A Repeat Protein-Based DNA Polymerase Inhibitor for an Efficient and Accurate Gene Amplification by PCR

Da-Eun Hwang,1 Yong-Keol Shin,2 Palinda Ruvan Munasingha,1 So-Yeon Park,2 Yeon-Soo Seo,1 Hak-Sung Kim1

1Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Korea; telephone: +82-42-350-2616; fax: +82-42-350-2610; e-mail: hskim76@kaist.ac.kr
2Enzynomics, Inc., Yuseong-gu, Daejeon, Korea

ABSTRACT: A polymerase chain reaction (PCR) using a thermostable DNA polymerase is the most widely applied method in many areas of research, including life sciences, biotechnology, and medical sciences. However, a conventional PCR incurs an amplification of undesired genes mainly owing to non-specifically annealed primers and the formation of a primer-dimer complex. Herein, we present the development of a Taq DNA polymerase-specific repebody, which is a small-sized protein binder composed of leucine rich repeat (LRR) modules, as a thermolabile inhibitor for a precise and accurate gene amplification by PCR. We selected a repebody that specifically binds to the DNA polymerase through a phage display, and increased its affinity to up to 10 nM through a modular evolution approach. The repebody was shown to effectively inhibit DNA polymerase activity at low temperature and undergo thermal denaturation at high temperature, leading to a rapid and full recovery of the polymerase activity, during the initial denaturation step of the PCR. The performance and utility of the repebody was demonstrated through an accurate and efficient amplification of a target gene without nonspecific gene products in both conventional and real-time PCRs. The repebody is expected to be effectively utilized as a thermolabile inhibitor in a PCR.


© 2016 Wiley Periodicals, Inc.

KEYWORDS: repebody; thermolabile inhibitor; DNA polymerase; hot start PCR

Introduction

A polymerase chain reaction (PCR), a method for amplifying a specific gene, is most widely used in biosciences, biotechnology, and medical sciences (Erlich et al., 1991; Mackay et al., 2002; Yamamoto, 2002). A thermostable DNA polymerase from Thermus aquaticus enables a robust and efficient PCR to be conducted at high temperature, and consequently mostly employed (Chien et al., 1976; Saiki et al., 1988). A PCR consists of three repeated processes: denaturation, primer annealing, and extension. In a conventional PCR, primers are known to be non-specifically annealed to a template or/and a primer-dimer complex formed during the early stage of DNA denaturation, and the resulting complex acts as a template for other primers (Das et al., 1999; Huang et al., 2014). This usually leads to an amplification of undesired genes by Taq DNA polymerase at lower temperature than normal DNA extension temperature, resulting in a reduction in the efficiency and accuracy of the PCR.

As an approach to avoid the amplification of undesired genes, many methods have been developed, which are collectively termed a hot start PCR (Paul et al., 2010). The earliest attempts were to pre-heat PCR components at 95°C, followed by addition of a Taq DNA polymerase (D’Aquila et al., 1991) or to rely on a physical separation of PCR components using paraffin wax or agarose beads (Chou et al., 1992; Hébert et al., 1993). Despite a hot start effect, these methods required an additional handling step and are difficult to achieve, mainly owing to a re-hardening problem of the wax or beads used during the thermal cycling process. The use of a chemically inactivated Taq DNA polymerase was revealed to have a hot start effect to a certain degree (Birch, 1996; Moretti et al., 1998), but requires a longer period of time (10–15 min at 95°C) to reactivate the Taq DNA polymerase activity, resulting in depurination and a cleavage of the DNA (Cheng et al., 1994). Neutralizing antibodies against a Taq DNA polymerase have been employed as thermolabile inhibitors (Dahiya et al., 1995; Kellogg et al., 1996).
et al., 1994; Mizuguchi et al., 1999; Scalise et al., 1994). However, antibodies have certain drawbacks, including a high production cost, difficulty in engineering, and a tendency to aggregate.

