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Biochemical and Biophysical Research Communications 328 (2005) 265–272
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High fidelity PCR with an off/on switch mediated by proofreading polymerases combining with phosphorothioate-modified primer

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Received 17 December 2004
Available online 6 January 2005

Abstract

In the initial report, introducing a single phosphorothioate modification at the very 3′ terminus of the oligodeoxynucleotide primer has been shown to effectively protect the oligodeoxynucleotide degradation due to the 3′ exonuclease activity. In this study, we reported a novel finding that phosphorothioate modification at the 3′ end of primers could not only effectively prevent the primer from degradation, but could also mediate an off-switch extension by *Pfu* polymerase when primers also carry single or multiple mismatched bases located in the first eight bases of the 3′ terminus. This suggests that the combination of 3′ phosphorothioate-modified primers with exo$^+$ polymerases such as *Pfu* constituted an on/off switch, which allows perfectly matched primers to be extended but not mismatched primers. Furthermore, we found that polymerases with different fidelities showed different efficiencies in turning off mismatched-primer mediated extension. So we described here a SYBR green-based real-time quantitative PCR assay for the detection of abundance level of gene expression that did not require fluorescently labeled gene-specific probes or complicated primer combinations. The emergence of real-time quantitative RT-PCR technology is thus suited for a diverse application with a need for high-throughput methods to detect and quantify different gene expressions by way of simplicity, versatility, and accuracy, and thus could complement global microarray-based expression profiling strategies.

Keywords: Fidelity polymerases; Polymerase chain reaction; Phosphorothioate

With the success of the sequencing of the human genome and the concurrent advances in genomics, there arose an increasing need for cost-effective, high-throughput, and accurate technologies for probing the correlation between genotype and phenotype [1–3]. At the most basic level, mRNA is present in all of the genes expressed in a cell at a given time. RT-PCR allows for the simultaneous assessment of the transcription levels of tens of thousands of genes and has a wide dynamic range. Polymerase chain reaction (PCR) has been demonstrated to be a powerful method to amplify as little as a single copy of gene sequence from an entire mammalian genome. In theory, one single oligonucleotide primer could adequately represent a corresponding

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doi:10.1016/j.bbrc.2004.12.159

gene, so gene expression profiles could be satisfactorily characterized by checking their PCR products. The main problems of PCR, however, are its fidelity and specificity [4–7]. In regular PCR, primer can couple with kinds of templates. When the 3′ end of a primer complements its template perfectly the targeted product will be available. Whereas, the extension of a mismatched primer will produce non-specific products [8,9]. By using *Taq* polymerase without a proofreading function, the mismatched nucleotides will be kept away from occurring in extended primer products [10,22]. Proofreading enzymes utilize their 3′ to 5′ exonuclease activity to remove incorrectly incorporated bases. This activity, however, can also edit primers during setup and the start of PCR. 3′ mismatched-primer extension in assays of gene express profiling always produces false positive results, even under the strictly optimized conditions [11].

In the previous study, the primer at the 3′ end with a single phosphorothioate modification could effectively protect the oligodeoxynucleotide degradation from proofreading polymerase with 3′ terminal exonuclease activity [11] and increase product yields in multiply primed rolling circle amplification by Φ29 DNAP [12]. Thus, primers with 3′ phosphorothioate modification were chosen in SNP assay development, which markedly improved the specificity [13–15]. However, the potentially precise mechanism is still unclear. In our study, accumulating evidences demonstrated that, if a 3′ to 5′ exonuclease resistant reaction were introduced, the proofreading polymerase could effectively mediate an off switch of DNA polymerization, and only perfectly matched primers could extend from the on-switch effect. With the combination of perfectly matched and single or multiple mismatched primers, sophisticated amplicons are amplified using 4 types of DNA polymerases with or without internal 3′ to 5′ exonuclease activities, such as *Pfu*, Pyrobest, Vent, and *Taq* DNA polymerases. Sequence data confirmed that the application of the off/on switch in gene expression profiling not only helps to maintain the minimal misintegration during primer extension, but also obviously decreases the mispriming in the initiation stage of primer extension. The mispriming is well known to be a major mistake that cannot be avoided in conventional PCR, especially in multiplex template extension.

