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Single-base Discrimination Mediated by Proofreading Inert Allele Specific Primers

Chen Lin-Ling[‡], Jia Zhang[†,‡], Steve S. Sommer[§] and Kai Li[‡,§,*]

[†]Genomapping Inc., Tianjin, China

[‡]SNP Institute, Nanhua University, Hengyang, China

[§]Molecular Genetics and Human Genomics, City of Hope National Medical Center, Duarte, USA

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The role of 3' exonuclease excision in DNA polymerization was evaluated for primer extension using inert allele specific primers with exonuclease-digestible ddNMP at their 3' termini. Efficient primer extension was observed in amplicons where the inert allele specific primers and their corresponding templates were mismatched. However, no primer-extended products were yielded by matched amplicons with inert primers. As a control, polymerase without proofreading activity failed to yield primer-extended products from inert primers regardless of whether the primers and templates were matched or mismatched. These data indicated that activation was undertaken for the inert allele specific primers through mismatch proofreading. Complementary to our previously developed SNP-operated on/off switch, in which DNA polymerization only occurs in matched amplicon, this new mutation detection assay mediated by exo$^+$ DNA polymerases has immediate applications in SNP analysis independently or in combination of the two assays.

Keywords: Exo$^+$ polymerase, Inert primer, Off/on switch, Proofreading, SNP

Introduction

It is well known that the fidelity of DNA replication by proofreading polymerases is at least ten-fold higher than that by DNA polymerases lacking a proofreading function (Kunkel *et al*., 1987; Mattila *et al*., 1991; Goodman and Fygenson, 1998). However, in single nucleotide polymorphism (SNP) analysis, polymerases used in laboratory practice were exclusively exo$^-$ polymerases until 2003 (Li and Zhang, 2003; Zhang *et al*., 2003a). We recently advocated the use of exo$^+$ polymerases in SNP analysis and reduced practice of two SNP assays mediated by exo$^+$ polymerases: the 3' terminal labeled primer extension and the SNP operated on/off switch assay (Zhang and Li, 2001; Zhang *et al*., 2001; 2004). The latter is achieved by exo$^+$ polymerase via proofreading allele specific primers with a nuclease-resistant 3' terminal modification (Guo *et al*., 2003; Zhang and Li, 2003a; 2003b; Zhang *et al*., 2003b; 2003c). The advantage of high fidelity DNA polymerases in SNP assays and for mutation detection has now been recognized and applied to a variety of platforms, including microarrays, by several groups (Cahill *et al*., 2003; Di Giusto and King, 2003; 2004).

During the development of SNP assays utilizing exo$^+$ polymerases, we screened 3' terminal labeled, 3' phosphorothioate modified, nucleic acid locked, and 3' inactived allele specific primers by having a ddNMP. According to our results, the inert allele specific primers in combination with exo$^+$ polymerases sensitively detected single nucleotide mutations. Unlike the previously described SNP operated on/off switch, inert allele specific primers behave in a way complementary to that of 3' phosphorothioate-modified primers. In the case of inert primers, matched amplicons turn off and mismatched amplicons turns on DNA polymerization. Since inert allele specific primers efficiently block non-specific primer extension, this off/on switch sensitively and reliably detects mutations.

Materials and Methods

The amplicon set used in this study was obtained from Genomapping (Tianjin, China), and included two templates, one regular and one inert allele specific sense primer, and one regular antisense primer. The templates of these amplicons were in vitro synthesized, as previously described (Zhang *et al*., 2003d), and had the following sequences (polymorphisms are in italicized bold font): 5'-GGCTGAGGCAGGAGAATGGCGTGAACCCGGGAG

*To whom correspondence should be addressed.
Tel: 858-385-0216
E-mail: kaili34@yahoo.com

Table 1. An off/on switch for DNA polymerization using proofreading inert allele specific primers

	Template 1	Template 2
	5'…GATCTGGG...3'	5'…GATCTGGC…3'
exo⁻ pol	not extended	not extended
exo⁺ pol	not extended	extended

GCGGAGCTTGCAGTGA (***G/C***) CCAGATCCCGCCACTGCAC TCCAGCCTGGGCGACAG-3'.

The sense primer had the sequence 5'-GGAGGCGGAGCTTGC AGTGA***C***-3', and the inert sense primer had the same sequence, but the 3 terminal nucleotide dCMP was replaced by ddCMP. Inert primers were prepared from a pro-primer with the sequence GGAGGCGGAGCTTGCAGTGA by single base extension using terminal transferase in the presence of ddCTP, followed by PAGE separation and gel extraction, as previously described (Liu and Sommer, 2000). The antisense primer had the sequence 5'-GCCCA GGCTGGAGTGCAGTG-3' with a 3' terminal phosphorothioate-modification.

