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In Vitro Nucleotide Sequence Editing Using Single-Primer Reactions In Parallel PCR.

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ABSTRACT

Correct nucleotide sequence is essential for coding recombinant protein or befitting function of DNA/RNA in *cis* acting level. Polymerase Chain Reaction (PCR) is common method for in vitro amplification or isolation of DNA in molecular biology or recombinant DNA technology work. Mutation or wrong base incorporation can be occur during PCR process. Hence, editing process should be done to obtain correct sequence. This study was aimed to use single-primer reaction in parallel (SPRINP) PCR for editing nucleotide sequence of incorrect PCR product in plasmid in vitro. Two mutant primers were designed to add TA nucleotides into our target DNA fragment in plasmid and were used in separate tube in PCR process. Pairing of DNA strand from both separate tube was done by combining them in one tube and gradually decreasing the temperature. Parental plasmid strands were degraded by Dpn1 enzymatic reaction. New correct DNA strands were transformed into Escherichia coli. Isolated plasmids from transformants were sequenced to confirm editing result. DNA sequencing showed that TA nucleotides have been incorporated correctly to the sequence. In conclusion, SPRINP PCR can be used for editing DNA sequence in vitro.

Keywords: mutation, nucleotide editing, PCR, SPRINP.

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INTRODUCTION

Therapeutic proteins or biopharmaceuticals become a common medication for advance treatment nowadays. They are produced in recombinant form in many different host cells depending on their characteristics. Since protein profile is determined by its amino acids sequence and they are coded by DNA, the correct sequence of nucleotide base in DNA fragment is crucial for production of therapeutic protein. Not only in coding region of a gene, correct nucleotide in non coding region, i.e. promoter, terminator, regulation region, is also needed for optimal function of those gene element. The amount of protein possibly affected if the function of each elements in non coding region are not optimal. To get intended DNA fragment or gene in recombinant DNA technology or molecular biology work, Polymerase Chain Reaction (PCR) is commonly used. Unfortunately spontaneous mutation or incorrect nucleotide incorporation might be happened during PCR process, moreover if a non high proof reading DNA polymerase enzyme is used or repeat sequence present in the DNA fragment [1, 2]. In such case, editing process of incorrect DNA sequence must be done by site directed mutagenesis (SDM) approach. Generally SDM is used for protein engineering or studying biological function of a protein.

QuikChange™ from Stratagene, a PCR-based SDM, is a very famous used method [3]. Basically two primers that contain altered nucleotide in the middle of primer sequence were used and called mutant primer. Both primer were used in PCR by using high proof reading DNA polymerase. Then, PCR product was processed further with *Dpn*1 restriction enzyme in order to destroy parental template that possess original DNA sequence. Many modification on this method were done to hinder primer dimer problem. Among them were modification of mutant primer design [4] and performing the PCR by using forward and reverse mutant primer in separate tube [5]. In this study we combined both modification to make PCR-based SDM method more efficient and simple. We used it to insert T and A nucleotide for editing and generate a stop codon TAA up stream *Nde*1 site of modified pET-16b plasmid.

MATERIALS AND METHODS

Materials

Modified pET-16b plasmid (Novagen) was used as a template plasmid that will be edited by inserting T and A base nucleotide in SDM PCR. Mutant primers forward (5'-CATCATCATTAA**CATATG**CTCGAGGAT-3') and reverse (5'-CGAG**CATATG**TTA ATGATGATGATGATG-3') were design by using PrimerSelect program (DNAStar) and synthesized commercially by IDTDNA. Bold nucleotides were *Nde*1 site and inserted T and A nucleotide were underlined. KAPA HiFi Hotstart was used as DNA polymerase and purchased from KAPABiosystem. *Dpn*1 restriction enzyme was purchased from Thermo Fisher Scientific.

Plasmid Isolation

Plasmid that used as template in SDM PCR and to be analyzed for migration and sequencing analysis was isolated from *E. coli* TOP10 by using High-Speed Plasmid Mini Kit (Geneaid) according to manufacture protocol.

Polymerase Chain Reaction

SDM SPRINP PCR reaction was performed separately between forward and reverse mutant primer. A total volume of 25 µl PCR mixture was consist of 200ng template plasmid, 0.4µM forward mutant or reverse mutant, 0.4µM dNTP, 5 µL KAPA HiFi Hotstart Fidelity Buffer 5x, 0.5U KAPA HiFi Hotstart, 1.5µl ethylene glycol. PCR cycle was done 2 min of 95°C for predenaturation, followed by 30 cycles of amplification. Each amplification cycle was consist of 95°C for 30 sec, 60°C for 40 sec, 72°C for 4 min. Final elongation was done at 72°C for 10 min. PCR product from both PCR tubes was combine and denatured at 95°C for 5 min. Temperature was then decreased gradually 90°C for 1 min, 80°C for 1 min, 70°C for 30 sec, 60°C for 30 sec, 50°C for 30 sec, 40°C for 30 sec and 37°C for 30 sec. Combined PCR product was digested by *Dpn*1 [5].

