Millipore®

High Throughput Screening Protocol

Eshmuno® CMX Resin

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Contents

Introduction

Biologics process development using mixed mode chromatography resins can be more challenging than traditional resins due to potential overlapping interactions between the target molecule and functional groups on the chromatography resin. In mixed mode chromatography, increasing the conductivity simultaneously enhances the hydrophobic interaction between the solute and the stationary phase (unlike traditional cation exchange chromatography when increasing conductivity facilitates elution). This can result in the inability of the solute, such as a protein, to elute from the column. Therefore, it is essential to identify the optimal process conditions for bind and elution for each target protein based on its properties.

The emergence of new formats like bispecific antibodies or antibody-drug conjugates (ADCs) introduces additional downstream processing challenges due to increased hydrophobicity (Koehnlein et al., 2022).

A high throughput screening approach can be employed to identify suitable process conditions. Small lab-scale screenings can be performed manually or using an automated liquid handling station. This approach offers the advantage of reducing the amount of target protein and chromatography resin required while enabling the screening of multiple conditions in a short time. This ultimately helps in reducing costs and time for process development.

Eshmuno® CMX is a mixed mode resin based on the established Eshmuno® resin technology. It possesses unique selectivity due to its weak cation exchange group and moderate hydrophobicity.

By combining the advantages of enhanced selectivity based on the target molecule's pI and hydrophobicity, this resin can achieve:

- > 90% recovery rates.
- Product binding capacity of >60 mg/mL.

Before the Experiments

- For robotic screening the microplate reader should be able to measure at least 280 nanometer (nm) absorbance for total protein quantification. To overcome variances in liquid level per well, a wavelength correction at 900 nm and approximately 977 nm absorbance is recommended. Consult the microplate reader manufacturer for additional instructions.
- A UV/VIS photometer capable of measuring 280 nm is sufficient for manual experiments.
- It is important to plan experiments in advance, especially when using a robotic system. Measurements should be performed in duplicate or triplicate.
- For static binding capacity (SBC) measurements, UV extinction measurements at 280 nm are sufficient when using a highly pure target protein.
- Additional analytics should be selected according to target protein and impurities (e.g. size exclusion chromatography, host cell protein content, host cell DNA content).
- During all incubation steps, ensure proper mixing of the sample within the microplate or tube. Adjust agitation speed to maintain suspension of resin particles, but avoid excessive shaking.
- Check the total working volume per well before measurement. The maximum working volume per well may vary depending on the microplate and shaker in use. Total working volume should not exceed 200 µL for the recommended microplate.
- An appropriate calibration curve needs to be measured for mass calculation by extinction of the protein solution, which can be performed beforehand or directly on the plate.

Materials

Robotic Measurements

- Microplate reader for extinction measurements at 280 nm absorbance and optional pathlength correction (consult the microplate reader manufacturer instructions).
- Orbital shaker for plates
- Centrifuge or vacuum manifold
- Pipettes and multichannel pipettes
- Graduated cylinder
- Glassware
- Filter plate (e.g. MilliporeSigma, MSDVN6550, MultiScreenHTS-DV, 0.65 µm, transparent)
- UV compatible 96-well collection plate (e.g. MilliporeSigma, MSCPNUV40, 96-well MultiScreenHTS)
- UV compatible Cuvettes (e.g. BrandTech Scientific Microcuvettes, 759200, optional)
- Additional analytics according to impurity profile.

Manual Experiments

- Photometer, extinction measurements at 280 nm absorbance
- Orbital shaker for spin columns
- Centrifuge or vacuum manifold
- Pipettes and multichannel pipettes
- Graduated cylinder
- Glassware
- Filter units (e.g. Corning Costar® Spin-X® Tubes – Filter Units, 8163)
- Tubes (e.g. Corning Costar® Tubes 2 mL – Tubes, 3213)
- UV compatible Cuvettes (e.g. BrandTech® Scientific Microcuvettes, 759200)
- Additional analytics according to impurity profile.

