DNA Sequencing

POST SEQUENCING REACTION PURIFICATION IN 96-WELL GEL FILTRATION COLUMN PLATES

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Introduction

Capillary electrophoresis (CE) is an excellent approach for meeting the increasing demand for high-throughput, automated DNA sequencing. However, it does represent a substantial change in DNA sequencing technology. The presence of unincorporated dye-labeled ddNTP and salts in the sample can have a significant negative effect on the quality of sequencing data generated by CE. Post-reaction purification methods that effectively remove the offending molecules are required. Furthermore, as throughput demands continue to increase, the need for effective purification methods that are less time-consuming and less laborious becomes greater.

The standard ethanol precipitation method used to remove unincorporated nucleotides and salts is very effective but, unfortunately, time-consuming and labor-intensive. In this bulletin, we compare three column purification methods for post-reaction purification with ethanol precipitation. These column purification methods use 96-well gel filtration column plates. Data from tube ethanol precipitation and the gel filtration methods are shown.

Materials and Methods

Template Preparation

Three different plasmid DNA templates were used in this study: pUC18 (2.7 kb), a large (12 kb) plasmid, and a (63.9%) GC-rich plasmid. These plasmid DNA templates were prepared using commercially available kits from either QIAGEN (QIAprep*) or Promega (Wizard*). The manufacturer's instructions were followed in each case.

Templates were quantitated by running restriction-digested aliquots on agarose gels and comparing the ethidium bromide staining intensity to that from known standards.

Sequencing Reactions

Sequencing reactions were set up as described in the Beckman Coulter DTCS Sequencing Protocol.[1] For all the templates except the pUC18 control, a simple preheating procedure[2] was carried out to improve the sequencing reaction. Cycling conditions were 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes.

Post-Sequencing Reaction Purification by Ethanol Precipitation

Cleanup of post-sequencing reaction samples by ethanol precipitation was carried out as described in the Beckman Coulter DTCS Sequencing Protocol.)

Post-Sequencing Reaction Purification Using the Millipore Plates

Dry Sephadex G50* medium (Pharmacia/Amersham) was loaded into a Millipore HV plate using the

* QIAprep is a registered trademark of Qiagen, Inc.
Wizard is a registered trademark of Promega Corporation.
Sephadex G50 is a trademark of Amersham Pharmacio Biotech, Inc.

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MultiScreen* 45 µL Column leader as described in the instructions from Millipore (Bedford, MA), after which 300 µL of deionized water was added to each well. The resin was allowed to swell at room temperature for 3 hours. A MultiScreen Centrifuge Alignment Frame was placed on the bottom of the MultiScreen HV plate and the entire setup was placed on top of a 96-well microtiter plate (Beckman Coulter, P/N 373660). To pack the columns, the entire microplate setup was centrifuged at 1100 × *g* (3000 rpm) for 5 minutes in a GS-15R centrifuge (Beckman Coulter). The columns were washed once with 150 µL of deionized water.

Sequencing reactions were transferred onto individual wells of the prepared MultiScreen HV plates. The HV plate/Alignment Frame was placed on top of a 96-well CEQ™ 2000 DNA Sequencer sample plate (Beckman Coulter). The plates were aligned (matching the well positions of the top and bottom plates properly) and held together with tape. The entire microplate setup was centrifuged at 1100 × *g* (3000 rpm) for 5 minutes in a GS-15R centrifuge (Beckman Coulter). The purified samples were dried in a DNA SpeedVac** (Savant). The samples were resuspended in 40 µL of deionized formamide prior to loading on the CEQ 2000 DNA Sequencer.

* MultiScreen is a registered trademark of Millipore Corp.

