

2.2.2.2 Mustang® Ion Exchange Membrane

The high capacity Mustang Q and S membranes, made from polyethersulfone (PES) material modified with anion and cation exchange chemistry, have been put into an AcroPrep™ 96-well plate format. Mustang ion exchange membranes deliver efficient and rapid flow rates with a convective pore structure resulting in processing times that are much shorter and more efficient than conventional resin-based technology. Mustang devices have throughputs of up to 100 times that of traditional bead-based media with no associated loss of capacity. This multi-well format can be used to carry out parallel, high throughput ion exchange pre-fractionation of complex samples, such as serum or plasma. The multi-well ion exchange plate specifications are summarized in Table 2.13 for Mustang Q and S chemistries. This convenient methods development format can be directly scaled up to syringe-based Acrodisc® devices (see Section 4.1, page 297) and larger capsules with Mustang Q membrane for large-volume bioprocess applications. These 96-well plate and Nanosep® centrifugal device applications are equivalent to batch-mode chromatography using stepwise elution conditions with a great deal of flexibility in the number of steps employed by the user.

Table 2.13

Specifications of the AcroPrep 96 Filter Plates with Mustang Ion Exchange Membrane

Specification	Parameter
Materials of Construction Membrane Device	Mustang Q or S modified Supor® PES Polypropylene
Effective Membrane Area	0.25 cm ²
Dimensions	
Length	12.78 cm (5.03 in.)
Width	8.51 cm (3.35 in.)
Height (Plus Lid)	1.66 cm (0.655 in.)
Height (without Lid)	1.44 cm (0.565 in.)
Tip Length	0.53 cm (0.21 in.)
Capacity/Well	
Maximum Well Volume	0.35 mL
Recommended Volume	0.30 mL
Maximum Centrifugal Force	3,000 x g
Centrifuge	Swinging bucket rotor
Operating Vacuum	25.4 cm Hg (10 in. Hg)

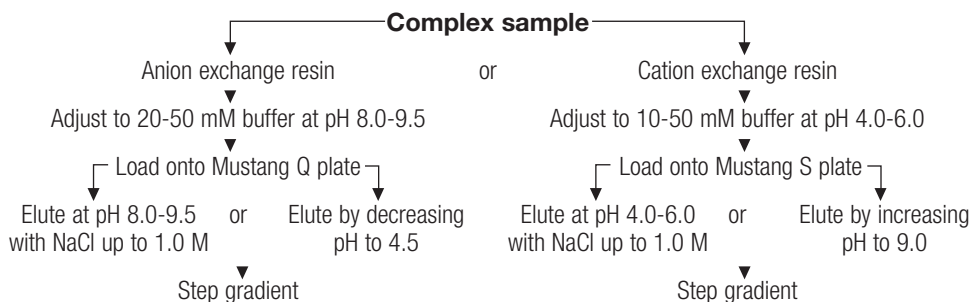
The capture of proteins for ion exchange is typically done in the presence of low salt. Due to the nature of Mustang membranes, they are more salt sensitive than the same chemistries on beads. This should be considered when choosing the buffers for protein capture and elution. In addition, they should not be overloaded with protein since the separation under these conditions is much less reproducible and predictable. For

2.2 – Section 2.2.2.2

proteomics applications where the goal is complexity reduction in user-defined number of fractions, the binding step is often performed at relatively low stringency so the majority of proteins will bind. Then stepwise elution is used to selectively elute specific subsets of proteins, based on their pI (pH elution) or charge density (salt elution). Elution based on pI is achieved with buffers of increasingly higher stringency (lower pH for anion exchange and higher pH for cation exchange). For salt-based elution (most common for traditional ion exchange chromatography), a buffer with increasing salt concentrations is used. Thus proteins elute from the ion exchange surface due to a change in charge state so they are no longer capable of stable salt bridge formation with the ion exchange chemistry (pH elution) or displacement due to the presence of a small counter ion in the form of salt. The two approaches are useful in developing appropriate pre-fractionation strategies in multi-well formats and are summarized in Table 2.14. When downstream steps are salt sensitive, pH elution is preferred since this eliminates the need for a salt removal step. The number of intervals in a stepwise elution (i.e., number of different pH or salt concentrations) is largely determined by the expected pI range in the sample, the complexity of the sample, and the total number of fractions desired. Either strategy or a combination of both can be applied to the purification of components from a complex sample in the AcroPrep™ 96 filter plate.

