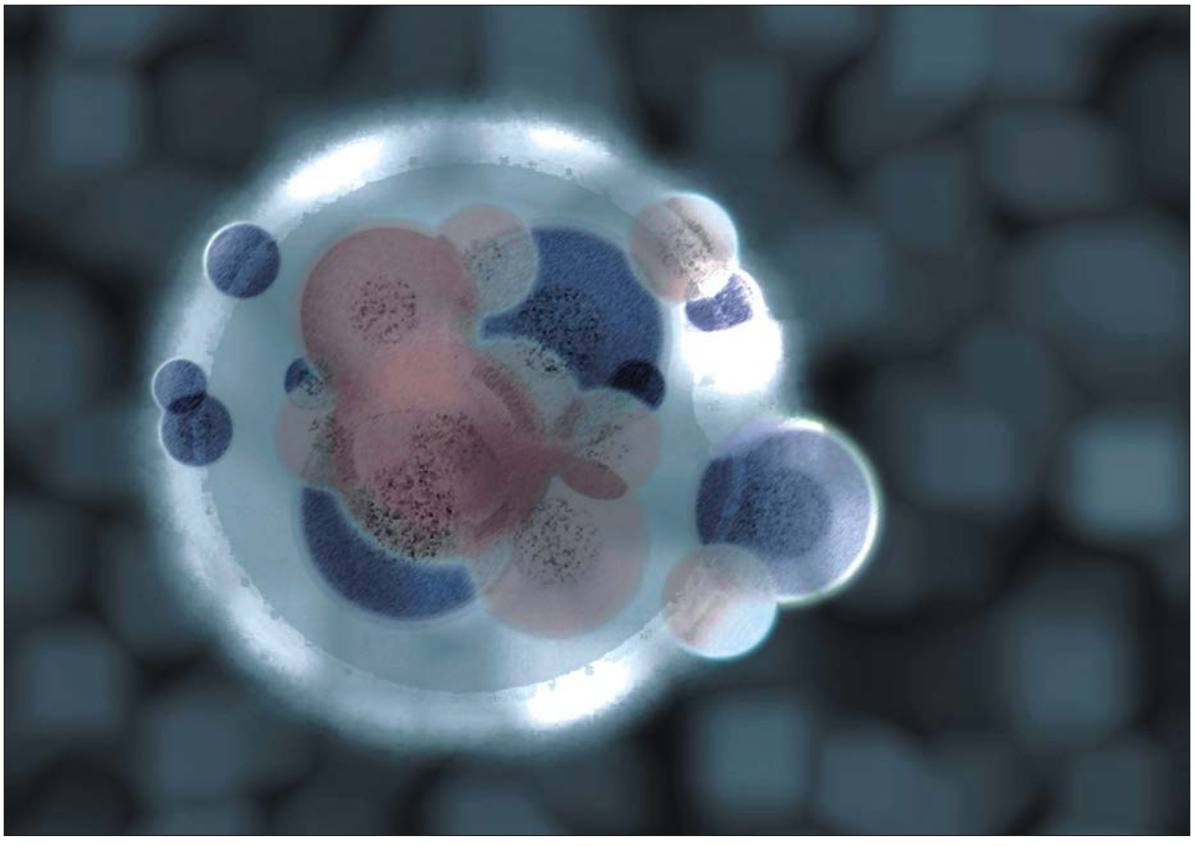

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Optimization of the Sample Preparation Method for DNA Sequencing

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Abstract: This study reports on the modification and optimization of the sample preparation method for analyzing nucleotide sequence in plant species by capillary electrophoresis. The experimental study was aimed at developing possible ways of increasing the throughput of the method. The optimization conditions modified were purification of the PCR product, concentration adjustment of cycle sequencing kit BDT v3.1 from half to quarter reactions for economic analysis. Concentrations of the tamplet, primer and program of thermocycler were also optimized. Finally the volume of Hi-Di form amide was adjusted for the required sequencing. The accuracy of analysis was greater than 99.99%. The modified method enables quality sequencing results with higher average signal intensities compared to the standard recommended method. With the optimized electrophoresis conditions reported here, greater than 700 base pairs can be analyzed within 75 min using POP7. It was found that deviation from the recommended method consistently delivers higher signal-to-noise ratios and signal intensities and longer Phred20 read lengths. It is more reproducible than that of other methods and enables rapid, more cost effective robustness for nucleotide sequencing in the samples of plant origin received from research laboratory sources.

