Technical Overview

Tips for Optimizing Microplate Vacuum Filtration Results

For vacuum-to-waste procedures

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Introduction

Effective vacuum filtration of assay reagent material from a microplate is a function of many variables including filtration plate design factors such as membrane material, membrane pore size, well size and well shape, and assay parameters such as the total volume and viscosity of reagents to be removed from the wells. Additionally, test procedure base line settings for vacuum time and pressure may be given assuming only a single vacuum source recommended for the assay, and different vacuum filtration manifold architecture (or example waste tray depth and shape) can influence vacuum draw point patterns across the plate, leading to variable results system to system for the same settings. Achieving the goal of even and reproducible distribution of results across the plate is also dependent on the ability to accurately determine when the microplate wells are dry (devoid of contaminating or excess substances that can interfere with assay functionality or detection instrument performance, thereby effecting final result determination).

While this technical overview offers tips on optimizing vacuum filtration results as applied in DNA sequencing reaction cleanup using an Agilent BioTek ELx405 vacuum filtration module and Millipore Montage SEQ96 Sequencing Reaction Clean-up kit, many of these tips are universal to vacuum filtration using membrane-based microplates. These tips may prove especially useful when a vacuum manifold other than the recommended model is used, when vacuum distribution appears uneven through variable material removal patterns, membrane pore sizes are unknown or unavailable, and time of vacuum is critical to maintain assay performance and sample viability. This technical overview assumes basic knowledge of using vacuum filtration onboard the Agilent BioTek microplate washers, and is intended to provide tips for good practice and technique, rather than provide instruction for performing a particular assay or instrument function. Differences between the EL406 and ELx405 vacuum filtration modules, or any other manufacturer model, are not noted and are left to the discrimination of the user.

1. **Establish the recommended vacuum pressure for the assay.** An online conversion tool was used to translate the assay-recommended 23 to 25 inHg to 500 to 600 mmHg units as used by the ELx405 specifications.
2. **Establish the membrane pore size for the supplied filtration plate.** In the referenced Millipore assay, this is proprietary information, however, even if the exact pore size is unavailable a range of pore size can generally be obtained. For the DNA sequencing cleanup kit, the membrane pore size was verified as being less than 0.45 µM (the smallest pore size specified for the Agilent BioTek vacuum filtration module).
3. **Using the recommended vacuum pressure and pore size, find the closest match using the Agilent BioTek selectable vacuum levels, or those provided by the vacuum manifold manufacturer.**

Table 1. Selectable vacuum levels.

	0.45 µm		1.2 µm	
	96-well (mmHg)	384-well (mmHg)	96-well (mmHg)	384-well (mmHg)
Lowest – 0.047 in	111	108	117	117
Low – 0.032 in	200	196	203	198
Medium – 0.020 in	405	371	409	387
High – 0	561	563	550	450

4. **Identify any other parameters that can be manipulated to optimize for the assay materials** after configuring the manifold for the correct pressure setting, "High" in the case of the ELx405 manifold. For the DNA sequencing cleanup procedure, unspecified membrane pore size would have to be validated as a function of duration or other technique as no further increase in vacuum pressure could be implemented for pore sizes less than 0.45 µm on the ELx405.
5. **Perform a test run using the chosen vacuum pressure at the assay-recommended durations using only the wash or other filtration reagent specified for the test.** Generally, assay kits provide enough excess reagents for this purpose without the added expense of using real samples or other costly procedures for results determination (capillary electrophoresis in the case of DNA sequencing). Here are some recommendations for the test run:
    a. **Weigh the empty filtration plate before loading any reagent.**
    b. **Add the total amount of reagent to be removed to all wells of the plate (include volume of source material even though it is not used for the test run).**
    c. **Weigh the plate again before filtration.**
    d. **Perform the vacuum filtration following the manufacturer's instructions for time.** Even if not required or recommended, blot the underside of the plate during or at the end of the cycle. Fewer blot steps are better, but lower residuals greatly improve assay performance.
    e. **Weigh the dry plate following a filtration step.** This can provide a good guideline specification for assistance in determining a dry plate. For the Millipore procedure a visual inspection of the bottom of the wells (not the underside) should result in observation of a shiny surface indicative of a dry well. This could also be gauged by a color change or other measure as noted by a given assay.
    f. **Adjust time intervals until the fastest vacuum duration within range for the assay produces the lowest residual when the plate is weighed (that weight closest to the dry plate from step 5a).** The residual difference could be quite high (≥1.2 g, for example) and is highly dependent on absorbency rating of membrane material, reagent profile, and duration time (itself depending on factors such as total volume in the well and reagent viscosity).