Table 2.14

Summary of Pre-fractionation Options for Mustang® Ion Exchange Membrane in an AcroPrep 96 Filter Plate



In this application section, protocols will be described for pre-fractionation of complex samples employing multi-well, small-volume devices.

Protocol for Mustang Ion Exchange Membrane

Collection of fractions from Mustang ion exchange plates can be done under vacuum or in a centrifuge. Both methods are described below. Experience indicates that fraction collection in a centrifuge is somewhat more reproducible even though it takes slightly more time.

A. Materials Required

1. AcroPrep 96 filter plate, 350 µL and 1 mL volume filter plates with Mustang Q and S membranes. For specifications, see Table 2.13.
2. Collection plates [e.g., Axygen 96 well polypropylene V bottom, 0.5 mL (P96450V) or round bottom, 1.64 mL (PDW20)]

3. High purity binding/equilibration buffer such as 25 mM Tris HCl, pH 8.5 (Mustang® Q membrane) and 10 mM MES-NaOH, pH 5.5 (Mustang S membrane)
4. High purity elution buffers
5. Separation apparatus
 - a. Source of vacuum capable of 25.4 cm Hg (10 in. Hg) (vacuum manifold, PN 5017); or
 - b. Centrifuge fitted with a swinging bucket rotor with multi-well plate adapters and able to spin up to 3,000 x g

B. Vacuum Manifold Processing

1. During use, hold the plate so that the outlets on the bottom of the plate are not touched to prevent liquid flow due to wicking. The easiest way to accomplish this is to place the Mustang plate on a receiver plate.
2. Place an appropriately sized receiver plate into the vacuum manifold.
3. Place the filter plate in the vacuum manifold. Add 0.2 mL equilibration buffer.
4. Apply vacuum to manifold to initiate liquid flow. Recommended vacuum is minimum required to initiate flow, up to 25.4 cm Hg (10 in. Hg) for 96-well plates. DO NOT exceed 38.1 cm Hg (15 in. Hg). DO NOT leave the plate under vacuum for longer than necessary or it will dry out and protein will be unrecoverable.
5. Release vacuum and discard the first filtrate. Gently tap the plate to remove any hanging droplets.

Tip: Do not release vacuum by pulling the corner of the plate as it will degrade the manifold gasket.

6. Add the sample to the plate.
7. Place a receiver plate into the vacuum manifold sized to collect the volume of the filter plate.
8. Apply vacuum to manifold to initiate liquid flow, as directed above. Use minimal vacuum to achieve flow and maximize the protein-membrane interaction time.
9. Release vacuum from the manifold and recover unbound proteins (flow through sample) from the collection plate.

C. Processing by Centrifugation in a Swinging Bucket Rotor

1. Place the AcroPrep™ 96 filter plate on top of a receiver plate sized to collect the appropriate volume from the filter plate.
2. Insert the two plates into a standard multi-well plate swinging bucket rotor assembly.
3. Place a duplicate pair of plates matching the weight of the test plate (add water to the receiver plate and match weight of the test plate).

Tip: An imbalance can result with a single test plate if no counter balancing plate is used. If different volumes of sample are used in multiple plates, they will need to be balanced in pairs by addition of water to empty wells.

4. Centrifuge at 500 x g for 2 minutes.

Tip: The centrifugal force and time parameters can be varied to optimize the filtration rate of fluids in contact with the well of the plate.

2.2 – Section 2.2.2.2

D. General Fractionation Protocol

Tip: Although the binding capacity of the Mustang® membrane is quite high per unit volume, there are only 2 layers of membrane in the current Mustang AcroPrep™ 96-well plate. As a result, the net binding capacity is limited. We recommend loading < 0.2 mg protein per well. If over capacity, the binding is much less reproducible. It is very important to allow the protein sample sufficient time to interact with the membrane during the fraction collection step. For this reason, low vacuum and low centrifugation speeds are highly recommended.

1. Place an appropriately sized collection plate into the vacuum manifold.
2. Place a Mustang ion exchange membrane plate onto the vacuum manifold.
3. Pipette 0.3 or 0.5-0.8 mL of equilibration/loading buffer into the well of the plate (depending on plate capacity).

