Removal of the Remaining PCR Primers and dNTPs for Cycle Sequencing Reaction Using Exo-Alp PCR Cleanup Mix

Abstract

As an alternative to ExoSAP-IT PCR Product Cleanup Reagent, Exo-Alp PCR Cleanup Mix is used to remove the remaining primers and dNTPs from PCR templates before Sanger sequencing. We show comparable sequencing performance after either Exo-Alp or ExoSAP-IT for PCR product cleanup. We also show comparable sequencing data between the SupreDye™ and BigDye® cycle sequencing kits after Exo-Alp treatment.

INTRODUCTION

PCR products are the most common template for Sanger sequencing. However, the PCR products usually need further cleanup because the remaining PCR primers and dNTPs can interfere with the cycle sequencing reactions, leading to noisy sequencing background in the electropherogram and wrong base-calling.

The most commonly methods for the cleanup of PCR products prior to Sanger sequencing use either spin columns, magnetic beads, or enzymatic digestions. Although spin columns and magnetic beads can achieve good results, they are time consuming and often lose a portion of samples due to incomplete column or bead binding, multiple washing steps, or inefficient elution steps. The enzymatic method, on the other hand, is easy and simple, needs less hands-on time, requires no change of reaction tubes, and causes no sample loss; there is no need for further quantification before cycle sequencing.

The most popular product using the enzymatic methods is ExoSAP-IT PCR Product Cleanup Reagent from Thermo Fisher Scientific. ExoSAP-IT is made to work in the completed PCR reactions without adding any buffer. By incubation at 37°C for 30 minutes followed by 80°C for 30 min, the remaining PCR primers are removed, the remaining dNTPs dephosphorylated, and the enzymes inactivated. The comparison between the treated and untreated samples shows that the sequencing background has been significantly reduced and the sequencing quality has greatly improved after the ExoSAP-IT treatment.

We have developed Exo-Alp PCR Cleanup Mix, a close alternative to ExoSAP-IT PCR Product Cleanup Reagent to purify the PCR template for Sanger sequencing. Here, we compare and see similar sequencing quality after purification using these two products. We also achieve comparable performance from sequencing using BigDye® Terminator Cycle Sequencing Kit and SupreDye™ Cycle Sequencing Kit after Exo-Alp PCR Cleanup.

MATERIALS AND METHODS

PCR Reactions

A 50 µl of PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystem) in a 0.5 ml PCR tube containing 1X standard Taq reaction buffer, 200 µM dNTPs, 0.2 µM Forward primer and Reverse primer, 10 ng plasmid DNA, and 2.5 Units Taq polymerase. The PCR protocol was as follows: initial denaturation at 95°C for 2 minutes, followed by 25 cycles of denaturation at 55°C for 20 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 2min, and a final extension for 5 minutes.

ExoSAP-IT and Exo-Alp Products

ExoSAP-IT PCR Product Cleanup Reagent was purchased from Thermo Fisher Scientific. Exo-Alp PCR Cleanup Mix was manufactured at AdvancedSeq.

Cleanup of the PCR Products

For PCR product cleanup, either the ExoSAP-IT or Exo-Alp product was used according to the protocol in each user manual. Briefly, to every 5 µl of completed PCR product reaction, 2 µl of the product was added. The mix was incubated at 30°C for 30 minutes and inactivated at 80°C for 30 min. The reactions were diluted for 7-fold after the EXOSAP-IT or Exo-Alp treatment. The control PCR product (not cleanup) was diluted for 10-fold in TE buffer. Two µL of the diluted PCR product was used for a cycle sequencing reaction.

Sanger Sequencing

A 10 µl sequencing reaction containing 4.75 µl H_2O, 2 µl diluted PCR product, 0.5 µl BigDye or SupreDye, 1 µl of 2 µM sequencing primer, 1.75 µl 5X buffer was carried out in a thermal cycler (GeneAmp PCR System 9700), starting at 95°C for 1 minutes, followed by 30 cycles at 95°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. The sequencing reaction was cleaned up by adding 2 µl 1x magnet beads and 50 µl 85% ethanol, mixing well, incubating for 5 minutes followed by incubating on magnet tube holder for 5 minutes. After washing of the beads with 100 µl 85% ethanol, the sequencing products were eluted with 60 µl elution buffer and loaded on a 3730 Genetic Analyzer. The sequencing data were analyzed using Sequencing Analysis software v5.4.

RESULTS AND DISCUSSION

Exo-Alp and ExoSAP-IT Show Similar Performance in Reduction of the Sequencing Background

We first compared the PCR template sequencing quality after treatment with either of the two products, Exo-Alp and ExoSAP-IT. Figure 1 shows the comparison of the two products for their effect on quality of sequencing using the SupreDye cycle sequencing kit. It is obvious that the sequencing background for the untreated PCR products is significantly higher than the two treated samples byExo-Alp or Exo-SAP-IT. The samples treated with Exo-Alp or ExoSAP-IT does not show much difference in sequencing quality. In addition, the treated samples show longer reading length and better resolution at 550 bp area compared to the untreated samples. Our result indicates that the Exo-Alp PCR Cleanup Mix is a close alternative to ExoSAP-IT PCR Product Cleanup Reagent for PCR product cleanup using the same procedure. For cost-effective concern, Exo-Alp is a high-quality alternative to ExoSAP-IT.

