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RESEARCH ARTICLE

An economical and efficient method of post reaction cleanup of labeled dye terminator sequencing products

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Abstract

Sanger dideoxy DNA sequencing method is the most popular and simple DNA sequencing method and the most economic method available in the market as per as the throughput is concerned. The good quality of sequences depends not only on the quality of the template but also on efficient removal of unincorporated fluorescent labeled dideoxy nucleotides (ddNTPs). The present study reports an economical and efficient method of post reaction cleanup of labeled unincorporated ddNTPs. The efficiency of the method was compared with different methods. EDTA - Isopropanol method gave very good sequence compared to other methods used in the present study. The EDTA - Isopropanol method gave good quality (QV≥ 20) sequence of 86 bases out of 120 bases of initial region of the sequence which covers both dye blobs. The enhancement of the quality of sequences especially at the initial region of 120 bases not only makes the analysis part easy but also reduces the use of number of primers. Subsequently, reducing the cost of sequencing.

Introduction

Sanger dideoxy DNA sequencing method (Sanger et al., 1977) is the most popular and simple DNA sequencing method and the most economic method available in the market. Overall scientific grants and funding for basic research have come down globally. At this stage only solution left with us is to reduce the cost of experimentation by making use of reagents most economically. Here we are addressing the issue of post reaction cleanup of labeled dye terminator sequencing products. There are different methods available in the market like X dye terminators, Na acetate alcohol method etc. But these are expensive and introducing foreign substances inside the DNA analyzers which affect the life of capillary array in long run. Hereby we tested a simple method of purifying the PCR product before sequencing that is not only cost effective but also simple and easy to perform. We obtained clean sequences free of dye blobs and spike especially in the region 90-120, which most of the purification protocols mentions as universal problem. By this method we are addressing this universal problem. Additionally, this method further reduces the cost by decreasing the number of primers required and hence reducing the number of reactions.

Materials and methods

Except Isopropanol and molecular grade water (Sigma) rest of the chemicals and Sequencing plates were purchased from Life technologies, USA.

Template preparation

Plasmid with known sequence information was used in this study. The plasmid DNA template was prepared using Plasmid DNA isolation kit (Quiagen, Germany) following the manufacturer's instructions. The plasmid DNA was quantified using Nano drop (JH Bio India). Hundred nanogram of plasmid template was taken for the sequencing PCR reaction.

Cycle sequencing PCR and product purification

A cycle sequencing PCR master mix was prepared using Big dye terminator v 3.1 cycle sequencing kit (Applied Biosystems USA) which includes Big dye terminator reaction mix, 5x sequencing buffer, Plasmid DNA (100 ng) single primer and remaining molecular grade DNase free water. In thermalcycler (GeneAmp PCR system 9700, Applied Biosystems USA) the following program was executed: an initial denaturation of 95^{o}C for 1 min followed by 25 cycles of 95^{o}C for 10 sec: 50^{o}C for 5 sec: 60^{o}C for 4 min and hold at 4^{o}C. After cycle sequencing PCR, the product was split into four tubes and the PCR product was purified as mentioned below (Figure.1).

a. Isopropanol precipitation

Freshly prepared 80 µl of 80 % isopropanol was added to sample containing well and allowed to incubate for 15 min in dark and centrifuged at 4000 rpm for 45 min. After centrifugation the plate was taken out from the centrifuge, septa was removed, a three folds of paper towels were placed on the plate and gently inverted along with holding paper towels and placed it in to the centrifuge. It was centrifuged for 1 min at 600 rpm. The plate was then taken out. The resulted pellet was re-suspended in 10 µl of Hi Di Formamide (Applied Biosystems, USA) heat shocked at 95^{o}C for 2 min and snap cooled.

b. Sodium acetate-Ethanol precipitation (Das et al., 2006)

Two microlitre of 3M Sodium acetate (NaOAc pH 5.2) was added to the well containing PCR product, mixed thoroughly and allowed to incubate for 15 min in dark. To this a 60 µl of 95% ethanol was added and Centrifuged at 2000xg for 30 min. The invert plate centrifuge step was followed as mentioned above. A 150 µl of 70 % ethanol was added to the well and centrifuged at 2000xg for 30 min. The above mentioned invert plate centrifuge step was performed by covering the plate with three folds of paper towel. Air dried the pellet and re-suspended it in 10 µl of Hi Di Formamide (Applied Biosystems, USA) heat shocked at 95^{o}C for 2 min and snap cooled.