Tip: At this stage, loading buffer optimization should be carried out as suggested in Table 2.14. The 96-well filter plate facilitates replicates and parallel testing of many variables in one experiment.

4. Discard the equilibration buffer from the collection plate.
5. Add up to 0.3 or 0.8 mL equilibration/binding buffer to the wells.

Tip: The total protein load on these plates should be < 0.2 mg per well (equivalent to 2-3 μ L whole plasma) in a minimum of 20 μ L. Dilute samples if necessary. For best results samples should be prefiltered with an Acrodisc® syringe device or an AcroPrep 96 filter plate, 0.45 μ m, with GHP membrane (see Section 2.5, page 198); or hard spun (5-10 minutes in microfuge at maximum speed) to remove particulate material.

6. Collect the unbound proteins [flowthrough fraction (FT)] as indicated above.

Tip: The vacuum should be controlled to allow sufficient time for protein interaction with the mustang membrane surface. We recommend at least 1-2 minutes.

7. For best results, after liquid has passed through the membrane, release vacuum and reload the filtrate into the wells of the plate and repeat Step 6 above.

Tip: This recycling should be optimized since the Mustang ion exchange membranes exhibit very rapid adsorption/desorption kinetics due to the efficient convective pore structure of the modified PES microporous membrane.

8. Add 0.1-0.3 or 0.5-0.8 mL of equilibration buffer to the wells and repeat Steps 5-7 to wash away residual unbound proteins.

Tip: At this stage, these wash fractions can be retained, combined with the flowthrough fractions, or discarded. If the protein concentration of the combined flowthrough and wash is too dilute, the sample can be concentrated with an AcroPrep 96 filter plate with a 10K MWCO UF membrane (see Section 2.4, page 152).

9. Elute bound proteins using salt or pH change as described in Table 2.14. Combinations of both variables should be considered to fine tune elution conditions when necessary.
10. Fractions are eluted by adding 0.1-0.3 or 0.2-0.8 mL of elution buffer to the wells and repeating Steps 5-7 above. Each step should be saved in a separate collection plate and processed for analysis.

11. After the final NaCl or pH elution step, tightly bound material can be eluted with 1% (w/v) sodium dodecyl sulfate (SDS) in water and, if necessary, recovered by acetone precipitation or SDR detergent removal (see Section 2.3, page 141). Other strong denaturants compatible with downstream steps can also be used. After an SDS detergent elution, the plate should be discarded.

Application Data for Mustang® Ion Exchange Membrane

Mustang Ion Exchange Membrane Protein Binding Capacity in an AcroPrep™ 96 Filter Plate

The current Mustang ion exchange membrane in an AcroPrep filter plate contains two layers of modified Supor® PES membrane and can be used to pre-fractionate a complex sample, such as serum or plasma. To estimate protein binding capacity of ion exchange membrane, it is common practice to use saturation binding with purified standard proteins, such as BSA for an anion ion exchange and lysozyme for a cation ion exchange surface. An example of a study to measure the BSA binding across a whole Mustang Q AcroPrep plate is summarized in Table 2.15. The results showed a high degree of reproducibility across the 96 wells of the plate and a saturation binding capacity of 0.17 mg/well under these conditions, which corresponds to 24.4 mg/mL of Mustang Q membrane volume. This protein binding capacity is sufficient for small scale fractionation (e.g., proteomics applications) and purification methods development to investigate purification parameters for larger scale processes employing Mustang Q and S Acrodisc® syringe filters (see Sections 4.1.2, page 298 and 4.1.3, page 303).

Table 2.15

Saturation BSA Binding Capacity for Mustang Q in a 350 µL AcroPrep 96 Filter Plate on a Vacuum Manifold

Binding Capacity (mg BSA/well)*				
Membrane	Elution #1 (+/- SD)	Elution #2 (+/- SD)	Total	mg BSA /mL media**
Mustang Q	0.16 ± 5.9%	0.01 ± 7.3%	0.17	24.3