By using SYBR green I fluorescence dye as fluorescent reporter of the quantitative real-time PCR, we calculated the amplification efficiencies of mispriming through modified and unmodified primers with *Pfu* polymerase. This novel off-switch mechanism of extension ensures that products all come from perfectly matched primers and are amplified with an extremely low error rate. Combined usage of phosphorothioate-modified primers with *Pfu* polymerase and real-time RT-PCR offers a powerful tool to quantify the expression of differential genes with advantages of simplicity, versatility, and low price. Thus, the new off switch could complement global microarray-based expression profiling strategies.

Materials and methods

DNA polymerases. *Pfu* DNA polymerase was purchased from BBI, *Taq* polymerse and Vent DNA polymerase were purchased from New England Biolabs, Pyrobest polymerase was purchased from TaKaRa, and PCR amplifications were performed based on the manufacturer's protocols. The conditions for each enzyme described above are the optimal conditions suggested by the manufacturer.

Cell culture and samples. All cells (HEPG2 and ECV304) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% defined FBS (HyClone Laboratories), 100 U/ml each penicillin and streptomycin, and 2 mM GlutaMAX-1 (Gibco-BRL) in a humidified 37 °C incubator with 5% CO_2. Fresh tissue samples including vascular, liver, and lung were collected from 20 mice.

RNA preparation and reverse transcriptase-polymerase chain reaction. Total RNA of mouse liver, lung, vessels, and cells (HEPG2 and ECV304) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, respectively. The RNA concentration was determined by measuring the absorption at 260 nm. cDNAs were synthesized using 'SuperScript First-Strand Synthesis System for RT-PCR' (Invitrogen): 1 μg of total RNA was reverse-transcribed to cDNA by incubation for 60 min at 50 °C in a RT reaction mixture consisting of 5 pmol oligo($dT_{12–18}$) primer, 0.5 mM deoxynucleotide triphosphate, 50 U RNAsin, 50 U Superscript III reverse transcriptase, 5 mM $MgCl_2$, 1× RT buffer (50 mM KCl, 20 mM Tris–HCl, pH 8.4) (Invitrogen), and DEPC-treated water to a total volume of 20 μl.

Oligonucleotide primers and template sites. Caveolin-1 gene sequences between human and mice were compared, and their homogeneity was shown up to 92%. In primer design, we select some sequences that show difference at the 5′ or 3′ end between human and mice caveolin-1 genes. So primers from human caveolin-1 gene were considered to be mismatched primers of mouse caveolin-1 gene, and vice versa. The oligonucleotide primers used in this study are described in Tables 1, 2, 3. All phosphorothioate-modified and unmodified primers were synthesized by Sangon (Shanghai, China).

Primer extension reactions. *Taq* polymerase (NEB) or *Pfu* polymerase (BBI) with 3′ to 5′ exonuclease activity was used in a two-directional primer extension with annealing temperatures ranging from 50 to 65 °C. A 285 bp region of the mouse caveolin-1 gene was amplified by gradient RT-PCR with two phosphorothioate (PS)-modified primers: the sense primer (FP1: 5′ AGA CGA GGT GAC TGA GAA GC 3′) and the antisense primer (AP1: 5′ CCA GAT GTG CAG GAA GGA 3′) [Gi: 41223399]. The reactions included 10 pmol primers, 200 μM of each deoxynucleotide triphosphate, 5 μl buffer, 1 U *Taq* polymerase or *Pfu* polymerase, and 1 μl cDNA template in a final volume of 50 μl. These reactions were initiated with 2 min denaturation at 95 °C for 2 min, primer extension was then carried out for 30 cycles as follows: 30 s denaturation at 94 °C, 30 s annealing, and 40 s extension at 72 °C, and finally ended with a 10 min extension at 72 °C. PCR products were visualized using 2% agarose gel electrophoresis running at 5 V/cm. Selected PCR products were sent for sequence analysis.