Buffer Preparation

- Sodium dihydrogen phosphate - hydrate (MilliporeSigma, 1.06346)
- Disodium hydrogen phosphate - dihydrate (MilliporeSigma, 1.06580)
- Acetic acid (MilliporeSigma, 1.00063)
- Sodium acetate (MilliporeSigma, 1.06268)

Preparation

Target Protein Solution

The start concentration is calculated from the 200 µL protein working solution per well or tube.

Depending on the initial concentration of the target protein solution, it may need to be concentrated to approximately 50 mg/mL. The solution is then diluted at least 1:10, reducing matrix effects to approximately 5 mg/mL.

If adjusting the target protein concentration is not possible due to stability concerns, dialysis into different buffers can be performed.

The prepared sample can be utilized for protein stability determination, static binding capacity (SBC) measurements, and experiments to determine optimal separation conditions.

Stability Study of the Protein

To determine the static binding capacity (SBC), it is essential to assess the protein's stability under different conditions. This is achieved by diluting the protein in various loading buffers (refer to Target Protein Solution) and measuring the extinction at 350 nm or 600 nm absorbance to monitor precipitation.

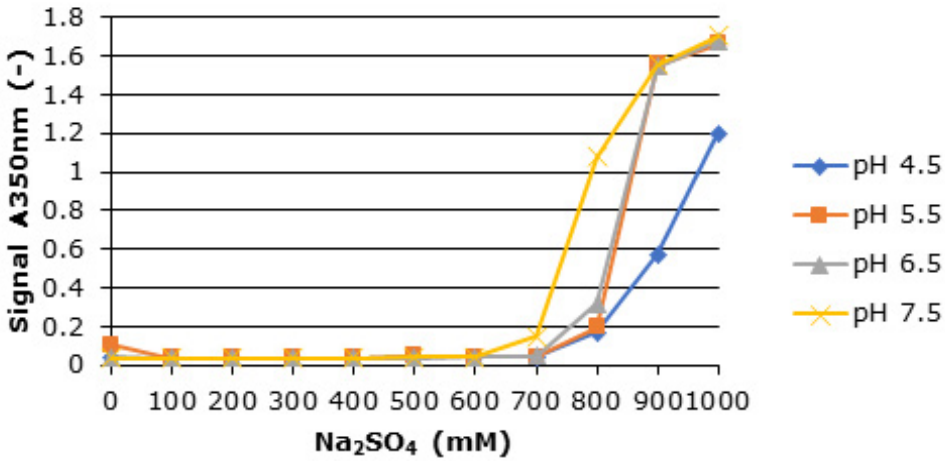

Figure 1: Stability study of a mAb across varying pH levels and Na_2SO_4 concentrations.

Figure 1 shows the stability study of a mAb in a given buffer system. The UV signal at 350 nm was measured across four pH levels and eleven Na_2SO_4 concentrations. At pH 7.5, an increasing absorbance at 350 nm was observed, indicating mAb precipitation at 700 mM Na_2SO_4 concentration. Additionally, high absorbance signals at 800 mM Na_2SO_4 indicated an unstable buffer condition for the protein. Based on these findings, conducting an experiment plan ranging from 0 to 700 mM Na_2SO_4 at all pH values specifically for this protein is recommended.

Resins

- Let the resin settle overnight in a graduated cylinder.
- Adjust the settled slurry to 20% (v/v) slurry by adding or removing storage solution liquid from the cylinder.

Buffer System

The pH and salt concentration of the buffers can be adjusted based on the pI (isoelectric point) and hydrophobicity of the target protein. In addition, the choice of salt can be modified according to the target protein's hydrophobicity, following the principles of the Hofmeister series.

