Using Design of Experiments (DOE) with Nuvia cPrime to Elucidate Capture Conditions of a Recombinant Protein

Introduction

Here we describe the use of a Design of Experiment (DOE) approach using spin columns in the initial screening of chromatographic conditions for purification of a recombinant protein expressed in *E. coli* on Nuvia cPrime Hydrophobic Cation Exchange Resin. The ligand on this media has three major functionalities: a weak carboxylic acid end group, an aromatic hydrophobic ring, and an amide bond serving as a potential hydrogen bond donor/acceptor. Under specific purification conditions, one or more such interaction modes may be involved in the binding or repulsion between the target protein and the chromatographic media. Therefore, the behavior of a protein during purification by mixed-mode chromatography often cannot be predicted based on its isoelectric point or amino acid sequence.

JMP Software was used in these DOE studies to identify optimal binding and elution conditions on Nuvia cPrime for the target protein contained in the *E. coli* control lysate. The effects of four parameters were evaluated using a response surface matrix custom design provided by JMP Software, including three center points and a total of 18 experiments:

- pH binding conditions
- conductivity binding conditions
- pH elution conditions
- conductivity elution conditions

Our studies show that, with a limited amount of protein sample and chromatography media, a simple DOE setup can be used to determine the effects of buffer pH and conductivity on selectivity, recovery, and robustness of purification on Nuvia cPrime. Working conditions established by such scale-down studies can be used for the purification of a target protein on a preparative scale.

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Using Design of Experiments (DOE) with Nuvia cPrime to Elucidate Capture Conditions of a Recombinant Protein

DOE with JMP Software

1. Open JMP Software (v10.0.2).

2. Open the DOE Custom Design dialog and add responses (binding, yield, purity) and factors as indicated in the image. Adjust limits and values as needed. The values of the factors are as follows:
   - pH range protein binding: 4.75–6.75
   - NaCl concentration protein binding: 0–400 mM
   - pH range protein elution: pH 6–8.75
   - NaCl concentration protein elution: 0–1,000 mM

3. Continue and click RSM (response surface matrix). In contrast to screening experiments, researchers use RSM when they already know which factors are important.

4. Adjust the number of center points to three, limit the number of runs to 18, and continue by clicking Make Design.

5. JMP Software now provides a table for the 18 spin column experiments.

6. After the spin column experiments and SDS-PAGE analysis of the individual fractions, add the corresponding data to the JMP design table and begin data evaluation with the JMP Software. A good start is to design contour plots to display the influence of pH and conductivity on target binding, yield, and purity (see example). For more information, refer to the JMP Software manual.
Sample and Spin Column Preparation

Materials

<table>
<thead>
<tr>
<th>Item</th>
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<td>7326207</td>
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<td>Microtube Racks</td>
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<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>2 ml microcentrifuge tubes, capless</td>
<td></td>
</tr>
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</table>

Additional Materials Required

- Vortex mixer
- Centrifuge
- Pipets and pipet tips
- Laboratory gloves
- Deionized water

1. Add 15 ml of deionized water to the E. coli control lysate\(^4\) and shake well until all lyophilized material is dissolved. Set the sample aside at room temperature and do not put on ice. The total protein concentration is about 2 mg/ml.

2. Snap off the tips of the 18 spin columns and seal the bottoms again.

3. Place the columns in 2 ml microcentrifuge tubes.

4. Remove the top caps of the microcentrifuge tubes.

5. Shake the Nuvia cPrime bottle to obtain a homogenous suspension.

6. Carefully apply 200 µl of the resin suspension to the center of all spin columns.

7. Remove the bottom seal from the columns and then put the columns back into the microcentrifuge tubes.

8. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.

9. Discard the buffer and seal the bottoms of the columns.

10. Place the columns in a 2 ml microcentrifuge tube.

11. Apply 500 µl of appropriate binding buffer to the columns and apply the top seal (see Appendix 1).

12. Carefully vortex the columns for 1 min.

13. Remove the top and bottom seals and place the columns in a 2 ml microcentrifuge tube.

14. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.

