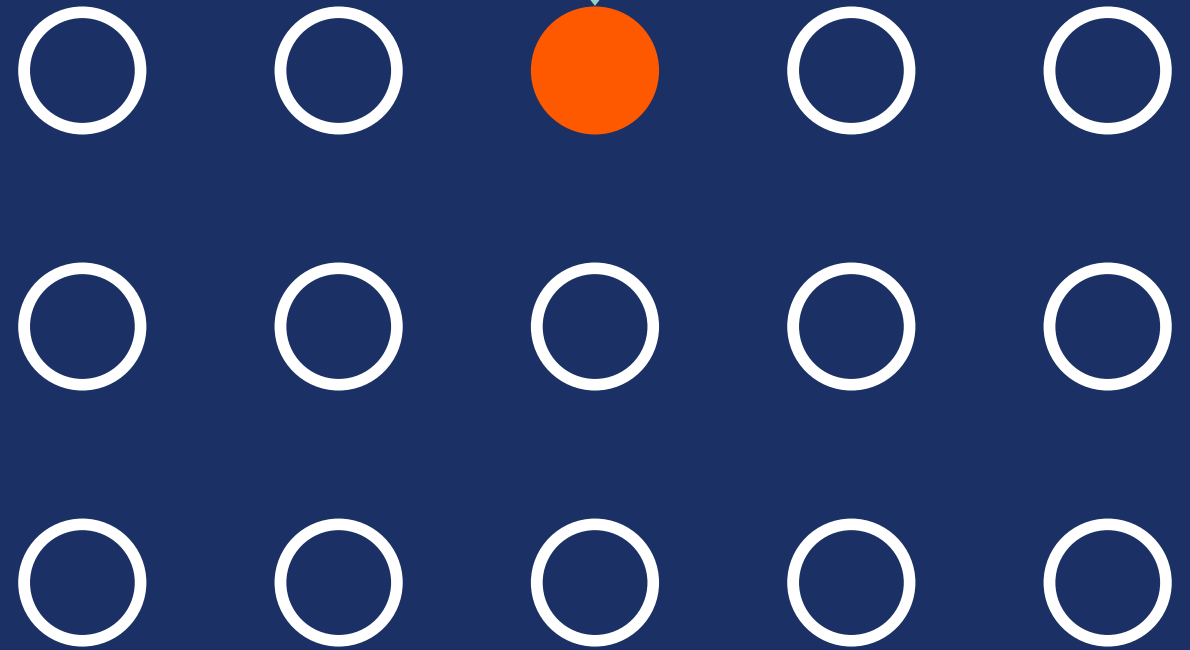


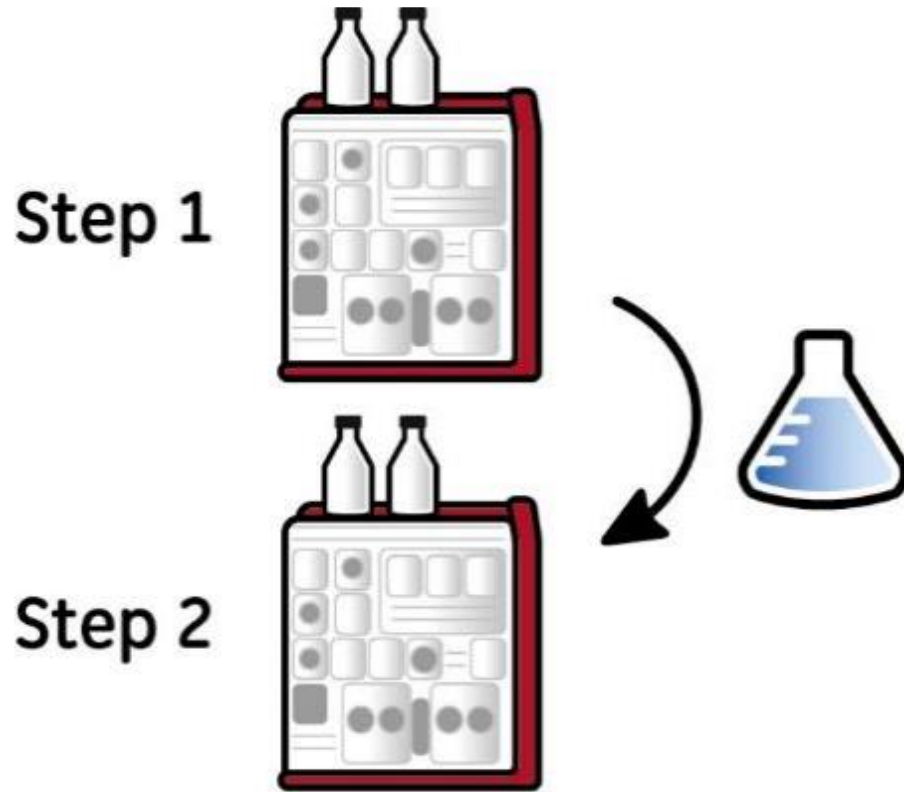


Getting started with automated two-step protein purification



Benefits of automated two-step purification

Typically, manual intervention is needed



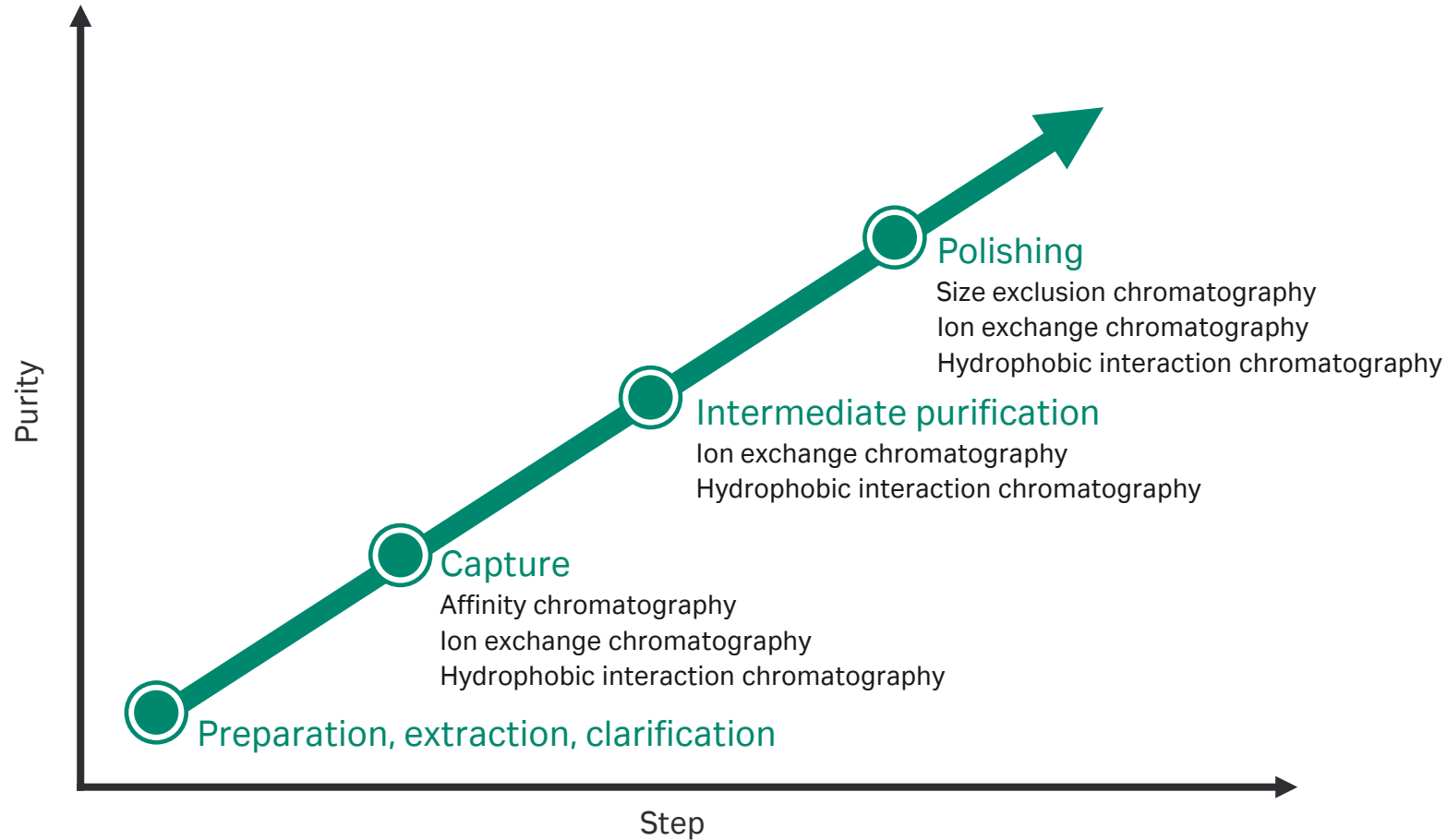
Reduce manual interactions

- Increase throughput
- Reduce labor
- Improve process consistency

Eliminate hold steps

- Ensure product stability
- Eliminate or reduce hold containers

Apply multistep purification on a wide range of purification schemes



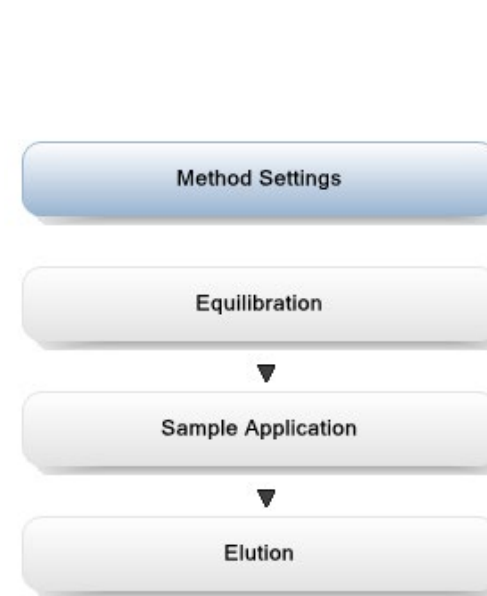
Example application: Automated two-step purification using predefined methods

1. Capture



2. Polishing

Automatic transfer of target protein (peak) and start of second method



Ways of working

1. Select purification protocol
2. Set up ÄKTA™ pure with components to support selected protocol
3. Set up capture method in UNICORN™ Method Editor
4. Set up polishing method in UNICORN Method Editor
5. Set up **Method queue**
6. Run the methods first with buffer and then with sample
7. Evaluate results

The following slides will demonstrate a workflow for a two-step purification with intermediate loop collection.

1. Select purification protocol



Select purification protocol

- Discuss with colleagues
- Web searches
- Reference literature
- Discuss with suppliers

Set up the protocol to combine a capture and a polishing step in one run.

