal 3′-OH group was modified by the phosphoryl group, was employed. The result of studies with this system shows that the quite low fluorescence signal is observed from the

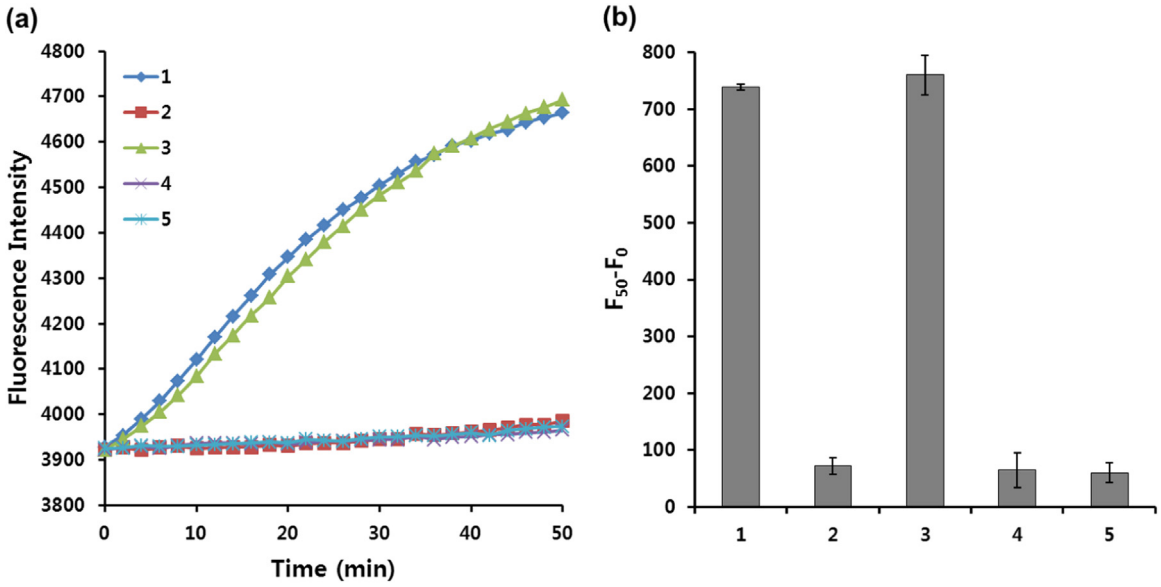

Fig. 3. Fluorescence signals produced by target-triggered polymerase activity for Exo I assay. (a) Time-dependent fluorescence intensities from TaqMan probe during a primer extension reaction. (b) Fluorescence signal change (F_{50}-F_0), where F_{50} and F_0 are the fluorescence intensities from TaqMan probe measured at 50 min and 0 min, respectively. (1) Exo I detection probe, (2) Exo I detection probe+Exo I blocker, (3) Exo I detection probe+Exo I blocker+ Exo I, (4) Exo I detection probe+control Exo I blocker, and (5) Exo I detection probe+control Exo I blocker+Exo I. The final concentrations of DNA polymerase, Exo I detection probe, Exo I blocker, and Exo I are 5.5 nM, 200 nM, 6 nM, and 5 U/mL, respectively.

solution containing a control Exo I blocker that hybridizes to the overhang sequence of Exo I detection probe (4, Fig. 3). As expected, the fluorescence signal was not enhanced by the treatment with Exo I because the 3′ phosphoryl group protects the control Exo I blocker from being cleaved by Exo I (5, Fig. 3). This observation indicates that Exo I activates DNA polymerase by specifically excising nucleotides from 3′-OH of the Exo I blocker DNA and hindering the stabilization of Exo I detection probe.

Next, the denaturation in overhang region of Exo I detection probe promoted by Exo I was further investigated by melting curve analysis. As presented in Fig. S3, the melting peak corresponding to the duplex DNA formed between overhang region of Exo I detection probe and blocker was observed at 43 °C (2, Fig. S3), but this melting peak completely disappeared after the treatment with Exo I (3, Fig. S3). In contrast, when Exo I blocker containing 3′ phosphoryl group was employed as a control, the melting peak at 43 °C was retained regardless of the treatment with Exo I (4 and 5, Fig. S3). Overall, these observations demonstrate that target enzyme, Exo I could be reliably assayed based on this novel strategy to modulate DNA polymerase activity.