15. Repeat buffer exchange twice.

16. Seal the bottoms and tops of the spin columns.

17. Following centrifugation, the resin is now in the binding buffer.

18. Prepare 2% SDS solution in water.

Spin Column Experiment

Materials

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<tr>
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<td>Microtube racks</td>
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<td>Binding buffers (see Appendix 2)</td>
<td></td>
</tr>
<tr>
<td>Elution buffers (see Appendix 2)</td>
<td></td>
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<td>2 ml microcentrifuge tubes</td>
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</table>

Additional Materials Required

- End-over-end shaker
- Vortex mixer
- Centrifuge
- Pipets and pipet tips
- Laboratory gloves
- Deionized water

Overview of the spin column experiment

1. Add 500 µl of sample solution and incubate for 10 min and centrifuge.

2. Add 167 µl of binding buffer and vortex and centrifuge. Repeat twice.

3. Add 500 µl of binding buffer and vortex and centrifuge. Discard solution.

4. Add 167 µl of elution buffer and vortex and centrifuge. Repeat twice.

5. Add 250 µl of elution buffer and vortex and centrifuge.

6. Add 167 µl of 2% SDS solution and vortex and centrifuge. Repeat twice.

1. Pipet 300 µl of each sample buffer into a 1.5 ml microcentrifuge tube and add 300 µl of E. coli sample dissolved in water. Prepare the samples just before beginning the spin column experiments. Close the tubes and mix well.

2. Open the top cap of the spin columns and add 500 µl of the sample solution to the center of all spin columns.

3. Seal all columns and mix well for 10 min using an end-over-end rotator.

4. Place columns in 2 ml microcentrifuge tubes and remove the top and bottom seals.

5. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.

6. Keep flow through (U) after centrifugation.

7. Seal the bottom of all columns and place columns in 2 ml microcentrifuge tubes.

8. Add 167 µl of binding buffer to wash the resin and apply the top seal.

\(^2\) Note: 1.2 ml; bed height: 2.8 cm; overall length: 3.3 cm.

\(^3\) Note: 50% v/v slurry in 20% ethanol.

\(^4\) Note: Quantity per bottle: 30 mg lyophilized total protein, including 10 mg of target protein. pI target protein: 5.47 (calculated) and 5.9 (isoelectric focusing); MW target protein: 51 kD. Grand average of the hydropathicity (GRAVY) of the target protein: –0.275.
9. Vortex three times for 5 sec each and remove the top and bottom seals.
10. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.
11. Keep the wash solution after centrifugation.
12. Seal the bottom of all columns and place them in 2 ml microcentrifuge tubes containing the previous wash solution(s).
13. Repeat steps 9–13 twice. The total volume of wash solution (W) is 500 µl.
14. Seal the bottom of all columns and place columns in unused 2 ml microcentrifuge tubes.
15. Add 500 µl of binding buffer to wash the resin and apply the top seal.
16. Vortex three times for 5 sec and remove the top and bottom seals.
17. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.
18. Discard this wash solution after centrifugation.
19. Seal the bottom of all columns and place them in unused 2 ml microcentrifuge tubes.
20. Add 167 µl of elution buffer to elute the bound proteins and apply the top seal.
21. Vortex three times for 5 sec and remove the top and bottom seals.
22. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.
23. Keep the elution solution after centrifugation.
24. Seal the bottom of all columns and place them in 2 ml microcentrifuge tubes containing the previous elution solution(s).
25. Repeat steps 19–23 twice. The total volume of elution solution (E1) is 500 µl.
26. Seal the bottom of all columns and place them in unused 2 ml microcentrifuge tubes.
27. Add 250 µl of elution buffer to elute the bound proteins and apply the top seal.
28. Vortex three times for 5 sec and remove the top and bottom seals.
29. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.
30. Keep the elution solution after centrifugation.
31. The total volume of elution solution (E2) is 250 µl.
32. Seal the bottom of all columns and place them in unused 2 ml microcentrifuge tubes.
33. Add 167 µl of 2% SDS solution to elute the bound proteins and apply the top seal.
34. Vortex three times for 5 sec and remove the top and bottom seals.
35. Centrifuge for 1 min in a microcentrifuge at 1,000 x g.
36. Keep the elution solution after centrifugation.
37. Seal the bottom of all columns and place them in 2 ml microcentrifuge tubes containing the previous elution solution(s).
38. Repeat steps 32–36 twice. The total volume of elution solution (E3) is 500 µl.