2. Set up ÄKTA pure with components to support selected protocol using intermediate loop collection

The flexible system configuration allows for an optimized flow path

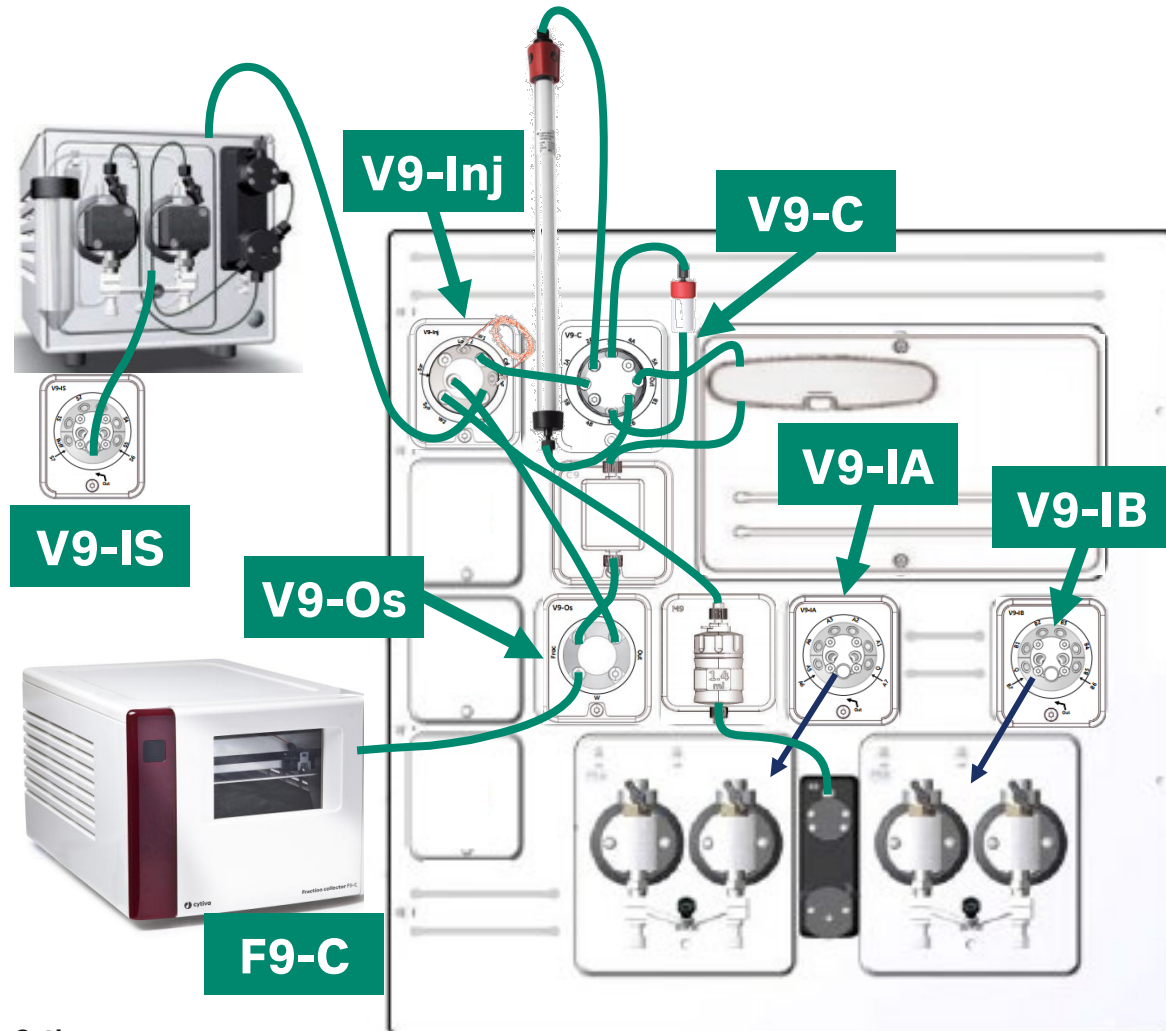
Components needed	Name
Sample inlet valve	V9-IS [‡]
Inlet valve	V9-IAB
Inlet valves*	V9-IA [‡] / V9-IB [‡]
Injection valve	V9-Inj [‡]
Column valve	V9-C [‡]
UV monitor 1	U9-M [‡] /U9-L
Conductivity	C9n [‡]
Outlet valve	V9-O V9-Os [‡]
Versatile Valve [†]	V9-V
Fraction collector	F9-R [‡] /F9-C
Sample pump	P9S [‡]

* Enables additional inlet buffers

† Use a versatile valve in front of injection valve if syringe position is needed for manual loop filling

‡ Used in the example described in the following slides

2. Set up ÄKTA pure with components to support selected protocol (cont.)



Optimized flow path with intermediate loop collection used in the application example

A sample pump facilitates sample loading in capture step.

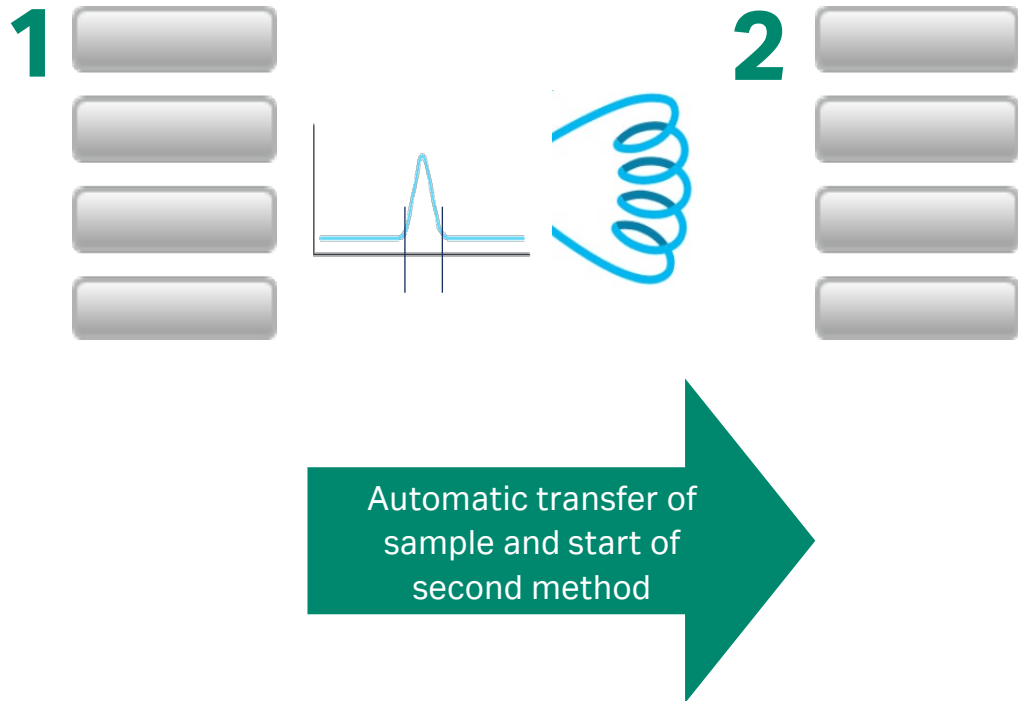
Connect outlet position from Outlet valve to Syringe port of injection valve.