Key words: PCR product, BDT cycle sequencing kit v3.1, Hi-Di Form amide

INTRODUCTION

DNA sequencing has become a major tool for the analysis of genes. Reliable sequencing systems that allow the determination of a fragment of about 1000 and higher nucleotides are available (Belen'kit, 2000; Zhou *et al*., 2000)). Large-scale technical projects on the sequencing of DNA require high-throughput analyzer with high sequencing accuracy, low cost and less time. This in turn has stimulated the modification and optimization of recommended methods in terms of analytical device, methods and reagents for analyzing nucleic acids (Belen'kit and Kurochkin, 2003). ABI 3130 Genetic Analyzer recommended the BigDye Terminator v3.1 Cycle Sequencing kit which has a new formulation that delivers increased robustness; more even peak height and longer read length (ABI Prism BigDye, 2002; Seo *et al*., 2005). The Alcohol precipitation is required to clean template to obtain well separated sequencing peaks. The primer used to amplifying the PCR products is efficiently removed before the product is sequenced. This can be achieved in a number of ways, including excision from agarose gels and the use of size exclusion membranes (The QIAGEN Guide, 1998). The deleterious effect of template DNA on the separation of sequencing fragments was suppressed in the presence of salt in a concentration above 100 μM in the sample solution (Salas *et al*., 1998). The use of form amide is known to reduce the melting temperature of the DNA duplex by weakening hydrogen bonds (Ryeon and Kim, 1997). For efficient electro kinetic injection of sequencing product onto the capillaries, it is an important factor that the medium that they are suspended in has a low conductivity (Hideki *et al*., 1998) Standard Tamplet Suppressor Reagent (TSR) contains excessive ionic materials, which prevents efficient transfer of sequencing product onto the capillaries (Rochelear *et al*., 1992).

In this research we report a simpler optimized sample preparation method for DNA sequencing in plant species. The modified method was applied for quality sequencing results with higher average signal intensities and compared to the standard recommended method, which showed 99.99% accuracy (Ewin *et al*., 1998). It was also observed that the deviation from the use of recommended volume of Hi-Di form amide provided well-separated evenly spaced peaks and reduced baseline noise (Zipperlen *et al*., 2005).

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MATERIALS AND METHODS

Commercially available Microcon Centrifugal Filter device, BigDye Terminator v3.1 cycle sequencing kit, BigDye Terminator v3.1 Sequencing Standard kit, 10 x buffer, Hi-Di Form amide and POP7 were received from ABI. And the samples (PCR products) were purified by Microcon Centrifugal Filter device. All other reagents used were of analytical grade. Plant samples were received from the corresponding research laboratories, Department of Biochemistry and Molecular Biology, University of Dhaka. Sequencing was performed in ABI Prism 3130 Genetic Analyzer at the Centre of excellence, University of Dhaka.

Purification of PCR product: The PCR product was purified by using commercially available Microcon Centrifugal Filter device. Microcon sample reservoir was inserted into a screw cap vail. Then pipetted 0.5 mL of sample solution into sample reservoir, without touching the membrane with the pipette tip and sealed with the attached cap. The vail assemble was centrifuged at 12,000 rpm for 12 min. The assemble was removed and the sample vail was separated from the reservoir. Sample reservoir was placed upside down in a new vail, then spined at 10,000 rpm for 3 min to transfer to concentrate vail. Finally the sample reservoirs were separated and snap sealing cap shut to store the product for use (ABI Prism BigDye 1997; Patrick and Mullan, 2000).

Cycle sequencing: The cycle sequencing program was optimized for this experiment. The following parameters were modified: 4 and 2 μL of BigDye Terminator v3.1 were used; DNA tamplet concentrations used for 1000 nucleotides were 10, 15, 25, 30 and 40 ng μL^{-1}, respectively. Primer concentration used for this reaction is 3.2 pmol μL^{-1} for half reaction and 1.8 pmol μL^{-1} for quarter reaction. Total volume was made up to 20 and 10 μL by adding nano-pure water. The solution was mixed well by gentle pipeting without forming air bubbles and modified thermocycler program was set as follow:

Initial denaturation	96°C	2 min	
Denaturation	96°C	10 sec	25 cycles
Annealing	50°C	5 sec	25 cycles
Extension	60°C	4 min	25 cycles
Termination	60°C	1 min	

The cycle sequence programme was also changed for 2 μL of the BigDye v3.1 terminator. We continued 35 cycles instead of 25 cycles (Meis, 2000).