Example: 50 mM Acetate/ Sodium phosphate buffer with Na_2SO_4

pH: 4.5 , 5.5 , 6.5 , 7.5

Na_2SO_4: 0, 100, 200, …, 900, 1000 mM

Table 1: Preparation of four buffer stock solutions

Molar Concentration	Substance	Cat Number	Molar Mass (g/mol)
A1: low salt and low pH (pH after dissolving ~ 3.4)			
50mM	$NaH_2PO_4*H_2O$	1.06346	137.99
50mM	Acetic acid, glacial	1.00063	60.05
B1: low salt and high pH (pH after dissolving ~ 9.1)			
50mM	$Na_2HPO_4*2H_2O$	1.06580	177.99
50mM	NaOAc	1.06268	82.03
A2: high salt and low pH (pH after dissolving ~ 3.6)			
50mM	$NaH_2PO_4*H_2O$	1.06346	137.99
50mM	Acetic acid, glacial	1.00063	60.05
1M	Na_2SO_4	1.06649	142.04
B2: high salt and high pH (pH after dissolving ~ 8.4)			
50mM	$Na_2HPO_4*2H_2O$	1.06580	177.99
50mM	NaOAc	1.06268	82.03
1M	Na_2SO_4	1.06649	142.04

Use the four buffer stock solutions to prepare a set of 11 buffers at a fixed pH value from 0 -1 M Na_2SO_4 concentration. Create pH stock buffers at low salt concentrations (A1 and B1 buffers) and high salt concentrations (A2 and B2 buffers). Target four pH values.

To achieve the desired salt concentration, dilute the created pH stock buffers while maintaining a fixed pH.

This will create a set of 44 individual buffers of four pH values and eleven different Na_2SO_4 concentrated (0 M Na_2SO_4 included).

Target pH 4.5 and 500 mM Na_2SO_4

Prepare a pH 4.5 solution with no salt by titrating stock A1 with stock B1. Prepare a pH 4.5 solution with 1M Na_2SO_4 by titrating stock A2 with stock B2. Combine an equal amount of 0 M and 1 M Na_2SO_4 at pH 4.5 for a 500 mM Na_2SO_4.

Target pH 7.5 and 800 mM Na_2SO_4

Prepare a pH 7.5 solution with no salt by titrating stock B1 with stock A1. Prepare a pH 7.5 solution with 1M Na_2SO_4 by titrating stock B2 with stock A2. Combine two part of 0 M with eight part of 1 M Na_2SO_4 at pH 7.5 for a 800 mM Na_2SO_4 at pH 7.5.

Note Check and confirm the pH of the solution.

Measurement of SBC

Liquid Handling Systems

1. Calculate the amount of resin and the corresponding slurry volume needed per well. 5 to 10 µL resin per well is recommended. Example: for 10 µL resin/well, 50 µL of a 20% (v/v) slurry per well is needed. Total working volume per well should not exceed 200 µL.
2. Calculate the difference between total working volume and slurry volume per well. Pre-fill each filter plate well with calculated volume (e.g. 150 µL with equilibration buffer according to experiment plan).
3. Add resin slurry into filter plate. Properly resuspend slurry, transfer into a shallow container (e.g. multi-channel pipette reservoir or equivalent), and pipette via multichannel pipette.

   Note Pre-wet pipette tips with slurry before pipetting. Ensure the resin is completely resuspended during the pipetting process.
4. For automated liquid handling stations: Prepare deep well plate with loading buffers according to experiment plan and prepare reservoir, or deep well plate, for wash and elution buffers.
5. Evacuate supernatant via vacuum (e.g. 350 mbar for 1 minute) or centrifugation (e.g. 1200 rcf for 3 minutes); adjust parameter if residual liquid remains in filter plate.
6. Add 200 µL of equilibration buffer to the each well of the filter plate.
7. Shake for 2 minutes.
8. Remove supernatant via vacuum or centrifugation.
9. Discard supernatant.
10. Repeat steps 6 to 9 (3 equilibration steps in total).
11. Load the protein by adding 200 µL of the prepared protein solution to each well according to the experiment plan. Cover the filter plate with a lid, or an adhesive foil, to reduce evaporation during loading phase.
12. Incubate for 2 hours while shaking. The incubation time can be adjusted, but ensure the resin is visibly swirling the well for a minimum of 2 hours (recommended) for SBC.
13. Collect flow-through fraction via vacuum or centrifugation into an empty UV compatible 96-well plate.
14. Measure UV extinction of the new 96-well plate.