When using V9-Os a fraction collector must be used.

Note: As an alternative, the sample can be loaded using the system pump and a mixer by-pass valve.

3. Method setup: Automated two-step purification with intermediate loop collection

Include first and second method in a *Method queue*



1. Set up your methods for the two steps as individual methods using the predefined protocols
 - **Capture step**
Use peak fractionation (via the outlet valve) to direct the eluted peak from the capture step to the loop
 - **Polishing step**
Fractionate normally to Fraction collector or V9-O
2. Create **Method queue** and start the run in System Control

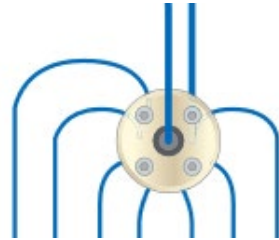
3. Method setup

Method 1: Capture step

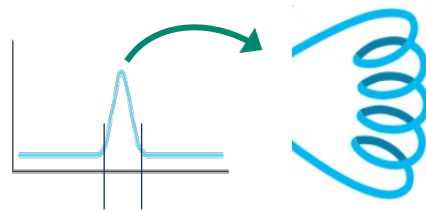
Load sample for the capture step



Eluted peak is automatically directed from outlet valve via syringe port to injection valve loop

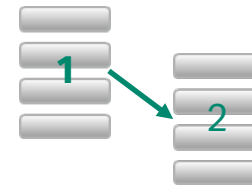


Sample (peak) from capture is stored in loop between first and second method

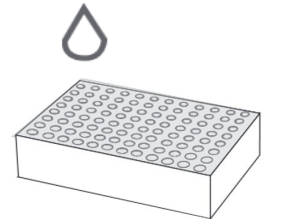


Method 2: Polishing step

Method queue function automatically starts second method



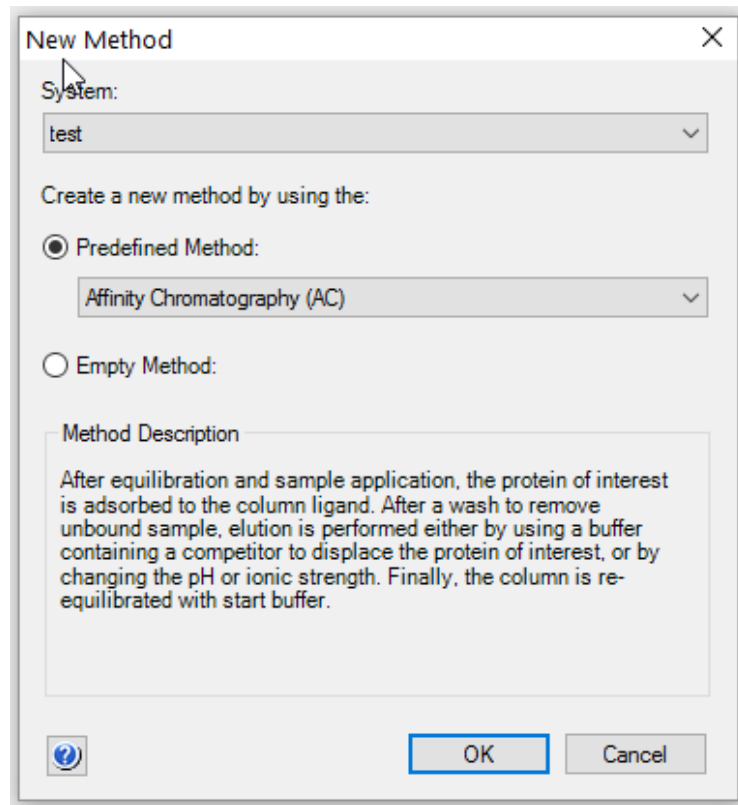
Target molecule is fractionated



3. Method setup: Capture

Select adsorption technique

Select predefined method in *Method editor*:



New Method

System:
test

Create a new method by using the:

☒ Predefined Method:
Affinity Chromatography (AC)

☐ Empty Method:

Method Description

After equilibration and sample application, the protein of interest is adsorbed to the column ligand. After a wash to remove unbound sample, elution is performed either by using a buffer containing a competitor to displace the protein of interest, or by changing the pH or ionic strength. Finally, the column is re-equilibrated with start buffer.

