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

Protocol

Protein Purification

Bulletin 7128

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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.[1] 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.

[1] Bio-Rad Bulletin 6418. Purification of Recombinant Proteins on Nuvia cPrime Hydrophobic Cation Exchange Media: A Simple Approach to Method Development

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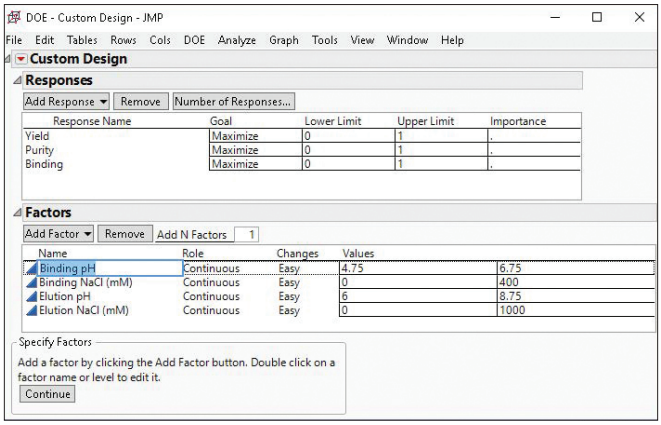
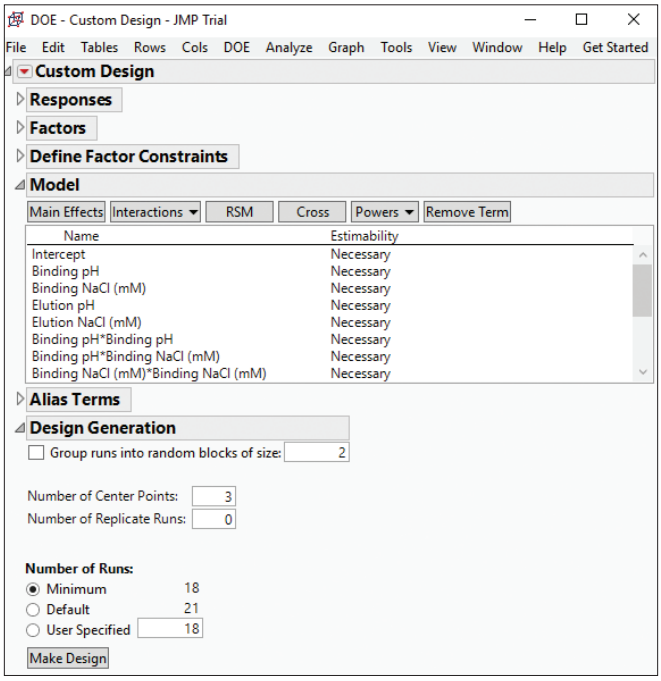
DOE with JMP Software

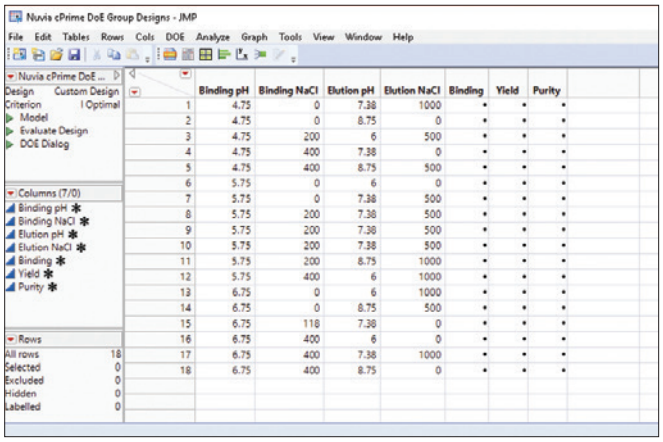
1. Open JMP Software (v10.0.2).
2. Open the DOE Custom Design dialog and add responses (binding, yield, purity) and factors as indicated in the image. Adjust limits and values as needed. The values of the factors are as follows:
   - pH range protein binding: 4.75–6.75
   - NaCl concentration protein binding: 0–400 mM
   - pH range protein elution: pH 6–8.75
   - NaCl concentration protein elution: 0–1,000 mM