We previously developed a repeat protein scaffold, called a repebody, which is composed of leucine rich repeat (LRR) modules, as an alternative to antibodies (Lee et al., 2012). The repebody scaffold was shown to have desirable properties as a protein binder, including a high expression level in bacteria, a high degree of stability, and ease of engineering through the use of a modular evolution approach (Heu et al., 2014; Lee et al., 2014, 2015). Herein, we present the development of a Taq DNA polymerase-specific repebody as a thermolabile inhibitor for an efficient and accurate amplification of a target gene by PCR. We selected a repebody that specifically binds to a Taq DNA polymerase through a phage display and increased its affinity to up to 10 nM using a modular evolution approach. We demonstrated the performance and utility of the repebody by showing an efficient and accurate amplification of a target gene without undesired gene products in both conventional PCR and real-time PCRs. The details are reported herein.

**Experimental Section**

**Construction and Phage Display of a Repebody Library**

We constructed a phage-displayed repebody library as described in our previous work (Lee et al., 2012). Briefly, the primers used for the library construction of LRRV2 and LRRV3 modules are the same as those of our previous study (Lee et al., 2007), and four variable sites on each LRRV1 and LRR1 modules were randomized using mutagenic primers with the following sequences:

LRRV1 module: GC ATT CAG TAT CTG CCG AAT GTT CGT CAG TAT CTG CTG NNK CTG NNK CTG NNK AAC AAA CTG CAT GAC ATC TCG GCA C

LRR1 module: GTC ACG CAA AAT GAA CTG AAC AGT ATT GAC NNK ATC NNK GNK NNK TCC GAT ATC AAA TCA GTG CAA GGC ATT CAG T

The DNA fragments of the repebodies were cut using EcoRI and Xhol, and cloned into a pBEL118 N vector, followed by a transformation into *Escherichia coli* (E. coli) strain XL1-blue (Stratagene, La Jolla, CA) through electroporation. The cells were grown until OD600 reached 0.6, and superinfected with a VCSM13 helper phage (Stratagene) overnight at 4°C. Following incubation with TPBSA (PBS containing 0.05% Tween 20 and 1% BSA) for 2 h at room temperature, 1 mL of a phage solution (1012 cfu/mL) was incubated for 2 h at room temperature. After washing with TPBS three times, the phages were eluted by adding 1 mL of 0.2 M glycine-HCl (pH 2.2) and neutralized with 60 μL of Tris–HCl (pH 9.0). *E. coli* XL1-blue was infected by the eluted phages, and the infected cells were grown on 2XYT agar plates containing 50 μg/mL of carbenicillin, 10 μg/mL of tetracyclin and 1% glucose (2XYT/ATG) overnight at 30°C. The cells were used for an estimation of the phage titers and the next round of panning. After the fifth round, 96 colonies were randomly selected and grown in a 2XYT/ATG medium in a 96-well plate (Nunc, Roskilde, Denmark). Following helper phage infection, kanamycin was added at a final concentration of 50 μg/mL, and cells were incubated overnight at 30°C. The phage-displayed repebodies in the supernatant were applied to a phage enzyme-linked immunosorbent assay (ELISA) for the selection of repebodies specific to Taq DNA polymerase.

**Selection of Repebodies Specific for Taq DNA Polymerase**

Repebodies specifically binding to Taq polymerase were selected through a panning process described in our previous work (Lee et al., 2012, 2014). Briefly, 1 mL of a Taq DNA polymerase (100 μg/mL) was immobilized in 5 mL immune-tubes (Greiner, Germany) overnight at 4°C. Following blocking with TPBSA (PBS containing 0.05% Tween 20 and 1% BSA) for 2 h at room temperature, 1 mL of a phage solution (1012 cfu/mL) was incubated for 2 h at room temperature. After washing with TPBS three times, the phages were eluted by adding 1 mL of 0.2 M glycine-HCl (pH 2.2) and neutralized with 60 μL of Tris–HCl (pH 9.0). *E. coli* XL1-blue was infected by the eluted phages, and the infected cells were grown on 2XYT agar plates containing 50 μg/mL of carbenicillin, 10 μg/mL of tetracyclin and 1% glucose (2XYT/ATG) overnight at 30°C. The cells were used for an estimation of the phage titers and the next round of panning. After the fifth round, 96 colonies were randomly selected and grown in a 2XYT/ATG medium in a 96-well plate (Nunc, Roskilde, Denmark). Following helper phage infection, kanamycin was added at a final concentration of 50 μg/mL, and cells were incubated overnight at 30°C. The phage-displayed repebodies in the supernatant were applied to a phage enzyme-linked immunosorbent assay (ELISA) for the selection of repebodies specific to Taq DNA polymerase.