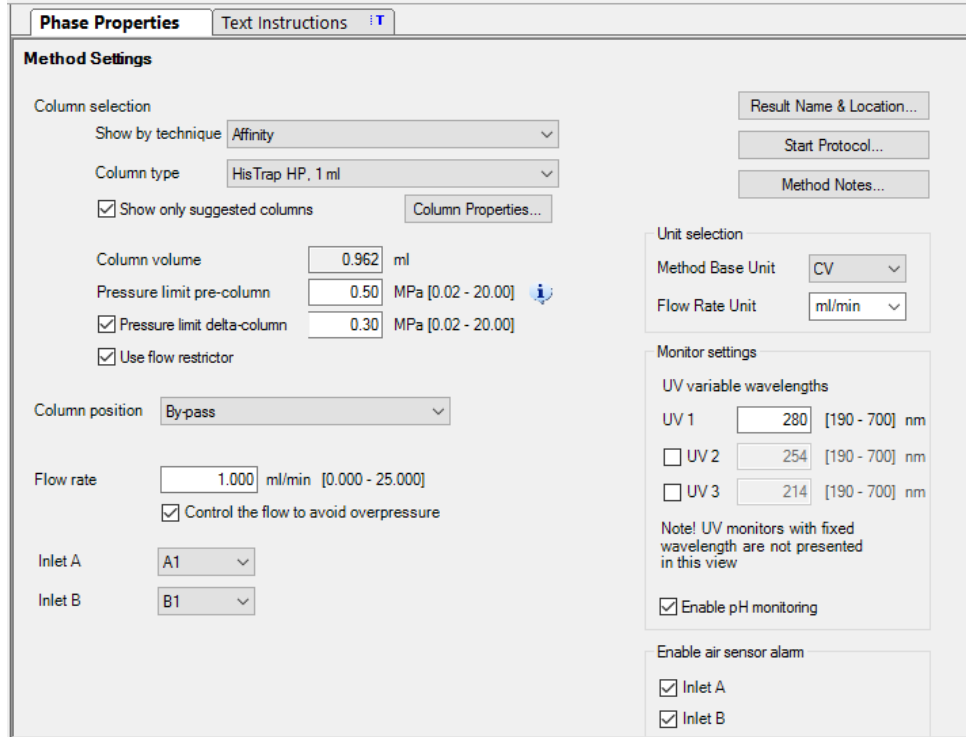
OK Cancel

Different phases (steps) will be displayed in *Method outline*



3. Method setup: Capture Adjust **Phase properties**

Define parameters for each phase in *Phase properties* tab



The screenshot shows the 'Phase Properties' tab in a software interface. It contains several sections for configuring method settings:

- Method Settings**
 - Column selection**: 'Show by technique' is set to 'Affinity'. 'Column type' is 'HisTrap HP, 1 ml'. There is a checkbox for 'Show only suggested columns' and a 'Column Properties...' button.
 - Column volume**: Set to 0.962 ml.
 - Pressure limit pre-column**: Set to 0.50 MPa [0.02 - 20.00].
 - Pressure limit delta-column**: Set to 0.30 MPa [0.02 - 20.00].
 - Use flow restrictor**: Checked.
 - Column position**: Set to 'By-pass'.
 - Flow rate**: Set to 1.000 ml/min [0.000 - 25.000]. A checkbox 'Control the flow to avoid overpressure' is checked.
 - Inlet A**: Set to 'A1'.
 - Inlet B**: Set to 'B1'.
- Unit selection**
 - Method Base Unit**: Set to 'CV'.
 - Flow Rate Unit**: Set to 'ml/min'.
- Monitor settings**
 - UV variable wavelengths**: 'UV 1' is set to 280 nm [190 - 700]. 'UV 2' is set to 254 nm [190 - 700]. 'UV 3' is set to 214 nm [190 - 700].
 - Note!** UV monitors with fixed wavelength are not presented in this view.
 - Enable pH monitoring**: Checked.
 - Enable air sensor alarm**: Two checkboxes, 'Inlet A' and 'Inlet B', are both checked.

Buttons on the right side include 'Result Name & Location...', 'Start Protocol...', and 'Method Notes...'.

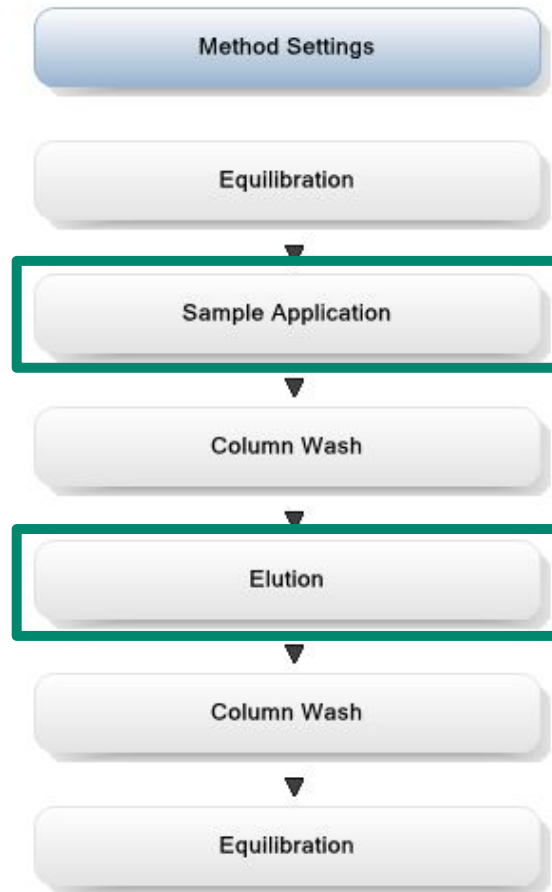
Settings available in *Phase properties*

- Flow rate and pressure limits for selected column
- Column position
- Inlets to be used
- Base unit
- Monitor settings



3. Method setup: Capture

Sample application and Elution properties



Sample application

Load sample using a sample pump.

Elution

Use peak fraction to collect the eluting peak in a loop.

The screenshot shows the 'Phase Properties' window with the 'Text Instructions' tab selected. The 'Sample Application' section is active, showing the following settings:

- ☒ Use the same flow rate as in Method Settings
- Flow rate: 5.000 ml/min [0.000 - 25.000]
- ☐ Inject sample from loop
- ☒ Inject sample directly onto column
- Sample inlet: S2
- ☐ Inject fixed sample volume: 20.00 ml
- ☒ Inject all sample using air sensor
- ☒ Set maximum volume to: 15.00 ml
- ☒ Wash sample flow path with buffer
- ☐ Prime sample inlet with: 6.00 ml
- ☐ Wash sample flow path with buffer after sample application.

Notes: Buffer inlet on Sample Inlet valve will be used to finalize sample injection. Buffer inlet on Sample Inlet valve will be used to wash the sample flow path.

