APPLICATION INFORMATION

Post-Reaction Cleanup

A RAPID AND EFFICIENT METHOD FOR THE POST-REACTION CLEANUP OF LABELED DYE TERMINATOR SEQUENCING PRODUCTS USING THE AVANTI® J-E CENTRIFUGE WITH THE ALLSPIN JS-5.3 ROTOR

Introduction

Post-reaction cleanup of labeled DNA sequencing products to remove both the unincorporated dye terminators and residual salts is a critical step for all capillary electrophoresis-based automated sequencing technologies. This is because: 1) the unincorporated dye terminators tend to form "dye blobs" (usually within the first 80 bases) compromising the accuracy of base calling, and 2) excess salts and other ion-carrying molecules in the sequencing mix act to lower signal intensity by competing with the dye-labeled DNA sequencing products for migration into the capillaries during the electro-kinetic injection sample loading process.

Ethanol plate precipitation is an efficient and cost-effective way of removing unincorporated dye terminators and salts from sequencing reactions. Compared to commercially available kits, this method provides significant savings in cost per sample and operation time and produces higher signal intensities due to better DNA recovery (Ref. 1).

The Allegra® 25R centrifuge with the S5700 rotor has been widely used for ethanol plate precipitation sequencing cleanup (Ref. 1). We describe here another powerful tool – the Avanti J-E centrifuge coupled with the JS-5.3 rotor from Beckman Coulter, Inc., for rapid and efficient ethanol plate precipitation with even higher throughput and shorter centrifugation turnaround time.

Materials and Methods

Template Preparation

pUC18 (2.7 Kb), a large 12 Kb GC-rich (63.9%) plasmid with known sequence information was used in this study. The plasmid DNA template was prepared using QIAGEN (QIAprep*) following the manufacturer's instructions. The plasmid DNA template was quantitated with the DU® 800 Spectrophotometer (Beckman Coulter, Fullerton, CA) and adjusted to a concentration of 160 fmole/µL.

Sequencing Reactions

A master sequencing reactions mix was set up according to the protocol in Beckman Coulter's CEQ™ DTCS Kit (P/N 608000). The mix was then split into eight individual reactions each containing 80 fmole pUC18 DNA template, 3.2 pmole –47 primer, an appropriate amount of 4 dNTPs, ddATP, ddCTP, ddGTP, ddUTP, thermal stable sequencing polymerase and sequencing buffer in a volume of 20 µL. The reaction mixes were incubated with 35 cycles of 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for four minutes. After the thermal cycling incubation, the reaction mix was kept at 4°C until the next step was performed.

Ethanol Plate Precipitation

After thermal cycling, the eight reactions were divided into two groups each with four reactions in two separate sample plates for comparison of ethanol precipitation sequencing product cleanup using two different centrifuges. One set of the reactions was cleaned up using the Allegra® 25R with the S5700 rotor as described previously (Ref. 1). The other set was cleaned up using the Avanti® J-E with the JS-5.3 rotor as described bellow:

1. Add in each sample well 5 µL of freshly made stop solution containing:

3 M NaOAc, pH 5.2	2 µL
100 mM Na2EDTA, pH 8.0	2 µL
20 µg/uL glycogen	1 µL

   Stock Solutions:
   - 3M Sodium Acetate, pH 5.2 (0.2 µm nylon filter sterilized)—Sigma Catalog # S7899; store at room temperature.
   - 0.5M Na2EDTA, pH 8.0 (0.2 µm nylon filter sterilized)—Sigma Catalog # E7889; store at room temperature.

2. Cap the wells and vortex to mix completely.

3. Open the caps and add 60 µL 95% EtOH (ice-cold). Cover plate with SEAL AND SAMPLE™ Aluminum Foil Lids (Beckman Coulter, P/N 538619). Vortex the plate to mix completely. See Figure 1.

4. Centrifuge in the JS-5.3 at 5,300 rpm for two minutes at 4°C with maximum acceleration and deceleration. Save this setting as Program 1 on the Avanti J-E.

5. After centrifugation, remove the plates and place three to four folds of paper towels on the centrifuge plate holder. Carefully remove the foil lid and gently invert the plate to remove the supernatant (DO NOT turn the plate right-side up before spinning, as this may disrupt the DNA pellet). See Figure 2.