    Note Ensure the absorption value is in range of the calibration curve. If not, dilute accordingly (e.g. 1:4 dilution, 50 µL samples + 150 µL buffer). Refer to Appendix for an example.

    IMPORTANT

    Always work with the same liquid level per well as performed with calibration curve.
15. Add 200 µL of the desired equilibration buffer, or a wash buffer, to each well in the filter plate.
16. Shake for 2 minutes.
17. Transfer supernatant into empty UV compatible 96-well plate.
18. Measure the UV extinction of the wash plate at 280 nm absorbance.
19. The UV absorbance from step 18 should be the same absorbance as the the equilibration buffer. If not, repeat steps 15 to 18 for additional washing steps.
20. Add 200 µL of elution buffer to each well of the filter plate.
21. Shake for 5 to 10 minutes.
22. Transfer supernatant into empty UV compatible 96-well plate.
23. Measure the UV extinction of the elution plate. Ensure the absorption value is in range of the calibration curve. If not, dilute accordingly (e.g. 1:4 dilution, 50 µL samples + 150 µL buffer).

Manual Handling

1. Calculate the amount of resin and the corresponding slurry volume per tube needed. 5 to 10 µL resin per tube is recommended. Example: for 10 µL resin/well, 50 µL of a 20% (v/v) slurry per tube is needed. Total working volume per tube should not exceed 200 µL.
2. Calculate the difference between total working volume and slurry volume per tube. Pre-fill each filter unit with calculated volume (e.g. 150 µL with equilibration buffer according to experiment plan).
3. Add resin slurry into filter unit. Properly resuspend slurry, transfer into a shallow container (e.g. multi-channel pipette reservoir or equivalent), and pipette via multichannel pipette.

   Note Pre-wet pipette tips with slurry before pipetting. Ensure the resin is completely resuspended during the pipetting process.
4. Remove supernatant via vacuum (e.g. 350 mbar for 1 minute) or centrifugation (e.g. 1200 rcf for 3 minutes); adjust parameter if residual liquid remains in filter plate.
5. Add 200 µL of equilibration buffer to the each tube.
6. Shake for 2 minutes.
7. Remove supernatant via vacuum or centrifugation.
8. Discard supernatant.
9. Repeat steps 5 to 8 (3 equilibration steps in total).
10. Transfer filter unit into a new tube.
11. Load the protein by adding 200 µL of the prepared protein solution to each filter unit according to the experiment plan. Close the lid of the tubes to reduce evaporation during loading phase.
12. Incubate for 2 hours while shaking. The incubation time can be adjusted, but ensure the resin is visibly swirling the well for a minimum of 2 hours (recommended) for SBC.
13. Collect flow-through fraction via centrifugation in the tube.
14. Measure UV extinction of the flow through.

    Note Ensure the absorption value is in range of the calibration curve. If not, dilute accordingly (e.g. 1:4 dilution, 50 µL samples + 150 µL buffer).
15. Transfer filter unit into new tube.
16. Add 200 µL of the desired equilibration buffer, or a wash buffer, to each tube.
17. Shake for 2 minutes.
18. Transfer supernatant to a tube.
19. Measure the UV extinction of the wash fractions at 280 nm absorbance.
20. The UV absorbance from step 19 should be the same absorbance as the the equilibration buffer. If not, repeat steps 15 to 19 for additional washing steps.
21. Transfer filter unit into new tube.
22. Add 200 µL of elution buffer to each tube.
23. Shake for 5 to 10 minutes.
24. Transfer supernatant to a tube..
25. Measure the UV extinction of the elution plate. Ensure the absorption value is in range of the calibration curve. If not, dilute accordingly (e.g. 1:4 dilution, 50 µL samples + 150 µL buffer).

