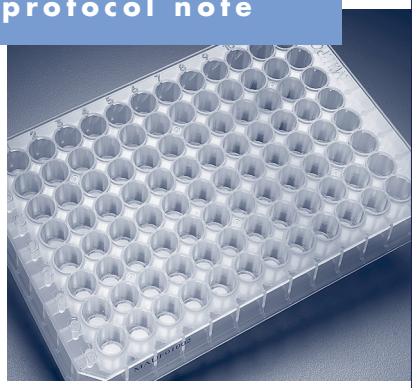
protocol note



MultiScreen® Filter Plate with Ultracel®-10 Membrane

Protein Retention, Recovery, Volume Recovery, and Guidelines for Concentration and Desalting

Introduction

MultiScreen Filter Plate with Ultracel-10 membrane (MultiScreen Ultracel-10 filter plate) provides a new method for high throughput sample preparation. The ultrafiltration-based filter plate is designed for automation-compatible sample purification, concentration and desalting of biological solutions, as well as protein removal from samples prior to instrument analysis. The 96-well MultiScreen filter plate incorporates the Ultracel-10 membrane (10,000 nominal molecular weight limit regener-ated cellulose) for ultra low-binding, high recovery results. It is designed for use with centrifugation.

This protocol note includes guidelines for protein retention, recovery and concentration/desalting using MultiScreen Ultracel-10 filter plates in centrifugal mode. Ultrafiltration is shown to be a highly effective and rapid means for these applications. The 96-well filter plate increases sample throughput and is compatible with standard microtiter plates, instrumentation and liquid handling equipment.

Part 1: **Protein Retention**

Part 2: **Protein Recovery**

Part 3: **Protein Desalting**

Part 4: **Ultrafiltration Time and Volumes**

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Part 1: Protein Retention

Objective: The goal of this experiment was to determine the protein retention performance, as well as ultrafiltration reliability across the plate as measured by protein recovery. FITC-BSA (67 kDa) and Cytochrome c (12 kDa) were used as model molecules in this experiment.

Materials

Reagents

- Milli-Q® water
- Phosphate Buffered Saline (PBS) (Sigma-P 3813, pH 7.4)
- Cytochrome c (Sigma C-8266), 0.5 mg/mL solution in PBS
- Bovine Albumin – Fluorescein Isothiocyanate labeled (FITC-BSA) (SIGMA A-9771), 0.5 mg/mL solution in PBS

Equipment and Materials

- Centrifuge equipped with a 96-well plate rotor
- Molecular Devices 96-well plate reader set to 410 nm wavelength. Reader must be equipped with auto mixer and Pathcheck™.
- SOFTMax® Pro 3.1 software for Molecular Devices reader (Reader must be equipped with auto mixer and Pathcheck).
- Wallac/Victor™ Fluorescence Detector or comparable instrument
- 96-well collection plates (Costar 9017 or equivalent)
- Black 96-well collection plates (Costar 3915 or equivalent)
- Plate shaker

Protocol (Part 1 Protein Retention)

1. Place a MultiScreen Ultracel-10 filter plate on top of a 96-well collection plate (Costar 9017).
2. Add 200 µL/well of Cytochrome c or 300 µL/well of FITC-BSA to the MultiScreen Ultracel-10 filter plate.
3. Centrifuge at 3000 x g for 1 hour, until wells are empty.
4. Evaluate the concentration of protein in the filtrate as follows.
   a. Determine FITC-BSA concentration by reading plates at 485Ex and 535Em using a Fluorescence Detector.
   b. Determine Cytochrome c con centration by reading plates at 410 nm wavelength using a spectrophotometer.
5. After generating a standard curve for both proteins, calculate protein concentrations in both the retentates and filtrates from the standard curve. Calculate percent protein retention based on the amount of protein in the filtrate divided by the initial protein concentration of 0.5mg/mL.

Results

The results show average Cytochrome c retention greater than 97% and average BSA retention greater than 99.9% (limit) with excellent well-to-well reproducibility (see Figures 1 and 2).

Figure 1. Average protein retention of Cytochrome c (n = 76 per well position, sd <10%).