Post-Sequencing Reaction Purification Using the Edge Biosystems Plates

Use of the AGTC** 96-well gel filtration block (columns) from Edge Biosystems (Gaithersburg, MD) was carried out following the manufacturer's instructions. Briefly, the 96-well column block was centrifuged at 750 × *g* (2500 rpm) for 2 minutes in

** DNA SpeedVac is a registered trademark of Savant Instruments, Inc. AGCT is a registered trademark of Edge Biosystems.

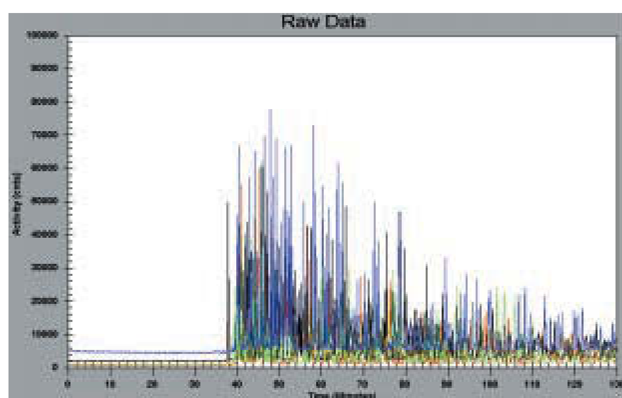

Figure 1a. *Raw data profile for pUC18 control DNA sequencing sample processed by ethanol precipitation.*

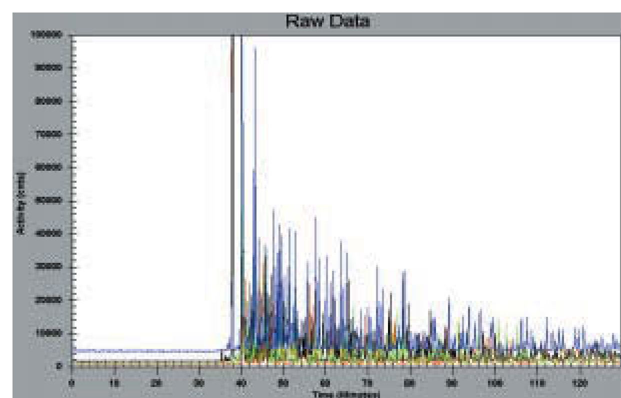

Figure 1b. *Raw data profile for pUC18 control DNA sequencing sample processed by gel filtration using Sephadex G50 in the Millipore MultiScreen plate.*

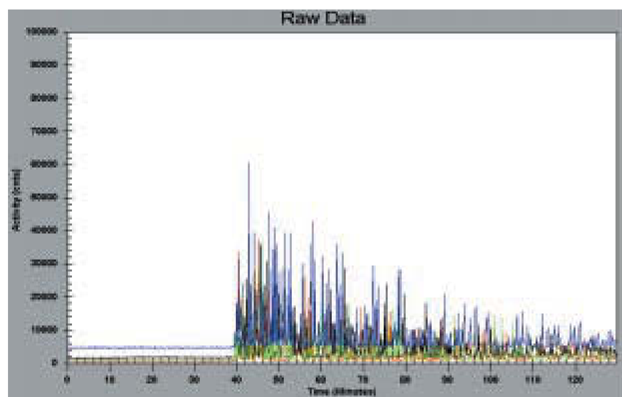

Figure 1c. *Raw data profile for pUC18 control DNA sequencing sample processed by gel filtration using the AGTC 96-well gel filtration block from Edge Biosystems.*

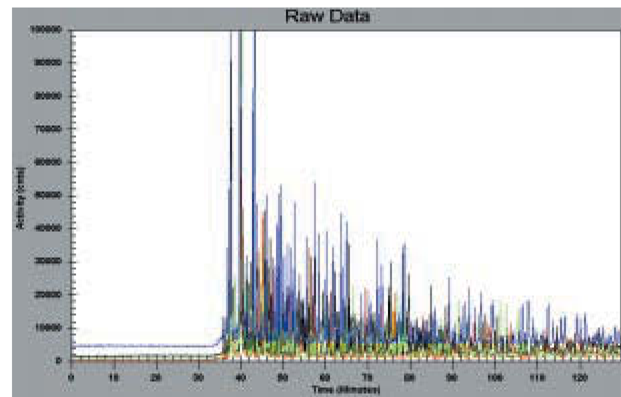

Figure 1d. *Raw data profile for pUC18 control DNA sequencing sample processed by gel filtration using the CentriSep 96 filter plate from Princeton Separations.*

a GS-15R centrifuge (Beckman Coulter). The column eluate was discarded. The sequencing reactions (usually about 20 µL, not to exceed 50 µL) were loaded onto the individual columns. The 96-well column block was then centrifuged again at 750 × *g* for 2 minutes. The purified samples were collected and dried in a DNA SpeedVac (Savant). The samples were resuspended in 40 µL of deionized formamide prior to loading on the CEQ™ 2000 DNA Sequencer.

