

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 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 filtration module of a combination micro plate washer to perform DNA sequencing reaction cleanup.

BioTek Instrumentation

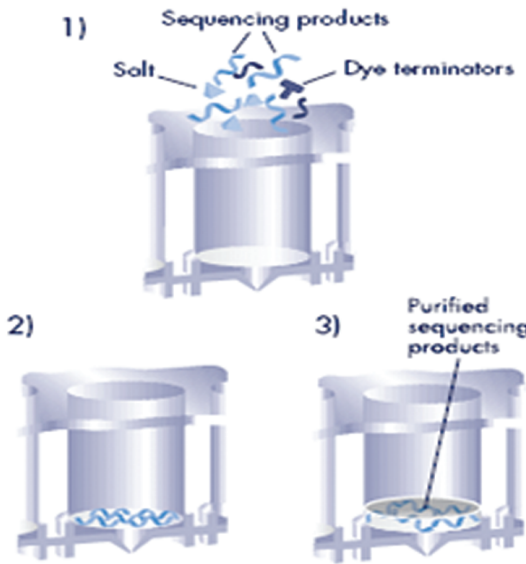


Figure 1 – The adjustable vacuum filtration module used for this demonstration is available on both the BioTek ELx405 and EL406 Microplate Washers (top and bottom respectively). In addition to membrane based size exclusion technologies, the module can be used for bead based assays, such as the Luminex xMAP® technology. The versatile washers are also equipped to perform other common laboratory techniques including ELISA and cell based assay procedures such as microplate washing, reagent dispensing, cell dispensing and cell media exchanges in 96-, 384- and 1536-well plate format depending on the model.

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 2 – Millipore™ Montage™ SEQ₉₆ Sequencing Reaction Cleanup Kit protocol. 96- and 384-well formats are available.^[1]

Materials and Method

1. Sequencing Reaction Setup – 1/8x

Final Reaction Volume	15 µL/well
Template	Plasmid (150-400 ng/well)
Primer	pGEM®-3Z(+)/350 ng
BDT Premix	ABE BigDye™ Terminator v3.1
H₂O	Cycle Sequencing Chemistry

2. Cycle Sequencing

Step	Action
1	Place the plate in a thermal cycler and set the volume to 15 µL
2	96°C for 1 minute
3	Repeat the following for 25 cycles: 1. 96°C for 10 seconds 2. 50°C for 5 seconds 3. 60°C for 4 minutes
4	Hold at 4°C until ready to purify
5	Spin down the plate in a microcentrifuge

3. Cleanup

Vacuum Filtration	Millipore Montage® SEQ ₉₆ Sequencing Reaction Cleanup Kit, PN LSK-99804
Montage® Wash Solution	PN LSK-99500
ELx405™ RSMF	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, 8 m+30 s 5.0 pmol 4.5 m+45 s, 8 m+15 s
Thermo Plate Genie Shaker	Run#1 = Speed 2, 10 m
BarnsteadLabLine Shaker	Run#2 = Speed 8, 10 m

Gel Filtration	2% SDS in deionized water to 0.2% final
EdgeBio Performa® DTR V3 96-Well Short Plates	PN 405020
EDTA	to 0.1-0.15 mM final

4. Run

Instrument	ABI 3130XL
	50 cm 16 capillary array
	Injection v = 1.6 kv
	Injection time = 15 s
	Run Time = 6000 s @ 8.5 kv
	Polymer = POP7

Two separate runs were completed. Run #1 was designed to gauge BioTek vacuum performance using the Millipore kit recommended protocol, except a 3.2 pmol primer concentration was used as recommended by ABI. Differences between manual pipetting and shaking for the resuspension step were also evaluated. 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.

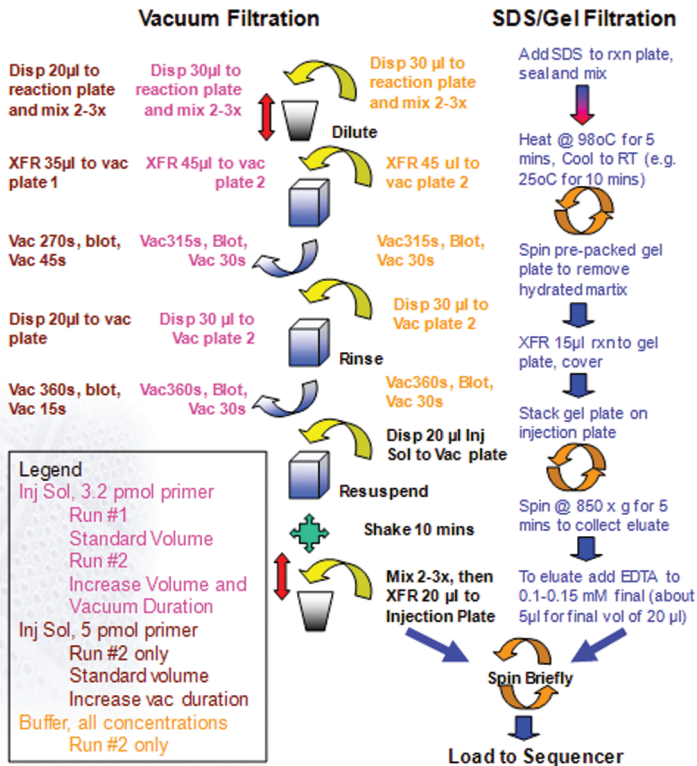


Figure 3 – Experiment workflow showing side-by-side of vacuum and gel filtration cleanup methods.