**Phage-Based Enzyme-Linked Immunosorbent Assay (Phage-ELISA)**

A 96-well plate (Nunc) was coated with 1 μg/mL of Taq DNA polymerase overnight at 4°C. The plate was blocked with TPBSA for 1 h at room temperature, and repebody-displaying phages were incubated for 1 h. Following a washing using TPBS, an HRP-conjugated anti-M13 monoclonal antibody (GE healthcare, Buckinghamshire, UK) was incubated for 1 h at room temperature. After washing with TPBS five times, 100 μL of a 3,3',5,5'-tetramethylbenzidine solution (TMB; Sigma–Aldrich, St. Louis, MO) was added for signal generation. The reaction was stopped by adding 100 μL of 1 M sulfuric acid, followed by an absorbance measurement at 450 nm using an Infinity M200 plate reader (Tecan, Durham, NC).

**Competitive Phage-ELISA**

A competitive phage-ELISA was carried out using a procedure similar to that of a phage-ELISA. For the competition, JumpStart Taq antibody (Sigma–Aldrich, Yongin, Korea) was added to the solution of phage-displayed repebodies at a final concentration of 100 μg/mL, and the absorbance was measured at 450 nm through a similar procedure as a phage ELISA using an Infinity M200 plate reader (Tecan).

**Protein Expression and Purification**

Repebodies were cloned into a pET21a vector (Invitrogen, Carlsbad, CA) with a C-terminal His6 tag for purification, and transformed into *E. coli* Origami B (DE3). The transformed cells were seeded and cultured until OD600 reached 0.5–0.7. After the addition of 0.5 mM
IPTG for induction, the cells were grown further at 18°C for 16 h. Cells were harvested and disrupted using sonication. Following centrifugation at 16,000 rpm for 1 h, supernatants were incubated with Ni-NTA resin (Qiagen, Chatsworth, CA), and eluted using a Tris–HCl buffer containing 200 mM NaCl and 250 mM imidazole. The eluted proteins were further purified through size exclusion chromatography using Superdex 75 (GE healthcare) in a 20 mM HEPES-KOH buffer (pH 7.9) supplemented with 200 mM KCl and 1 mM EDTA.

A full-length Taq DNA polymerase was purified as described previously with a slight modification (Datta and LiCata, 2003). The gene encoding for the full-length Taq DNA polymerase was cloned into a pET21a vector between the Ndel and XhoI restriction sites and transformed into E. coli BL21 (DE3)-RIL cells (Stratagene). The cells were disrupted through heating and subjected to centrifugation, and the supernatant was loaded onto a Q sepharose (Lyamichev et al., 1999) and TaqC (amino acids 290–329) Taq DNA polymerase derivatives, TaqN (amino acids 1–83), were resuspended in T300 (50 mM Tris-HCl (pH 7.8), 300 mM NaCl) and additional 3 h and collected through centrifugation. A cell pellet was pre-incubated with a slight modification at 16,000 rpm for 1 h, supernatants were incubated with Ni-NTA column. The column was washed successively with 50 mM NaCl in 50 mM Tris (pH 7.8). The activity of Taq DNA polymerase was measured, and active fractions were pooled. The pooled fractions were stored at −70°C until use. The truncated Taq DNA polymerase derivatives, TaqN (amino acids 1–293) (Lyamichev et al., 1999) and TaqC (amino acids 290–832), were amplified using a PCR, and the resulting genes were inserted into Ndel and XhoI sites in plasmid pET28a with an N-terminal His6 tag.

Isothermal Titration Calorimetry (ITC) Analysis
The binding affinity was determined using MicroCal iTC200 (GE healthcare) at 25°C. Typically, 0.2 mM of a repebody was titrated with 0.02 mM of a Taq DNA polymerase in a 20 mM HEPES buffer. The data were analyzed using MicroCal Analysis Software (GE Healthcare), and fitted to a “one-set binding model.”

Circular Dichroism (CD) Analysis
CD spectra of the repebodies were recorded from 190 to 270 nm at 25°C with a J-815 CD spectrometer (Jasco, Japan) to check their secondary structures. For the thermal denaturation experiments, 1 mg/mL of repebodies were brought from 30°C to 80°C, and the molar ellipticity at 222 nm was measured. The melting temperature was determined using a thermal denaturation analysis program (Jasco).