c. EDTA -Isopropanol Precipitation

For 10 µl reaction volume, 2 µl of 1x EDTA (Ethelenediaminetetra acetic acid) buffer was added and centrifuged briefly in order to mix the content uniformly and allowed to incubate for 5 min. Freshly prepared 80 µl of 80 % Isopropanol was then added and further incubated for 15 min in dark and centrifuged at 4000 rpm for 45 min. The above mentioned invert plate centrifuge step was performed to get rid of isopropanol. The pellet was re-suspended in 10 µl of Hi Di formamide (Applied Biosystems, USA) and heat shocked at 95^{o}C for 2 min and snap cooled.

d) Magnetic bead method

For magnetic bead separation method HighPrep DTR (Magbio Genomics Inc, USA) kit was used. Ten micro liters of highprep DTR reagent and 40 µl of 85% Ethanol were added to 10 µl reaction volume and rest of the procedure performed as per the kit insert.

DNA sequencing

The Sanger DNA sequencing was performed in 3130*xl* DNA analyzer (Applied Biosystems Pvt. Ltd Foster USA). After entering the sample details in plate manager module the plate containing samples was kept in auto-sampler block and run command was executed. Two hours standard run was performed for 50 cm long array with injection voltage at 2.0kV and sample injection volume 1µl. The similar analysis was also performed in 3730*xl* DNA analyzer (data not shown).

Data analysis

The Ab1 file thus generated was opened in sequencing analysis software v5.4 (Applied Biosystems Pvt. Ltd, USA) for sequence quality check and also for comparison with sequences obtained by four different purification methods employed in the present study.

Results and Discussion

Among four different methods of cycle sequencing product purification performed in the present study the combination of Isopropanol-EDTA gave very good sequence. The dye blob intensity was reduced, which is evident by seeing the electropherograms (Figure. 2). The dye blob 1 observed between 60 to 75 bp was present in all the three electropherograms except magnetic bead method, where as the dye blob 2 observed between 105 to 120 bp was absent in EDTA-Isopropanol purified sample. The number of good quality bases (quality value assigned by the sequence analysis software were QV ≥20) were counted within the first 120 bases which covers both the dye blobs. Compared to other three methods the EDTA and Isopropanol method gave 86 bases with quality value ≥20 (Table 1). Even though magnetic bead method gave dyeblob free sequence but the good quality sequence in the initial 120 bp region is 77 only which is less than the EDTA-Isopropanol method but relatively more than the other two methods (Table.1).

The Sanger dideoxy sequencing method or capillary electrophoresis is a well established, highly reliable, most economic and simple method of DNA sequencing available for the researcher. There are different array of instruments available in the market. By introducing foreign substances like Sodium acetate we are sacrificing the capillary array life and also increasing the cost of sequencing reaction, as the charged molecule Sodium acetate is injected and interferes with the DNA migration in the capillary (http://www.roswellpark.edu). We used 1x EDTA

buffer which is also present in the machine in buffer jar and in buffer tray. Our method is not adding any new chemical to the sequencing machine and as per Isopropanol is concerned it is easily removed by invert spin. This method has proven to be effective and inexpensive as compared to other conventional methods. Compared to NaOAc-Ethanol purification, plane Isopropanol purification, EDTA-Isopropanol purification method gave very good, dye blob free sequences, even though the magnetic bead method gave dye blob free sequence but quality wise and cost wise EDTA-Isopropanol will be a better choice. The increase in the quality of the sequences especially at the initial region of 120 bases not only makes the analysis part easy but also reduces the use of number of primers. There are number of attempts has been made by different labs around the world to reduce the cost of sequencing per sample. For example Ma and Difazio (2008) reported purification method for PCR products before sequencing, they have used standard agarose gel combined with 0.1 % -0.2 % low-melting point (LMP) agarose, but they have not addressed the issue of cycle sequencing product purification which is not possible through the above mentioned method. Combining present method with method developed by Ma and Difazio (2008) the cost of sequencing per sample can be reduced further.

If the genomic DNA isolated through the method developed by Looke et al. (2011) which includes the use of lithium acetate (LiOAc)–SDS and both are relatively very much cheaper than genomic DNA isolation kits.

Author contributions: RRK works as contract person at sequencing facility and reports to VN and has worked on the concept in the laboratory. VN proposed the concept and guided RRK for the laboratory work. RRK and VN have jointly written the manuscript.

Table.1 comparison of sequences obtained from different sequencing PCR product purification methods.

Parameters	Isopropanol	Sodium acetate and Ethanol	Isopropanol and 1x EDTA buffer	Magnetic bead
Dye blob 1 (60 -75 bp)	Present	Present	present	Absent
Dye blob 2 (105-120 bp)	Present	Present	Absent	Absent
Dye blob 1 intensity	>28000	> 7000	>2600	--
Dye blob 2 intensity	>2500	>1800	--	--
Good quality Initial sequences * (out of 120)	64	71	86	77

* Sequence quality value ≥20

Figure.1 Experimental design: the cycle sequencing PCR product was split into four tubes and performed four different purification methods.

