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Application Note

June, 2021

Keywords or phrases:
Mixed mode resins, 96 well-plate, Screening method

Method for High Throughput Screening of MEP, HEA, PPA, and CMM HyperCel Mixed-Mode Chromatography Resins Using 96-Well Plates

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1. Abstract

This document provides a protocol for the high throughput screening of mixed-mode chromatography resins.
A method is provided for the rapid screening of both binding and elution conditions using 96-well plates.

2. Screening Strategy

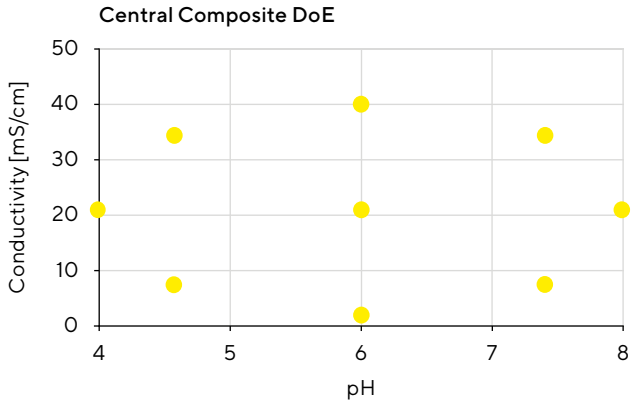
The Sartorius range of mixed-mode resins supply both ion exchange and hydrophobic interaction functionalities. This affords the resins unique selectivities. Therefore, it is necessary to screen both binding and elution conditions to leverage this selectivity and allow for contaminant removal. Optimization of conditions may involve multiple steps. Initially, a resin may be selected due to its superior performance in a 96-well plate high throughput screen. Once a resin has been selected, an additional round of 96-well screening may be performed to refine operating conditions before transferring to the column chromatography. Small scale column chromatography is performed to verify the operating conditions and determine capacity in dynamic mode.If the target protein is not well characterized, it can be worthwhile to screen a broad range of pH and conductivities to identify a binding condition that allows the target protein to be captured, whilst contaminants flow through. To screen binding and elution conditions, a design of experiments (DoE) approach is recommended. An example central composite design is shown in Table 1 and Figure 1. Alternatively, if DoE software is not available, a more simple approach can be employed and the data analyzed as contour plots. Conditions for a typical broad range screening experiment are shown in Table 2 and Figure 2.

Find out more: www.sartorius.com/en/products/process-chromatography/chromatography-consumables/mixed-mode-chromatography

Table 1 and Figure 1

Example central composite design

pH	Buffer Type	Conductivity [mS/cm]
4.0	50 mM acetate	21.0
4.6		7.6
4.6		34.4
6.0	25 mM phosphate	2.0
6.0		21.0
6.0		21.0
6.0		21.0
6.0		21.0
6.0		21.0
6.0		40.0
7.4		7.6
7.4		34.4
8.0	25 mM Tris	21.0

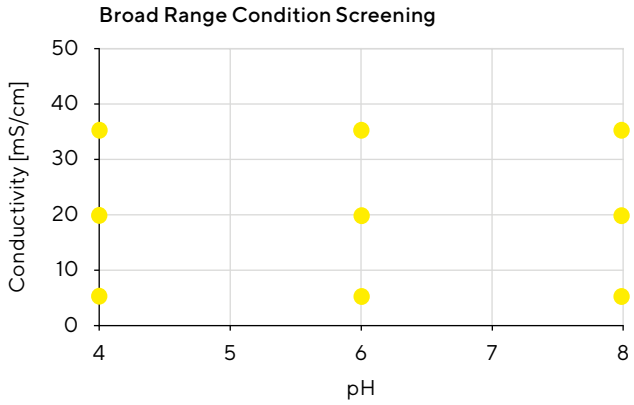


Note that the center point is repeated 5 times. This allows for the statistical significance of the experiment to be determined.