Equation 1: Calculation of static binding capacity (SBC) based on flow-through fraction

$$SBC_{FT}\left(\frac{mg}{mL\ resin}\right) = \frac{(c_0 - c_{end_{FT}}) \cdot V_{liquid}}{V_{resin}}$$

Parameter	Description
SBC_{FT}	Static binding capacity based on flow-through fraction
c_0	Protein load concentration (mg/mL)
$c_{end\ FT}$	Protein concentration after 2 h incubation (mg/mL)
V_{liquid}	Load volume per well (mL)
V_{resin}	Resin volume per well (mL resin)

Identification of Optimal Separation Conditions

To identify optimal separation conditions and facilitate further analytics, it is recommended to load 80% of the determined static binding capacity (SBC) value for each specific condition. In cases where the SBC value is not determined, a loading of 30 mg protein/mL resin can be selected, although the results may be suboptimal.

To maintain consistency, the load volume should remain constant at 200 µl. If there is a need to reduce the amount of loaded protein, the load can be diluted with the appropriate binding buffer.

Liquid Handling Systems

1. Calculate the amount of resin and the corresponding slurry volume per well needed. 5 to 10 µL resin per well is recommended. Example: for 10 µL resin/well, 50 µL of a 20% (v/v) slurry per well is needed. Total working volume per well should not exceed 200 µL.
2. Calculate the difference between total working volume and slurry volume per well. Pre-fill each filter plate well with calculated volume (e.g. 150 µL) with equilibration buffer according to experiment plan.
3. Add resin slurry into filter plate. Properly resuspend slurry, transfer into a shallow container (e.g. multi-channel pipette reservoir or equivalent), and pipette via multichannel pipette.

   Note Pre-wet pipette tips with slurry before pipetting. Ensure the resin is completely resuspended during the pipetting process.
4. For automated liquid handling stations: Prepare deep well plate with loading buffers according to experiment plan and prepare reservoir, or deep well plate, for wash and elution buffers.
5. Remove supernatant via vacuum (e.g. 350 mbar for 1 minute) or centrifugation (e.g. 1200 rcf for 3 minutes); adjust parameter if residual liquid remains in filter plate.
6. Add 200 µL of equilibration buffer to the each well of the filter plate.
7. Shake for 2 minutes.
8. Remove supernatant via vacuum or centrifugation.
9. Discard supernatant.
10. Repeat steps 6 to 9 (3 equilibration steps in total).
11. Load the protein by adding 200 µL of the prepared protein solution to each well according to the experiment plan. Cover the filter plate with a lid, or an adhesive foil, to reduce evaporation during loading phase.
12. Incubate for 2 hours while shaking. The incubation time can be adjusted, but ensure the resin is visibly swirling the well for a minimum of 2 hours (recommended) for SBC.
13. Collect flow-through fraction via vacuum or centrifugation into an empty UV compatible 96-well plate.
14. Perform analytics according to impurity profile.
15. Add 200 µL of the desired equilibration buffer, or a wash buffer, to each well in the filter plate.
16. Shake for 2 minutes.
17. Transfer supernatant to an empty UV compatible 96-well plate.
18. Perform analytics according to impurity profile.
19. The UV absorbance from step 18 should be the same absorbance as the the equilibration buffer. If not, repeat steps 15 to 18 for additional washing steps.
20. Add 200 µL of elution buffer to each well of the filter plate.
21. Shake for 5 to 10 minutes.
22. Transfer supernatant to an empty UV compatible 96-well plate.
23. Perform analytics according to impurity profile.

Manual Handling

1. Calculate the amount of resin and the corresponding slurry volume per tube needed. 5 to 10 µL resin per tube is recommended. Example: for 10 µL resin/well, 50 µL of a 20% (v/v) slurry per tube is needed. Total working volume per tube should not exceed 200 µL.
2. Calculate the difference between total working volume and slurry volume per tube. Pre-fill each filter unit with calculated volume (e.g. 150 µL) with equilibration buffer according to experiment plan.
3. Add resin slurry into filter unit. Properly resuspend slurry, transfer into a shallow container (e.g. multi-channel pipette reservoir or equivalent), and pipette via multichannel pipette.