Two DNA-dependent polymerases were purchased from New England Biolabs (Beverly, USA), 'Deep Vent⁻' and 'Deep Vent'. Deep Vent is the wild-type that has strong 3' to 5' exonuclease activity, the potential of which was evaluated by SNP analysis. On the other hand, Deep Vent⁻ contained a point mutation that resulted in a loss of proofreading function, and was used as a control. Two-directional primer extensions were carried out at annealing temperatures of 53 and 57°C. The primer extension was performed using both a matched and mismatched amplicons. The matched amplicon consisted of the template harboring the sequence 5'… GATCTGGG …3', and a matched inert primer having its 3' terminal ddCMP as mentioned earlier, which formed a C-G match. In the contrary, the mismatched amplicons included the template harboring the sequence 5'… GATCTGGC …3', which provided a single-base mismatched amplicon of C-C mismatch between the template and the inert primer (Table 1).

Following denaturation at 95°C for 2 min, primer extension was achieved using 30 cycles of 15 s denaturation at 95°C, 30 s annealing at 53 or 57°C, and 30 s extension at 72°C. After extension the reaction mix was cooled to 4°C. The primer extensions were performed in a total volume of 20 µl with 15 pg of template, 0.25 mM deoxynucleotide-triphosphate (dNTP), 0.01 U/ml of polymerase, 10 pmol/ml of both sense and antisense primers, and 2 µl of 10× NEB polymerase reaction buffer. This provided a final concentration of 10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 2 mM magnesium sulfate ($MgSO_4$), and 0.1% Triton X-100. PCR products were visualized using 2.5% agarose gel electrophoresis at 5 V/cm.

Results and Discussion

Blockade of non-specific primer extension by inert primers DNA polymerases without a proofreading function yielded primer-extended products from regular primers. Regardless of whether the amplicons were matched or mismatched, no primer extension occurred using the inert allele specific primers. In these conventional conditions without the administration of extra PPi, inert primers cannot be activated. Thus, the inert allele specific primers lacking a 3' free hydroxyl group blocked the integration of dNTP substrate at the polymerase polymerization domain.

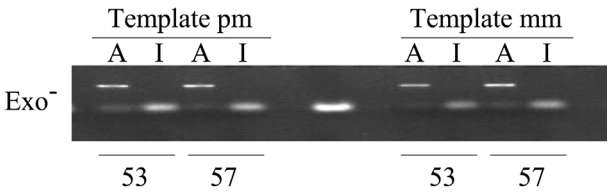

Fig. 1. Gel electrophoresis graphs showing the blockade of primer extension by inert allele specific primers. Regular active primers (A) were efficiently extended by polymerases without proofreading activities; whereas few PCR products were observed using the inert allele specific primers (I). The figures 53 and 57 represent annealing temperatures. pm, perfect match; mm, mismatch.

In the case of conventional allele specific primer extension, such as for the regular control primers used in the present study, mismatched amplicons are occasionally efficiently extended. The inability of inert allele specific primers to extend works as an initial switch to minimize errors. Theoretically, inert allele specific primers may be activated by pyrophosphorolysis, however the dynamics of polymerization exceeds that of pyrophosphorolysis by some 1,000 times. Therefore, in the absence of additional PPi, inert allele specific primers efficiently blockade primer extension by DNA polymerases without a proofreading function.

Mismatch proofreading turned on DNA polymerization by activating inert primers In the case of an amplicon containing a single-base mismatch between the 3' termini of the inert allele specific primers and the templates (templates harboring the sequence 5'… GATCTGGC …3'), DNA polymerases with or without a proofreading function were found to have opposite effects on primer extension. As a control, DeepVent⁻, a DNA polymerase lacking a proofreading function, failed to produce primer-extended products from inert allele specific primers (Fig. 1). However, exo⁺ DNA polymerases efficiently yielded PCR products from the inert allele specific primers when there was a mismatch between the primer and the template (Fig. 2). This mismatch triggered DNA polymerization worked in the 2 annealing temperatures (53 and 57°C), indicating its wide practical applicability for mutation detection.

For matched amplicons, DNA polymerases with and without 3' to 5' exonuclease activity produced similar results by failing to extend the inert allele specific primers. These similar results were obtained through different underlying mechanisms. For exo⁻ DNA polymerases, inert primers cannot be activated due to the lack of 3' to 5' exonuclease activity. However, for exo⁺ polymerases, no enzymatic excision occurs

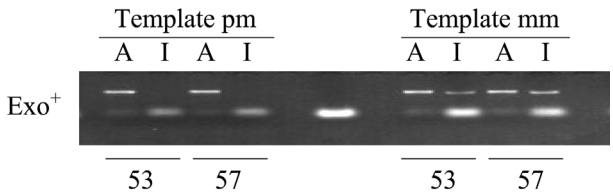

Fig. 2. An off/on switch effect was obtained using proofreading inert allele specific primers. A matched amplicon turned off DNA polymerization and a mismatched amplicon turned on the primer extension process by activating the inert primers. Similar off/on effects were observed at two different annealing temperatures of 53 and 57°C.

as no proofreading is required by matched amplicons. Under both conditions, little pyrophosphorolysis was observed as no primer extended products were yielded by matched amplicons using the inert allele specific primers. For the exo$^+$ DNA polymerases, perfectly matched amplicons restrained the inert allele specific primers within the polymerization domain, preventing its proofreading at the exo nuclease domain of the DNA polymerases. Clearly, the matched amplicon turns off the DNA polymerization from inert allele specific primers.