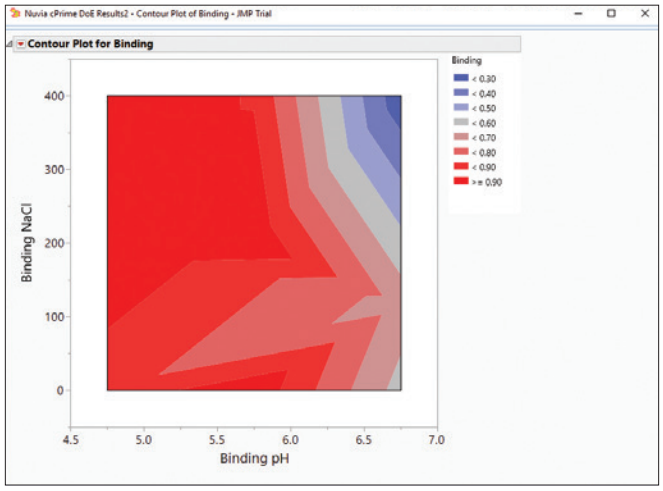
3. Continue and click **RSM** (response surface matrix). In contrast to screening experiments, researchers use RSM when they already know which factors are important.



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

Item	Catalog #
E. coli Control Lysate	6200233
Mini Bio-Spin Chromatography Columns[2]	7326207
Nuvia cPrime Hydrophobic Cation Exchange Resin[3]	1563401
Microtube Racks	1660481
Binding buffers (see Appendix 2)	–
1.5 ml microcentrifuge tubes	–
2 ml microcentrifuge tubes, capless	–

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

Item	Catalog #
Sample buffers (see Appendix 1)	–
Microtube racks	1660481
Binding buffers (see Appendix 2)	–
Elution buffers (see Appendix 2)	–
10% SDS solution, 250 ml	1610416
1.5 ml microcentrifuge tubes	–
2 ml microcentrifuge tubes	–

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	Incubate for 10 min and centrifuge.
2. Add 167 µl of binding buffer	Vortex and centrifuge. **Repeat twice**.
3. Add 500 µl of binding buffer	Vortex and centrifuge. **Discard solution**.
4. Add 167 µl of elution buffer	Vortex and centrifuge. **Repeat twice**.
5. Add 250 µl of elution buffer	Vortex and centrifuge.
6. Add 167 µl of 2% SDS solution	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 flowthrough (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 the 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

Item	Catalog #
Gel Doc EZ Imaging System	1708270
Stain Free Sample Tray	1708274
2x and 4x Laemmli Sample Buffer	1610747
2-mercaptoethanol	1610710
4–20% Criterion TGX Stain-Free Protein Gel, 18 well, 30 µl	5678094
Precision Plus Protein Unstained Standards	1610363
Criterion Cell (3x)	1656001
10x TGS Buffer	1610732
PowerPac Universal Power Supply	1645070
1.5 ml microcentrifuge tubes	–

Additional Materials Required

Heat block[5]
Centrifuge
Pipets and pipet tips
Deionized water
Laboratory gloves

1. Prepare 2x and 4x Laemmli buffer.[6]
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]

[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.[9]
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.[10]
15. Pour a few milliliters of water on the Blot/UV/Stain-Free Tray and place the gel on the tray.[11] 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).

Appendix 1: Spin Column Sample Buffers[12] (100 mM)

#	pH	NaCl, mM	Buffer
1	4.75	0	Citrate-Na
2	4.75	0	Citrate-Na
3	4.75	400	Citrate-Na
4	4.75	800	Citrate-Na
5	4.75	800	Citrate-Na
6	5.75	0	MES-Na
7	5.75	0	MES-Na
8	5.75	400	MES-Na
9	5.75	400	MES-Na
10	5.75	400	MES-Na
11	5.75	400	MES-Na
12	5.75	800	MES-Na
13	6.75	0	MOPS-Na
14	6.75	0	MOPS-Na
15	6.75	236	MOPS-Na
16	6.75	800	MOPS-Na
17	6.75	800	MOPS-Na
18	6.75	800	MOPS-Na

Appendix 2: Spin Column Binding and Elution Buffers (50 mM)