Table 2 and Figure 2

Simple broad-range experimental design analyzed using contour plots

pH	Buffer Type	Conductivity [mS/cm]
4.0	50 mM acetate	5
4.0		20
4.0		35
6.0	25 mM phosphate	5
6.0		20
6.0		20
6.0		20
6.0		20
6.0		35
8.0	25 mM Tris	5
8.0		20
8.0		35



Alternatively, if a conductivity meter cannot be used, recommended buffers are detailed in Appendix 1.

The binding experiment is performed by adjusting the pH and conductivity of the load to the conditions indicated for the DOE. The binding experiment can be assessed in two ways. After the binding step, the flow through fractions can be analyzed directly to determine the amount of target protein and contaminants in each of the flow through fractions. Alternatively, if an elution condition is known, the bound proteins can be eluted and the amount of target and contaminant proteins in the elution can be determined. Both data sets can be separately analyzed using statistical software to determine the statistical significance of the data and generate response surfaces.

Response surfaces help to visualize tends in the data to select conditions preferred for binding. Once a binding condition has been established, a separate DoE may be executed to establish conditions ideal for elution at the selected condition for binding.

It is recommended to investigate a broad range DOE for an uncharacterized protein. Additional DOEs may be generated, focusing on a more narrow range after establishing a general range of conditions for operation.

3. Required Materials

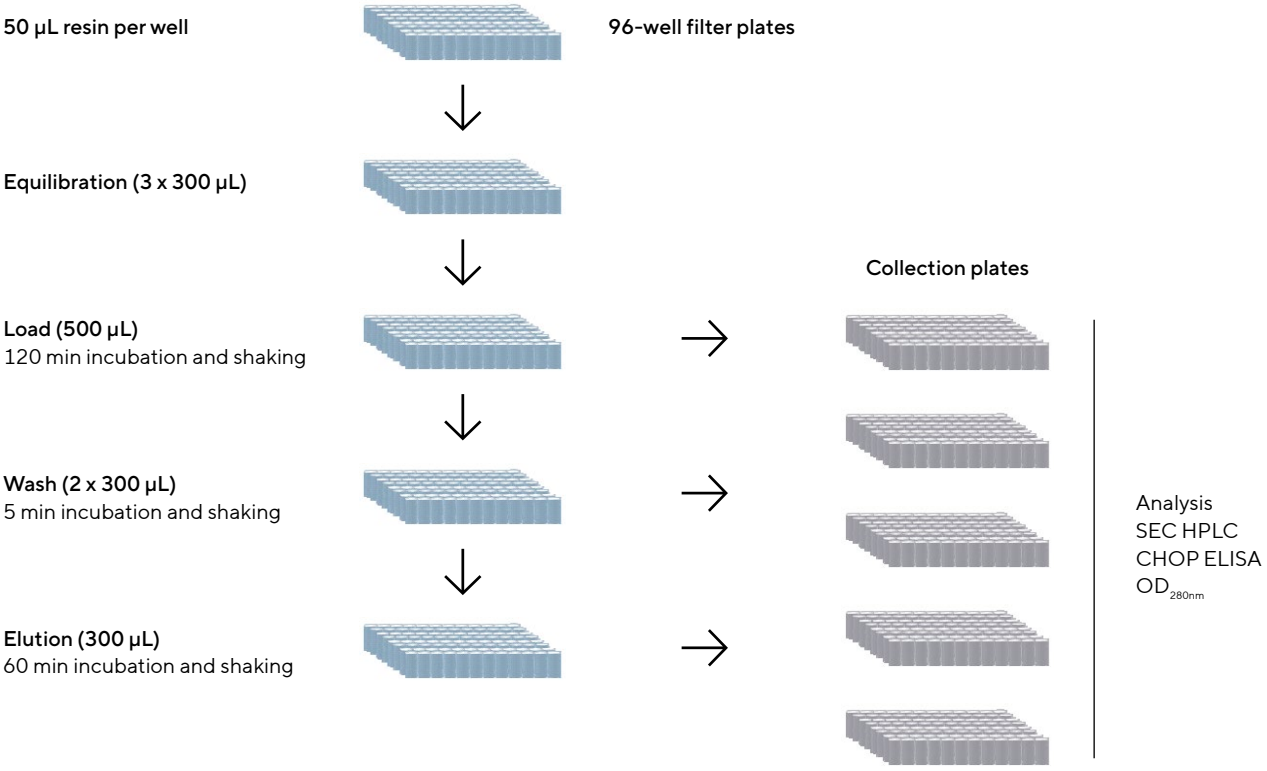
- Selected HyperCel mixed-mode resins (see part numbers [P/N] in Appendix 2)
- 300 µL multi-channel pipette
- Stirring plate or an orbital shaker adaptable to microplates
- Vacuum manifold (Pall multi-well plate vacuum manifold, P/N 5017) or a centrifuge
- AcroPrep™ Advance filter plate Pall P/N 8129
- Flat bottom 96-well collection plates (2 mL deep-well depending on the size of the fractions to collect e.g., Axygen Scientific, P/N P-2ML-SQ-C
- Buffer | solutions volume: Plan in excess 50 mL of buffers, NaOH, deionized (DI) water. Volumes depend on the chosen methodology