Confirming the offlon switch. We tested whether extension could be stopped by phosphorothioate-modified primers with mismatched nucleotides at or near the 3′ terminus combined with polymerase with or without proofreading polymerase. The plasmid pcDNA3.1-caveolin-1 was chosen as the template. While the plasmid pcDNA3.1-caveolin-1 was used as a template, the sense primer designed for mouse caveolin-1 (FP1: 5′ AGA CGA GGT GAC TGA GAA GC 3′) did not perfectly match the human derived pcDNA3.1-caveolin-1, and showed

Table 1
Compared with homogenous caveolin-1 gene sequence between human and mouse in primer design; primer–template sequences used for measuring extension for matched and mismatched primer 3′ terminal bases

```
FP4         atgtctgggggcaaatacgtagactcggagggacatctctacac-3'
FP2     caccatgtctgggggcaaatacgta-3'
human: 1    atgtctgggggcaaatacgtagactcggagggacatctctacaccgttcccatccgggaa 60
           ||||||||||||||||||||||||| ||||||||||||||||| ||||||||||||||
mouse: 1    atgtctgggggcaaatacgtagactccgagggacatctctacactgttcccatccgggaa 60

                                          FP3  agacgagctgagcgagaagc-3'
human: 61   cagggcaacatctacaagcccaacaacaaggccatggcagacgagctgagcgagaagcaa 120
           ||||||||||||||||||||||||||||||||||||||||||||||| |||  |||||||||
mouse: 61   cagggcaacatctacaagcccaacaacaaggccatggcagacgaggtgactgagaagcaa 120
                                          FP1  agacgaggtgactgagaagc-3'

                                        AP4    ctcgaaggactaactctaagtca-5'
      AP5  agaaaggacgtgtagacc-5'         AP3  attctcgaaggactaactctaag-5'
human: 367 tctttcctgcacatctgggcagttgtaccatgcattaagagcttcctgattgagattcagt 426
           || |||||||||||||||| |||||||| ||||| ||||||||||||||||||||||||
mouse: 367 tccttcctgcacatctgggcggttgtaccgtgcatcaagagcttcctgattgagattcagt 426
       AP1 aggaaggacgtgtagacc-5'

                                    AP6 gtagttgaacgtctttctttatatt-5
                                  AP2 ggcgtagttgaacgtctttctttat-5
human: 481 gaagctgttgggaaaatattcagcaatgtccgcatcaacttgcagaaagaaatataa 537
           |||||| |||| || ||||||||||  ||||||||| |  ||||||||| |||| |
mouse: 481 gaagctattggcaagatattcagcaacatccgcatcagcacgcagaaagagatatga 537
```

Table 2
Different results from PS-modified matched human caveolin-1 gene primers extended by polymerases *Pfu* and *Taq* with the cDNA template of mice tissues

Sense primer		Antisense primer		*Pfu*	*Taq*
FP2 caccatgtctgggggcaaatacgta		AP4 actgaatctcaatcaggaagctc		+	+
FP2 caccatgtctgggggcaaatacgta		AP3 gaatctcaatcaggaagctcttA	−1	−	+
FP2 caccatgtctgggggcaaatacgta		AP5 ccagatgtgcaggaaAga	−3	−	+
FP2 caccatgtctgggggcaaatacgta		AP6 tTatatTtctttctgcAAgTtgatg	−6, 8, 9	−	+
FP3 agacgagcTgagCGagaagc	−8, 9, 13	AP4 actgaatctcaatcaggaagctc		−	+
FP2 caccatgtctgggggcaaatacgta		AP2 tatTtctttctgcAAgTtgatgcgg	−9, 11, 12	+	+

The capitalized nucleotide is the base mismatch at the 3′ end of primer from the mouse caveolin-1 template. Digit stands for mismatched position of primer 3′ terminus.

3′ mismatches on the −8, −9, and −13 base from the 3′ end of the primer. The antisense primer designed for mouse caveolin-1 (AP1: 5′ CCA GAT GTG CAG GAA GGA 3′) showed a mismatch on the third base pair from the 3′ end of primer comparing with the human caveolin-1 sequence. The expected size of product is about 285 bp. The phosphorothioate-modified and unmodified mismatched primers were used to amplify with two kinds of exo+ polymerases (*Pfu* and Vent) and an exo$^-$ polymerase (without proofreading activity), respectively. The plasmid DNA was diluted step by step in geometric proportion with 1:3.5 folds to form 9 kinds of templates. A 540 bp fragment of the human caveolin-1 gene was amplified by *Pfu* polymerase as positive control [GI: 16307193] with two perfectly matched human phosphorothioate-modified primers (FP2: 5′ CACC ATG TCT GGG GGC AAA TAC GTA 3′; AP2: 5′ TAT TTC TTT CTG CAA GTT GAT GCGG 3′) under the same template concentration, respectively.