Figure 1a. Sequence Centered at 100 bp

Figure 1b. Sequence Centered at 300 bp

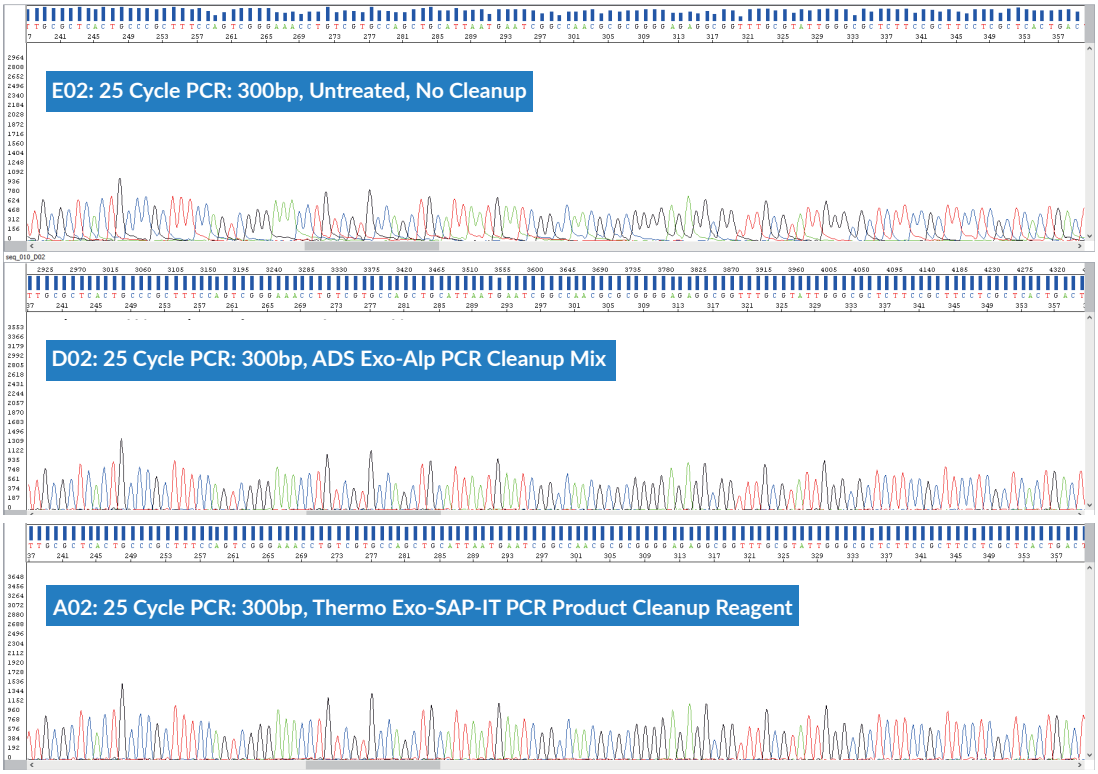

Figure 1c. Sequence Centered at 550 bp

Figure 1. Comparison of Exo-Alp and ExoSAP-IT for PCR product cleanup. 1a. The sequencing comparison at 100 bp region; 1b. The sequencing comparison at 300 bp region; 1c. The sequencing comparison at 550 bp region.

Exo-Alp Cleanup Reduces Sequencing Backgrounds for Both BigDye and SupreDye Chemistry Cycle Sequencing Reactions

We then purified the PCR template with Exo-Alp and sequenced it with BigDye Terminator Cycle Sequencing Kit and SupreDye Cycle Sequencing Kit, respectively. Our results indicate that Exo-Alp treatment reduces background for both BigDye and SupreDye cycle sequencing reactions compared to the untreated conditions, especially in the 1 to 240 bp region (Figure 2a). In addition, the Exo-Alp treatment shows better peak resolution at the 460 to 700 bp region compared to the untreated sample (Figure 2b).

The data also showed that both BigDye and SupreDye chemistry produces comparable sequencing quality for the PCR product.

In summary, we have shown that the Exo-Alp is a close alternative to ExoSAP for PCR template cleanup before cycle sequencing. Both products significantly reduce the sequencing background and improve the resolution of peak separation. Therefore, it is strongly recommended that the PCR products be cleaned up before cycle sequencing to improve Sanger sequencing quality. Our results have demonstrated again that SupreDye chemistry can replace BigDye chemistry for Sanger sequencing.

Figure 2a


Untreated FKS1: 1-240bp
ABI BigDye v3.1 Cycle Sequencing Kit
Untreated FKS1: 1-240bp
ADS SupreDye v3.1 Cycle Sequencing Kit
EXO-Alp Treated FKS1: 1-240bp
ABI BigDye v3.1 Cycle Sequencing Kit
EXO-Alp Treated FKS1: 1-240bp
ADS SupreDye v3.1 Cycle Sequencing Kit

Figure 2b

Figure 2. Exo-Alp treated PCR product for Sanger sequencing using either BigDye or SupreDye cycle sequencing kit. The 1 to 240 bp region (2a) and 460 to 700bp (2b) region were compared.