Table 3
Recommendations of buffer in loading conditions in bind | elute or flow through conditions

Loading pH	4.0	5.0	6.0	7.0	8.0	9.0
HEA, PPA HyperCel			Binding			
		Flow Through | Elution				
MEP HyperCel			Binding			
		Flow Through | Elution				
CMM HyperCel			Binding			
				Flow Through | Elution		

The bind | elute mode and flow through mode conditions are given as a rough guide and will strongly depend on the isoelectric point and hydrophobicity of the targeted molecule along with the conductivity. For more information, refer to the corresponding instructions for use (see Appendix 3).

Figure 2
96-well plate screening protocol



4. Protocol

4.1. Plate Processing

The 96-Well Plate

The filter plate enables separation of liquid and resin by drawing liquid through the filter using a vacuum or centrifugation. The wells of the plate have a 1 mL volume, but volumes over 500 µL are not recommended as they may lead to weeping from the filter at the bottom of the wells, as well as splashing when using a shaker plate for sample incubation. To keep the filter plate clean and avoid contamination, it is recommended to keep the filter plate on a 2 mL collection plate at all time, including during shaking.

Vacuum Manifold vs. Centrifugation

For pulling liquid through the plate, multi-well plate vacuum manifold is recommended at -0.3 to -0.5 barg (225 to 375 mm Hg). Alternatively, a swing-rotor centrifuge can be used at 500 x g for 1 to 2 minutes.

Table 4
Characteristics of 96 well plates

Plate size	128 x 86 x 33 mm
Plate material	Polypropylene
Number of wells	96
Well volume	1 mL
Filter media and type	0.45 µm hydrophilic polyethersulfone membrane
Mode of operation	Vacuum using manifold or centrifuge
Volume of chromatography resin per well	50 µL in a total volume of 800 µL
Storage solution per well	750 µL of 1 M NaCl with 20% (v/v) ethanol
Working volume per well	100 to 500 µL
Maximum centrifugation force	700 x g
Maximum vacuum	0.5 bar g (375 mm Hg)

Preparing the Resin and Filling the Plate

Gently suspend the resin in the storage solution and pour approximately 20-40 mL of the slurry into a 50 mL conical tube. The resin can be pelleted in a centrifuge operating at 3000 x g for 2 minutes or by gravity until resins are settled. The supernatant can be poured off and then new solution added. It is recommended to employ this strategy to prepare the resin for use to:

1. Wash with water to remove the storage solution
2. Wash with 1 M NaOH to remove any bound material that might have UV absorbance
3. Wash with buffer to bring the pH to neutral. This normally requires 3 – 4 wash steps.

At this point, the resin can be aliquoted. To aliquot effectively, it is necessary to bring the resin to a 50% (or lower) slurry. To accurately assess the resin volume, the resin can be allowed to settle overnight or centrifuged for 10 minutes at 1000 x g.

To aliquot the resin, it is best to mix thoroughly by end over end mixing in the 50 mL tube followed by decanting into a 50 mL reservoir. Because the resin settles quickly, it is necessary to work quickly and to constantly mix the resin in the reservoir by rocking (if the resin settles out, return to the 50 mL tube and mix end over end again). To pipette the resin, a multi-channel pipette is used. It is best to use wide-bore pipette tips or to trim the ends of regular tips so that the resin does not clog the tip when aspirated.