   Note Pre-wet pipette tips with slurry before pipetting. Ensure the resin is completely resuspended during the pipetting process.
4. Remove supernatant via vacuum (e.g. 350 mbar for 1 minute) or centrifugation (e.g. 1200 rcf for 3 minutes); adjust parameter if residual liquid remains in filter plate.
5. Add 200 µL of equilibration buffer to the each tube.
6. Shake for 2 minutes.
7. Remove supernatant via vacuum or centrifugation.
8. Discard supernatant.
9. Repeat steps 5 to 8 (3 equilibration steps in total).
10. Transfer filter unit into new tube.
11. Load the protein by adding 200 µL of the prepared protein solution to each filter unit according to the experiment plan. Close the lid of the tubes to reduce evaporation during loading phase.
12. Incubate for 2 hours while shaking. The incubation time can be adjusted, but ensure the resin is visibly swirling the well for a minimum of 2 hours (recommended) for SBC.
13. Collect flow-through fraction via centrifugation in the tube.
14. Perform analytics according to impurity profile.
15. Transfer filter unit into a new tube.
16. Add 200 µL of the desired equilibration buffer, or a wash buffer, to each tube.
17. Shake for 2 minutes.
18. Transfer supernatant to a tube.
19. Perform analytics according to impurity profile.
20. If needed, repeat steps 15 to 19 for additional washing steps.
21. Transfer filter unit into new tube.
22. Add 200 µL of elution buffer to each tube.
23. Shake for 5 to 10 minutes.
24. Transfer supernatant to a tube.
25. Perform analytics according to impurity profile.

Elution Screening

If an elution screening is planned, the target protein can be bound and washed with fixed conditions (e.g. pH 5 and no salt). The binding and washing procedure should follow the steps described earlier. During the elution stage, buffers will be added based on the experiment plan, and the relevant parameters will be analyzed.

For further information on the determination of optimal elution conditions from screening, navigate to page 12.

Example for SBC Measurement of a Hydrophobic Antibody

Table 2: Exemplary table for an experiment plan

	1	2	3	4	5	6	7	8	9	10	11	12
A B	pH 4.5 - 0M	pH 4.5 - 0.1M	pH 4.5 - 0.2M	pH 4.5 - 0.3M	pH 4.5 - 0.4M	pH 4.5 - 0.5M	pH 4.5 - 0.6M	pH 4.5 - 0.7M	pH 4.5 - 0.8M	pH 4.5 - 0.9M	pH 4.5 - 1.0M	Buffer Blank
C D	pH 5.5 - 0M	pH 5.5 - 0.1M	pH 5.5 - 0.2M	pH 5.5 - 0.3M	pH 5.5 - 0.4M	pH 5.5 - 0.5M	pH 5.5 - 0.6M	pH 5.5 - 0.7M	pH 5.5 - 0.8M	pH 5.5 - 0.9M	pH 5.5 - 1.0M	Buffer Blank
E F	pH 6.5 - 0M	pH 6.5 - 0.1M	pH 6.5 - 0.2M	pH 6.5 - 0.3M	pH 6.5 - 0.4M	pH 6.5 - 0.5M	pH 6.5 - 0.6M	pH 6.5 - 0.7M	pH 6.5 - 0.8M	pH 6.5 - 0.9M	pH 6.5 - 1.0M	Buffer Blank
G H	pH 7.5 - 0M	pH 7.5 - 0.1M	pH 7.5 - 0.2M	pH 7.5 - 0.3M	pH 7.5 - 0.4M	pH 7.5 - 0.5M	pH 7.5 - 0.6M	pH 7.5 - 0.7M	pH 7.5 - 0.8M	pH 7.5 - 0.9M	pH 7.5 - 1.0M	Buffer Blank

Table 3: Exemplary results for the SBC measurement of Eshmuno® CMX resin and a hydrophobic mAb

Results as measured by plate, average of duplicates*:

	1	2	3	4	5	6	7	8	
A B	21	19	16	15	19	22	22	23	pH 4.5
C D	48	27	16	10	12	11	17	23	pH 5.5
E F	25	2	4	3	2	2	4	7	pH 6.5
G H	0	0	0	0	1	2	1	5	pH 7.5
mM Na_2SO_4	0	100	200	300	400	500	600	700	

*Due to protein stability issues only 0 to 0.7M Na_2SO_4 were measured.