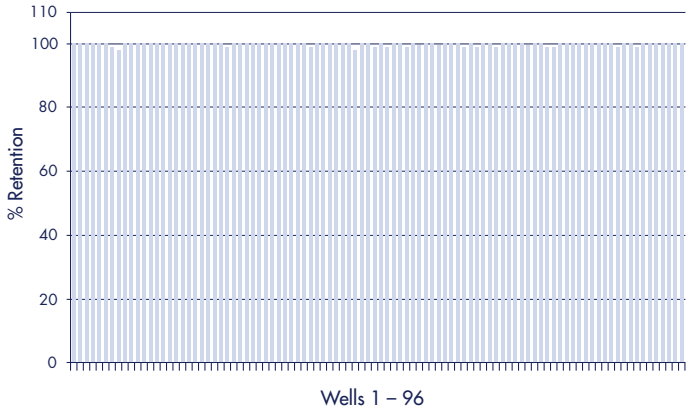
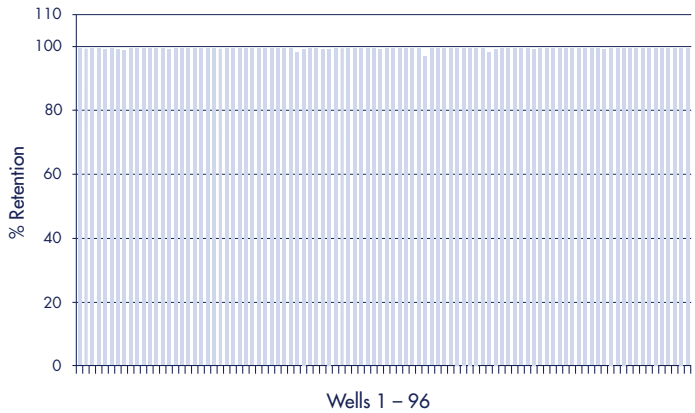


Figure 2. Average protein retention for BSA-FITC (n = 76 per well position, sd <10%).



Part 2: Protein Recovery

Objective: Ultrafiltration provides an effective means for the rapid concentration of biological fluids and protein solutions. With Ultracel-10 ultrafiltration membrane, it is expected that any protein over 10 kDa will be retained by the membrane. The goal of this experiment was to determine protein recovery, as well as ultrafiltration reproducibility across the plate as measured by protein recovery. FITC-BSA (67 kDa) and Cytochrome c (12 kDa) were used as a model molecules in this experiment.

Method

Reagents

- Milli-Q water
- Phosphate Buffered Saline (PBS) (Sigma P 3813, pH 7.4)
- Cytochrome c (Sigma C-8266), 0.5 mg/mL solution in PBS
- Bovine Albumin – Fluorescein Isothiocyanate labeled (FITC-BSA) (SIGMA A-9771), 0.5 mg/mL solution in PBS

Equipment and Materials

- Centrifuge epuipped with 96 well plate rotor
- Molecular Devices 96 well plate reader set to 410 nm wavelength. Reader must be equipped with auto mixer and Pathcheck.
- SOFTMax Pro 3.1 software for Molecular Devices reader
- Wallac/Victor Fluorescence Detector or comparable instrument
- 96-well collection plates (Costar 9017 or equivalent)
- Black 96-well collection plates (Costar 3915 or equivalent)
- Plate shaker

Protocol (Part 2 Protein Recovery)

1. Place the MultiScreen Ultracel-10 filter plate on top of the 96-well collection plate (Costar 9017).
2. Add 200 µL/well of Cytochrome c or 300 µL/well of FITC-BSA to the MultiScreen Ultracel-10 filter plate.
3. Centrifuge at 3000 x g for 1 hour, until wells are empty.
4. Add 200 µL/well or 300 µL/well PBS to each well with a multi-channel pipettor.
5. Agitate the plates for 3 minutes on a plate shaker.
6. Transfer the resuspended retentate into a clear 96-well collection plate for Cytochrome c and a black collection plate for FITC-BSA analysis.
   a. Determine FITC-BSA concentration by reading plates at 485Ex and 535Em using a Fluorescence Detector.
   b. Determine Cytochrome c concentration by reading plates at 410 nm wavelength using a spectrophotometer.
7. After generating a standard curve for both proteins calculate protein concentrations in both the retentate and filtrates from the standard curve. Calculate percent protein recovery based on the initial protein concentration of 0.5 mg/mL.

Table 1. Average protein recovery for Cytochrome c and FITC-BSA after concentration in MultiScreen Ultracel-10 filter plates (n=96).