The off/on switch fits well for mutation detection. As illustrated in Fig. 3, a binary output of assay results is reached in response to the input of mixed templates. Inert primers of a perfectly matched amplicon are not sent to the 3' exo domain of the high fidelity DNA polymerases. In this situation, inert primers stay inactivated and no DNA polymerization can occur. Therefore, similar results of no product extension were observed for perfectly matched amplicons using either low fidelity or high fidelity DNA polymerases. However, the inert primers of mismatched amplicon trigger the 3' exonuclease excision process, which removes the mismatched 3' terminal. Actually, the proofreading of the mismatched primer is designed to activate the inert primer in this off/on switch system. After removing the 3' terminal nucleotide from the original inert primer, the product of 3' exonuclease digestion possesses an active 3' hydroxyl group, which can be used for DNA polymerization. Technologically, negative results with no PCR products indicate that the template perfectly matches the 3' terminal of the inert primer, that is a one to one complementary relationship in nucleotide detection. One of the beauties of the off/on switch is that the positive results can detect all the three mismatched nucleotides other than the complementary one, which is powerful in mutation detection.

Actually, this third mutation assay mediated by exo$^+$ polymerases was initially reported as proof-reading PCR by Bi and Stambrook (1998). As shown in Table 2, many kinds of blockade can be used to reach the similar results. In our understanding, the widespread application of the proof-read PCR is largely restrained by the reliability of the inert primers to be used. In a similar genetic assay using inactivated primers, the pyrophosphorolysis activated polymerization, the quality of inert primers have been a key determinant limiting its practical application (Liu and Sommer, 2000). Obviously,

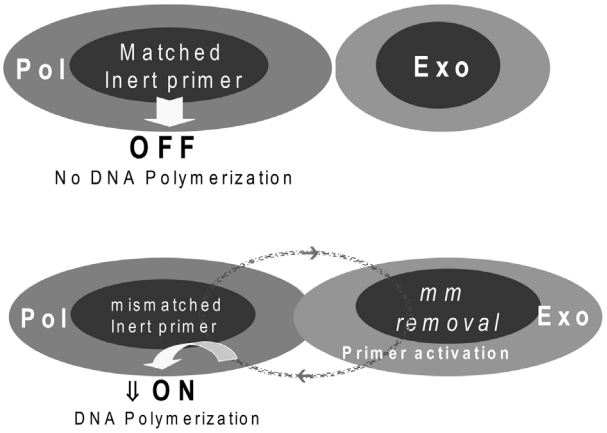

Fig. 3. The off/on switch consisting of inert primers and high fidelity DNA polymerases with 3' exonuclease activity. When inert primer matches the template the 3' exo domain is not involved, whereas a mismatched primer is recognized by the proofreading procedure and the 3' terminal nucleotide is then excised. This 3' excision activates the inert primer and DNA polymerization can then occur. Where mm represents mismatched nucleotides.

Table 2. Comparison of different inert primers in their specificities and sensitivities in the proofreading PCR

	Extension in matched amplicon	Extension in mismatched amplicon
-3-NH2*	none	yes
-C3-SH*	none	yes
-Pi*	none	yes
-Pi	yes	yes
-C3-H	almost none	yes

*Results from the reference of Bi and Stambrook (1998). Some other blockade methods, such as acyNMP, are to be evaluated.

more experiments in large-scale evaluation of different types of inert primers are required. The availability of sensitive and reliable inert primers would eventually help to pave the way to exo$^+$ polymerases mediated genetic analysis.

As compared with the other two SNP assays mediated by exo$^+$ DNA polymerases, this SNP operated off/on switch works in a way complementary to proofreading 3' phosphorothioate-modified or 3' labeled primers. With the introduction of inert allele specific primers, matched amplicons turn off and mismatched amplicons turn on DNA polymerization. One particularly important feature of the previously developed on/off switch and this new off/on switch is that identical reaction conditions are required by the two types of exo$^+$ DNA polymerase mediated primer extensions. In large scale SNP scanning, the application of two complementary assays within one platform such as a multi-well plate or microarray will help minimize wrongly genotyped SNP sites due to special local sequence content. Although an increasing number of SNP assays have been

developed, SNP assays remain a technical challenge for modern personalized medicine (Chicurel, M., 2001). In the application of the off/on switch using inert primers and an on/off switch using the phosphorothioate-modified primers, their complementary effects will help to increase assay sensitivity and reliability in genetic analysis. The on/off switch offers assays that can precisely detect the site and type of a mutation, whereas, the off/on switch provides a very powerful and efficient assay for unknown mutation scanning.

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