Single or multiple mismatched primers' RT-PCR. In order to confirm that the polymerase proofreading activity and PS modification of primer are two key factors for off/on switch, a variety of primers were designed to match specifically to human caveolin gene different regions. However, when mouse cDNA is used as amplification template, there would be a single base or multiple bases' mismatch occurring in different positions of primers' 3′ terminus (Table 2), the corresponding region of the mouse caveolin-1 gene would be amplified by RT-PCR with *Pfu* and *Taq* polymerase, respectively.

Single mismatched-primer extension. To further prove the off/on-switch mechanism, modified and unmodified primers with a single base mismatch from the 1st base to the 12th base of their 3′ ends were used for primer extension. cDNAs from human cells (HEPG2, ECV304) and mouse tissue specimens including blood vessels, liver, and lung were used as templates, respectively. As illustrated in Table 3, PCR products were extended by polymerases with various proofreading activities (*Pfu*, Vent, Pyrobest, and *Taq* polymerases). For each enzyme, its efficiency was measured with templates in three kinds of concentrations. Furthermore, we also calculated the off/on-switch amplification

Table 3
Different results from PS-modified primers extended by polymerases with and without 3′ exonuclease activity

Mismatch position	PS-modified primer sequences	RT-PCR products			
		Pfu	Vent	Pyrobest	*Taq*
—	atgtctgggggcaaatacgtagactcggagggacatctctacac	+	+	+	+
−1 mm	atgtctgggggcaaatacgtagactcggagggacatctctacaG	−	−	−	+
−2 mm	atgtctgggggcaaatacgtagactcggagggacatctctacTc	−	−	−	+
−3 mm	atgtctgggggcaaatacgtagactcggagggacatctctaGac	−	−	−	+
−4 mm	atgtctgggggcaaatacgtagactcggagggacatctctTcac	−	−	+	+
−5 mm	atgtctgggggcaaatacgtagactcggagggacatctcAacac	−	−	+	+
−6 mm	atgtctgggggcaaatacgtagactcggagggacatctGtacac	−	−	+	+
−7 mm	atgtctgggggcaaatacgtagactcggagggacatcActacac	−	−	+	+
−8 mm	atgtctgggggcaaatacgtagactcggagggacatGtctacac	−	+	+	+
−9 mm	atgtctgggggcaaatacgtagactcggagggacaActctacac	+	+	+	+
−10 mm	atgtctgggggcaaatacgtagactcggagggacTtctctacac	+	+	+	+
−11 mm	atgtctgggggcaaatacgtagactcggagggaGatctctacac	+	+	+	+
−12 mm	atgtctgggggcaaatacgtagactcggagggTcatctctacac	+	+	+	+

The antisense sequence is AP4 (5′ actgaatctcaatcaggaagctc). The capitalized nucleotide is the single base mismatch at the 3′ end of primer.

efficiency of *Pfu* polymerase by using quantitative real-time PCR. Testing was performed by using SYBR green I detection on Stratagene's Mx4000 multiplex quantitative PCR system (Roche Applied Sciences).

Results

RT-PCR specificity comparison of PS primers with exo$^+$ or exo$^-$ DNA polymerases

To examine the specificity of PCR using exo$^+$ and exo$^-$ DNA polymerases, a two-step gradient PCR experiment was performed at various annealing temperatures from 50 to 65 °C. As shown in Fig. 1, PS primers combined with *Pfu* polymerase produced a single band at the designated molecular weight (lanes 1–5). The identity of the PCR product was confirmed by sequence analysis. However, multiple non-specific PCR products were produced by *Taq* polymerase, which was varied with different annealing temperatures (lanes 6–10 in Fig. 1). Although a conclusive reason for the production of these non-specific amplification products has not been identified, the results were extremely reproducible at those temperatures under a variety of reaction conditions (data not shown), and those non-specific amplification products were likely due to mispriming. However,

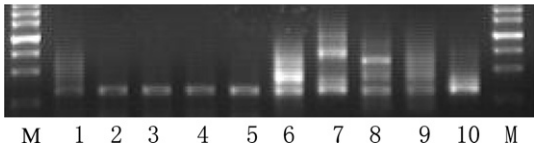

Fig. 1. Extension of perfectly matched primers with PS modification by exo$^-$ or exo$^+$ polymerase, respectively. Lanes 1–5: PS primer extension products at annealing temperatures of 50, 55, 58, 62, and 65 °C with *Pfu* exo$^+$ polymerase; lanes 6–10: PS primer extension products by *Taq* polymerase at corresponding annealing temperatures. All products from *Pfu* were with high specificity, on the contrary, a lot of side products were obtained with *Taq* polymerase.

phosphorothioate-modified primers coupled with *Pfu* polymerase could switch off the mispriming.