	Cytochrome c	FITC-BSA
Plate 1	85%	88%
Plate 2	92%	87%

Results

Table 1 shows the results of protein recovery for 200 µL of 0.5 mg/mL of Cytochrome c and FITC-BSA, based on 2 plates (192 wells) analyzed for each protein. The protein solutions were spun to dryness and resuspended in 50 µL of PBS. Figure 3 shows individual recovery numbers for 96- wells tested with Cytochrome c. The results demonstrate that over 85% of either protein can be recovered after 1 hour spin-to-dryness, and that recovery is consistent throughout all 96 wells of MultiScreen Ultracel-10 filter plates.

Even higher recoveries of the protein can be achieved with increased resuspension volume and time on a shaker. Figure 4 shows that 98.5% of protein can be recovered if the protein is resuspended in 200 µL, and the plate is agitated for 30 minutes for resuspension.

Figure 3. Percent retentate starting from 0.5 mg/mL Cytochrome c.

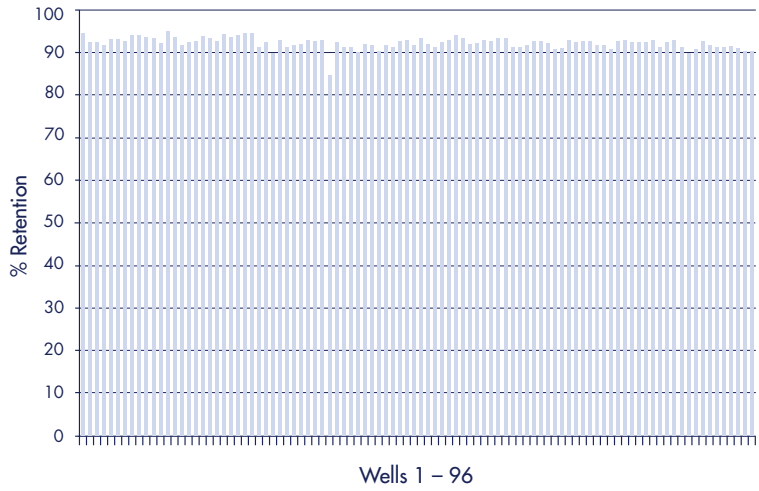


Figure 4. The effect of resuspension volume and shake time.

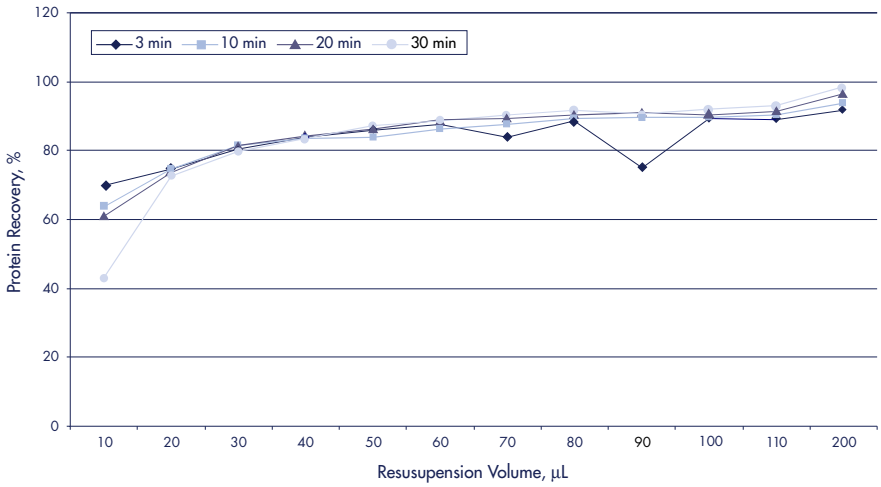


Table 2. Well-to-well reproducibility of Cytochrome c recovery.

avg % retentate	92.5	min	84.9
std dev	1.3	max	95.4

Part 3: Protein Desalting

Objective: The MultiScreen Ultracel-10 filter plates is an efficient tool for the removal of salts, unincorporated radioisotopes, dye and other small molecules from protein solutions. Usually it requires more than one round of ultrafiltration to remove a majority of salt and exchange protein into low salt buffer. To achieve this, concentrated protein gets re-dissolved in water or low molarity buffer and concentrated a second time. Multiple dilution/concentration steps may be required until salt is removed. It is important that proteins be recovered from the membrane after repeated centrifugations. In this experiment, we demonstrate MultiScreen Ultracel-10 filter plate performance for desalting of proteins.

