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Effects of Different PCR Product Purification Methods on DNA Sequencing

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Farklı PCR Ürünü Saflaştırma Yöntemlerinin DNA Dizilemeye Etkisi

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Abstract

Direct sequencing of polymerase chain reaction (PCR) products without cloning is a rapid and efficient way of sequence analysis. Prior to direct sequencing, it is necessary to purify the PCR products from excess primers, nucleotides and enzymes that could interfere with the sequencing reaction. There are several PCR product purification methods such as spin column-based purification, enzymatic purification, ethanol precipitation and gel extraction. In this study, it is aimed to evaluate the efficiency of ethanol-ammonium acetate (EtOH-NH_4Ac) and polyethylene glycol (PEG) precipitation methods for purification of PCR products prior to the dye terminator cycle sequencing. A 741 bp region of the Toll-Like Receptor (TLR) 4 gene was amplified from bovine genomic DNA using PCR. After analyzing the PCR product using agarose gel electrophoresis, it was purified with one of the following methods: a) PEG precipitation, b) EtOH-NH_4Ac precipitation and c) ExoSAP-IT PCR product cleanup reagent. ExoSAP-IT reagent was used as a standard PCR product cleanup protocol. Sanger sequencing of PCR samples purified with different purification methods was performed on a Beckman Coulter CEQ8800 Genetic Analysis System. The sequence data were analyzed using Sequencing Analysis software implemented within the system. The quality check and alignment of sequences were performed using BioEdit software. The sequencing results of PCR products purified with different purification methods were compared with each other. It was found that PCR products purified with both purification methods provided good-quality sequencing templates like that of purified with ExoSAP-IT reagent.

Keywords: *DNA sequencing; PCR Product; PEG; EtOH-NH_4Ac; ExoSAP-IT; CEQ8800 genetic analysis system..*

Öz

Polimeraz zincir reaksiyonu (PCR) ürünlerinin klonlama olmadan doğrudan dizilenmesi, dizi analizinin hızlı ve etkili bir yoludur. Doğrudan dizilemeden önce PCR ürünlerinin, dizileme reaksiyonuna olumsuz anlamda müdahale edebilecek fazla primerlerden, nükleotidlerden ve enzimlerden arındırılması gerekir. Spin-kolon bazlı saflaştırma, enzimatik saflaştırma, etanol çöktürmesi ve jel ekstraksiyonu gibi çeşitli PCR ürünü saflaştırma yöntemleri vardır. Bu çalışmada PCR ürünlerinin boya sonlandırıcı döngü dizilemesi öncesinde saflaştırılmasında etanol-amonyum asetat (EtOH-NH_4Ac) ve polietilen glikol (PEG) çöktürme yöntemlerinin etkinliğinin değerlendirilmesi amaçlanmıştır. Toll-benzeri reseptör (TLR) 4 geninin 741 bç'lik bölgesi, sığır genomik DNA'sından PCR ile çoğaltılmıştır. PCR ürünü agaroz jel elektroforezi kullanılarak analiz edildikten sonra aşağıdaki yöntemlerden biri ile saflaştırılmıştır: a) PEG çöktürmesi, b) EtOH-NH_4Ac çöktürmesi ve c) ExoSAP-IT PCR ürünü temizleme reaktifi. ExoSAP-IT reaktifi standart PCR ürünü temizleme yöntemi olarak kullanılmıştır. Farklı saflaştırma yöntemleriyle saflaştırılan PCR örneklerinin Sanger dizi analizi Beckman Coulter CEQ8800 Genetik Analiz Sistemi ile gerçekleştirilmiştir. Dizi verileri sistemde yer alan Dizi Analizi yazılımı kullanılarak analiz edilmiştir. Dizilerin kalite kontrolü ve hizalanması BioEdit yazılımı ile gerçekleştirilmiştir. Farklı saflaştırma yöntemleriyle saflaştırılan PCR ürünlerinin dizileme sonuçları birbirleriyle karşılaştırılmıştır. Her iki saflaştırma yöntemiyle saflaştırılan PCR ürünlerinin ExoSAP-IT reaktifi ile saflaştırılana benzer şekilde iyi kalitede dizileme kalıbı sağladığı bulunmuştur.

