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

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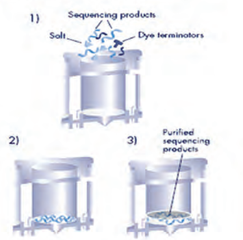
Introduction

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 DNA 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 cleanup procedure, where contaminating artifacts of the sequencing reaction are removed prior to capillary electrophoresis. There are currently a number of viable DNA sequencing cleanup methods available using either filtration, precipitation, or sequestering. Each method has its own costs and benefits and is a proven way of purifying reaction samples. In collaboration with a comprehensive DNA Analysis Core Facility that utilizes state-of-the-art sequencing chemistires and technology, a microplate washer fitted with an integrated vacuum filtration module was used to perform DNA sequencing reaction cleanup. Results were substantiated against a gel filtration method currently used by the collaborator. Evidence provided by this demonstration support the efficacy of the microplate washer demonstrated here to contribute to the genomic workflow typical of many molecular biology laboratories and core facilities.

Membrane-Based Size-Exclusion Protocol via Vacuum Filtration

Patented Size-exclusion Technology

Montage SEQ Sequencing Reaction Cleanup kits follow a 10-minute, vacuum-driven protocol that is easily integrated with automation.



1. Add sample and injection solution to well.
2. Filter using vacuum manifold until wells are empty. DNA is retained on the membrane surface while smaller contaminants are filtered to waste.
3. Wash sample once, resuspend, and recover.

***Figure 1** – Millipore™ Montage™ SEQ$_{96}$ Sequencing Reaction Cleanup Kit protocol. 96- and 384-well formats are available. Montage vacuum procedures are also available for PCR and Plasmid Miniprep applications.[1]*

BioTek Instrumentation

***Figure 2** – The BioTek 405™ Touch Microplate washer equipped with its available vacuum filtration module was used for DNA sequencing reaction cleanup before loading samples to capillary electrophoresis for analysis.*

Materials and Methods

1. **Sequencing Reaction Setup – 1/8x**

Final Reaction Volume	15 µL/well
Template Plasmid (150-400 ng/well)	pGEM®-3Zf(+) 350 ng
Primer M13 reverse (Run#1, Run#2)	3.2 pmol
M13 reverse (Run #2 only)	5.0 pmol
BDT Premix ABI® BigDye™ Terminator v3.1 Cycle Sequencing Chemistry	3.4 µL 1/8x BD mix
H_2O	q.s.

2. **Cycle Sequencing**

1. Place the plate in a thermal cycler & set volume
2. 96ºC for 1 minute
3. Repeat the following for 25 cycles:
a. 96ºC for 10 seconds
b. 50ºC for 5 seconds
c. 60ºC for 4 minutes
4. Hold at 4ºC until ready to purify
5. Spin down the plate in a microcentrifuge

3. **Cleanup**

Vacuum Filtration	Gel Filtration
Millipore Montage® SEQ$_{96}$ Sequencing Reaction Cleanup Kit, P/N LSK 509604	2.2% SDS in deionized water to 0.2% final
Montage® Wash Solution, P/N LSK BW 500	EdgeBio Performa® DTR V3 96-Well Short Plates P/N 4050203
ELx405™ Models w/Vacuum Filtration Vacuum Setting High (609 mmHg/24" Hg) Vac Duration: Run#1, 3.2 pmol 4 m, 4 m Run#2, 3.2 pmol 5.25 m+30 s, 6 m+30 s 5.0 pmol 4.5 m+45 s, 6 m+15 s	EDTA to 0.1-0.15 mM final
Thermo Plate Genie Shaker Run#1 = Speed 2, 10 m	
Barnstead/LabLine Shaker Run#2 = Speed 8, 10 m	

4. **Run**

Instrument	Settings
ABI 3130xL	50 cm 18 capillary array Injection v = 1.6 kv Injection time = 15 s Run Time = 6000 s @ 8.5 kv Polymer = POP7

Materials and Methods (*continued*)

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 resuspension 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. Figure 3 shows the complete workflow for the experiment.

