Efficacy of Using a Combination Microplate Washer for Vacuum-Based DNA Sequencing Reaction Clean-up

Demonstrated with BioTek's ELx405™ Microplate Washer and Millipore Montage® SEQ$_{96}$ Sequencing Reaction Clean-up Kit in comparison to EdgeBio Performa® DTR V3 Gel Filtration Method

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BigDye® Terminator Clean-up

BigDye Terminator v3.1

Size Exclusion Separation

96-well Sequencing Clean-up

384-well Sequencing Clean-up

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The ability to determine the specific pattern of base pairs in DNA molecules is an indispensable part of contemporary molecular biology. Over the past 10-12 years, the evolution of market-leading dye terminator methods and automated capillary electrophoresis instrumentation has largely standardized the procedure for automated Sanger-based sequencing, quickly making it more accessible, less resource intensive, and easier to perform at many different throughput levels. A critical component of this genomic workflow is the sequencing reaction clean-up procedure, where contaminating artifacts of the sequencing reaction are removed prior to capillary electrophoresis. There are currently a number of viable sequencing clean-up methods available using either filtration, precipitation, or sequestering as a process of choice. Each method has its own costs and benefits and is a proven way of purifying reaction samples. Where dedicated instrumentation is not required, value can be gained by the use of multi-functional instruments that can perform tasks across many application areas, contributing to optimal resource sharing, typical of many molecular biology laboratories and core facilities. In collaboration with a comprehensive DNA Analysis Core Facility using state-of-the-art sequencing chemistries and instrumentation, we demonstrate an example of the merit of hybrid instrumentation by using an integrated vacuum-to-waste filtration module of a combination microplate washer to perform DNA sequencing reaction clean-up.

Introduction

Even with rapid technological advances in genomics and proteomics, an integral part of these workflows remains almost universal – sample prep protocols that require a clean-up procedure. Clean-up is used at different points of sample processing to separate and remove contaminants that can interfere with sensitive downstream procedures like capillary electrophoresis used in Sanger-based sequencing or amplification and normalization of PCR fragments for re-sequencing using Next Generation Sequencing (NGS). Microplates are commonly used for clean-up as they are automation friendly and versatile, lending themselves, as in the case of dye terminator clean-up, to a host of different methods including centrifugation or vacuum filtration, SPRI bead, applied pellet, or anion exchange charged sequestering, and precipitate methods using ethanol and/or sodium acetate. According to survey results of DNA Sequencing Facilities conducted by the DNA Sequencing Research Group (DSRG) of The Association of Biomolecular Resource Facilities (ABRF) sequencing clean-up using spin columns (resin or gel wasn't differentiated) has declined from 67% to only 3% of users from 2003 to 2006, replaced largely by similar methods performed in microplates. The 2009 statistics indicate there has been little or no change from the 2006 results [1].

Microplate instrumentation that can perform a combination of methods is especially useful where multiple purification or other clean-up procedures are employed for reasons of effectiveness for different applications, cost benefit, efficiency, simplicity, or during periods of transition to new or supplemental technologies.

This Application Note describes the use of a vacuum filtration module available on a microplate washer to perform size exclusion clean-up in a 96-well microplate fitted with a patented membrane technology to rid samples of contaminating substances following an ABI BigDye® Terminator v3.1 DNA sequencing reaction. This method is compared to the EdgeBio Performa® DTR V3 96-well Short Plate gel filtration procedure used by the Vermont Cancer Center DNA Analysis Facility for their BDT clean-up.

Theory of Operation: Size Exclusion Filtration

Dye terminator sequencing reaction clean-up using size exclusion filtration can be achieved by:

1. Directing contaminants to waste by applying vacuum to a microplate fitted with a porous membrane, leaving purified samples behind to be eluted from the microplate wells (Figure 1); or

2. Dispensing samples into microplate wells packed with a gel matrix followed by centrifuging or vacuuming purified samples to a receiver plate, leaving contaminants behind in the matrix.

The first was used by the BioTek/Millipore method and the second by the comparative method via centrifugation.