Polymerase Activity Assay
Taq polymerase activity was determined using a trichloroacetic acid (TCA) precipitation assay with a slight modification (Kong et al., 1993). The polymerase-repebody complex was prepared through a pre-incubation of a Taq DNA polymerase (200 ng) and various amounts of repebody on ice for 15 min. The polymerase-repebody complex was mixed with a reaction mixture (50 μL) containing 1 nmol of dNTP each, 12.15 pmol of [3H]dTTP (0.1 Ci/mmol), and 0.2 mg/mL of activated calf thymus DNA (Aposhian and Kornberg, 1964) followed by incubation at 37°C for 30 min. The reaction was stopped by the addition of an 11X stop buffer (110 mM EDTA, 2.2% SDS), and the polymerase activity was determined using a scintillation counter Tri-Carb 2910TR (Perkin Elmer, Norwalk, CT).

Gene Amplification by PCR
For a standard PCR to evaluate the performance of selected repebodies, a primer set was designed based on the human GAPDH region (forward primer, 5′-CAAGGAATTTCGCTAGCA-3′; reverse primer, 5′-AGGGTCTACATGGCAACTG-3′). A reaction mixture (20 μL) contained 10 mM Tris–HCl (pH 8.3), 20 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 pmol/μL of a forward and reverse primer, and 1 unit of a Taq polymerase-repebody complex (molar ratio 1:10). Serially diluted human cDNA was added to a reaction mixture followed by a conventional PCR in a T100 Thermo Cycler (Bio-rad) through the following program: 95°C for 1 min; 45 cycles of 95°C for 10 s, 60°C for 15 s, 72°C for 20 s. A reaction mixture containing 0.5X SYBR green I dye (Final conc., Invitrogen) was used for the real-time PCR with additional melting curve analysis step (CFX96 Real-Time PCR Detection System, Bio-rad). To determine the reactivation time of a Taq DNA polymerase-repebody complex, a TaqMan probe real-time PCR was carried out using the following oligonucleotides: forward primer, 5′-CCTGGACACC-CAGCACAAT-3′; reverse primer, 5′-GCGTATCCATGCTGTTGAAA-3′; and TaqMan probe, 5′-FAM-ATCAAGATCATGGTCTCCTCTGAGCCGCGCGTTTAMARA-3′. The reaction conditions were the same as a standard PCR with a modification of 50 amplification cycles.

Results and Discussion
Selection of Repebodies Specific for Taq DNA Polymerase Using a Phage Display
We selected repebodies specifically binding to Taq DNA polymerase using a phage display. A phage library was constructed by introducing random mutations into three variable sites on each the LRRV2 and LRRV3 module (Fig. 1a). To select polymerase clones specific for a Taq DNA polymerase, individual phage-displayed repebodies were analyzed using a phage-ELISA. Through a phage display process, we isolated 45 positive clones showing more than a 20-fold higher signal compared to bovine serum albumin (BSA) and determined their DNA sequences. Among them, r-G6 was selected based on sequence clustering, and its specificity of r-G6 toward the Taq DNA polymerase-repebody complex was mixed with a reaction mixture (50 μL) containing 1 nmol of dNTP each, 12.15 pmol of [3H]dTTP (0.1 Ci/mmol), and 0.2 mg/mL of activated calf thymus DNA (Aposhian and Kornberg, 1964) followed by incubation at 37°C for 30 min. The reaction was stopped by the addition of an 11X stop buffer (110 mM EDTA, 2.2% SDS), and the polymerase activity was determined using a scintillation counter Tri-Carb 2910TR (Perkin Elmer, Norwalk, CT).
Affinity Maturation by a Modular Evolution