### Electrophoresis

#### Materials

<table>
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<td>1708270</td>
</tr>
<tr>
<td>Stain Free Sample Tray</td>
<td>1708274</td>
</tr>
<tr>
<td>2x and 4x Laemmli Sample Buffer</td>
<td>1610747</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>1610710</td>
</tr>
<tr>
<td>4–20% Criterion TGX Stain-Free Protein Gel, 18 well, 30 µl</td>
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<tr>
<td>Precision Plus Protein Unstained Standards</td>
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<td>Criterion Cell (3x)</td>
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<tr>
<td>10x TGS Buffer</td>
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</tr>
<tr>
<td>PowerPac Universal Power Supply</td>
<td>1645070</td>
</tr>
<tr>
<td>1.5 ml microcentrifuge tubes</td>
<td>–</td>
</tr>
</tbody>
</table>

#### Additional Materials Required

- Heat block
- Centrifuge
- Pipets and pipet tips
- Deionized water
- Laboratory gloves

1. Prepare 2x and 4x Laemmli buffer.⁵
2. Dissolve the samples from the spin column experiment with 2x or 4x Laemmli buffer in 1.5 ml micro test tubes according to Appendix 3.
3. Vortex for 5 sec and heat the sample for 10 min at 70°C.
4. Centrifuge for 30 sec at 10,000 x g.
5. Remove the comb and tape from the gel.
6. Wash the wells with 1x TGS buffer.⁷
7. Place the gel in a Criterion Cell.
8. Fill with 1x TGS buffer to the fill line.⁸

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⁵ **Note:** If you need to order one, we recommend the Bio-Rad Digital Dry Bath (#1660571).

⁶ **Recipe:** 2x Laemmli Buffer: Mix 300 µl of 2-mercaptoethanol, 2,700 µl of 4x Laemmli Buffer, and 3,000 µl of ultrapure water. 4x Laemmli Buffer: Mix 100 µl of 2-mercaptoethanol with 900 µl 4x Laemmli Buffer.

⁷ **Recipe:** Tris/glycine/SDS electrophoresis buffer: To prepare 1 L of buffer, mix 100 ml of 10x TGS buffer and 900 ml of ultrapure water.

⁸ **Tip:** Ensure that there is sufficient running buffer in the electrophoresis cell (as indicated by the fill lines, depending on the number of gels), especially when running at high voltages in order to achieve rapid run times.
9. Load samples (20 µl) in the following order:
   - E. coli sample in binding buffer S
   - Unbound sample solution U
   - Wash solution W
   - Elution solution E1
   - Elution solution E2
   - Elution solution E3

10. Each Criterion Gel (18 well) can run samples for three spin column experiments.

11. Run at 50 V for 15 min and 250 V until the dye front reaches the indicator line at the bottom of the gel.

12. Switch off the power supply and disconnect the electrical leads.

13. Remove the gel from the Criterion Cell.

14. Open the gel cassette using the aluminum lever.

15. Pour a few milliliters of water on the Blot/UV/Stain-Free Tray and place the gel on the tray. Avoid trapping air bubbles under the gel.

16. Choose the following settings to activate and image the stain-free gel:
   - Application: Protein Gels, Stain-Free Gel
   - Gel Activation: 2.5 min
   - Manually set the Image Exposure time to 0.5–1.0 sec.
      - Note: the exposure time is dependent on the gel and imager and will vary
   - Make sure that gel activation time and image exposure time are identical for all gels

17. Click Run Protocol to begin the activation and exposure.

18. Start data analysis using Image Lab Software (see Appendix 4).

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### Appendix 1: Spin Column Sample Buffers

<table>
<thead>
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<th>NaCl, mM</th>
<th>Buffer</th>
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</thead>
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<td>Citrate-Na</td>
</tr>
<tr>
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<td>800</td>
<td>Citrate-Na</td>
</tr>
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<tr>
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### Appendix 2: Spin Column Binding and Elution Buffers (50 mM)

<table>
<thead>
<tr>
<th>Spin column</th>
<th>Binding pH</th>
<th>Binding NaCl, mM</th>
<th>Buffer</th>
<th>Elution pH</th>
<th>Elution NaCl, mM</th>
<th>Buffer</th>
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Note: Typically 30 min.

Note: No longer supplied with boxes of gels; order separately (#4560000).

Tip: Keep gloves wet when handling the gels.