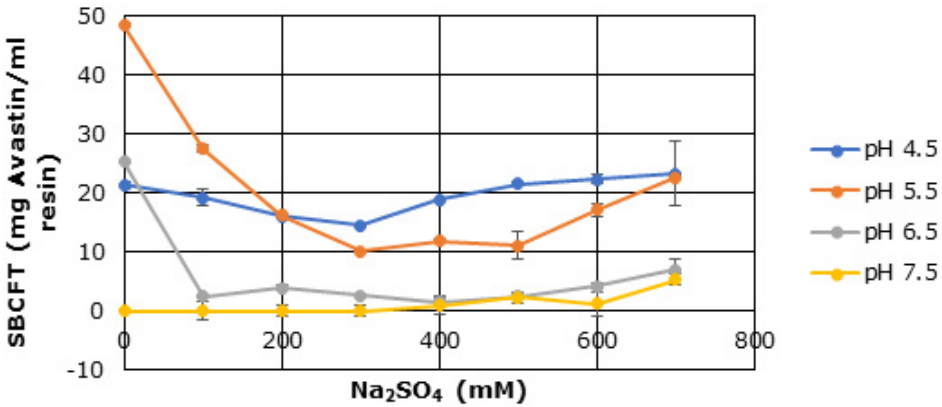

*Figure 2: Diagram with Error Bars of Duplicates (0 - 0.7M Na2SO4)**

*Due to protein stability issues only 0 to 0.7M Na_2SO_4 were measured.

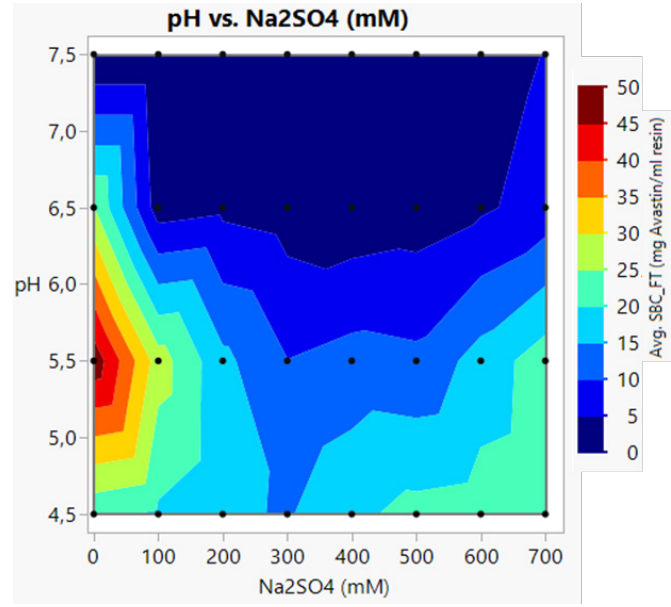

*Figure 3: Heat plot**

*Due to protein stability issues only 0 to 0.7M Na_2SO_4 were measured.

In the heat plot, red areas indicate the highest binding capacity, while blue areas represent conditions where no protein binds to the column, offering opportunities for elution.

These heat plots can be generated for both the target protein and measured impurities. To select the optimal process, identify areas of high binding for the target protein. In some cases, there may also be opportunities for certain impurities to flow through.

Once the protein is bound to the resin under the selected conditions (e.g., pH 5.5 with no added salt), impurities can be washed off. The wash conditions should be chosen based on the comparison of heat plots between the target protein and impurities. Repeat the washing procedure for each impurity.

After washing off the impurities, select an elution condition that maximizes recovery. In this case, an elution condition at pH 7.5 with a moderate increase in salt concentration to 300 mM Na_2SO_4 is used.