Post-Sequencing Reaction Purification Using the Princeton Separation Plates

Use of the CentriSep* 96-well filter plates from Princeton Separations (Adelphia, NJ) was carried out following the manufacturer's instructions with slight modifications. Briefly, the 96-well plate was centrifuged at 1100 × *g* (3000 rpm) for 5 minutes in a GS-15R centrifuge (Beckman Coulter). The column eluate was discarded. The sequencing reactions (usually about 20 µL, not to exceed 50 µL) were loaded onto the individual wells. The 96-well plate was then centrifuged again at 1100 × *g* for 5 minutes. The purified samples were collected and dried in a DNA SpeedVac (Savant). The samples were resuspended in 40 µL of deionized formamide prior to loading on the CEQ 2000 DNA Sequencer.

Sequencing on the CEQ 2000 DNA Sequencer

The purified sequencing reaction samples were run on the CEQ 2000 DNA Sequencer using the DTSC-2 method. Separation was at 35°C, 8 kV, for 130 minutes. Data analysis was carried out using the default settings.

* CentriSep is a trademark of Princeton Separations, Inc.

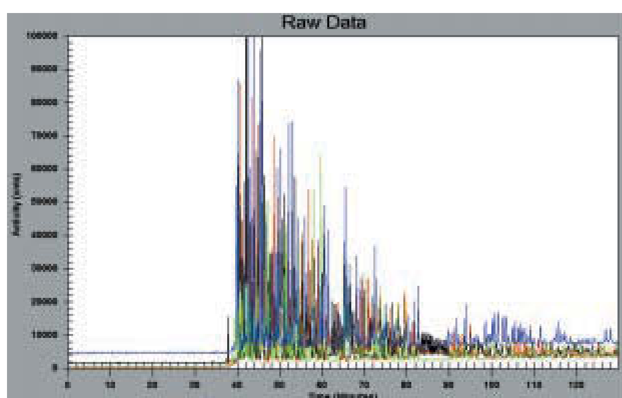

Figure 2a. *Raw data profile for a large (12 kb) plasmid DNA sequencing sample processed by ethanol precipitation.*

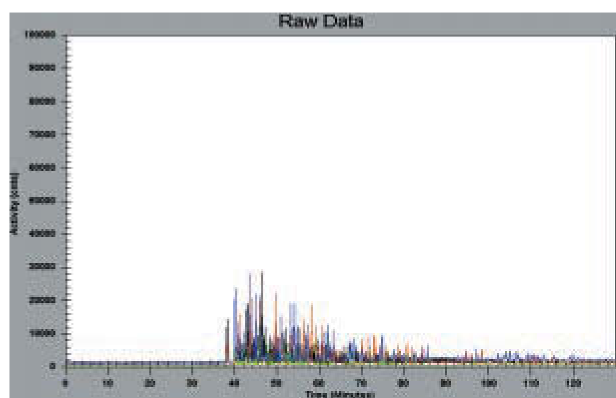

Figure 2b. *Raw data profile for a large (12 kb) plasmid DNA sequencing sample processed by gel filtration using Sephadex G50 in the Millipore MultiScreen plate.*