Anahtar Kelimeler: *DNA dizileme; PCR ürünü; PEG; EtOH-NH_4Ac; ExoSAP-IT; CEQ8800 genetik analiz sistemi*

1. Introduction

Direct sequencing of PCR products without cloning is a rapid and efficient way of sequence analysis. The excess primers, nucleotides and enzymes from the PCR reaction mixture could interfere with the sequencing reaction and affect the accuracy of the sequencing results significantly (Rao 1994). Since the purity of the DNA template is one of the most critical factors to get reliable and accurate sequencing reads the PCR product needs to be purified prior to sequencing. There are several PCR product purification methods, each with its own advantages and disadvantages, such as gel or column-based purifications, enzymatic cleanup, different precipitation methods and magnetic bead separation. PCR products are analyzed by gel electrophoresis for assessing yield and purity before purification. If there are undesired by-products of the PCR reaction such as primer dimers and non-specific amplification products, the target PCR band first must be isolated, usually from an agarose gel, followed by cleanup procedures prior to sequencing (Seelan and Grossman 1991, Leonard et al. 1998). If a single desired band is amplified, in this case the PCR product can be directly

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sequenced after removal of residual primers and dNTPs. The enzymatic cleanup of PCR products is a simple and fast method for removal of residual primers and dNTPs. It contains two hydrolytic enzymes: Exonuclease I (ExoI) and Shrimp Alkaline Phosphatase (SAP). ExoI enzyme removes residual primers and any single-stranded DNA produced during PCR while SAP dephosphorylates unincorporated dNTPs from the PCR product, preventing them from being used in subsequent reactions. There are several commercially available enzymatic PCR cleanup reagents. ExoSAP-IT PCR Product Cleanup Reagent is a one of the most widely used and efficient one for DNA sequencing and single nucleotide polymorphism analysis (Berger 2001, Dugan 2002). This reagent simplifies the PCR cleanup process by combining two essential enzymatic activities into a single reaction without product loss, saving time and reducing the risk of contamination so that it is an effective alternative to classical PCR cleanup methods prior to sequencing (Werle 1994, Bell 2008). However, the presence of common by-products of PCR such as primer dimers and non-specific amplification products limits the use of these enzymatic purification methods because of the inability of these enzymes to remove such by-products.

EtOH purification is one of the most commonly used method for nucleic acid concentration and purification in aqueous solutions (Maniatis et al. 1982). In the presence of salts such as ammonium acetate, sodium acetate and sodium chloride, ethanol induces a structural transition in nucleic acid molecules, which causes them to aggregate and precipitate from solution (Eickbush and Moudrianakis 1978). The washing of the pellet with 70% ethanol solution effectively desalt DNA.

Precipitation by PEG is a general method of concentrating a variety of biological macromolecules including DNA. The minimal concentration of PEG required for precipitation is dependent on the size of DNA molecule. Higher molecular mass DNA precipitates at lower PEG concentrations than lower molecular mass DNA (Lis and Schleif 1975). The concentration of NaCl has also a significant influence on the amount of DNA precipitated. PEG precipitation can be performed with basic laboratory equipment without expensive chemicals or complex procedures (Lis 1980). Sanger sequencing, also known as chain termination sequencing or dideoxy sequencing, is the most commonly used method for DNA sequencing since its invention by Dr. Frederick Sanger and his colleagues in 1977 (Sanger et al. 1977). This method is based on the detection of labeled chain-terminating nucleotides that are incorporated by a DNA polymerase during the replication of a template. It was the first method used to sequence genomes, including the Human Genome Project (International Human Genome Sequencing Consortium 2004). The modern and automated version of Sanger sequencing called dye terminator sequencing uses fluorescent-labeled dideoxy nucleotide chain terminators, which permits sequencing in a single reaction. The synthesized strands are separated by size using capillary electrophoresis and an optical system detects the dye fluorescence, outputting the data as a fluorescent peak trace chromatogram. This sequencing technology produces high-quality reads up to 850 bp (Smith et al. 1985 and 1986).

The present study aimed to evaluate the efficiency of PEG and ethanol precipitation methods for purification of PCR product prior to the dye terminator cycle sequencing. Here we report and compare the sequencing results of PCR products purified with different purification methods.

2. Materials and Methods

All PCR reagents were purchased from Promega. ExoSAP-IT PCR Product Cleanup Reagent was provided by Thermo Fisher Scientific, Inc. Beckman Coulter, Inc. provided GenomeLab DTCS Quick Start Kit and Agencourt CleanSEQ beads.