Purification of the cycle sequencing products: Purification was carried out using standard ethanol precipitation method (ABI Prism BigDye, 2002). The entire sample content was dissolved in 16 μL of Hi-Di form amide. The samples were immediately denatured at 95°C for 3 min in a thermal cycler. The samples were preserved in ice immediately before use for sequencing. 10 μL of sample aliquot was used for analysis (Salas *et al*., 1998; Meis, 2000).

Sequencing: The purified cycle sequencing product is analyzed by electrophoresis in the ABI-Prism 3130 genetic analyzer.

RESULTS AND DISCUSSION

DNA samples analyzed with the modified method provided well-resolved peaks with stable base line. There is no signal noise following the method. Reproducible standard sequencing results were obtained with 8 μL BigDye v3.1 Terminator kit that was prepared according to ABI recommended method (Fig. 1). Modification of the plant sample preparation method using 4 μL had no significance changes (Fig. 2). Comparison of the results confirmed that the sequencing pattern of both the standard and the sample are the same, even though the concentration of sequencing kit v3.1 has reduced to half reaction (4 μL). Further modification of the cycle sequencing program had no effect on sequencing results. The time required for the cycle sequencing program is less than that of recommended method. In the experimental procedure, the volume of cycle sequencing kit v3.1 was further reduced to quarter reaction (2 μL). Here, the results showed the same sequencing pattern with higher average signal intensities. And additional observation was made that the small fragment less than 500 bp can be read very clearly without any signal noise (Fig. 3). The results were compared with a longer run of fragment size greater than 1000 bp. It was found that the sequence beyond 500 bp, base call is not confident (Fig. 4). The peaks become broader and are not well resolved. There is also less space between base call beyond 500 bp. Some of the space at the top is regular, which is reliable but as the sequence progress is further the spacing become irregular. There were no background bands, which indicates that the sample preparation was perfect following the modified method. Also if the sample contains impurities, extra bands inserted in between two normal peaks, or may be superimposed on another band, producing a two-color peak. Figure 5 shows that the modified method consistently delivers higher signal-to noise ratios and

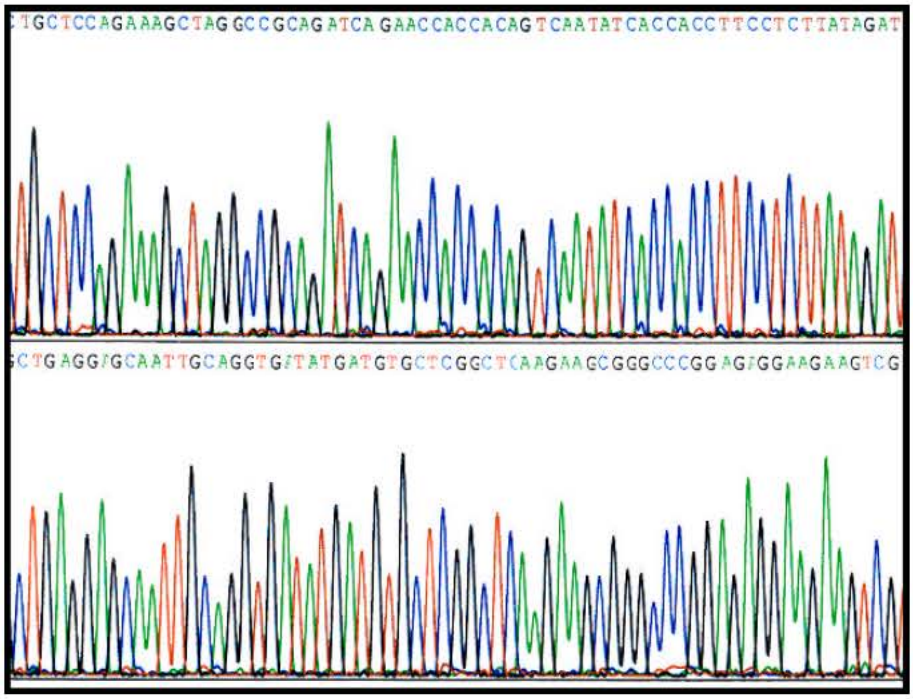

Fig. 1: Sequencing of standard DNA fragment (1200 bp) following ABI recommended sample preparation method using 8 μL BigDye Terminator v3.1