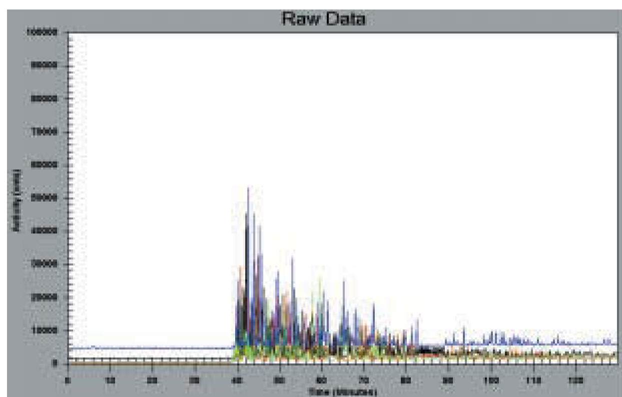

Figure 2c. *Raw data profile for a large (12 kb) plasmid DNA sequencing sample processed by gel filtration using the AGTC 96-well gel filtration block from Edge Biosystems.*

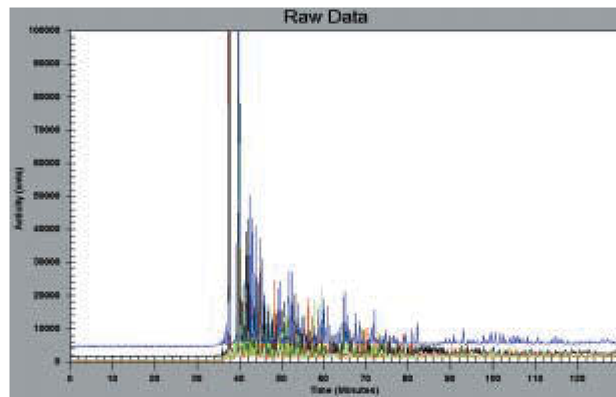

Figure 2d. *Raw data profile for a large (12 kb) plasmid DNA sequencing sample processed by gel filtration using the CentriSep 96 filter plate from Princeton Separations.*

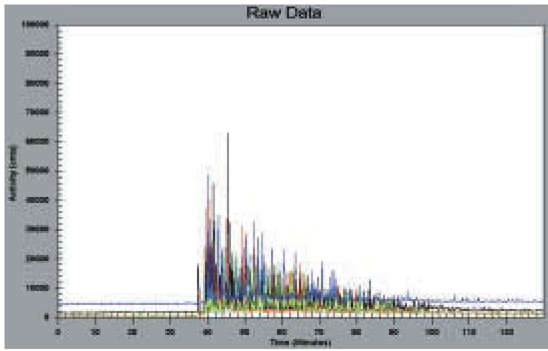

Figure 3a. *Raw data profile for a GC-rich plasmid DNA sequencing sample processed by ethanol precipitation.*

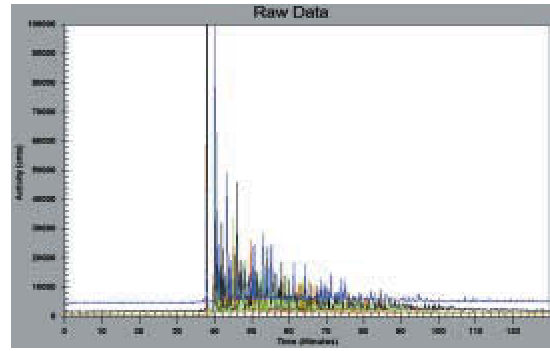

Figure 3b. *Raw data profile for a GC-rich plasmid DNA sequencing sample processed by gel filtration using Sephadex G50 in the Millipore MultiScreen plate.*

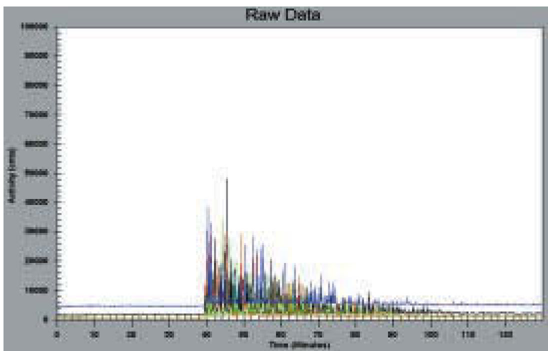

Figure 3c. *Raw data profile for a GC-rich plasmid DNA sequencing sample processed by gel filtration using the AGTC 96-well gel filtration block from Edge Biosystems.*