Spin column	Binding pH	Binding NaCl, mM	Buffer	Elution pH	Elution NaCl, mM	Buffer
1	4.75	0	Citrate-Na	7.38	1,000	MOPS-Na
2	4.75	0	Citrate-Na	8.75	0	Tricine-Na
3	4.75	200	Citrate-Na	6.00	500	MES-Na
4	4.75	400	Citrate-Na	7.38	0	MOPS-Na
5	4.75	400	Citrate-Na	8.75	500	Tricine-Na
6	5.75	0	MES-Na	6.00	0	MES-Na
7	5.75	0	MES-Na	7.38	500	MOPS-Na
8	5.75	200	MES-Na	7.38	500	MOPS-Na
9	5.75	200	MES-Na	7.38	500	MOPS-Na
10	5.75	200	MES-Na	7.38	500	MOPS-Na
11	5.75	200	MES-Na	8.75	1,000	Tricine-Na
12	5.75	400	MES-Na	6.00	1,000	MES-Na
13	6.75	0	MOPS-Na	6.00	1,000	MES-Na
14	6.75	0	MOPS-Na	8.75	500	Tricine-Na
15	6.75	118	MOPS-Na	7.38	0	MOPS-Na
16	6.75	400	MOPS-Na	6.00	0	MES-Na
17	6.75	400	MOPS-Na	7.38	1,000	MOPS-Na
18	6.75	400	MOPS-Na	8.75	0	Tricine-Na

[9] **Note:** Typically 30 min.
[10] **Note:** No longer supplied with boxes of gels; order separately (#4560000).
[11] **Tip:** Keep gloves wet when handling the gels.
[12] **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.

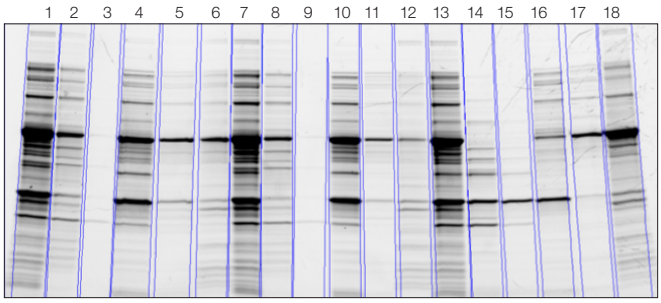
Dilute the samples as indicated below and heat for 10 min at 70°C.

- 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)

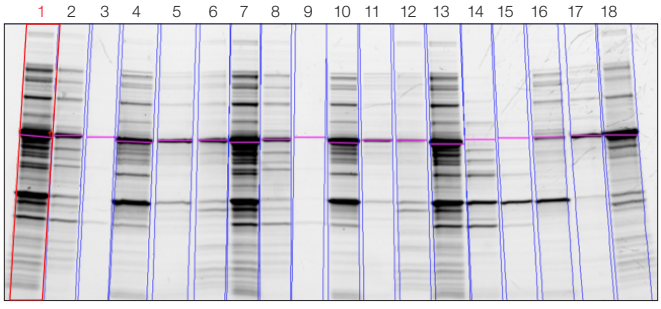
	S	U	W	E1	E2	E3
Sample, µl	50	50	60	50	50	50
2x Laemmli, µl	50	50	–	50	50	50
4x Laemmli, µl	–	–	20	–	–	–

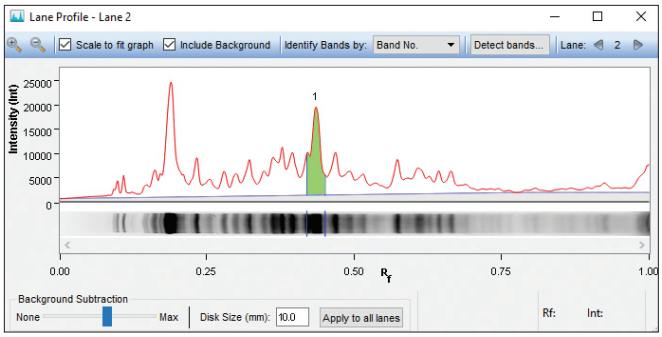
Appendix 4: Gel Analysis with Image Lab Software

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.

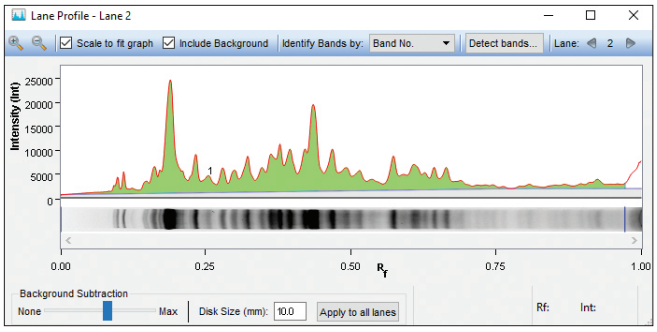


3. Detect target bands manually in each lane. Use the lane profile tool to make sure the target band is correctly detected.