Validity of the offlon switch

As shown in Fig. 2, 3′ phosphorothioate-modified primers in combination with exo$^+$ polymerase demonstrated an attractive off/on-switch system for identification of matched and mismatched primers. In this study, the template plasmid containing human caveolin-1 DNA was serially diluted with the ratio of 1:3.5 to produce 9 kinds of different concentrations, and the product was amplified by *Pfu*, Vent or *Taq* polymerases with mismatched modified or unmodified PS primers (lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17 in Figs. 2A–D). The expected product is 285 bp. We observed that, perfectly matched PS primers (as positive controls) with *Pfu* polymerases produced specific PCR product (540 bp) (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 in Figs. 2A–D). Of note, the level of products is decreased corresponding to the template dilution. The relative copy of template in lanes 17 and 18 was at least assumed to be 1, and the template copies in lanes 1 and 2 could reach 22,519. The PCR extension of matched modified primer was triggered even as little as few molecules of starting template (lane 18 in Figs. 2A–D). This indicated that the extension sensitivity of perfectly matched primer would not be affected by PS modification. However, PCR could be extended effectively using mismatched PS primers by exo$^-$ polymerase (*Taq*) (Fig. 2C) or using unmodified primers by exo$^+$ polymerase (*Pfu*) (Fig. 2D). Once the primers were modified with phosphorothioate at the 3′ terminus, mismatched primers were not extended by either *Pfu* or Vent exo$^+$ polymerases (lanes 5, 7, 9, 11, 13, 15, and 17 in Figs. 2A and B) except in the presence of excess template (lanes 1 and 3 in Figs. 2A and B). The extension of primers with multiple bases' mismatch in 3′ terminus was efficiently blocked, even in the presence of

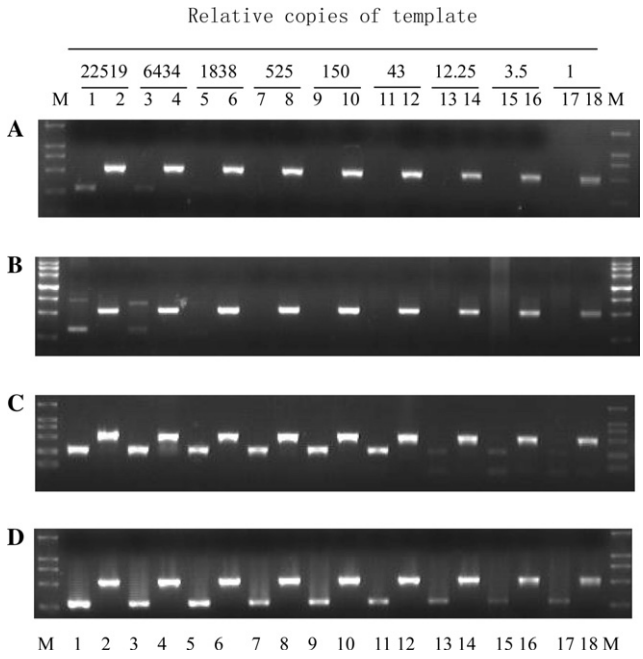

Fig. 2. Validity of the off/on switch. The template plasmid containing human caveolin-1 DNA was serially diluted with the ratio of 1:3.5 to produce 9 kinds of different concentrations. PCRs of even lane (lanes 2,4, 6, 8, 10, 12, 14, 16, and 18) in (A–D) were performed by using the perfectly matched PS primers (FP2 + AP2) and *Pfu* DNA polymerase. All matched PCR products were about 540 bp (shown in even lane). However, PCR amplifications of odd lane (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17) in (A–D) were performed by using mismatched primers (FP1 + AP1) with or without PS and different polymerases: (A) mismatched primers with PS + *Pfu*; (B) mismatched primers with PS + Vent; (C) mismatched primers with PS + *Taq*; (D) mismatched primers without PS + *Pfu*. All mismatched PCR products were about 285 bp (shown in odd lane). Lane M is shown as the DNA marker.

as many as 6434 copies of starting homologous template molecules. These observations allowed us to believe that, if a 3′–5′ exonuclease resistant reaction is introduced, proofreading polymerase is able to effectively mediate an off switch of mismatched DNA polymerization, and only perfectly matched primers can start the extension from the on-switch effect. The polymerase proofreading activity and PS modification are two key factors for off/on switch.