2.1 PCR Product Amplification

Bovine genomic DNA was used to generate the sequencing template by PCR. The amplification of a 741 bp region of the Toll-Like Receptor (TLR) 4 gene was performed using gene-specific forward (5'-CTACCAAGCCTTCAGTATCTAG-3') and reverse (5'-GGCATGTCCTCCATATCTAAAG-3') primers as described in (Mariotti et al. 2009). The PCR reaction was performed in a 30 µl volume containing 30 ng of genomic DNA, 1.6 pmol of each primer, 0.2 mM of each dNTP, 5X GoTaq Reaction Buffer (Promega, Madison, USA) and 0.2 units of GoTaq DNA polymerase (Promega, Madison, USA) on a Thermo Hybaid thermal cycler (Thermo Fisher Scientific, Inc.). The touchdown PCR program was given in Table 1.

For the evaluation of different PCR product purification methods, PCR was performed in five tubes and then all amplification reactions were mixed to create a uniform pool of starting material. An aliquot of the PCR product was visualized on a 1.5 % agarose gel stained with ethidium bromide. The remaining product was aliquoted and purified with one of the following methods; PEG precipitation, EtOH-NH_4Ac precipitation and ExoSAP-IT cleanup protocol. The concentration and quality of all purified samples were measured using NanoDrop 1000 spectrophotometer (Thermo Scientific, Inc.).

Table 1. PCR program for amplification of bovine TLR 4 gene.

Step	Temperature	Time	Stage and number of cycles
Initial denaturation	94 °C	5 min	
Denaturation	94 °C	30 sec	Stage 1 14 cycles
Annealing	47 °C + 7 °C (-0.5 °C per cycle)	45 sec	
Extension	72 °C	40 sec	
Denaturation	94 °C	30 sec	Stage 2 20 cycles
Annealing	47 °C	45 sec	
Extension	72 °C	40 sec	
Final extension	72 °C	5 min	

2.2 Purification of PCR Product

ExoSAP-IT PCR Product Cleanup

PCR product was cleaned with ExoSAP-IT reagent (Applied BioSystems, Thermo Fisher Scientific, Inc.) to remove residual primers and dNTPs according to the manufacturer's instructions. ExoSAP-IT, a proprietary mixture of ExoI combined with SAP in a specially formulated buffer, removes excess primers and unincorporated nucleotides from the PCR product, making it suitable for further analysis. The ExoI/SAP treatment was conducted by mixing 5 µl of a post-PCR reaction product with 2 µl of ExoSAP-IT, and incubating at 37°C for 15 min. After treatment, ExoSAP-IT was inactivated at 80°C for 15 min and the treated PCR product was used directly in sequencing reactions (Bell 2008).

PEG Precipitation

For purification of PCR product with PEG precipitation, 50 µl of PEG mixture (20% PEG 8000, 2.5 M NaCl) was mixed with 50 µl of PCR product in a 0.5 ml tube by pipetting up and down very well. The DNA-PEG mixture was incubated at 37°C for 15 min. After incubation, it was centrifuged at high speed (~15,000 xg) for 15 min at room temperature to pellet the DNA. The supernatant was carefully pulled off with a pipette tip and discarded. The pellet was washed twice with 125 µl of cold 80% EtOH. If the EtOH was added into the bottom of the tube, the tube was spun for two minutes. If it was placed onto the side of the tube, the supernatant was removed with a yellow tip after waiting for one minute. The pellet was air dried until residual EtOH was completely evaporated. The pelleted DNA was dissolved in 25 µl of TLE Buffer (10 mM Tris and 0.1 mM EDTA) (Glenn 2020).

EtOH- NH_4Ac Precipitation

The PCR product was precipitated by addition of 0.25 volumes of 10 M NH_4Ac and 2.5 volumes of ice-cold absolute ethanol. The DNA was recovered by centrifugation at 14000 rpm for 20 min at 4°C. The supernatant was decanted without disturbing the pellet. 400 µl of cold 70% EtOH was added to wash the pellet. After centrifugation at 14000 rpm for 5 min at 4°C, the supernatant was discarded carefully. The DNA pellet was air-dried until all EtOH was evaporated. The pelleted DNA was resuspended in ddH_20 (15 µl of ddH_20 was used per 25 µl of PCR product).