Based on the results summarized above, the detection sensitivity was determined by time-dependent measurement of fluorescence intensity from TaqMan probe as a function of Exo I concentrations. The results show that the fluorescence signal change (F_{50}-F_0), where F_{50} and F_0 are the fluorescence intensities from TaqMan probe measured at 50 min and 0 min, respectively, increases with increasing concentrations of Exo I up to 2 U/mL, and then reaches a plateau at concentrations over 2 U/mL (Fig. 4). An excellent linear relationship (R^2=0.9905) existed in the range of 0–2 U/mL and the limit of detection (LOD) (3σ/slope) was ca. 0.218 U/mL (Table S3), which is a little higher than previously reported methods for Exo I assay (Song et al., 2014; Zhang and Kong, 2013). However, this strategy based on target-triggered polymerase activity requires only 30 min for the enzymatic reaction as compared to 120 min in the previous methods, which is quite advantageous.

Next, in order to assess the specificity of the new assay method, other enzymes including heat-inactivated Exo I (3), uracil DNA glycosylase (UDG) (4), BamHI (5), Lambda exonuclease (6), Exonuclease III (7), and Ribonuclease H (8) were investigated for their capabilities to activate DNA polymerase, which were then compared with that of Exo I (2). Analysis of the plots displayed in Fig. 5 clearly shows that only Exo I very specifically induces the high fluorescence signal change while other enzymes are not able to activate DNA polymerase and quite reduced fluorescence signals are obtained even at ten times higher concentration than that of Exo I, indicating that this strategy is highly specific toward target enzyme, Exo I.

3.3. Assay of UDG activity

To verify the universal applicability of this strategy, uracil DNA glycosylase (UDG) that catalyzes the hydrolysis of N-glycosydic bond between uracil and sugar, consequently producing an apyrimidinic (AP) site is employed as our second model enzyme (Leung et al., 2013b; Liu et al., 2007; Ono et al., 2012). As outlined in Fig. S4, the uracil-containing blocker DNA is first hybridized with overhang sequence of UDG detection probe, which is then treated with UDG (1, Fig. S4). This process for target enzyme recognition is reported by the separated signal transduction step (2, Fig. S4) as explained for the determination of Exo I activity (2, Fig. 2). Since the AP sites produced by UDG decrease the duplex stability of overhang region within the UDG detection probe, DNA polymerase activity is recovered to produce the significantly enhanced fluorescence signal after the multiple extension reactions, which could be used to determine the activity of UDG.

In the experiments to prove the feasibility of this strategy, UDG detection probe in the absence of UDG blocker was not able to inhibit DNA polymerase activity, which resulted in the generation of a high fluorescence signal during the primer extension reaction (1, Fig. S5). On the other hand, the presence of UDG blocker stabilized UDG detection probe to effectively suppress DNA polymerase activity, consequently resulting in quite reduced fluorescence enhancement (2, Fig. S5 and Fig. S6). Most importantly, the inactivated DNA polymerase was completely restored to produce a highly enhanced fluorescence signal when the UDG detection probe stabilized by UDG blocker was treated by the target enzyme, UDG because the duplex overhang region within the UDG detection probe was destabilized through the hydrolysis of uracil nucleobase within UDG blocker (3, Fig. S5). These results were