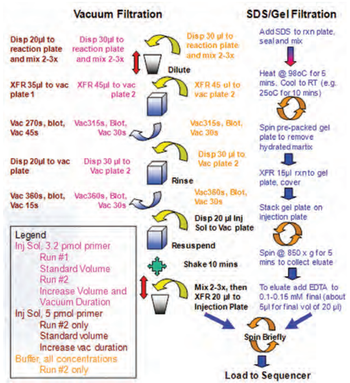


***Figure 3** – Experiment workflow showing side-by-side of vacuum and gel filtration DNA sequencing cleanup methods.*

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 for recommended vacuum settings, injection solution volumes, and vacuum duration were followed. No changes were made in the procedure to account for a starting sample volume 5 µL above the guideline in the kit protocol. Figure 4 shows representation of the dye blobs evident in vacuum filtration samples from the first run.

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.
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. Two sample groups utilized a Wash Buffer available from Millipore as an alternative solution during the rinse step of the cleanup procedure for 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.

Results

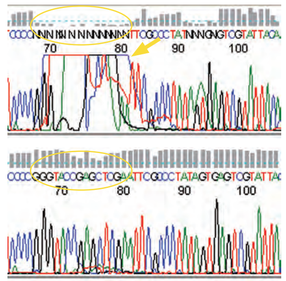


***Figure 4** – Snapshot of chromatograms captured through Finch TV[2] showing dye blobs after Run #1 using vacuum filtration (top). Following optimization of vacuum time and technique dye blobs are dramatically reduced as evidenced by absence of 'N' calls, little or no background trailer, and QV bars that support acceptable confidence of corresponding base calls (bottom).*

Results for common measures of quality sequencing on Run #2 samples is shown by Figure 5 for all samples of both methods except the sample groups using the Millipore Wash Buffer instead of injection solution.

- **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. e.g. a QV of 20 indicates a 1.0% probability of error in the base call, or 99% confidence the base call is correct, a QV of 40 indicates 0.001% 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 it's corresponding peak is also a quality indicator.

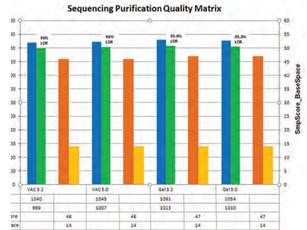


***Figure 5** – Run #2 Quality Matrix comparing average of total LOR, average number of HQV base calls, HQV as a percent of total LOR, Sample Score, and average base spacing for all samples in the group. Vac (n=16, n=15) Gel (n=8).*

Results (*continued*)

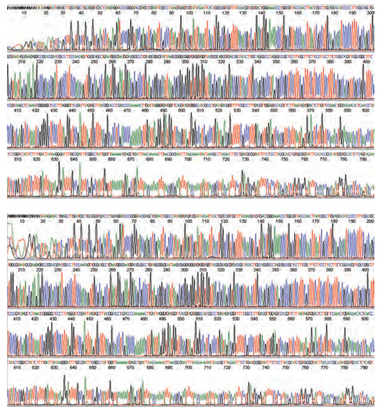


***Figure 6** – Representative chromatograms captured through Finch TV[2] of results achieved during Run #2 of the experiment. A typical sample result utilizing vacuum filtration is shown at top, and one using SDS/gel matrix filtration at bottom. Both samples at 5 pmol primer concentration.*

Conclusions

1. The vacuum filtration module available on the BioTek 405 Touch 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 shows acceptable results using both a 3.2 pmol and 5.0 pmol primer concentration.
   - Optimal settings for this demonstration were achieved by increasing vacuum duration times and introducing a blot step before drying the wells completely.
2. Value is added to the vacuum method from the instrumentation used to perform it.
   - In addition to genomic workflow capabilities, this microplate washer can perform bead based, cell based, and traditional ELISA assay procedures including washing, reagent dispensing, cell seeding and media exchanges.
   - This vacuum procedure can also be performed in 384-well plates.
   - The versatility of this instrumentation make it an excellent choice for resource sharing among laboratories or within core facilities.
3. Kits are available for PCR cleanup and plasmid miniprep from the same manufacturer using similar technique.

[1]http://www.millipore.com/publications.nsf/a73664f9f981af8c852569b9005b4eee/a640655c4b9de47885256f8900741e57/$FILE/MC1005ENUS.pdf | [2]FinchTV version 1.4.0 Copyright © 2004-2006 Geospiza, Inc.