Method

Reagents

- Milli-Q water
- Phosphate Buffered Saline (PBS) (Sigma P 3813, pH 7.4)
- Cytochrome c (Sigma C-8266), 0.5 mg/mL solution in PBS
- Bovine Albumin – Fluorescein Isothiocyanate labeled (FITC-BSA) (SIGMA A-9771), 0.5 mg/mL solution in PBS

Equipment and Materials

- Centrifuge equipped with 96-well plate rotor
- Molecular Devices 96 well plate reader set to 410 nm wavelength. Reader must be equipped with auto mixer and Pathcheck.
- SOFTMax Pro 3.1 software for Molecular Devices reader
- 96-well collection plates (Costar 9017 or equivalent)
- Black 96-well collection plates (Costar 3915 or equivalent)
- Plate shaker
- Conductivity meter

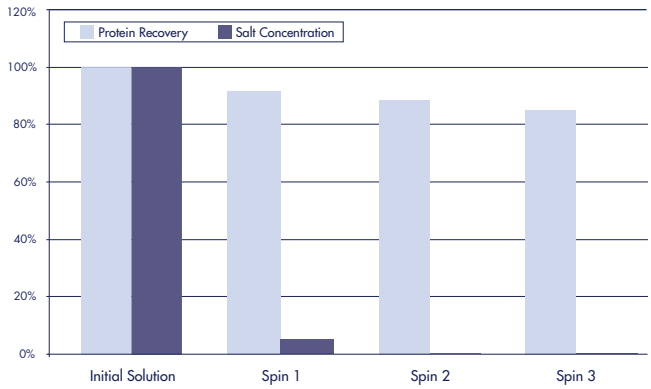
Protocol (Part 3 Protein Desalting)

1. Prepare 0.25 mg/mL solution of Cytochrome c in 0.5M NaCl.
2. Assemble three MultiScreen Ultracel-10 filter plates on top of 96-well collection plates.
3. Add 200 µL/well of protein solution to the MultiScreen Ultracel-10 filter plate.
4. Centrifuge at 3000 x g for 1 hour, until wells are empty
5. After the MultiScreen Ultracel-10 filter plate has spun, add 200 µL water to each well.
6. Shake the MultiScreen Ultracel-10 filter plate for 3 minutes.
7. Pool the protein solution from 96 wells of one plate together. Measure conductivity and determine protein concentration at 410 nm wavelength.
8. Repeat steps 4 – 7 for remaining two plates. Analyze the second plate after the second cycle and analyze the third plate after the third cycle.

Table 3. Salt removal and protein recovery using the MultiScreen Ultracel-10 filter plate. The starting solution was 0.25 mg/mL of Cytochrome c in 0.5M NaCl.

	% Protein Recovery	% Salt Remaining
Spin 1	92.8%	5%
Spin 2	89.7%	0.3%
Spin 3	85.8%	0.04%

Figure 5. Percentage of Cytochrome c and NaCl remaining in the Ultracel-10 wells after one, two, or three concentration/dilution cycles.



Results

Table 3 and Figure 5 show the results of salt removal and protein recovery in MultiScreen Ultracel-10 filter plates after 3 consecutive centrifugations of Cytochrome c solution with 0.5M NaCl. It is demonstrated that up to 95% of salt can be removed in the first cycle of concentration/desalting. As much as 99.9% salt can be removed after two centrifugations. It is important to note that protein recovery numbers are still high, with around 85% of protein being retained by the membrane after three centrifugation cycles.

Part 4: Ultrafiltration Time and Volumes

Objective: In this experiment, the time required to spin samples to dryness as a function of starting volume and protein concentration was determined.

Method

Reagents

- Milli-Q water
- Phosphate Buffered Saline (PBS) (Sigma P 3813, pH 7.4)
- Cytochrome c (Sigma C-8266), 0.5 mg/mL solution in PBS
- Bovine Albumin – Fluorescein Isothiocyanate labeled (FITC-BSA) (SIGMA A-9771), 0.5 mg/mL solution in PBS