2.3 DNA Sequencing and Data Analysis

DNA sequencing reactions were performed by using GenomeLab DTCS Quick Start Kit (Beckman Coulter Inc., California, USA) according to the manufacturer's instructions. Briefly, 50 fmol of PCR products were subjected to PCR in a 20 µl reaction mixture composed of 8 µl of DTCS QuickStart mix, 2 µl of one of the gene-specific primers (1.6 pmol/µl) used for template generation and ddH_2O under the following cycle conditions: 30 cycles of 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min. All cycle sequencing reactions were purified with Agencourt CleanSEQ beads (Beckman Coulter Inc., California, USA) according to the manufacturer's protocol. All samples were resuspended in 40 µl of sample loading solution (SLS) and analyzed on CEQ8800 Genetic Analysis System (Beckman Coulter Inc., California, USA) using LFR-1 method. The DNA sequences were analyzed using Sequencing Analysis software on CEQ8800 Genetic Analysis System (Beckman Coulter Inc., California, USA). The quality check and alignment of sequences was performed by BioEdit software (Hall 1999).

3. Results and Discussions

In this study, it is aimed at evaluating the effects of different PCR product purification methods on DNA sequencing. For this reason, a 741 bp region of the TLR4 gene was amplified from bovine DNA and used as a control template for sequencing. After amplification, an aliquot of this PCR product was visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and it was shown that the amplification produced a highly efficient single desired band (Figure 1).

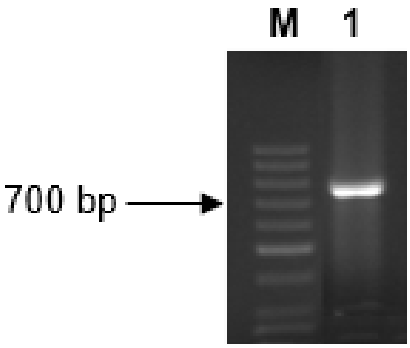

Figure 1. PCR product as a sequencing template.
M. GeneRuler 100 bp DNA Ladder (Cat. No: SM0241, ThermoFisher Scientific), **1.** The PCR product.

The PCR product was then purified using one of the following purification methods; a) PEG precipitation, b) EtOH-NH_4Ac precipitation and c) ExoSAP-IT reagent. Since ExoSAP-IT is known as simple and effective PCR clean-up reagent it was used as a standard clean-up protocol. After purification, PCR products were cycle sequenced using gene-specific amplification primers on CEQ8800 Genetic Analysis System (Beckman Coulter, California, USA). The sequencing reactions were repeated twice for each purification.

The cycle sequencing of PCR-generated templates purified with different purification methods gave good raw data signals. The raw data of each sequencing run was analyzed using the sequencing software implemented within the CEQ8800 genetic analysis system. All the chromatograms were opened with BioEdit software and visually inspected for quality analysis. Only a representative chromatogram was given for each purification method as an example. There were mostly evenly-spaced peaks and almost no baseline 'noise' as seen in Figures 2, 3 and 4.

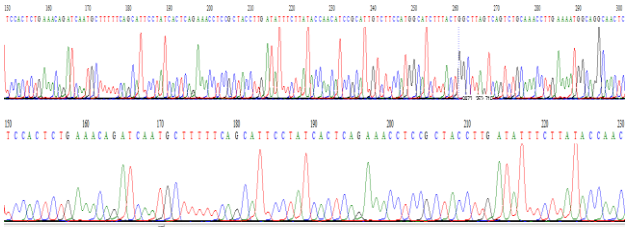

Figure 2. Chromatogram of the EtOH-NH_4.acetate purified PCR product.

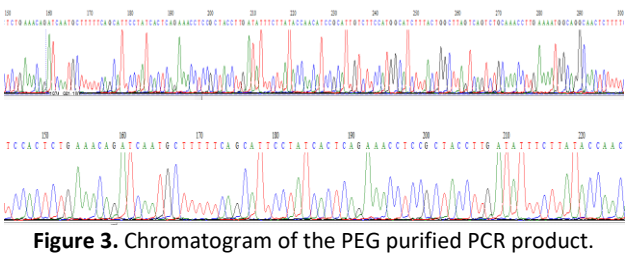

Figure 3. Chromatogram of the PEG purified PCR product.