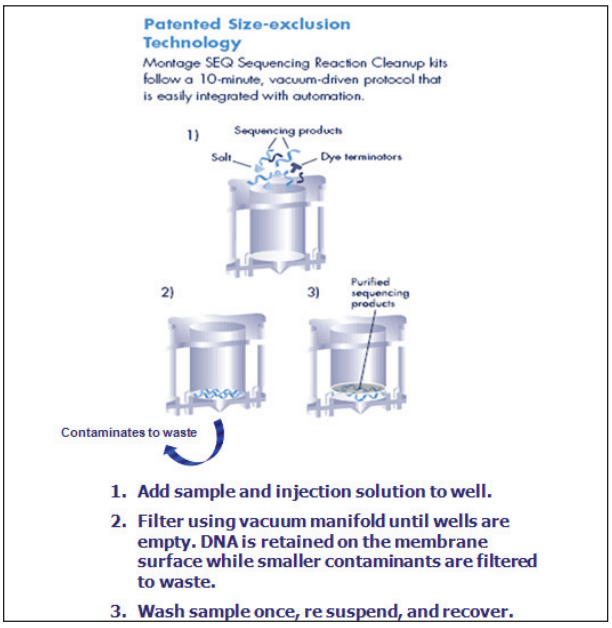

Figure 1. Protocol for the Millipore™ Montage™ SEQ$_{96}$ Sequencing Reaction Clean-up Kit used for the vacuum filtration demonstration on BioTek's ELx405™ Microplate Washer. 96- and 384-well formats of the kit are available, both compatible with the ELx405 vacuum module. Montage vacuum procedures are also available for PCR and Plasmid Miniprep applications. [2]

Materials and Methods

1. Setup for a 1/8x sequencing reaction at 15 µL final volume/well:
   - a. Template: 350 ng pGEM -3Zf(+)
   - b. Primer: 3.2 pmol and 5 pmol M13 reverse
   - c. Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Chemistry 3.4 µL 1/8x BD mix
   - d. H2O q.s.

2. Cycle Sequencing:
   - a. Place the plate in a thermal cycler and set the volume to 15 µL.
   - b. Run at 96°C for 1 minute.
   - c. Repeat the following for 25 cycles:
     - i. 96°C for 10 seconds
     - ii. 50°C for 5 seconds
     - iii. 50°C for 4 minutes
   - d. Hold at 4°C until ready to purify.
   - e. Spin down the plate in a microcentrifuge.

3. Clean-up:
   - a. Vacuum Filtration
     - i. Millipore® Montage SEQ$_{96}$ Sequencing Reaction Clean-up Kit P/N LSK 509604
     - ii. Montage® Wash Solution P/N LSK BW 500
     - iii. ELx405™ RSMF configured for a vacuum setting of High (609 mmHg_24" Hg_811.93 mbar)
     - iv. Thermo Plate Genie Shaker Speed 2
     - v. Barnstead/LabLine Shaker Speed 8
   - b. Gel Filtration
     - i. 2.2% SDS in deionized water to 0.2% final
     - ii. EdgeBio Performa® DTR V3 96-well Short Plates P/N 4050203
     - iii. EDTA to 0.1-0.15 mM final

4. Run:
   - a. ABI 3130xL using 50 cm 16 capillary array with a 1.6 kv injection voltage; 15 seconds injection time; run time of 6000 seconds @ 8.5 kv using a POP7 polymer.

Two separate runs were completed. Run #1 was designed to gauge BioTek vacuum performance using the Millipore kit recommended protocol and compare any differences between manual pipetting and shaking for the re-suspension step. Run #2 optimized results observed from the first run. Post cycle sequencing pGEM samples were divided between the gel filtration plate and the vacuum filtration plate. For quality control purposes, the injection plate protocol was defined to process one set of gel filtration samples first, followed by the vacuum samples, and finish with a final group of gel filtration samples. The gel filtration clean-up was performed by the Vermont Cancer Center collaborator for both runs. Figure 2 shows the workflow of both methods.