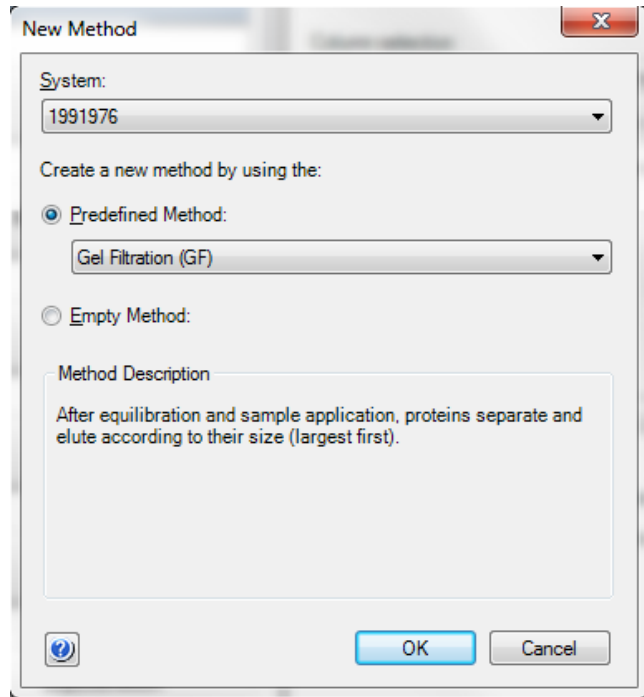
The 'Elution' section is also visible, showing the following settings:

- ☒ Gradient elution
- Start at: 0.0 % B [0.0 - 100.0]
- ☐ Fill the system with the selected buffer
- Table with 3 columns: Type, Target %B (0-100), Length (ml). Row 1: Step with fill, 100.0, 15.00.
- Buttons: Add Segment, Delete Segment.
- Note: A gradient delay is automatically added, provided that the last gradient segment is linear.
- Fractionate: ☐ in waste (do not collect), ☒ using outlet valve, ☐ using fraction collector.
- Fractionation settings: Fractionation type: Peak fractionation, Fractionation destination: Outlet 1, Peak fractionation destination: Outlet 1, Peak fractionation volume: 2.00 ml [0.01 - 20000.00].
- Buttons: Advanced Settings..., Peak Frac Settings...

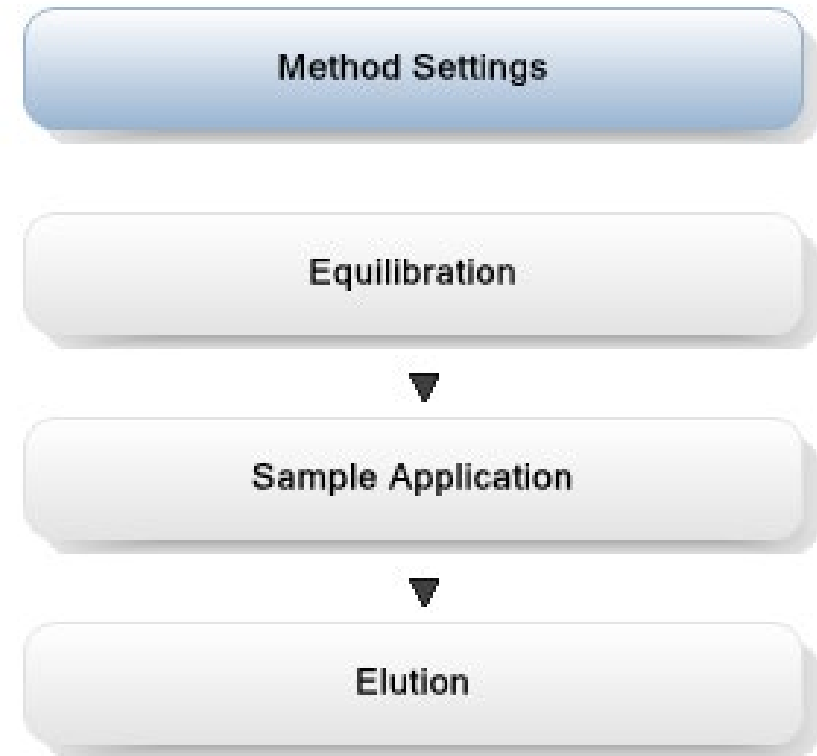
4. Method setup: Polishing

Select **SEC*/Desalting** in **Method editor**

Select predefined method: *SEC*/Desalting* in *Method editor*



Method will be displayed in *Method outline*



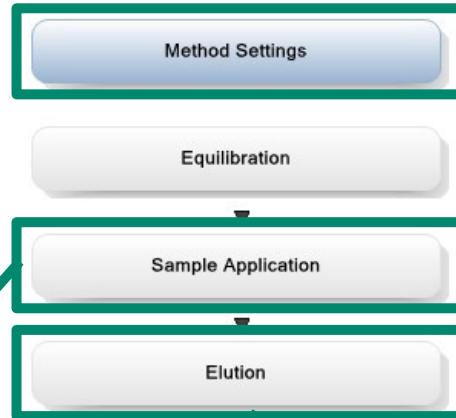
*Size exclusion chromatography, also called gel filtration (GF)

4. Method setup: Polishing Method settings, Sample application, and Elution properties

Re-inject sample

Select **Manual load**.

Note that the sample is already in the loop after the capture step.



Sample Application

☒ Use the same flow rate as in Method Settings
Flow rate: 0.750 ml/min [0.000 - 25.000]

☒ Inject sample from loop
☐ Inject sample directly onto column

Fill the loop using: **Manual load**
Loop type: **Capillary loop**

Sample inlet: S1

Fill loop with: 0.60 ml
Empty loop with: 1.00 ml
Sample volume: 0.00 ml

☒ Use the same inlets as in Method Settings
Inlet A: A2
Inlet B: B1 0.0 %

☐ Fill the system with the selected buffer

Elution

☒ Use the same flow rate as in Method Settings
Flow rate: 0.750 ml/min [0.000 - 25.000]

☒ Use the same inlets as in Method Settings
Inlet A: A2
Inlet B: B1

☐ Up flow

☒ Isocratic elution
Volume: 1.50 CV
0.0 % B [0.0 - 100.0]

☐ Gradient elution
Start at: 0.0 % B [0.0 - 100.0]