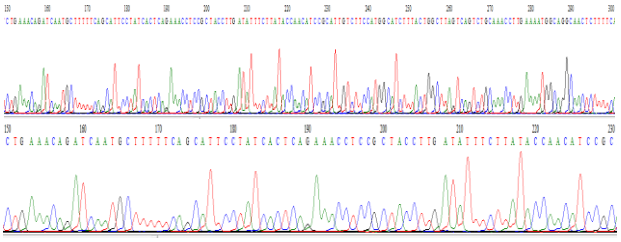

Figure 4. Chromatogram of the ExoSap-IT purified PCR product.

The manual check of the interpretation of the primary data showed predictable errors near the beginning and at the end of each sequencing run. The sequences of each run were easily interpreted from the 10^{th}-15^{th} nucleotide after the sequencing primer and extended hundreds of bases (Figures 2, 3 and 4). The dye-terminator cycle sequencing of PCR products purified with PEG and EtOH-NH_4Ac precipitations provided more than 650 bases of sequence with more than 95% accuracy, similar to the sequence analysis of the PCR product purified using ExoSAP-IT. It is well known that sequence analysis of ExoSAP-IT purified PCR products is performed with high accuracy even if the PCR product is very short in length (Bell 2008).

The sequences were checked by aligning against the reference sequence of Bos taurus TLR4 gene (GenBank accession number DQ839567.1) using ClustalW multiple sequence alignment tool (Thompson 2003) in Bioedit software. There were a few errors in base calling such as N (nt 580) and T (nt 657) in the chromatogram of EtOH-NH_4Ac; a missing T nucleotide (nt 619) in the chromatogram of PEG (Figure 5). These nucleotide positions were marked with asterisks in Figure 5.

It was found that PCR products purified with both purification methods provided good-quality sequencing templates. There was no significant sequencing result difference between the templates purified by EtOH-NH_4Ac precipitation, PEG precipitation and ExoSAP-IT purification.

To the best of the author's knowledge, no study has compared the sequencing results of the PEG precipitated and EtOH-NH_4Ac precipitated PCR products, as in this study. However, there are publications involving PCR product purification with different concentrations of PEG and EtOH-NH_4Ac than this study and their further analysis. In a study perfomed by Irwin et al. 2003, multiplex PCR products were cleaned with different techniques, including EtOH-NH_4Ac precipitation, before being subjected to fragment analysis by capillary electrophoresis and it was observed that EtOH-NH_4Ac precipitation was superior to other methods in clearing PCR contaminants without affecting the product analysis.

Figure 5. Multiple sequence alignment using the Bioedit software was performed using the reference TLR4 sequence.

PEG has the ability to precipitate nucleic acids differentially based on size (Lis 1980, Lis and Schleif 1975, Schmitz and Riesner 2006). Several publications have shown the use of PEG to precipitate PCR products. In a study aimed at large-scale production of DNA sequencing templates by microtitre PCR format, PEG precipitated PCR templates were sequenced using dye terminators with high accuracy (Rosenthal et al. 1993). In another study, PEG precipitation was performed using modified magnetic beads as solid phase in the purification of PCR products and the PCR products purified in this way were succesfully used in library preparation for high-throughput sequencing (Clarke 2014). All these example studies have shown that PEG and EtOH-NH_4Ac precipitated PCR products could be used as template for sequencing reactions as stated in the present study.

4. Conclusions

The efficient and cost-effective post-PCR purification method is very valuable especially in large number of samples present to be sequenced. There are many alternative post-PCR clean-up techniques. In this study, PEG and EtOH-NH_4Ac precipitation methods were used for post-PCR reaction clean-up and their effects were evaluated in the efficiency of sequencing reads. ExoSAP-IT was used as a standard PCR clean-up reagent since it gives very long and accurate sequencing results. It was found that the two mentioned purification methods gave similar results to the one obtained with ExoSAP-IT. These findings suggest that both purification methods can be used as efficient and cheap alternatives to the commercial enzymatic reagents like ExoSAP-IT when there is only desired PCR product in the amplification and there are large number of samples being cycle sequenced. However, PCR product size should be taken into consideration when choosing a precipitation method because the PEG mixture efficiently precipitates DNA fragments above 150 bp while EtOH precipitates very low molecular weight fragments efficiently (Paithankar and Prasat 1991).

Declaration of Ethical Standards

The author declares that she complies with all ethical standards.

Credit Authorship Contribution Statement

Author: Methodology/study design, Experiment and data analysis, Writing – original draft, Writing-review and editing

Declaration of Competing Interest

The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The author declares that the main data supporting the findings of this work are available within the article.

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