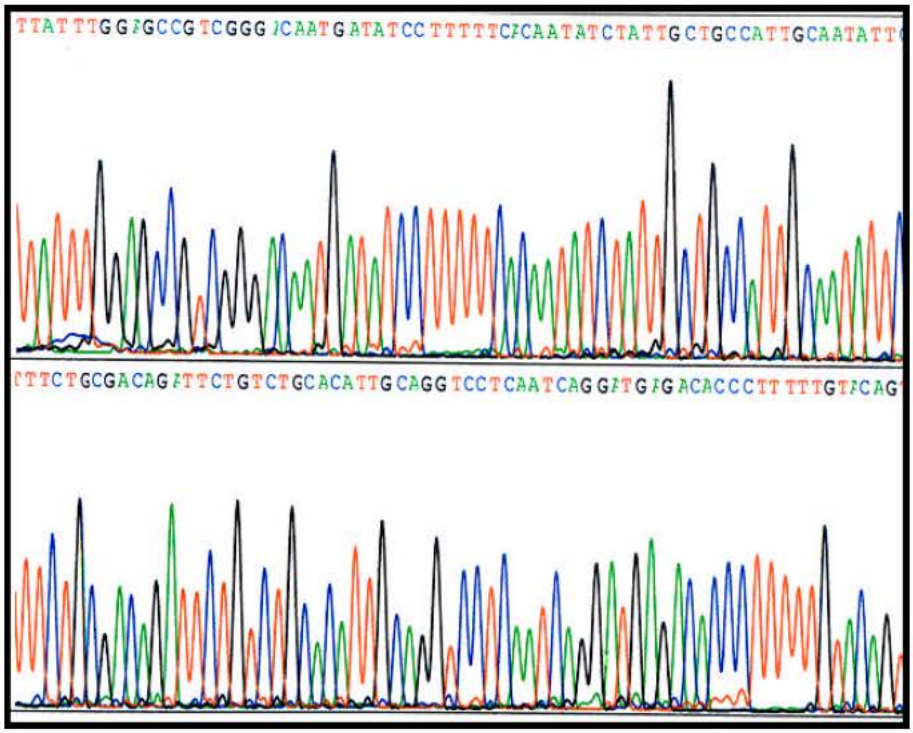

Fig. 2: Sequencing of plant DNA fragment (1200 bp) following modified sample Preparation method using 4 μL BigDye Terminator v3.1

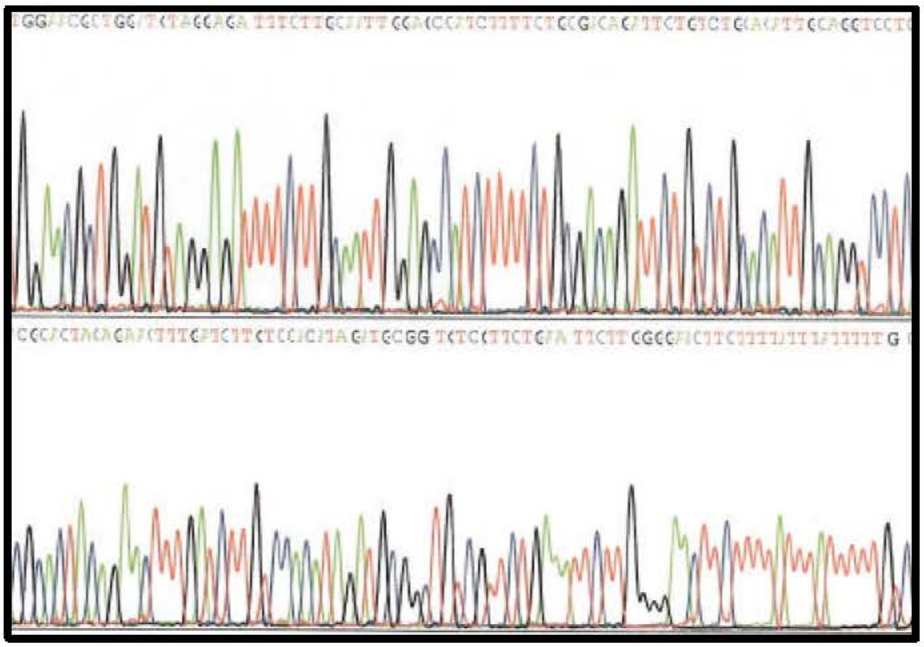

Fig. 3: Sequencing of plant DNA fragment (400 bp) following modified sample preparation method using 2 μL BigDye Terminator v3.1