☐ Fill the system with the selected buffer

Fractionate:
☐ in waste (do not collect)
☐ using outlet valve
☒ using fraction collector

Fractionation settings:
Fractionation type: **Peak fractionation**
Fractionation destination: **96 deep well plate**
Peak fractionation destination: **96 deep well plate**
Fixed fractionation volume: 2.00 ml [0.00 - 2.20]
Peak fractionation volume: 1.00 ml [0.00 - 2.20]

☒ Start fractionation after: 0.20 CV (only for isocratic elution)

Method Settings

Column selection: Gel Filtration
Column type: Superdex 200 Increase 10/300 GL
Column volume: 23.562 ml
Pressure limit pre-column: 5.00 MPa [0.02 - 20.00]
Pressure limit delta-column: 2.60 MPa [0.02 - 20.00]
☒ Use flow restrictor

Column position: 2

Flow rate: 0.750 ml/min [0.000 - 25.000]
☒ Control the flow to avoid overpressure

Inlet A: A2
Inlet B: B1

Unit selection:
Method Base Unit: CV
Flow Rate Unit: ml/min

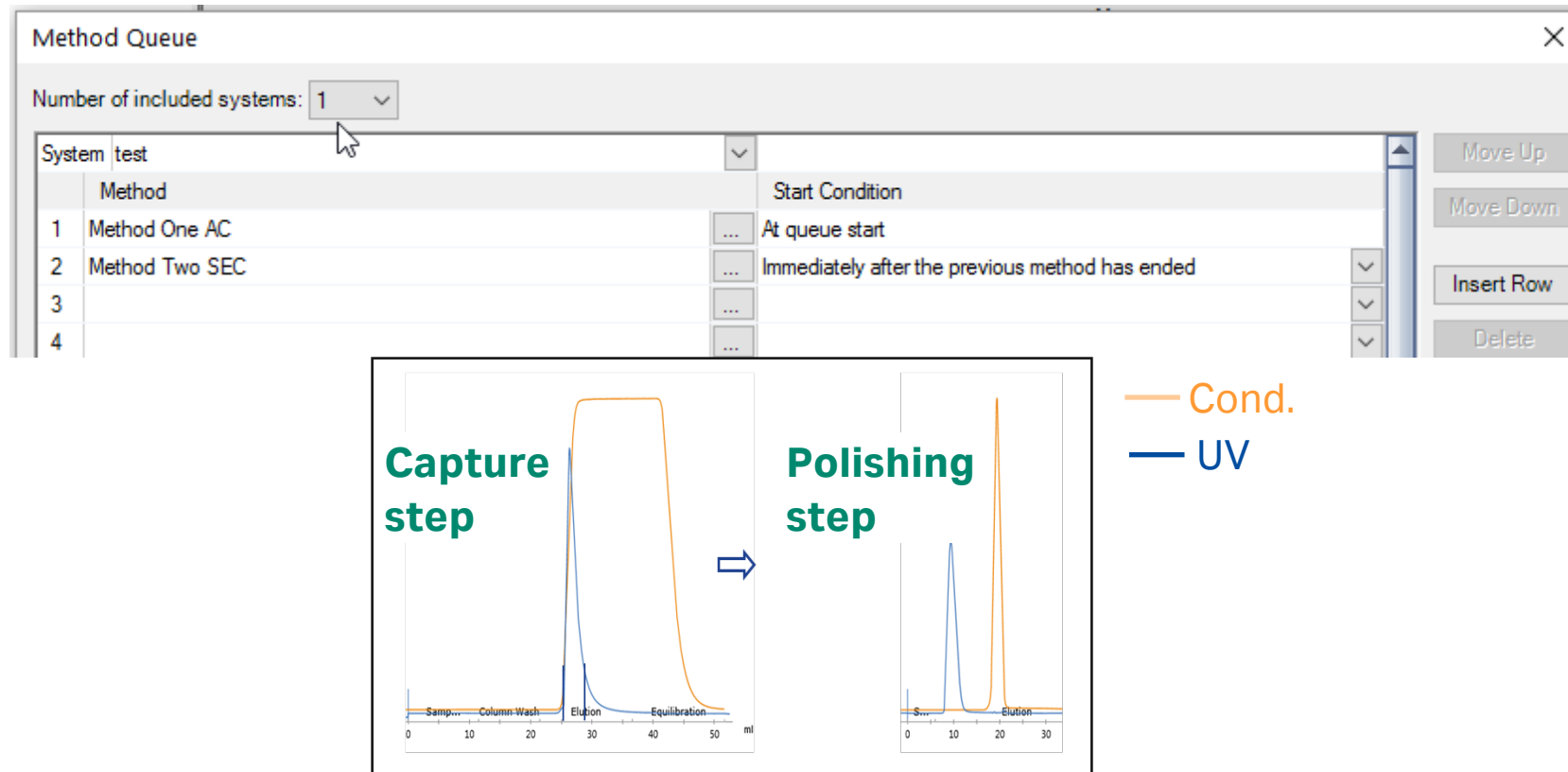
Monitor settings:
UV variable wavelengths:
UV 1: 280 [190 - 700] nm
UV 2: 254 [190 - 700] nm
UV 3: 214 [190 - 700] nm