**Binding capacity was measured using 5 mg/mL BSA in 25 mM Tris HCl, pH 8.5 as follows: a) equilibrate the membrane with the above buffer; b) add 0.3 mL of the BSA sample and adjust vacuum to give a flow time of 2 minutes; c) repeat process 5 times to load a total of 7.5 mg BSA per well, sufficient to saturate the binding sites on the Mustang membrane; d) wash with 10 x 0.3 mL of the loading buffer. Check absorbance at 280 nm of final filtrate on a plate reader to confirm eluate at baseline absorbance; and e) elute the bound BSA with 2 x 0.3 mL of 1 M NaCl in the above buffer. Measure eluted protein absorbance at 280 nm. Calculate BSA bound from the pooled fractions.*

***Membrane volume/well calculated to be 0.007 mL, assuming an area of 0.25 cm², and a membrane thickness of 0.014 cm with 2 layers per well.*

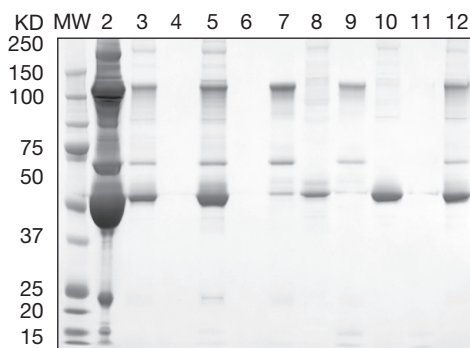
2.2 – Section 2.2.2.2

Pre-fractionation of Plasma by Mustang® Ion Exchange Membrane-based Chromatography

Resolution of complex samples such as plasma or serum by ion exchange chromatography leading to pre-fractionation can be investigated in the AcroPrep™ 96 filter plate. This methodology can be easily adapted to small volumes of samples and carried out in parallel using the multi-well plate format. An example of a preliminary bind and elute study to examine binding of plasma proteins to Mustang Q membrane in an AcroPrep filter plate is shown in a 1D SDS-PAGE in Figure 2.21. Binding conditions were varied while elution for all samples was done at pH 2.5. Under these circumstances a 1D gel will only show the most abundant proteins, but will give a sense of the separation possibilities. The results show that at physiological pH and ionic strength [PBS Lane 5 and 6, flowthrough (FT), and eluate], very little protein is retained as expected. When the ionic strength is lowered, some proteins are retained (Lane 8, eluate). When the capture is performed at pH 10 in the absence of salt, almost all proteins are bound. While in the presence of salt, some of the proteins show up in the FT fraction. Thus, the control of sample complexity in collected fractions occurs not only by elution buffer choice but also by capture conditions. This illustrates the utility of membrane-based ion exchange to rapidly explore a fractionation strategy in a multi-well filter plate. Some preliminary experiments to determine the optimal capture and elution conditions for appropriate complexity reduction are highly recommended.

Figure 2.21

Pre-fractionation of Human Serum on Mustang Q Membrane in an AcroPrep 96 Filter Plate



Pooled human serum proteins were captured on a Mustang Q membrane (AcroPrep 96 filter plate). The conditions for protein binding were varied in both pH (pH 10 carbonate buffer and pH 7 phosphate buffer, representing low and moderate stringency, respectively) and in the absence or presence of 150 mM NaCl. In all cases, bound proteins were eluted at pH 2.5 for SDS-PAGE analysis. Molecular weight markers and 1 µL of pooled human serum are in Lanes 1 and 2. The flowthrough (FT) and eluate (E) samples alternate from Lane 3-12, beginning with FT. Lane 3 = FT and Lane 4 = E from 10 µL of neat serum, which is not recommended due to poor reproducibility. Lanes 5 and 6 are FT and E from pH 7 + 150 mM NaCl binding. Lanes 7 and 8 are FT and E from pH 7, no salt capture conditions. Lanes 9 and 10 are FT and E from pH 10 + 150 mM NaCl binding. Lanes 11 and 12 are FT and E from pH 10, no salt capture conditions.

Ordering Information for Mustang® Ion Exchange Membrane

AcroPrep™ 96 Filter Plate, 350 µL Well

Part Number	Description	Pkg
5047	Mustang Q membrane, natural	10/pkg
5048	Mustang S membrane, natural	10/pkg

AcroPrep 96 Filter Plate, 1 mL Well

Part Number	Description	Pkg
5062	Mustang Q membrane, natural	5/pkg
5063	Mustang S membrane, natural	5/pkg