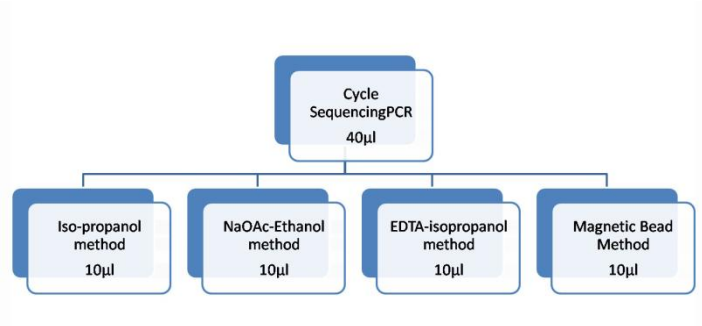
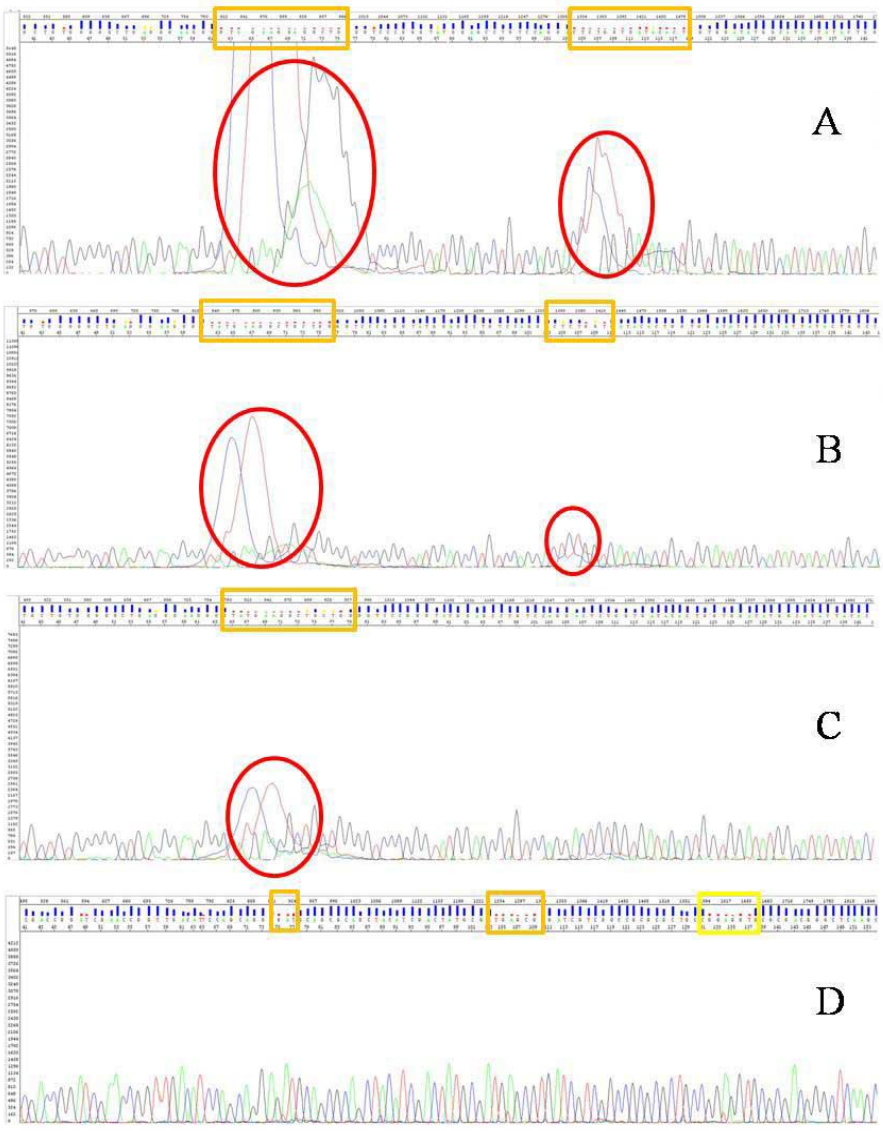


Figure.2 Electropherograms of DNA sample obtained after the purification using four different methods. (A) Sequencing products purified through Isopropanol method. (B) Sodium acetate and Ethanol method. (C) EDTA and Isopropanol method. (D) Magnetic bead method. The dye blob region was encircled and sequences with QV≤20 was represented in rectangular boxes.



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Competing interests: The authors declare no competing interests.

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