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Transformation and Confirmation of Edited Plasmid

Digested PCR product was transformed into competent *E. coli* TOP10 by heat shock method [6]. Plasmids from transformants that grow on 100 µg/ml ampicillin supplemented Luria Bertani agar medium were isolated and were analyzed their migration profile by agarose DNA electrophoresis and confirmed further for the nucleotide editing result by sequencing. DNA sequencing was performed using sequencing service of Macrogen, Inc. (South Korea).

Data Analysis

Sequencing result was aligned and analyzed by MultAlign program [7]. Original template plasmid sequence was used as comparison sequence.

RESULTS AND DISCUSSION

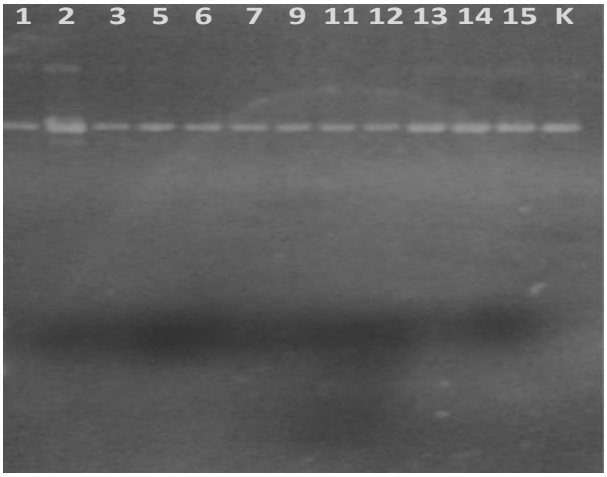

Figure 1: Migration profile of plasmid that isolated from several transformant colonies. Number indicated colony number, K = original template plasmid.

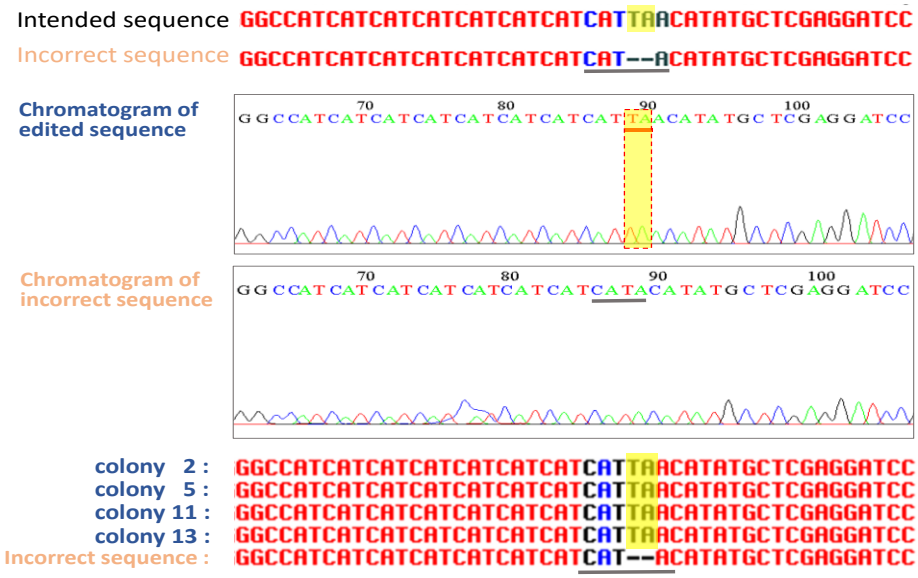

Figure 2: Chromatogram of sequencing result and alignment of edited sequence. T and A nucleotides (yellow box) have been successfully added into the edited sequences.

In this study, we have edited DNA sequence in vitro in a plasmid that should have T and A nucleotide in its sequence by using SPRINP method [5]. Modifications have been done from original protocol, i.e. position of altered nucleotide in mutant primer was near by 5' to make annealing to the template more possible [4]. In addition, KAPA HiFi was used as DNA polymerase instead of Pwo Master DNA polymerase that more cost efficient. Template amount was reduced from 500 ng to 200 ng and PCR cycles was made more from 18 to 30. The experiment was repeated three times.

Twelve colonies were randomly picked from grown transformants on ampicillin selected medium. Plasmid migration of those colonies were similar to original template plasmid (Figure 1). Additional two DNA nucleotide (T and A) did not change their migration profile compare to original template plasmid. DNA sequencing analysis showed that sequence from four colonies (colony nr. 2, 5, 11, 13) were successfully edited and have same sequence as intended one when analyzed using MultAlin program (Figure 2). T and A nucleotide have been incorporated correctly in their intended position.

CONCLUSIONS

In vitro editing of nucleotide sequence has been successfully done by using modified SPRINP method. Mutant primer design, DNA polymerase, template amount and PCR cycle have been modified in this study.

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