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 for common measures of quality sequencing on Run #2 samples is shown by Figures 5 and 6.

Results

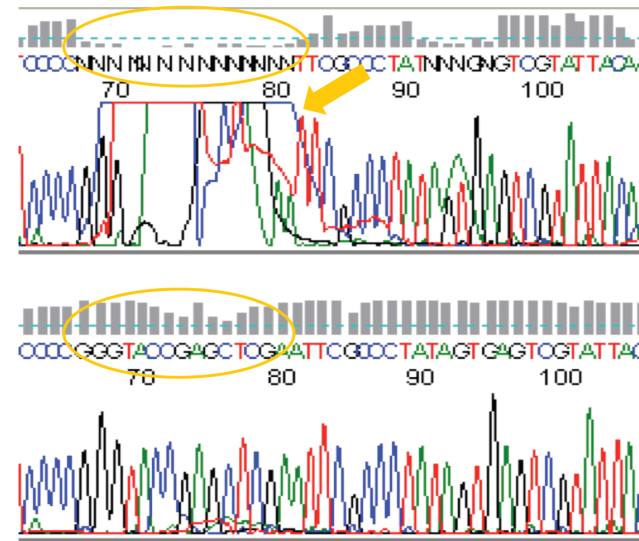


Figure 4 – Snapshot of chromatograms captured through Finch TV² showing dye blobs after Run #1 using vacuum filtration (top). Following optimization of vacuum time and technique dye blobs are dramatically reduced as evidenced by absence of 'N' calls, little or no background trailer, and QV bars that support acceptable confidence of corresponding base calls (bottom).

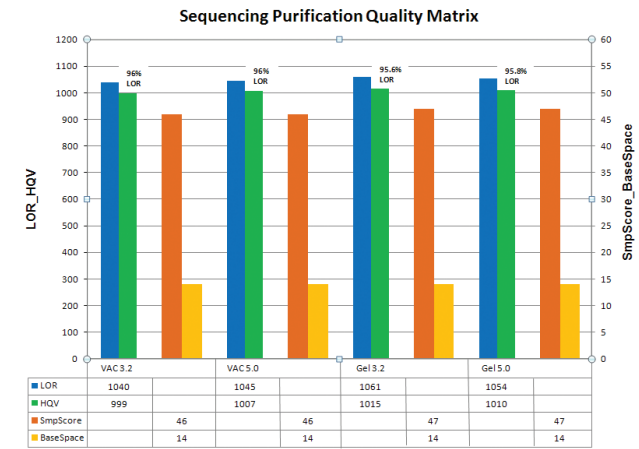


Figure 5 – Run #2 Quality Matrix comparing average of total LOR, average number of HQV base calls, HQV as a percent of total LOR, Sample Score, and average base spacing for all samples in the group. Vac (n=16, n=15) Gel (n=8).

- **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 .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.

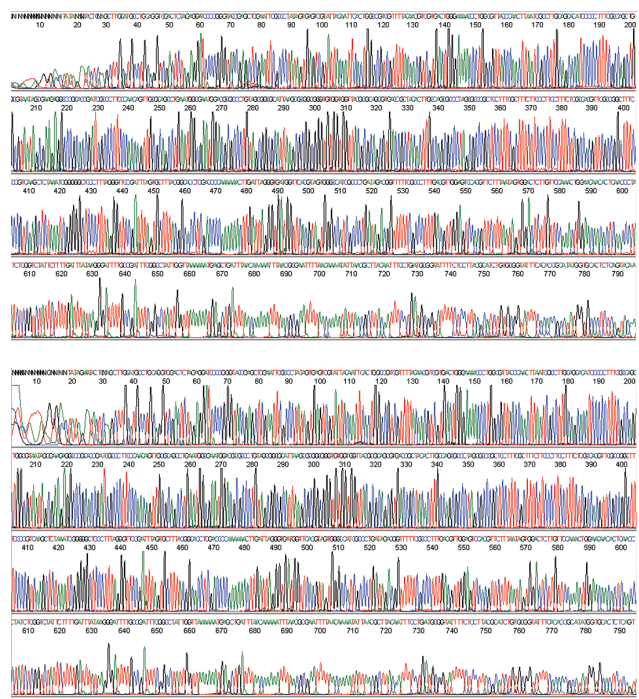


Figure 6 – Representative chromatograms captured through Finch TV² of results achieved during Run #2 of the experiment. A typical sample result utilizing vacuum filtration is shown at top, and one using SDS/gel matrix filtration at bottom. Both samples at 5pmol primer concentration.

Conclusions

1. The vacuum filtration module available on BioTek ELx405 and EL406 Microplate Washers 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, these combination microplate washers 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 lends itself to 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.