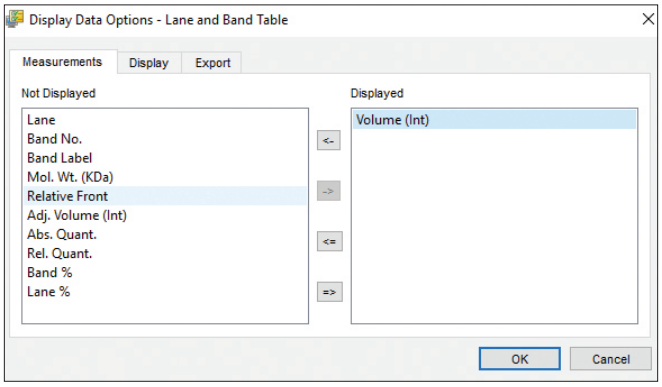




4. Save this dataset indicating the spin columns as well as that it is the target band. An example name would be Spin columns 1–3 SF gel data target analysis.
5. Manually adjust all 18 target bands so that they become a "total lane" band.



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*
   $[(U_{Target}/S_{Target}) + (W_{Target}/S_{Target})]$

   Target bound = 1 – Target unbound

   Target elution = sum of percentage target in elution 1, elution 2, and elution 3
   $[(E1_{Target}/S_{Target}) + (E2_{Target}/S_{Target}) + (E3_{Target}/S_{Target})]$

   Target yield = sum of percentage target in elution 1 and elution 2
   $[(E1_{Target}/S_{Target}) + (E2_{Target}/S_{Target})]$

   Target purity = Target yield divided by total lane intensity for elution 1 and elution 2
   $[(E1_{Target}/S_{Target} + E2_{Target}/S_{Target})/(E1_{Total\ Lane}/S_{Total\ Lane} + E2_{Total\ Lane}/S_{Total\ Lane})]$

   * 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.

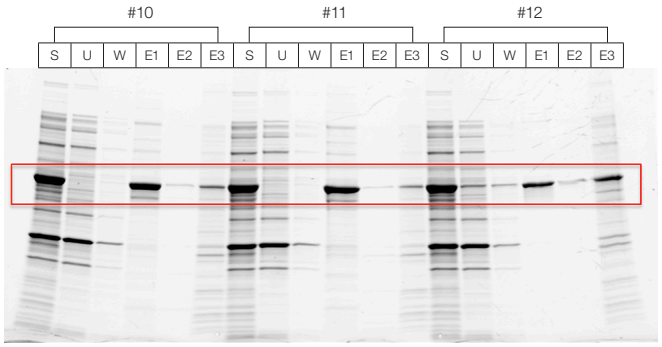
11. If data are within the acceptable range, then input calculated target bound, target yield, and target purity into JMP Software to analyze the data. The predictor profiler and contour plots should be the first visualization of the data.

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.



#	Binding pH	Binding NaCl, mM	Binding Target, %	Elution pH	Elution NaCl, mM	Yield Target, %	Purity Target, %
10	5.75	200	98	7.38	500	78	67
11	5.75	200	99	8.75	1,000	89	63
12	5.75	400	89	6.00	1,000	33	60

Appendix 6: Results

Spin Column	Binding pH	Binding NaCl, mM	Binding Target, %	Elution pH	Elution NaCl, mM	Yield Target, %	Purity Target, %
1	4.75	0	81	7.38	1,000	67	32
2	4.75	0	85	8.75	0	80	37
3	4.75	200	100	6.00	500	15	17
4	4.75	400	100	7.38	0	31	32
5	4.75	400	99	8.75	500	91	45
6	5.75	0	97	6.00	0	2	10
7	5.75	0	98	7.38	500	67	55
8	5.75	200	99	7.38	500	78	58
9	5.75	200	99	7.38	500	60	62
10	5.75	200	98	7.38	500	78	67
11	5.75	200	99	8.75	1,000	89	63
12	5.75	400	89	6.00	1,000	33	60
13	6.75	0	65	6.00	1,000	5	27
14	6.75	0	47	8.75	500	4	27
15	6.75	118	66	7.38	0	26	53
16	6.75	400	28	6.00	0	0	1
17	6.75	400	18	7.38	1,000	1	5
18	6.75	400	22	8.75	0	1	0

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