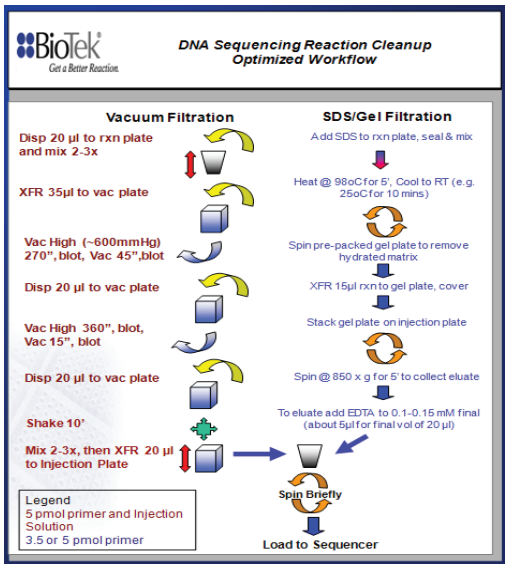

Figure 2. Experiment workflow showing side-by-side of vacuum and gel filtration clean-up methods.

On the first run, 20 µL of the Millipore injection solution was added to each 15 µL sample volume, and 35 µL of injection solution was added to remaining empty wells of the 96-well vacuum filtration plate. Instructions provided by the assay protocol were followed including recommended vacuum settings, injection solution volumes, vacuum duration time, and blotting of the underside of the microplate following each vacuum step. No changes were made in the procedure to account for a starting sample volume 5 µL above the guideline in the kit protocol. Figure 3 shows representation of the dye blobs evident in vacuum filtration samples from the first run.

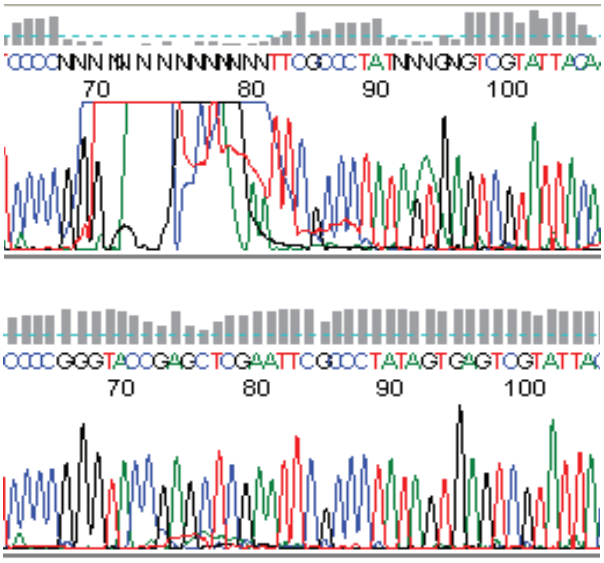

Figure 3. Snapshot of chromatograms captured through Finch TV[3] showing dye blobs after Run #1 using vacuum filtration (top). Following optimization of vacuum time and technique, dye blobs are dramatically reduced as evidenced by absence of 'N' calls, little or no background trailer, and QV bars that support acceptable confidence of corresponding base calls (bottom). The teal line through the bars represents QV20.

Optimization to decrease dye blobs was undertaken for the second run using 4 sample groups as follows:

1. On recommendation from Millipore, primer concentration of 5.0 pmol was run during the sequencing reaction. This sample group used the default 20 µL of injection solution, but an increase in vacuum duration time was added to account for the 15 µL sample volume.

2. Injection solution volume was increased by 10 µL on the 3.2 pmol samples and an increase in vacuum duration time was introduced to account for greater final well volume.

3. A Wash Buffer available from Millipore as an alternative solution during the rinse step of the clean-up procedure was introduced for two sample groups at both primer concentrations (data not shown).

4. A blot step was introduced after the first extended vacuum duration time and before an abbreviated dry vacuum time to clear the membrane for increased vacuum efficiency for all sample groups on both vacuum steps.

For additional procedural information refer to the Tech Note Tips for Optimizing Microplate Vacuum Filtration Results written to accompany this Application Note.

Results

Results for common measures of quality sequencing on Run #2 is shown by Figure 4 for all samples of both methods, except the sample groups using the Millipore Wash Buffer instead of injection solution. Chromatograms presented by Figure 5 for a sample from each of the methods (vacuum and centrifuge) provide further representation of the correlation achieved between methods.

- Length of Read (LOR): usable range of high-quality or high-accuracy bases, as determined by quality values.
- Quality Values (QV or Phred): confidence of base call accuracy. For example, a QV of 20 indicates a 1.0% probability of error in the base call or 99% confidence the base call is correct, while a QV of 40 indicates .001% probability of error in the base call or a 99.99% confidence the base call is correct. Higher QVs are better. QVs >= 20 are considered High QV (HQV).
- Sample Score: average QV of all base calls for the total LOR.
- Base Spacing: as reported here, the # of scan points from the crest of one peak to the crest of the next peak. On a chromatogram, the closer the alignment of a base call with its corresponding peak also indicates quality of the run.