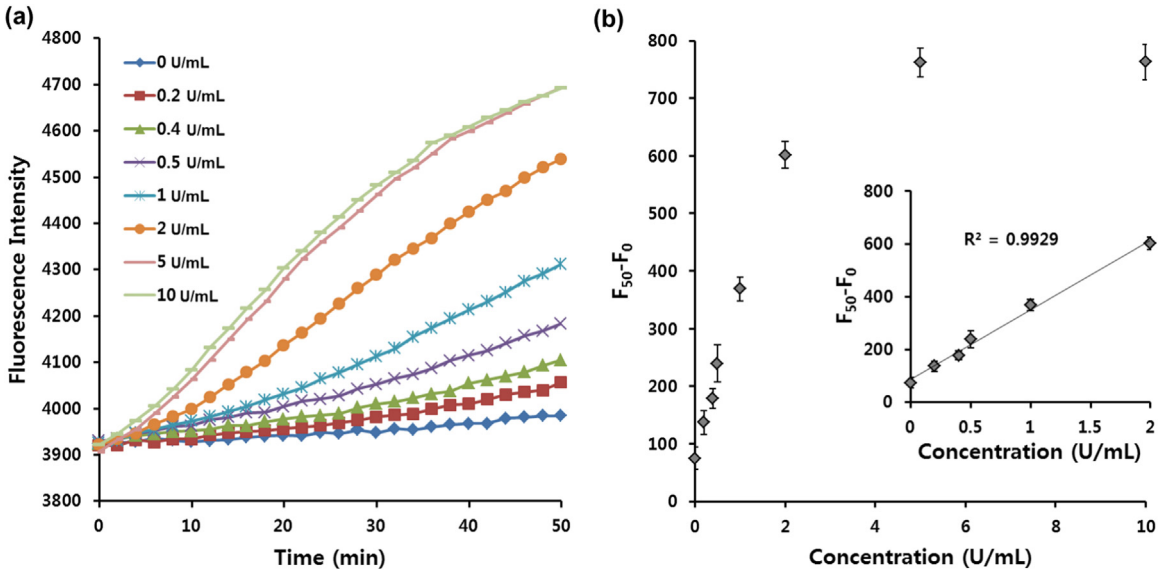

Fig. 4. Sensitivity of new Exo I assay. (a) Time-dependent fluorescence intensities from TaqMan probe during a primer extension reaction. Exo I blocker is treated with Exo I at varying concentrations and subjected to the assay procedures. (b) Fluorescence signal change (F_{50}-F_0) depending on the concentration of Exo I, where F_{50} and F_0 are the fluorescence intensities from TaqMan probe measured at 50 min and 0 min, respectively. Inset in (b): Linear range between (F_{50}-F_0) and Exo I (0–2 U/mL). The final concentrations of DNA polymerase, Exo I detection probe, and Exo I blocker are 5.5 nM, 200 nM, and 6 nM, respectively.

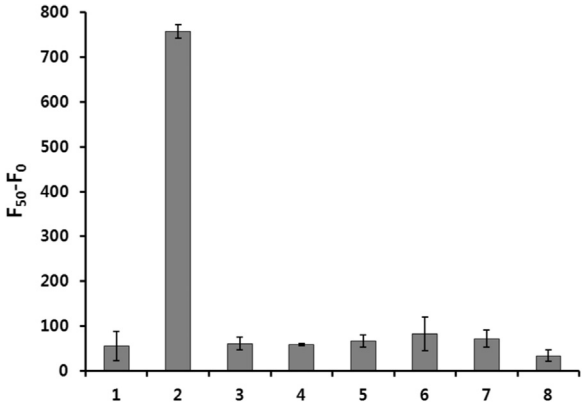

Fig. 5. Specificity of new Exo I assay. Fluorescence signal change (F_{50}-F_0) without any enzyme treatment (1) and with the treatment of Exo I blocker by Exo I (2), heat-inactivated Exo I (3), UDG (4), BamHI (5), Lambda exonuclease (6), Exonuclease III (7), and Ribonuclease H (8), where F_{50} and F_0 are the fluorescence intensities from TaqMan probe measured at 50 min and 0 min, respectively. Exo I blocker is treated with Exo I or the other enzymes. The final concentrations of DNA polymerase, Exo I detection probe, Exo I blocker, Exo I, and the other enzymes are 5.5 nM, 200 nM, 6 nM, 5 U/mL, and 50 U/mL, respectively.

further confirmed by electrophoretic analysis of the extension products (Fig. S7).

To verify that the observed fluorescence enhancement is induced by the specific catalytic activity of UDG, a control UDG blocker in which thymine replaces uracil was employed. The result of studies with this system shows that the fluorescence signal reduced due to the inactivated DNA polymerase is not enhanced by the introduction of UDG (4 and 5, Fig. S5), indicating that the thymine-replaced control UDG blocker is resistant against the catalytic activity of UDG. This observation verifies that UDG activates DNA polymerase by specifically excising uracil nucleobases from UDG blocker to generate AP sites and consequently lowering the duplex stability of the overhang region in the UDG detection probe.

Next, the proposal that the UDG-catalyzed removal of uracil nucleobase induces the denaturation in the overhang region of UDG detection probe was further confirmed by melting curve analysis. As presented in Fig. S8, the melting peak for the duplex DNA formed between UDG detection probe and blocker was observed at 37.5 °C (2, Fig. S8), but this melting peak disappeared after the treatment with UDG (3, Fig. S8). On the other hand, when UDG blocker containing thymine in place of uracil was employed as a control, the melting peak at 40.5 °C was retained even after the treatment with the UDG (4 and 5, Fig. S8). Overall, these observations demonstrate that target enzyme, UDG denatures the duplex overhang region of UDG detection probe by cleaving out uracil bases and consequently triggers DNA polymerase activity.