To develop a repebody that inhibits more strongly Taq DNA polymerase activity, we intended to increase the binding affinity of r-G6 for Taq DNA polymerase up to around 10 nM by taking into account previously reported antibodies (Mizuguchi et al., 1999; Scalice et al., 1994). In our previous study, we developed a modular evolution approach for the affinity maturation of a repebody, which leads to a step-wise optimization of the binding interface for a target in a module-by-module manner (Heu et al., 2014; Lee et al., 2014, 2015). For this, a repebody library was constructed by randomizing four variable sites on a nearby LRRV1 module of r-G6 (Fig. 1a), followed by phage display selection using a panning process. To select repebodies with a higher affinity, r-G6 was used as a competitor during the panning process. After five rounds, we selected three clones showing higher signals in a phage-ELISA. Among them, r-G6E1 was selected because it has the highest binding affinity of 37 nM for the Taq DNA polymerase (Fig. 1c and Supplementary Fig. S1b). To further increase the binding affinity of r-G6E1, a similar process was repeated for an additional LRR1 module of r-G6E1. Among seven clones, r-G6E1H8 was shown to have the highest binding affinity at about 10 nM (Fig. 1c and Supplementary Fig. S1c), which corresponds to a more than 13-fold increase compared to r-G6. This result demonstrates that a modular evolution approach is effective for increasing the binding affinity of a repebody for a Taq DNA polymerase owing to its structural feature.

Characterization of Selected Repebodies

We checked the specificity of r-G6E1H8 for a Taq DNA polymerase through a phage-ELISA using a phage-displayed r-G6E1H8. As shown in Figure 2a, r-G6E1H8 exhibited a high specificity toward the Taq DNA polymerase, giving rise to negligible cross-activity for the other proteins. Next, we characterized the biochemical properties of repebodies (r-G6, r-G6E1, and r-G6E1H8) selected at each round of modular evolution. After expression in E. coli, repebodies were purified using a Ni-NTA affinity chromatography and size-exclusion chromatography (SEC). The size-exclusion chromatogram and SDS–PAGE analyses showed that each of them displayed a monomeric peak on size in SEC, and appeared as a single band on the SDS–PAGE (Fig. 2b). In addition, circular dichroism (CD) analysis revealed that the repebodies have negligible changes in their secondary structure compared to the template scaffold (Fig. 2c), and the melting temperatures (Tm) of the repebodies were estimated to range from 60°C to 65°C (Fig. 2d). In particular, Tm of r-G6E1H8 was measured to be 62.0°C, implying that it acts as a desirable thermolabile inhibitor against the Taq DNA polymerase in a hot start PCR.

Inhibitory Effects of Repebodies Against Taq DNA Polymerase

In order for a repebody to be used in a hot start PCR, a repebody should suppress the Taq DNA polymerase activity at low temperature. In this regard, we assessed the inhibitory effect of repebodies with varying affinities against the Taq DNA polymerase through a TCA precipitation assay. The polymerase activity in the presence of a repebody was determined using an isotope-labeled dTTP and activated calf thymus DNA (Fig. 3a). As a result, r-G6E1H8 was shown to have the most significant inhibitory effect above 95%, whereas r-G6E1 and r-G6 resulted in a decrease in the polymerase activity by about 48% and 11%, respectively, which indicates an affinity-dependent inhibitory effect of a repebody. An off-target repebody as a negative control had no inhibitory effect (data not shown). To obtain insight into the inhibition mode of a repebody against a Taq DNA polymerase, we investigated the domain of the Taq DNA polymerase to which the r-G6E1H8 binds.

Figure 1. Selection and affinity maturation of a repebody specifically binding to Taq DNA polymerase. (a) Mutation modules and sites of a repebody for a library construction at each round of modular evolution. The modules used for library construction and the repebody selected at each round are shown. (b) Competitive phage-ELISA using a commercial antibody (A and b) as a competitor. (c) Changed amino acid sequences and the binding affinities of selected repebodies at each round of modular evolution. Binding affinities were determined by ITC.

<table>
<thead>
<tr>
<th>Repebody</th>
<th>Affinity (nM)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>r-G6</td>
<td>134.4</td>
<td>1.0</td>
</tr>
<tr>
<td>r-G6E1</td>
<td>37.5</td>
<td>3.6</td>
</tr>
<tr>
<td>r-G6E1H8</td>
<td>10.3</td>
<td>13.0</td>
</tr>
</tbody>
</table>
Taq DNA polymerase is known to have two different catalytic activities: 5' to 3' exonuclease and DNA polymerase activities. It is therefore likely that r-G6E1H8 inhibits Taq DNA polymerase activity by blocking either exonuclease or DNA polymerase activity. We constructed two differently truncated polymerases: TaqN (amino acids 1–293) and TaqC (amino acids 290–832) based on the structural analysis (Lyamichev et al., 1999). Each TaqN and TaqC possesses a domain responsible for a 5' to 3' exonuclease and DNA polymerase, respectively. As a result of a phage-ELISA, r-G6E1H8 was shown to have a high binding activity for TaqC, while exhibiting a much weaker interaction with TaqN (Fig. 3b). Based on the results, it seems that the repebody inhibits the DNA polymerase activity rather than the 5' to 3' exonuclease activity.