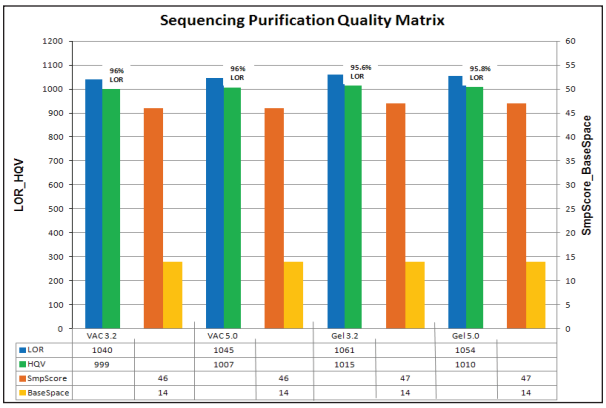

Figure 4. Run #2 Quality Matrix comparing average of total LOR; average number of HQV base calls; HQV as a percent of total LOR; Sample Score; and average base spacing for all samples in the group. Vac (n=16, n=15) Gel (n=8).

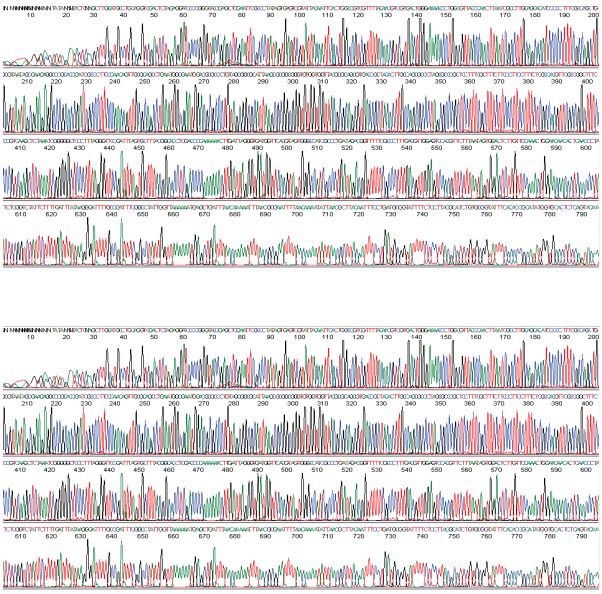

Figure 5. Chromatograms captured through Finch TV[3] of results achieved during Run #2 of the experiment. A typical sample result utilizing membrane-based vacuum filtration is shown at top, and one using SDS/gel matrix filtration at bottom. Both samples were extracted from the 5 pmol primer concentration group.

Discussion

The vacuum filtration module available on BioTek's ELx405™ Microplate Washer and EL406™ Microplate Washers / Dispensers effectively cleans contaminating artifacts from DNA sequencing reactions using membrane-based size exclusion technology. Results show confident correlation to a widely used comparative method; high LOR reads; a high percent of LOR QV >= 20; and high Sample Score averages on total LOR. Data also shows acceptable results using both a 3.2 pmol and 5.0 pmol primer concentration.

Optimal settings for this demonstration were achieved utilizing a 'High' vacuum setting on the ELx405 RSMF;,increasing vacuum duration times by 2 minutes above those recommended by the kit insert, and introducing a blot step before drying the wells completely. Although 3.2 pmol primer concentrations produced acceptable results, the 5.0 pmol results were marginally better and are recommended.

Value is added to the vacuum method from the instrumentation used to perform it. In addition to membrane-based size exclusion technologies, the BioTek ELx405 and EL406 vacuum module can be used for polystyrene bead-based filtration methods (the EL406 includes the benefit of gauge regulation of vacuum pressure). The washers are also fully equipped for magnetic bead assays, ELISA, mix and read, or cell-based assays and processes including microplate washing, reagent dispensing, cell dispensing, and cell media exchanges in 96-, 384-, and 1536-well standard and deep well plate formats, depending on the model. For higher throughput, this clean-up procedure is available in 384-well format, also fully compatible with the ELx405 and EL406 vacuum manifolds.

References

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