For further examples, refer to the publication "Purification of hydrophobic complex antibody formats using a moderately hydrophobic mixed-mode cation exchange resin" by Koehnlein et al., 2022.

Appendix

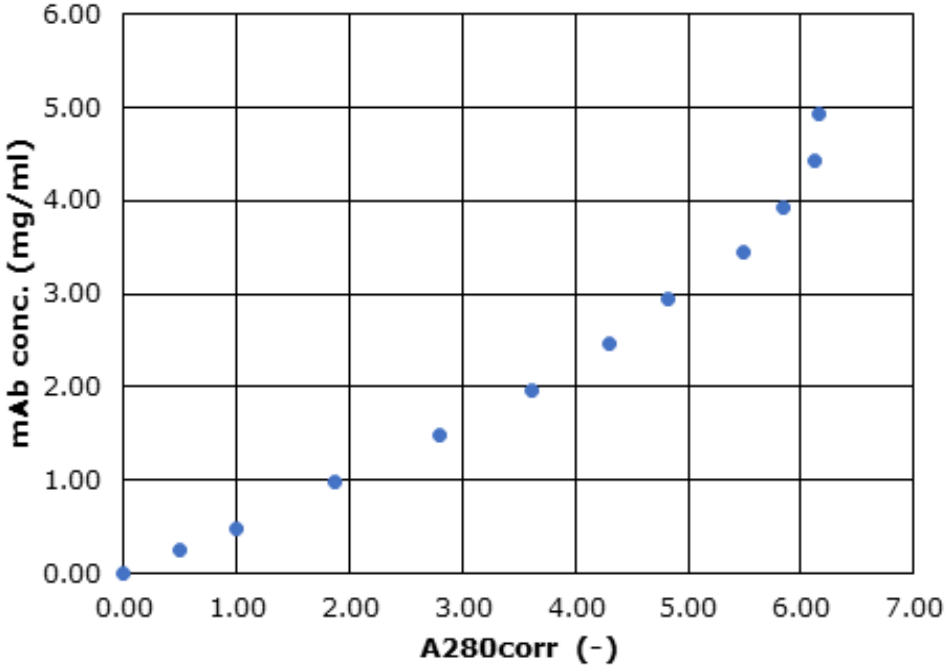

Figure 4: Exemplary calibration curve of a hydrophobic mAb.

Tested concentration of antibody versus the height corrected extinction at 280 nm. With an increase of concentration loss of linearity can be observed.

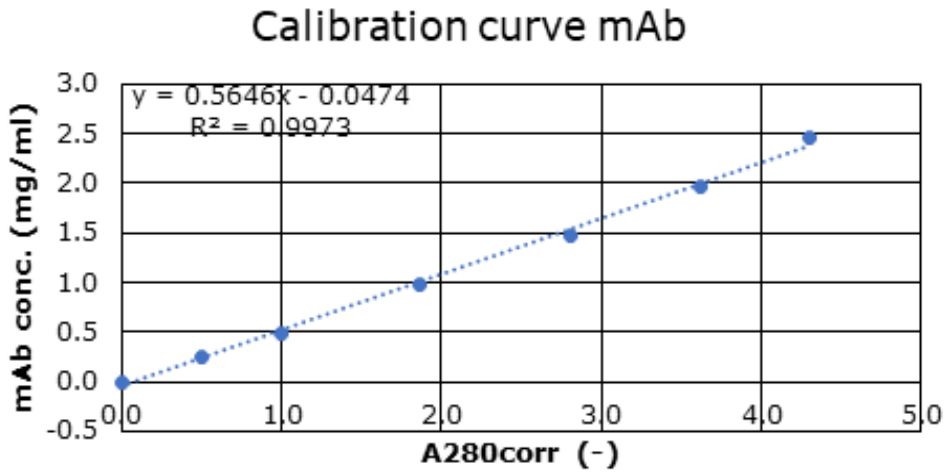

Figure 5: Selected linear area and calibration curve for the antibody.

Good linearity (R^2 = 0.9973) is shown in the select range of concentrations.

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