Usually, 100 µL of a 50% resin slurry is aliquoted into each well of the filter plate. If feedstock for the experiment is limited or at a low concentration, it is possible to use less resin. However, thought has to be taken to ensure that enough protein will be processed for analytic assays to be performed accurately.

To ready the resin in the plate for use, it is necessary to equilibrate to the same buffer condition that binding is going to be performed at (for an elution test where binding is all at the same buffer condition, the resin can be washed directly into the buffer of choice after the 1 N NaOH step). Three washes, each of 300 µL, are recommended to equilibrate the resin. To check the pH is lowered, the flow through fraction can be checked with a pH indicator strip. Between washes, the vacuum manifold can be used to draw the wash through the resin. Careful tapping of the vacuum manifold against the surface it rests upon can be employed to remove small droplets that stick to the side of the 96-well plate. The vacuum manifold can then be used to draw the remaining liquid through the resin. At this point, the resin is almost dry and load sample should be added as soon as possible (<30 minutes).

4.2. Recommendations for Sample Preparation

Loading Volume
Typically, 100 to 400 µL of sample protein is loaded per well. Lower load volumes are not recommended as efficient mixing might not be achieved.

Sample Preparation
As unclarified samples may clog the membrane at the bottom of the 96-well plate, it is recommended to filter the sample through a 0.2 µm filter. It may be possible to filter the load proteins before adjusting to the load condition as long as the pH and conductivity adjustment does not cause protein precipitation. Alternatively, proteins may have to be filtered directly before loading.

The sample to be loaded must be adjusted to the correct buffer loading conditions. If the load protein is concentrated, it may be diluted 1:10 or 1:20 in the correct buffer so that the load condition is approximately achieved. Alternatively, it may be necessary to adjust the pH and conductivity manually.

It is recommended that the binding experiments be performed at modest loading capacity (in the range of 5 to 10 mg/mL of resin. Thus, for 50 µl of resin, 0.5 mL of up to 1 mg/ mL solution can be loaded, for a total load of 0.5 mg per well).

This modest load capacity protects against false negatives for binding.

4.3. Plate Incubation
To ensure satisfactory mixing of resin and protein, orbital shaking at 1100 to 1200 rpm for 2 hours is required (this time may be shortened if control experiments are performed to show that binding equilibrium can be reached more quickly. For the control experiments, it is critical to test the same volume in the well as would be used in the actual experiment).

4.4. Sample Collection
After the binding step, the flow through fraction is captured in a 2 mL receiver plate (deep well block). Be sure to line up A1 on the filter plate and A1 on the receiver block.
(The plates will fit in an alternative 180 degrees orientation, but the wells do not line-up!)

4.5. Washing
After the flow through (post load and incubation) fractions are collected, the plate can be washed. Normally, two washes each of 300 µL are performed. The washes should be performed at the same buffer condition used for the binding. Both washes can be collected into a single 96-well block to be analyzed together. This is often required to close out a mass balance.

4.6. Sample Elution
The sample can be desorbed from the resin and collected by using two elution steps, each of 300 µL. Normally both elution volumes can be collected into a single 96-well block to be analyzed together.

4.7. Analysis
With the 96-well plate assay completed, the next step is to analyze the samples. For post Protein A monocolonal antibody (mAb) purifications, normally the target protein is >99% pure and the A_{280} can be used to determine the target protein concentration. For detecting alternate target proteins and contaminants, other analytics may be required, such as ELISA, size exclusion chromatography, gel electrophoresis or western blot. If the DoE approach is pursued, each category of protein | contaminant becomes a response. With the value of each response at all of the data points, DoE software can be used to generate overlaid contour plots which facilitate determination of the best conditions for the chromatography step. If DoE software is not available, the data can be analyzed manually and visualized as contour plots. The broad range screening should enable the visualization of conditions suitable for both binding and elution.