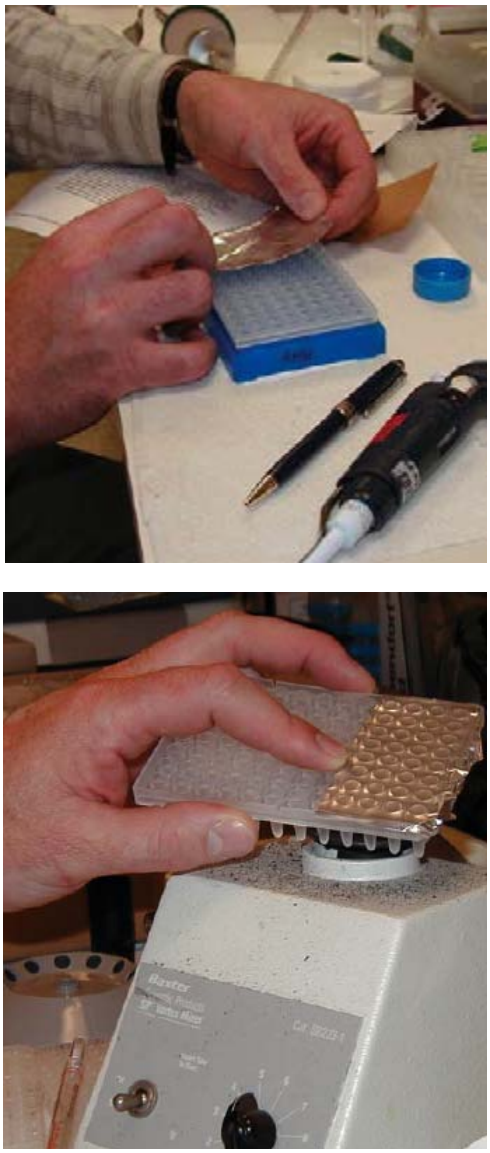

Figure 1. *(Top) Sealing the reaction plate with aluminum foil. (Bottom) Vortexing the plate.*

Figure 2. *Ensure the plate is kept inverted during the blotting process.*

6. Place the inverted plate onto the plate holder (containing folded paper towels) in the centrifuge (see Figure 3) and spin at 300 rpm (maximum acceleration and deceleration, save as Program 2) with proper balance. Carefully watch the speed indicator on the centrifuge since the Avanti J-E accelerates very quickly to 300 rpm. Once at speed, let it run for 10 seconds then press the stop button.

Figure 3. *The inverted plate containing the folded paper towels is placed into the rotor.*

7. Rinse the pellet with 200 µL ice-cold 70% EtOH/water (v/v). Do NOT mix or vortex the plate. See Figure 4.

8. Cover and centrifuge immediately at 5,300 rpm; 6,130 x *g* in the JS-5.3 at 5,300 rpm for two minutes at 4°C with maximum accelera tion and deceleration (Program 1).

9. After centrifugation, gently invert the plates to remove the supernatant. As before, do not turn the plates right side up. Place the invert ed plate into the centrifuge plate holder containing three to four folds of paper towels and spin at 300 rpm (Program 2). Again, be careful to watch the speed indicator. Once at speed, let it run for 10 seconds then press the stop button.

10. Repeat steps 7-9. This completes two washes with 70% EtOH along with the centrifugation steps.

11. Resuspend the pellets in 40 µL of sample load ing solution (SLS) provided in Beckman Coulter's CEQ™ DTCS Kit (608000) or CEQ DTCS Quick Start Kit (P/N 608120).

12. Overlay each of the resuspended samples with one drop of light mineral oil and load the sample plate into a CEQ Genetic Analysis System, such as a 2000XL, 8000 or 8800, following the instrument manual.

Results

Table 1 summarizes the preparation and centrifugation steps along with corresponding times for ethanol plate precipitation using the Avanti J-E centrifuge with the J-S5.3 rotor. All reactions and steps including thermal cycling were done in CEQ sample plates (P/N 609801) with the ethanol precipitation performed in the same plates.Figure 5 demonstrates the pUC18 plasmid sequencing results with sequencing product cleaned up using the Allegra 25R with the S5700 rotor (left) and the Avanti J-E with the JS-5.3 rotor (right). The data show that the ethanol precipitation cleanup method by the Avanti J-E as described in this report delivers comparable results as those by the Allegra 25R. The latter has been proven as one of the most effective methods for dye terminator cycle sequencing cleanup in terms of product recovery and data quality (Ref. 1).

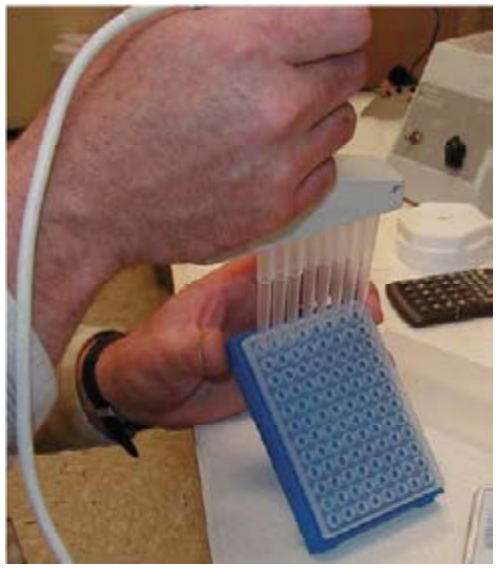

Figure 4. *Pellets are rinsed with a simple pipetting step. It is important not to mix or vortex the plate.*