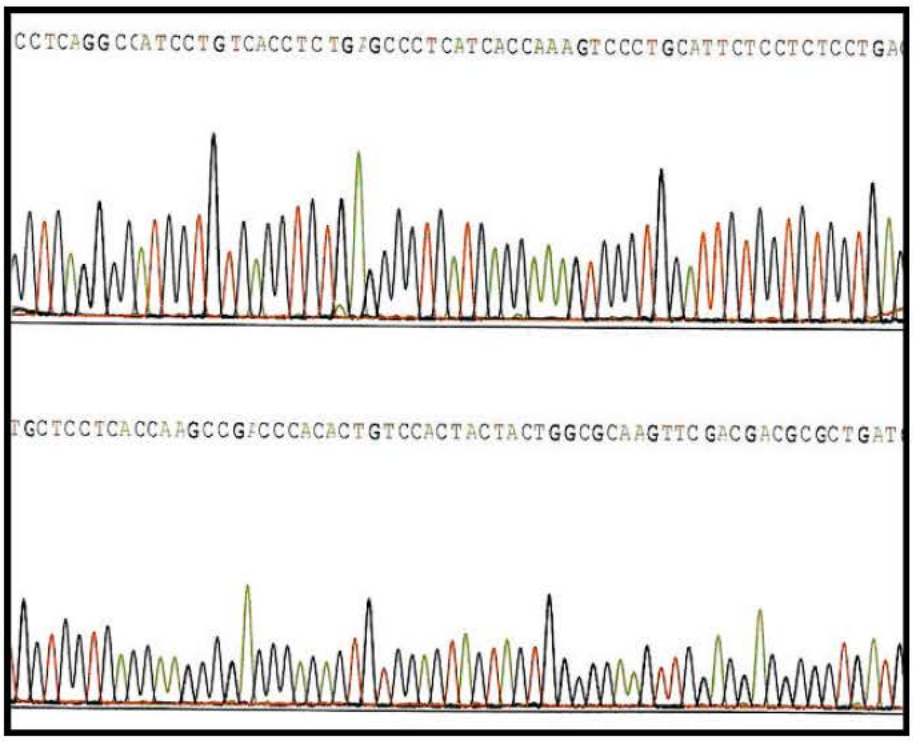

Fig. 4: Sequencing of the same plant DNA fragment size (1000 bp) following modified sample preparation method sing 2 μL BigDye Terminator v3.1

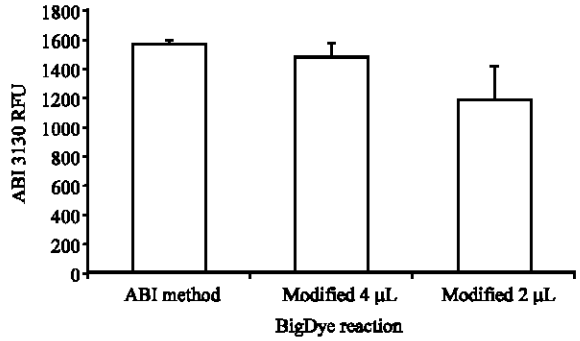

Fig. 5: Signal intensities of RFU with BigDye concentration reaction

signal intensities, longer Phred20 read length and produced Relative Fluorescence Unit (RFU) at the recommended range of 1000-2000 (Ewin *et al*., 1998; Richteriich *et al*., 1998).

In Fig. 2 and 3 there were no artifacts of peaks, which indicate that the template concentration used in the modified method was accurate. It was observed that for DNA fragment size 1000 bp, the concentration of template optimized was 40 ng μL^{-1} and for DNA fragment size 500 bp, the concentration of template optimized was 25 ng μL^{-1}. These values are different from the ABI recommended method. Results also showed that there was no broad stretch of multicolored peaks within the first 80 bases which indicates that unincorporated dye terminators have been removed successfully during ethanol precipitation reactions followed in this method. In this study, it was further observed that as the gel run progress with quarter reaction (2 µL) BigDye Terminator v3.1, it loses resolution above 500 bp for longer fragment. But for shorter fragment (400 bp) no broad peaks were observed. These samples were compared with standard run of control DNA provided by ABI. We found that for control DNA there was no broader peaks at the end of the gel run. The run was successful even with more than 800 bp.

From these results, it is concluded that the modified method valid for sequencing of DNA from different resources. In the experimental measurements, it was found that first 750 nucleotides are confirmed with less than 1% errors. Thus, the present modification and optimization of the recommended sample preparation method for DNA sequencing showed the best results and it can be followed for regular sequencing of any PCR products, single stranded and double stranded DNA, instead of using the regular time consuming method.

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