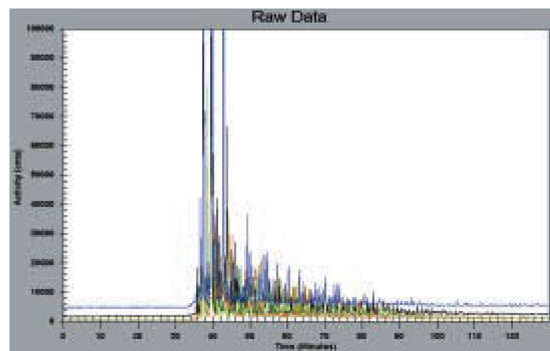

Figure 3d. *Raw data profile for a GC-rich plasmid DNA sequencing sample processed by gel filtration using the CentriSep 96 filter plate from Princeton Separations.*

*Table 1: Approximate Processing Times for Various Sample Clean-up Methods for CEQ™ 2000 DTCS Sequencing Reactions**

Clean-up Method	***Hands-On Time (minutes)***	***Total Time (minutes)***
Tube Precipitation (24 samples)	80	120
Plate Precipitation (96 Samples)	90	100
Spin Plates (96 samples)	15	45

* Times assume the use of a 96-well plate vacuum centrifuge system (SpeedVac). Other non-centrifuge-based vacuum drying methods may add as many as 90 minutes to the total times.

Results

Three different DNA templates were sequenced to demonstrate the application of the various purification procedures on templates prepared by standard commercial kits. As shown in the examples below, high-quality sequence calls were obtained with these post-reaction purification methods. Recovery of the samples by the Edge Biosystems and the Princeton Separations methods were about 40–60% and by the Millipore method was about 30–50% of the recovery with tube ethanol precipitation. Even though there were differences in the signal due to varying recovery, in all cases the accuracy at 500 bases for each of the three templates was greater than 98%.

Figure 1 shows the representative data profiles of pUC18 control template sequencing reactions purified by tube ethanol precipitation, gel filtration with Sephadex G50 in Millipore plate, AGCT gel filtration column block, and CentriSep filter plate (panels a, b, c, and d, respectively).

Figure 2 shows the raw data profiles of a large (12 kb) plasmid DNA template sequencing reactions purified by tube ethanol precipitation, gel filtration with Sephadex G50 in Millipore plate, AGCT gel filtration column block, and CentriSep filter plate (panels a, b, c, and d, respectively).

Figure 3 shows the raw data profiles of a GC-rich plasmid DNA template sequencing reactions purified by tube ethanol precipitation, gel filtration with Sephadex G50 in Millipore plate, AGCT gel filtration column block, and CentriSep filter plate (panels a, b, c, and d, respectively).

Conclusions

Ethanol precipitation is the most widely used post-sequencing reaction sample cleanup procedure for sequencing on CEQ™ 2000 DNA Sequencer. In this bulletin, we described alternative cleanup methods based on gel filtration in 96-well/column filter plates.

As shown in the data above, there is some loss of signal in the gel filtration methods. However, good quality calls (>98% accuracy at 500 bases) were made consistently with the Millipore, the Edge Biosystems, and the Princeton Separations plates. The major benefit of using these 96-well plate methods is the decrease in the time required for processing 96 sequencing reaction samples at a time (<20 minutes hands-on time). Ethanol precipitation, either by the tube or the plate format, requires more hands-on time (see Table 1).

Before using one of these gel filtration methods, we recommend that ethanol precipitation be used first to assess if sufficient signal is being generated from the particular template/primer combination in use. Strong raw data signal (preferably within a range of 50,000 to 60,000 counts) is desirable before these alternative cleanup methods are used.

References

1. Beckman Coulter DTCS Sequencing Protocol (part number 718119) may be obtained from Beckman Coulter, Inc., Fullerton, CA.
2. *Improved Sequencing of Plasmids on the CEQ 2000 by a Simple Template Preheating Procedure*. Mark Dobbs, Ph.D. Beckman Coulter, Inc., *Application Information Bulletin A-1872A.*

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