A reproducible weight with the lowest window between 5a and 5e, using a duration time closest to the assay specification, provides a satisfactory baseline for successful filtration. Duration sensitivity to recommended filtration times may not be available for an assay, and can be an art as well as a science to determine. Once a dry well is confidently determined, work backwards, if necessary, decreasing time in 20- to 45-second intervals until a filtration time within the specified range for the assay is achieved as closely as possible. For the DNA sequencing filtration, this was complicated by the fact that the volume of sample source material was greater than that assumed for the filtration time specified for the assay. Latitude in filtration time was required because there was more total volume to evacuate.

g. **Repeat steps 5b through 5f for the well volume and reagent required of each filtration cycle.** For example, if 20 µL of rinse solution is required for 15 µL of sample, verify the filtration time for 35 µL of rinse solution. If the second rinse step requires adding 20 µL of the same or a different solution to the wells, verify the time for 20 µL of the specified reagent even if it is the same reagent, as determining optimized duration time is highly dependent on well volume in addition to reagent, membrane, and manifold profiles.

6. **When confident removal of material for each volume and filtration time at each step of the assay is achieved, perform a "live" run using a known control for the assay.** Some recommendations for the live control run include:

   a. **Run enough control wells to give a broad indication of vacuum performance across the plate, but not too much to make the test run overly costly or tax any downstream instrumentation if something fails.** Capillary tubes typically used in DNA sequencing runs are very small and sensitive to even minute amounts of contamination or tiny air pockets, so minimizing the impact of any trace reaction artifacts is important not just for sample integrity, but for instrument care. To achieve this without sacrificing the ability to determine uniform vacuum distribution and thorough washing of the sample during filtration, the control was run on a broad spectrum of wells across the filtration plate, but then transferred to the fewest lanes that could hold all of the control wells on the capillary electrophoresis plate.

   b. **Add reagent to any empty wells in the same volume as control or sample wells.** The same total well volume should be equal across the plate. The best results are obtained when all wells of the plate contain the same volume and as much of the same reagent material as possible. Wrapping or covering unused wells of the filtration plate to prevent a vacuum leak should be avoided as it is not a true representation of uniform material removal in the plate.

   c. **Use a proven comparative method for test result validation.** This ensures confident confirmation of test method performance. An added benefit for the DNA sequencing demonstration was that by designing the experiment so that the comparative method strips were run both before and after the control strips, it was confirmed that the test wells did not adversely affect instrument performance.

   d. **Analyze results for those variables indicative of method performance.** Narrowing down any problems in data improves troubleshooting efficiency. Deviant results from a DNA sequencing run can indicate problems in the sample preparation or reaction procedure, not just the cleanup method. For cleanup, a good length of read and absence of dye blobs or interfering and overlaying peaks of color on the chromatogram, are two characteristic sequencing cleanup success indicators.

7. **Continue to optimize vacuum time and technique using controls until the comparative method and the test method show acceptable correlation.** The first control run on the DNA sequencing demonstration resulted in dye blobs clearly indicated on the vacuum filtration wells. This verifies a lack of reaction artifact removal. The pattern of dye blobs was fairly universal across the plate, so an uneven distribution pattern could not be clearly established.

   a. **Design follow-up experiments to implement possible solutions specific to each variable requiring optimization.** The following conditions were implemented to optimize removal and minimize interference of contaminating material (e.g., excess dye) for DNA sequencing cleanup:

i. **Using the default parameters for cleanup reagent volume, each vacuum filtration step was divided into a long filtration, followed by blotting the underside of the filtration plate, then a short dry filtration, followed by a final blot.** This allowed excess filtrate to be wicked from the membrane after the long filtration thus hastening the final removal of artifact during the short run. The membrane was then also cleaner for the next full filtration cycle that followed.

ii. The cleanup kit specifications assumed sample volumes of 5 and 10 µL, and the sample volume used for the test run was 15 µL. **Long vacuum time on the first filtration step was increased by 45 seconds to account for the higher sample volume.**

iii. A primer concentration of 5 pmol is recommended for the vacuum filtration method, but the primer concentration of 3.5 pmol used by the comparative method had been used for the test run. Both primer concentrations were implemented side-by-side on the second run. **It is helpful and useful to run original settings in parallel to new settings especially if more than one variable is being optimized.**

iv. The cleanup kit suggests use of a secondary buffer reagent for a rinse step if dye blobs are observed. This was implemented according to the kit insert using default volumes and vacuum times from the first run so as not to introduce additional variables. The buffer was also run at 5 µL higher volume and 45 second longer vacuum duration to match the new rinse solution parameters implemented by 7ii.

v. **Each test condition was run as a full strip to get the broadest possible distribution pattern range.** This helps in observing even vacuum distribution that may have been obscured by the dye blobs on the first run.

8. Steps used by 7i showed acceptable results on the second run for the DNA sequencing demonstration requiring no further optimization, but **it is highly recommended that if more than one filtration step is required for an assay that the plate be rotated between each step** as edge wells are generally more vulnerable to any effects of variable pressure points during applied filtration if they exist.

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