Note: Spin column sample buffers are 2x concentrated binding buffers.
Appendix 3: Sample Preparation for Protein Electrophoresis

The following samples are available for analysis after the spin column experiments.

Dilute the samples as indicated below and heat for 10 min at 70°C.

- 100 µl E. coli sample (~1 mg/ml) in different sample buffers (S: 1–18)
- 500 µl of unbound material in binding buffers (U: 1–18)
- 500 µl of wash solution in binding buffers (W: 1–18)
- 500 µl of eluted material in elution buffers (E1: 1–18)
- 250 µl of eluted material in elution buffers (E2: 1–18)
- 500 µl of eluted material in 2% SDS (E3: 1–18)

<table>
<thead>
<tr>
<th>Sample, µl</th>
<th>S</th>
<th>U</th>
<th>W</th>
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<th>E2</th>
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Appendix 4: Gel Analysis with Image Lab Software

1. Use the cropping tool to crop all images to the same size.
2. Use the lane tool to define the lanes of the gel.
3. Detect target bands manually in each lane. Use the lane profile tool to make sure the target band is correctly detected.
4. Save this dataset indicating the spin columns as well as that it is the target band. An example name would be Spin columns 1–3 SF gel data target analysis.
5. Manually adjust all 18 target bands so that they become a “total lane” band.
6. Save this dataset indicating the spin columns as well as that it is the total lane data. An example name would be Spin columns 1–3 SF gel data total lane analysis.
7. Repeat the procedure for the remaining gels and save the data accordingly (see example images in Appendix 5).
8. Prior to data export, adjust display data options as indicated.
9. Compile the intensity values acquired for both the target protein and total lane intensity into a master Excel (or similar software) spreadsheet. Target band intensities and total lane intensities should be used where indicated. Each experiment should be normalized to its own input or supernatant lane. The following calculations can then be made using these normalized intensity values:

Target unbound = sum of percentage target unbound and percentage target wash
\[
\frac{[M_{target}]}{S_{target}} + \frac{[W_{target}]}{S_{target}}
\]

Target bound = 1 – Target unbound

Target elution = sum of percentage target in elution 1, elution 2, and elution 3
\[
\frac{[E1_{target}]}{S_{target}} + \frac{[E2_{target}]}{S_{target}} + \frac{[E3_{target}]}{S_{target}}
\]

Target yield = sum of percentage target in elution 1 and elution 2
\[
\frac{[E1_{target}]}{S_{target}} + \frac{[E2_{target}]}{S_{target}}
\]

Target purity = Target yield divided by total lane intensity for elution 1 and elution 2
\[
\frac{[E1_{target}]}{S_{total lane}} + \frac{[E2_{target}]}{S_{total lane}}
\]

* Wash intensity for both target and total lane must be divided by 4 to account for the increased loading amount.

10. Check that data analysis is within the acceptable range by looking at center points as well as that \( U+W+E1+E2+E3 = S \) (± 5%). This should be true for both target intensities and total lane intensities.

11. If data are within the acceptable range, then input calculated target bound, target yield, and target purity into JMP Software to analyze the data. The predictor profiler and contour plots should be the first visualization of the data.

### Appendix 5: Stain-Free Imaging Technology Example Images

Spin column samples are run in sequential order:
- Sample in various binding buffers (S)
- Unbound (U)
- Wash (W)
- Elution 1 (E1)
- Elution 2 (E2)
- Elution 3 (E3)

Gel representing experimental data from samples 10–12.

### Appendix 6: Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Binding pH</th>
<th>Binding NaCl, mM</th>
<th>Binding Target, %</th>
<th>Elution pH</th>
<th>Elution NaCl, mM</th>
<th>Yield Target, %</th>
<th>Purity Target, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.75</td>
<td>200</td>
<td>98</td>
<td>7.38</td>
<td>500</td>
<td>78</td>
<td>67</td>
</tr>
<tr>
<td>11</td>
<td>5.75</td>
<td>200</td>
<td>99</td>
<td>8.75</td>
<td>1,000</td>
<td>89</td>
<td>63</td>
</tr>
<tr>
<td>12</td>
<td>5.75</td>
<td>400</td>
<td>89</td>
<td>6.00</td>
<td>1,000</td>
<td>33</td>
<td>60</td>
</tr>
</tbody>
</table>
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