Effects of the extent of mismatch on off switch by pfu and PS primers

In the analysis of gene expression profiling, the possibility of the mismatch between primer and template takes place in a random manner and not only exists at the last base of 3′ terminus. To investigate further the ability of exo$^+$ polymerase with PS-modified primers to turn off non-specific extension of mismatched primers, we examined the influence of primers with various mismatches to template. In this experiment, the PCR primers were designed to show single mismatch (−1 or −3 of 3′ terminus) or multiple mismatches (Table 2)

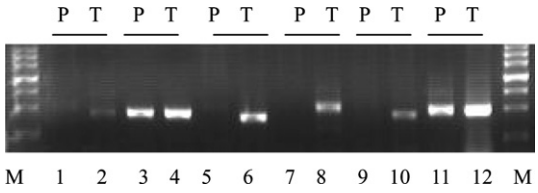

Fig. 3. Suppression comparison on DNA polymerization between *Pfu* and *Taq* with single or multiple mismatch PS primers. The PCR primers matched specifically to the human caveolin-1 gene at its 3′ terminus (Tables 1 and 2). cDNA templates from the mouse liver, lung, and blood vessel were utilized, and the PS primer with single or multiple mismatch at 3′ terminus and corresponding RT-PCR results are summarized as Table 2. *Pfu* was used in lanes 1, 3, 5, 7, 9, and 11; *Taq* was used in lanes 2, 4, 6, 8, 10, and 12. Primers FP2 + AP3 was used in lanes 1 and 2; FP2 + AP4 in lanes 3 and 4 as positive control; FP2 + AP5 in lanes 5 and 6; FP2 + AP6 in lanes 7 and 8; FP3 + AP4 in lanes 9 and 10; FP2 + AP2 in lanes 11 and 12.

when the cDNA from mouse liver, lung or blood vessel was used as templates. As shown in Fig. 3, PS-modified primers with mismatch started at −1, or −3, −6, −8 base of 3′ terminus were not extended by *Pfu* polymerase and no product was observed (lanes 1, 5, 7, and 9). However, PS-modified primers with multiple mismatches at −9, −11, and −12 bases of 3′ terminus could be extended by *Pfu* polymerase (lane 11), similar to perfectly matched primer (lane 3). In contrast, all types of mismatched primers could produce PCR products by *Taq* polymerase (lanes 2, 4, 6, 8, 10, and 12). This study suggested that off-switch effect of mismatched-primer extension contributing to pfu and PS-modified primers is related to the distance of mismatched base from 3′ terminus. That is, off-switch effect will disappear when the mismatched base is far away from the 3′ terminus (>−8).

Effects of single-base mismatch primer on off switch by various polymerases

To further investigate the influence of distance of mismatched base from 3′ terminus on off-switch ability of various exo$^+$ polymerases, we performed the PCR experiments using −1 to −12 single mutation mismatch primers with or without PS modification combining with exo$^-$ polymerase (*Taq*) or exo$^+$ polymerases (*Pfu*, Vent, and Pyrobest). As shown in Fig. 4, −1 to −12 single-base mismatched primers were extended by *Taq* polymerase (Fig. 4A) and sequence analysis showed that the extensive products were primer-dependent (data not shown). However, −1 to −12 single-base mismatch primers were extended by *Pfu* polymerase only when the primers were not modified with PS (Fig. 4B). Once the primers were modified with PS, those primers with mismatch site at −1 to −8 of 3′ terminus were not efficiently extended by *Pfu* polymerase (Figs. 4C and 5A), implicating that off-switch ability of *Pfu* polymerase is influenced by