4.8. Next Steps
At this point, it may be desirable to perform additional rounds of screening to further optimize both binding and elution conditions for contaminant removal. The conditions to be screened for binding should be chosen from the first broad range screen where at least 80% of the target molecule is bound. For elution, conditions should be selected based on the broad range screening where greater than 80% of the target molecule is in the flow through. The same high throughput screening strategy can be applied to these additional rounds of screening. Alternatively at this point screening and validation experiments can be performed with chromatography columns. There are a range of columns pre-packed with the mixed mode resins for speed and convenience.

For more information or support with screening these products, it is recommended to discuss with one of the technical experts at Sartorius.

5. Appendices

Appendix 1

Alternative buffer recommendations (see Table 2)

pH	Buffer Type	Conductivity [mS/cm]
4.0	50 mM acetate	5
4.0	50 mM acetate, 125 mM NaCl	20
4.0	50 mM acetate, 250 mM NaCl	35
6.0	25 mM phosphate	5
6.0	25 mM phosphate, 125 mM NaCl	20
6.0		20
6.0		20
6.0		20
6.0	25 mM phosphate, 250 mM naCl	35
8.0	25 mM Tris	5
8.0	25 mM Tris, 125 mM NaCl	20
8.0	25 mM Tris, 250 mM NaCl	35

Appendix 2

Part numbers for HyperCel mixed-mode resins (see Section 2)

Description	Size | Format	Part Number
MEP HyperCel resin	25 mL bulk resin	12035-010
	100 mL bulk resin	12035-028
	1 L bulk resin	12035-036
	5 L bulk resin	12035-040
	10 L bulk resin	12035-044
	PRC prepacked column 5x50, 1 mL	PRC05X050MEPHCEL
	PRC prepacked column 8x100, 5 mL	PRC08X100MEPHCEL
	RoboColumn®* MEP HyperCel 200 µL, row of 8	SR2MEP
	RoboColumn® MEP HyperCel 600 µL, row of 8	SR6MEP
HEA HyperCel resin	25 mL bulk resin	20250-026
	100 mL bulk resin	20250-033
	1 L bulk resin	20250-041
	5 L bulk resin	20250-042
	10 L bulk resin	20250-056
	PRC prepacked column 5x50, 1 mL	PRC05X050HEAHCEL
	PRC prepacked column 8x100, 5 mL	PRC08X100HEAHCEL
	RoboColumn® HEA HyperCel 200 µL, row of 8	SR2HEA
	RoboColumn® HEA HyperCel 600 µL, row of 8	SR6HEA

Appendix 2 (continued)

Description	Size | Format	Part Number
PPA HyperCel resin	25 mL bulk resin	20260-025
	100 mL bulk resin	20260-030
	1 L bulk resin	20260-040
	5 L bulk resin	20260-045
	10 L bulk resin	20260-052
	PRC prepacked column 5x50, 1 mL	PRC05X050PPAHCEL
	PRC prepacked column 8x100, 5 mL	PRC08X100PPAHCEL
	RoboColumn® PPA HyperCel 200 µL, row of 8	SR2PPA
	RoboColumn® PPA HyperCel 600 µL, row of 8	SR6PPA
CMM HyperCel resin	25 mL bulk resin	20270-025
	100 mL bulk resin	20270-031
	1 L bulk resin	20270-041
	5 L bulk resin	20270-055
	10 L bulk resin	20270-066
	PRC prepacked column 5x50, 1 mL	PRCCMMHCEL1ML
	PRC prepacked column 8x100, 5 mL	PRCCMMHCEL5ML
	RoboColumn® CMM HyperCel 200 µL, row of 8	SR2CMM
	RoboColumn® CMM HyperCel 600 µL, row of 8	SR6CMM

Appendix 3
Related documentation

Description
Datasheet: CMM HyperCel resin
Instructions for use: CMM HyperCel resin
Datasheet: MEP HyperCel resin
Instructions for use: MEP HyperCel resin
Datasheet: HEA and PPA HyperCel resins
Instructions for use: HEA and PPA HyperCel resins

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Last modified: 04 | 2026