**Rapid Recovery of Taq DNA Polymerase Activity From Inhibition**

As the repebody was revealed to efficiently inhibit the polymerase activity at low temperature, we next investigated whether the DNA polymerase activity is recovered from inhibition by a repebody as a thermostable inhibitor at a high temperature. A rapid and full recovery of the DNA polymerase activity from inhibition by a repebody during the initial denaturation step is critical to avoid the problems in a hot start PCR such as depurination and cut of DNA. We quantified the target gene amplification based on the cycle threshold (Ct) value under standard PCR conditions (10 mM Tris-HCl (pH 8.3), 20 mM KCl, and 1.5 mM MgCl2) with different initial denaturation times in the presence of r-G6E1H8. Non-specific amplification of a target gene might lead to experimental results. We thus employed a TaqMan probe assay with sufficient amount of a template DNA (10 ng of human cDNA). Given a predetermined template amount, the Ct value was fixed at 25–27 cycles, and the initial denaturation time varied from 0 to 15 min. The Taq polymerase without inhibitors was used as a control. As a result, r-G6E1H8 was shown to efficiently lead to the amplification of a human GAPDH gene in the presence of a Taq DNA polymerase with a fixed Ct value regardless of the initial denaturation time. In addition, the Ct values of the Taq polymerase in the presence of
r-G6E1H8 were shown to be almost same as that of the Taq DNA polymerase alone (Fig. 4), which indicates a rapid and full recovery of the polymerase activity from the inhibition by the repebody as temperature rises. A similar result was observed for another gene coding for human β-actin (Supplementary Fig. S2). Moreover, the time for a full restoration of Taq polymerase activity of the repebody was almost similar to that of commercially available antibody for amplification of human GAPDH and β-actin gene (Fig. 4 and Supplementary Fig. S2). It was reported that a full recovery of the polymerase activity usually requires at least 1 min in a hot start PCR in the presence of antibodies (Paul et al., 2010). On the other hand, when a chemically inactivated Taq DNA polymerase was used, at least 10 min was required to reach a full restoration of the polymerase activity under the same conditions (Fig. 4 and Supplementary Fig. S2). It is likely that r-G6E1H8 effectively inhibited the polymerase activity at low temperature through a non-covalent binding to the Taq DNA polymerase, and underwent denaturation at a temperature higher than its Tm, leading to a rapid and full recovery of the polymerase activity during the initial denaturation step of the PCR. A rapid restoration of the polymerase activity is essential to minimize the problems in a hot start PCR such as DNA damages and to shorten the total reaction time.

**Performance of a Repebody in a Hot Start PCR**

We further examined the performance of three repebodies (r-G6, r-G6E1, and r-G6E1H8) with different binding affinities for Taq DNA polymerase as a thermolabile inhibitor in a hot start PCR. The efficiency and accuracy of a target gene amplification are known to significantly decrease at a relatively small amount of a template. When the amount of a template is limited, thus a hot start PCR is widely employed. First, we conducted a conventional PCR using different amount of cDNA from HeLa cells as a template with GAPDH primers under the standard PCR conditions. Following the initial denaturation step at 95°C for 1 min, a 45-cycle PCR amplification was carried out using the following temperature profile: denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s. As shown in Figure 5, GAPDH gene was efficiently amplified in the presence of a repebody for a relatively sufficient amount of template DNA (100 pg). However, as the amount of template DNA decreased (10, 1, and 0 pg), the amplification of GAPDH gene was reduced mainly owing to the primer-dimer formation. The suppression became severe when 10 pg of a template DNA was used in the presence of r-G6 or r-G6E1. On the other hand, the addition of r-G6E1H8 resulted in specific amplification of the GAPDH gene even from 10 pg of the template DNA. The highest performance in the hot start PCR was observed through the use of r-G6E1H8 compared to the two other repebodies.
Figure 5. Performance of a repebody as a thermolabile inhibitor in a conventional block PCR. The performances of the repebodies in a hot start PCR were evaluated using various amounts of template DNA ranging from zero to 100 pg. Ninety ng of a repebody was added to a reaction mixture for a PCR. The GAPDH gene was amplified using the following profile: 95°C for 1 min, then 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. An antibody was used as a positive control.