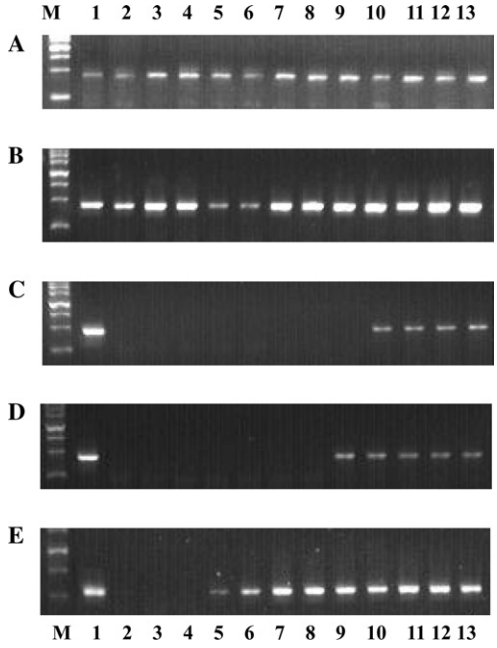

Fig. 4. Extension of 3′1–12 mono mismatch primer with *Pfu*, Vent, Pyrobest polymerase, and *Taq* polymerase. Lane 1 in (A–E) shows that perfectly modified match primer extension was used as a positive control; lanes 2–13 in (A) displays the results of *Taq* polymerase with 3′1–12 single mismatch PS primer; lanes 2–13 in (C–E) displays the results under the same condition as (A) except for *Pfu*, Vent, and Pyrobest exo$^+$ polymerases, respectively. Lanes 2–13 in (B) shows the results of *Pfu* polymerase with 3′1–12 single mismatch unmodified primer.

the distance of mismatched base from the 3′ terminus of primers. When the mismatched site is beyond −9 of the 3′ terminus, *Pfu* polymerase could trigger the extension of mismatched primer with PS modification the shown as a visible band in the gel (lanes 10–13 in Fig. 4C). Furthermore, real-time PCR analysis showed that the polymerization efficiency of *Pfu* polymerase decreased by showing that the peak-platform time was delayed from 13th cycle to 18th cycle (comparing the cover B with cover F in Fig. 5). However, mismatch at −10, −11, and −12 position of primer almost did not affect the amplification efficiency of PCR by comparing covers C, D, and E with cover F of positive control in Fig. 5, which implicated that the mismatch of primer at 5′ terminus did not significantly influence the extension efficiency. The practical significance of this finding is that, when we design a primer with 5-terminal mismatches, for instance the introduction of restriction endonuclease sites for gene cloning, the primer extension will run smoothly and will not be blocked by the off switch. Interestingly, different polymerases possess different abilities of turning off polymerization of mispriming. We observed that Vent polymerase could turn off the extensive reaction induced by −1 to −7 mismatched primers with PS modification (Fig. 4D). While Pyrobest polymerase only closed the polymerization induced by −1 to −3 mismatched primers (Fig. 4E). We referred to the Distance (nucleotide number) of the Mismatched Base from the 3′ Terminus of a primer (DMBT) as an index to evaluate the off-switch ability (OSA) of polymerases. The OSA of *Pfu*, Vent, Pyrobest, and *Taq* polymerases are summarized in Table 3.

Discussion

In this study, we found that most of the DNA polymerases could efficiently catalyze the extension of an unmodified primer with a single or multiple mismatched base pairs at the 3′ terminus. The exo$^-$ polymerase-catalyzed extension of mismatched primer produced a primer-dependent product, while the exo$^+$ polymerase-catalyzed extension of mismatched primer produced a template-dependent product [22]. Exo$^+$ polymerases with proofreading ability could fix the mismatched nucleotide of the 3′ terminus of a primer before extension according to the hybridized templates. Theoretically, enzymes with exonuclease activity first recognize the 3′

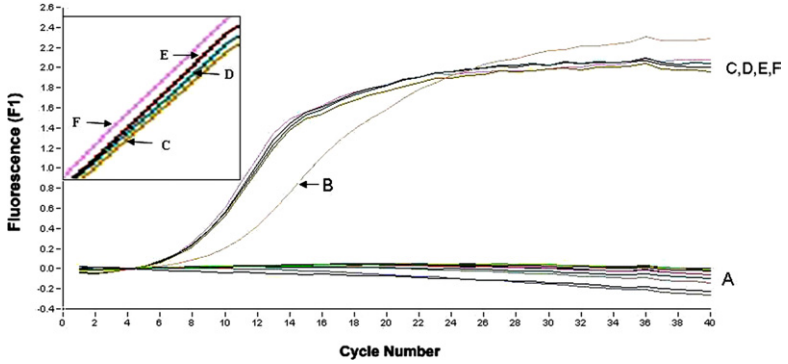

Fig. 5. Real-time RT-PCR with 3′ end of 1–12 mono mismatch PS primer by *Pfu*. (A) Fluorescence value of negative control and −1 to −8 position single mismatch primer extension respectively. (B) Fluorescence value of −9 position single mismatch primer extension. (C–E) Fluorescence values of −10, −11, and −12 position single mismatch primer extension, respectively. (F) Fluorescence value of perfectly matched PS primer extension (positive control).