Based on the results summarized above, the detection sensitivity was determined by time-dependent measurement of fluorescence intensity from TaqMan probe as a function of UDG concentrations. The results show that the fluorescence signal change (F_{50}-F_0) increases with increasing concentrations of UDG up to 0.3 U/mL, and then reaches a plateau at concentrations over 0.3 U/mL (Fig. S9). An excellent linear relationship (R^2=0.9888) exists in the range of 0–0.3 U/mL and the limit of detection (LOD) (3σ/slope) is ca. 0.024 U/mL (Table S4), a value that is comparable or lower than those reported from other UDG activity assays (Hu et al., 2011; Leung et al., 2013b; Liu et al., 2007; Ono et al., 2012).

Next, in order to assess the specificity of the new assay method, other enzymes including human alkyladenine DNA glycosylase (hAAG) (3), human 8-oxoguanine DNA glycosylase 1 (hOGG1) (4), formamidopyrimidine-DNA glycosylase (Fpg) (5), BamHI (6), Exo I (7), and Lambda exonuclease (8) were examined for their capabilities to activate DNA polymerase, which were then compared with that of UDG (2). Analysis of the plots displayed in Fig. S10 shows that only UDG induces the high fluorescence signal change while other enzymes are not able to activate DNA polymerase and produce quite reduced fluorescence signal change even at ten times higher concentration than that of UDG. This result clearly confirms the excellent specificity of our new assay toward target enzyme, UDG.

Finally, studies were carried out to verify the utility of this method in screening potential UDG inhibitors, which is important for the development of new therapeutic agents such as antibiotics and anticancer drugs (Goffin and Eisenhauer, 2002; Kazantsev and Thompson 2008). For this purpose, uracil glycosylase inhibitor (UGI) was employed as a model inhibitor and its inhibition against UDG was investigated using our new assay method (Leung et al., 2013b). The direct effect of UGI onto DNA polymerase was first examined and the control experimental results indicate that UGI has no influence on the activity of DNA polymerase (Fig. S11). We then measured the fluorescence response of this system as a function of UGI concentrations. As presented in Fig. S12, the relative activity of UDG, represented as a ratio of the fluorescence signal change (F_{50}-F_0) reduced by the presence of UGI to the initial fluorescence signal change in the absence of UGI, was found to decrease as the concentration of the inhibitor increased (Baek et al., 2013; Lee et al., 2015). These results demonstrate that our method could be successfully employed to study enzyme inhibition for the development of anticancer drugs. In addition, we applied our assay for the detection of UDG activities directly associated with cells by employing the human lung epithelial cancer (A549) cells (Liu et al., 2007). As shown in Fig. S13, cell-free extracts (2 μg) of A549 cells induced the high fluorescence signal change (2, Fig. S13), which was not observed when cell-free extracts were applied to the control UDG blocker in which thymine replaced uracil (3, Fig. S13). To further demonstrate the practical utility of this sensor, we also applied our method for the assay of the human serum samples which may contain a lot of biological substances interfering with the reliability of the assay. The results listed in Table S5 confirm that the system is still capable of reliably determining UDG activity in the serum sample (Lu et al., 2015).

4. Conclusions

In the study described above, we have developed a novel, universal, fluorescence turn-on system for enzyme assay. The method relies on the new finding that target enzymes could modulate DNA polymerase activity by destabilizing detection probe derived from DNA aptamer specific to DNA polymerase containing the overhang sequence and the complementary blocker DNA. This phenomenon is monitored by the separated signal transduction module, which relies on the primer extension reaction coupled with TaqMan probe. Based on this design principle, we have successfully determined the activities of two target enzymes, Exo I and UDG in a cost-effective manner. Importantly, since this system consists of the decoupled target recognition and signal transduction steps, it can be applied for the assay of numerous different enzyme activities by simply designing the detection probe to be destabilized by the corresponding enzymes, while keeping the same signal transduction element. We expect that this approach could be generalized to develop a new method to assay other DNA-related enzymes and even to detect other biological molecules such as proteins and cells.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.07.038.

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