Figure 6. Performance of a repebody as a thermolabile inhibitor in a real-time PCR. The performances of the repebodies in a hot start PCR were evaluated with varying amounts of template DNA: (a) 100 pg, (b) 10 pg, (c) 1 pg, and (d) zero. The PCR conditions were the same as those of a conventional PCR. SYBR green was added to the PCR mixture, and the PCR was carried out in the presence of repebodies and an antibody. A PCR without a repebody was conducted as a negative control (NC). Symbols in b–d are the same as in (a).
No amplification of the GAPDH gene was observed without a repebody when 1 pg of template DNA was used. It is noteworthy that r-G6E1H8 showed a comparable performance as a commercial antibody in a conventional PCR, preventing amplification of non-specific genes more effectively than a commercial antibody in the absence of a template DNA.

We next examined the performance of repebodies as a thermolabile inhibitor in a real-time PCR using SYBR green as a signal generator. The same template and target primers were used as in the same manner in the conventional PCR. Similar to a conventional PCR, when a large amount of template DNA (100 pg) was used, the amplification plots and melting peaks were shown to be almost the same regardless of the repebody used (Fig. 6 a and Supplementary Fig. S3a). A similar result was observed with a template DNA of 10 pg (Fig. 6b and Supplementary Fig. S3b). As the amount of template DNA decreased to 1 pg, the melting peaks started to appear at a lower Tm value than that of the GAPDH gene mainly owing to a non-specific amplification of the template DNA without a repebody and in the presence of r-G6 (Fig. 6c and Supplementary Fig. S3c). Unlike an antibody-based hot start PCR, the use of a repebody resulted in negligible gene products in the absence of the template DNA (Fig. 6d and Supplementary Fig. S3d). The results indicate that r-G6E1H8 with a higher binding affinity for a Taq DNA polymerase shows better sensitivity and specificity in both conventional, and real-time PCRs compared to a commercial antibody, leading to amplification of the target gene with high efficiency and accuracy. It is thus expected that the r-G6E1H8 repebody will result in a significantly reduced false-positive rate in a PCR-based diagnostic system.

**Conclusion**

We demonstrated that a repebody specific for a Taq DNA polymerase can be used as a thermolabile inhibitor for amplification of a target gene with high fidelity and accuracy by PCR. A modular evolution approach was proved to be effective for increasing the binding affinity of a repebody owing to its structural feature. The repebody was shown to strongly inhibit a Taq DNA polymerase at low temperature and undergo denaturation near the melting temperature, leading to a rapid and full recovery of the polymerase activity during the initial denaturation step of the PCR. The repebody was revealed to suppress the DNA polymerase activity mainly by binding to a domain with polymerase activity. The repebody exhibited a comparable and even better performance as a thermolabile inhibitor in a hot start PCR compared to an antibody and chemical method in terms of efficiency, and accuracy of gene amplification in both conventional and real-time PCRs. Based on the results, it is likely that the developed repebody will offer certain advantages over the currently used methods, such as a low cost, no additional process steps, a simple preparation of the PCR mixture, and a shortening of the total reaction time. It is expected that the repebody can be effectively used as a thermolabile inhibitor in a hot start PCR for amplifying a target gene with high accuracy and efficiency for many areas of research.

This research was supported by INNOPOLIS Foundation grant funded by the Ministry of Science, ICT & Future Planning through Enzymotics with grant number 2014DD0017, Global Research Laboratory (NRF-2015K1A1A203346), Mid-career Researcher Program (NRF-2014R1A2A1A01004198) funded by the Ministry of Science, ICT & Future Planning, and Global Ph.D. Fellowship Program (NRF-2011-0008211) and Brain Korea 21 plus funded by Ministry of Education.

**References**


Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.