terminus mismatch, then repair, and finally start to extend [16]. Thus, the polymerases containing internal 3′–5′ exonuclease activity can discriminate the non-complementary bases and keep the state of trying to excise them before extension by means of a 3′ to 5′ exonuclease resistant reaction. This provides an off-switch effect that blocks extension when primers were non-complementary to the template, and only allows perfectly matched primers to be extended. The stronger the proofreading activity of the polymerases is, the more powerful their ability of turning off mispriming. We further defined that the upstream regions of discrimination are in a pattern of enzyme-dependence and template-dependence. Our finding that *Pfu* polymerase could effectively discriminate the mismatched base within 8 nucleotides from the 3′ terminus of primers will provide a powerful tool to study specific gene expression profiling. Because the length of 4^8 nucleotides almost covers that of all human mRNA transcripts (about 20 kb), in theory, the use of off-switch platform consisting of exo$^+$ polymerases and primers with PS modification in RT-PCR can eliminate all non-specific products. Furthermore, since a pair of primers was usually used in PCR, 4^{16} nucleotides have just a binding site in the whole human gDNA, which may be the reason why other groups reported their magnitude improvement in SNP and SBE fidelity [10,13,14]. In conclusion, this off switch may provide a new sensitive and specific method in the analysis of gene expression. This method is specifically useful for gene expression profiling of low and very low-abundance gene products.

Primers used in the present experiments only contain one phosphorothioate-modified nucleotide at their 3′ end. Because conventional primer synthesis proceeds in the 3′ to 5′ direction, thus only four standard phosphorothioates would be required during synthesis [17]. The labeled primers could potentially be obtained from commercial oligonucleotide suppliers in a very cheap way. Nuclease-resistant modification is a critical requirement to enable the application of this off/on switch. Theoretically, a single Sp isomer at the last base of primer 3′ terminus is enough to be resistant to the proofreading exonucleases. However, we also found that a single PS-modified nucleotide could not completely block the proofreading effect of exo$^+$ polymerases [13,14,18,19] (data not shown). Therefore, more than one nucleotide modification at the 3′ terminus of mismatched primer would be required to avoid non-specific amplification due to non-absolute modification and self-degradation of primer (data not shown). It has been reported that there are additional feasibilities of synthesis of 3′ to 5′ exonuclease-resistant label [17,18]. We will explore these options in the near future.

Over the past 10 years, real-time PCR has been broadly used in the study of gene expression profiling because of its high specificity, efficiency, and reduplication, while its risk of carrying over contamination is minimized [20,21]. All real-time PCR systems rely on the detection and quantification of a fluorescent reporter used to detect PCR products as they accumulate within a closed reaction tube during real-time PCR. These include the non-specific DNA-binding fluorophores and the specific fluorophore-labeled oligonucleotide probes. However, the synthesis of specific detection probe (TaqMan or Molecular beacon) is very expensive, time-consuming, and hard to design in high-throughput and parallel format. SYBR green I fluorescence dye, which has the ability of binding specifically double DNA chain, is very cheap and easy to use. What will be the disadvantage is that SYBR green will bind to all types of double-stranded DNA in the reaction, such as primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. 3′ terminus of primer with phosphorothioate modification coupled with proofreading polymerase could switch off the extension reaction of mispriming and eliminate efficiently non-targeted products in PCR. Thus, SYBR green I combined with pfu and PS primers will be suitable and economic strategy to quantify gene expression levels, which is likely to be a cost-effective, unique high-throughput and high-accurate method.

Acknowledgments

The authors are indebted to Dr. Chen Yan in the University of Rochester for her critical discussions, suggestions, and valuable help in the preparation of the manuscript. This work was supported by the National Natural Science Foundation of China (30400265 and 30470719), the National Major Basic Research Program (973) of China (G2000056905) and the Natural Science Foundation of Hunan (03JJY3037).

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