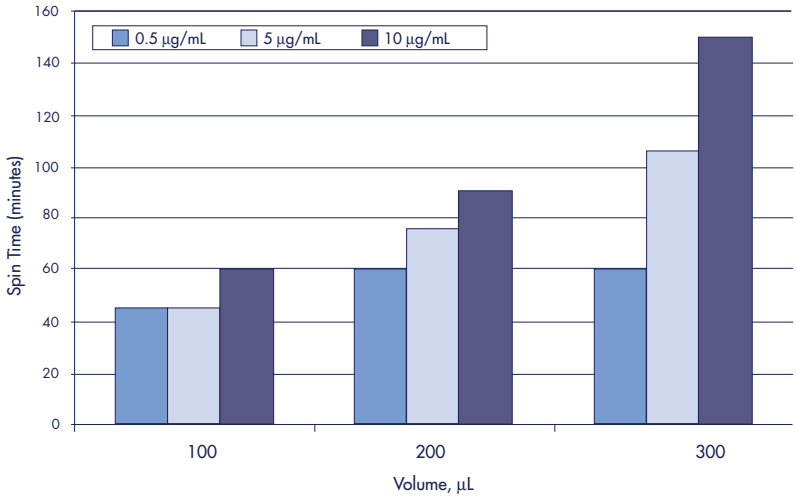
Equipment and Materials

- Centrifuge equipped with a 96-well plate rotor
- Molecular Devices 96-well plate reader set to 410 nm wavelength. Reader must be equipped with auto mixer and Pathcheck.
- SOFTMax Pro 3.1 software for Molecular Devices reader
- Wallac/Victor Fluorescence Detector or comparable instrument
- 96-well collection plates (Costar 9017 or equivalent)
- Black 96-well collection plates (Costar 3915 or equivalent)
- Plate shaker

Protocol

1. Prepare 0.5 mg/mL, 5 mg/mL and 10 mg/mL solutions of Cytochrome c in PBS.
2. Place a MultiScreen Ultracel-10 filter plate on top of a 96-well collection plate.
3. Add 100 µL/well, 200 µL/well and 300 µL/well of protein solution to different wells of a MultiScreen Ultracel-10 filter plate.
4. Place plates in centrifuge and spin at 3000 x g. Check for retentate volumes at 15, 25, 30, 45, 60, 75, 90, 105 and 150 minutes.

Figure 6. Times required to spin 3 concentrations of Cytochrome c solutions to dryness at 3000 x g, 25 °C.



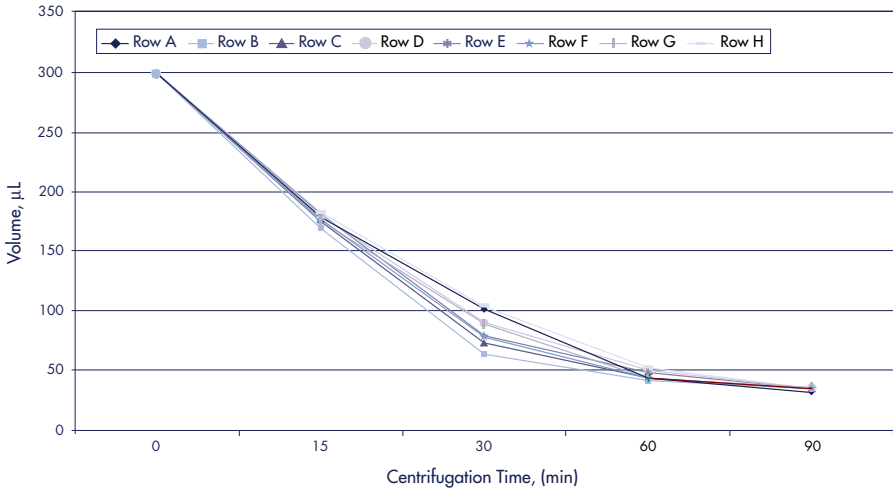
Results

Results of this study are shown in Table 4 and Figure 6. The time to dryness depends on the protein concentration and starting volume. As long as 150 minutes centrifugation may be required to concentrate 300 µL of 10 mg/mL Cytochrome c solution. Figure 7 demonstrates that volume reduction is reproducible throughout all 8 rows of wells of the MultiScreen Ultracel-10 filter plate.

Table 4. Times required to spin to 50 µL retentate at 3000 x g, 25 °C

Initial Volume of 0.5 mg/mL Cytochrome c (µL)	Time to Spin to 50 µL Retentate (minutes)
100 µL	15
200 µL	25
300 µL	30

Figure 7. Row-to-row variations of retentate volumes for Cytochrome c at various times at 25 °C and 3000 x g with 300 µL starting volume.



Summary and Conclusions

Protein retention, recovery, volume recovery, concentration and desalting can be accomplished rapidly and with good results using centrifugation and an ultrafiltration membrane. Results presented here and published separately demonstrate that MultiScreen Filter Plate with Ultracel-10 membrane is designed for high retention and low protein binding. It is an efficient tool for the parallel processing of 96 samples at once by centrifugation.

Related literature

PF2050EN00: MultiScreen Filter Plate with Ultracel-10 Membrane data sheet

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