Conclusions

The Avanti J-E with the JS-5.3 rotor is another powerful tool that can be used for ethanol plate precipitation, as described above, to effectively remove unincorporated dye terminators and salts resulting from dye terminator cycle sequencing reactions. Just like the method using the Allegra 25R, all the processes with the Avanti J-E are carried out in the original sample plate removing possible identification and contamination errors. This method has proven to be fast, effective and inexpensive when compared to commercially available sample cleanup kits (Ref.1). Compared to the Allegra 25R, the Avanti J-E provides higher throughput and shorter centrifugation turnaround time.

Table 1. Centrifugation and preparation times for ethanol plate precipitation using the Avanti J-E centrifuge with the J-S 5.3 rotor (max RPM: 5,300; 6,130 x *g*). Number of sample transfer steps is zero.

Total time is less than 25 minutes.

Centrifugation Step	Time (min)	Preparation (Hands On)	Time (min)
Initial Spin	0.50	Add Stop Solution	2.00
Precipitation Spin	2.00	Add 95% Ethanol	0.50
Invert Spin	0.17	Blot	0.33
Wash Spin	2.00	Add 70% Ethanol	1.00
Invert Spin	0.17	Blot	0.33
Wash Spin	2.00	Add 70% Ethanol	1.00
Invert Spin	0.17	Blot	0.33
		Dry	10.00
		Add Formamide	2.00
Total Centrifugation Time	7.01	Total Preparation Time	17.49

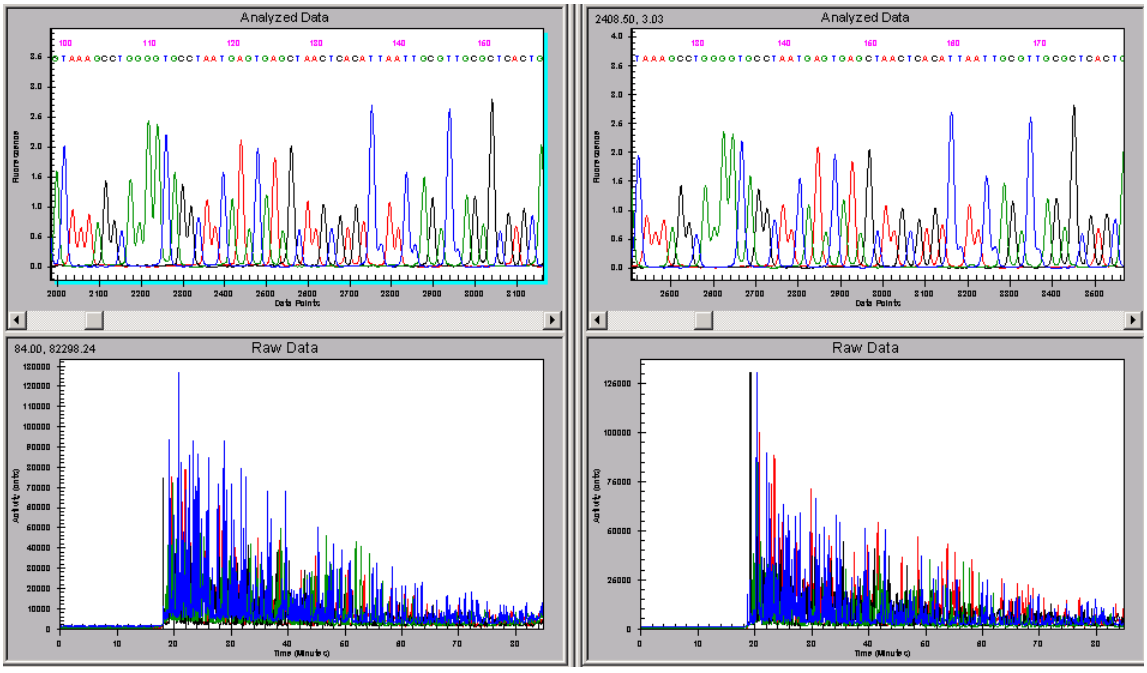

Figure 5. *Electropherograms of analyzed (upper) and raw (lower) data for pUC 18 sequence cleaned up by ethanol plate precipitation using the Allegra 25R centrifuge with the S5700 rotor (left) and the Avanti J-E with the JS-5.3 rotor (right)..*

References

1. Roby, K and Gull, H. *A Rapid and Efficient Method for the Post-reaction Cleanup of Labeled Dye Terminator Sequencing Products*. Beckman Coulter, Inc., Application Information Bulletin A-1903A (2001)

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