☒ Enable pH monitoring
☒ Enable air sensor alarm

5. Method setup: Create **Method queue**

Include the capture and polishing step in **Method queue**

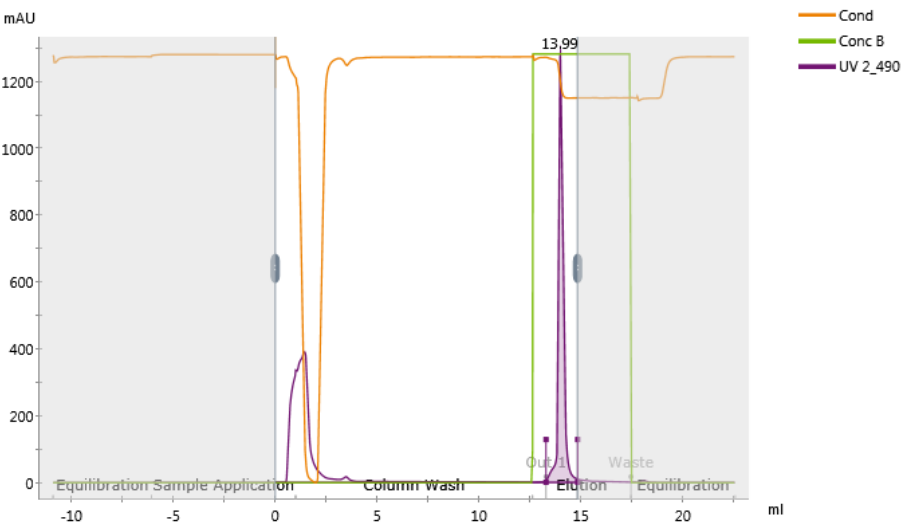
Method queue is set in UNICORN™ Method editor



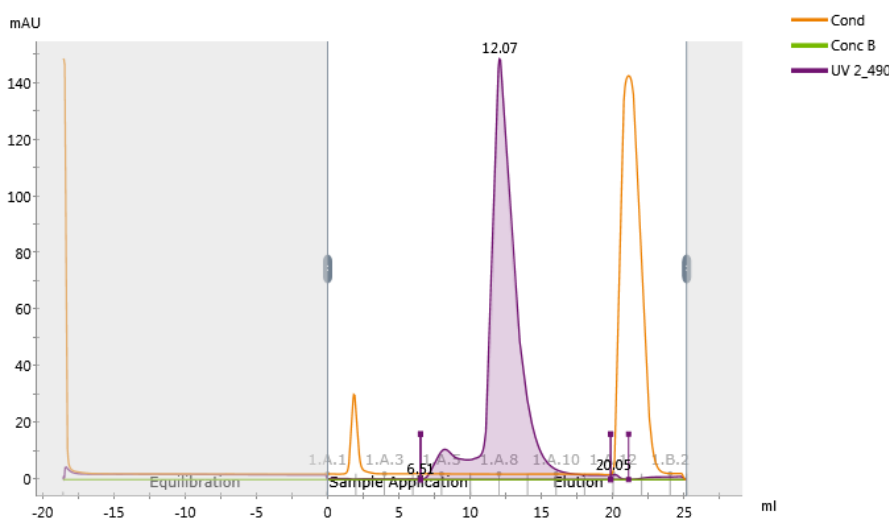
6. Running the methods

Automated two-step purification, example 1

Capture: Affinity chromatography with HisTrap™ HP 1 mL



Polishing: SEC* Superdex™ 75 Increase



Method	Peak	Retention (mL)	Area (mL*mAU)	Volume (mL)
AC	Peak A	13.990	313.2	1.549
SEC	Peak A	12.070	295.4	13.329

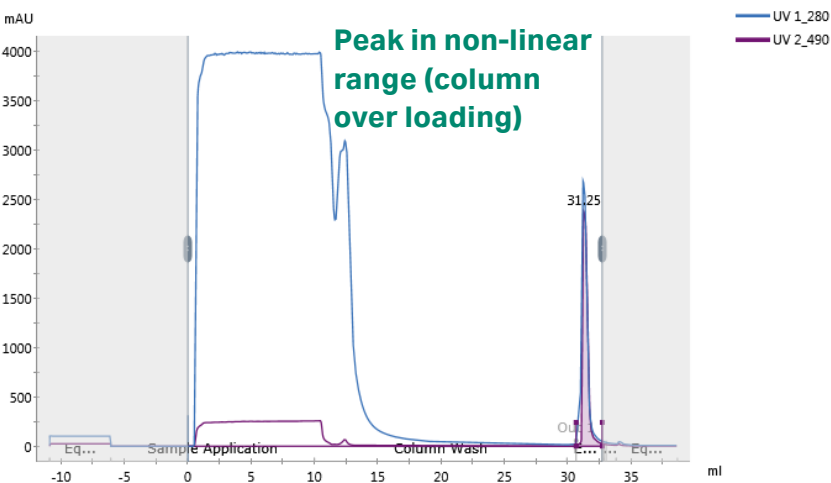
Purple peaks are similar in area indicating minimal sample loss.

Sample: GFP-His (clarified homogenate, 5.3 mg GFP-His/g *E. coli* cell pellet)
*Size exclusion chromatography, also called gel filtration (GF)

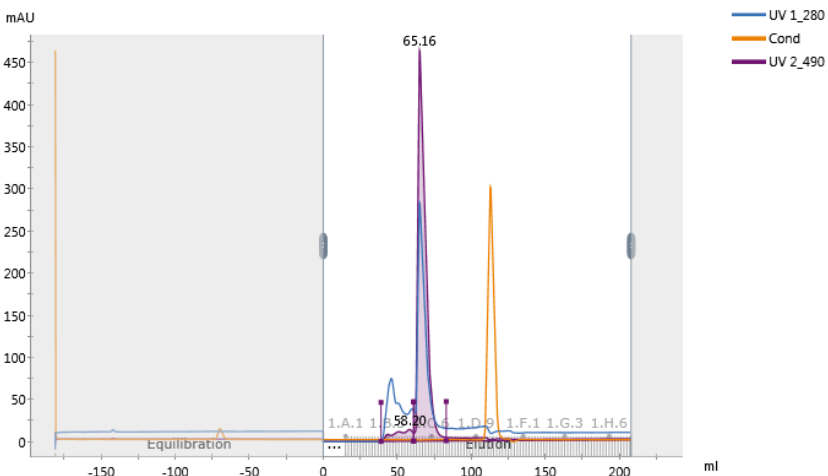
6. Running the methods

Automated two-step purification, example 2

Capture: Affinity chromatography with HisTrap™ HP 1 mL



Polishing: SEC* HiLoad™ 16/600 Superdex™ 75 pg



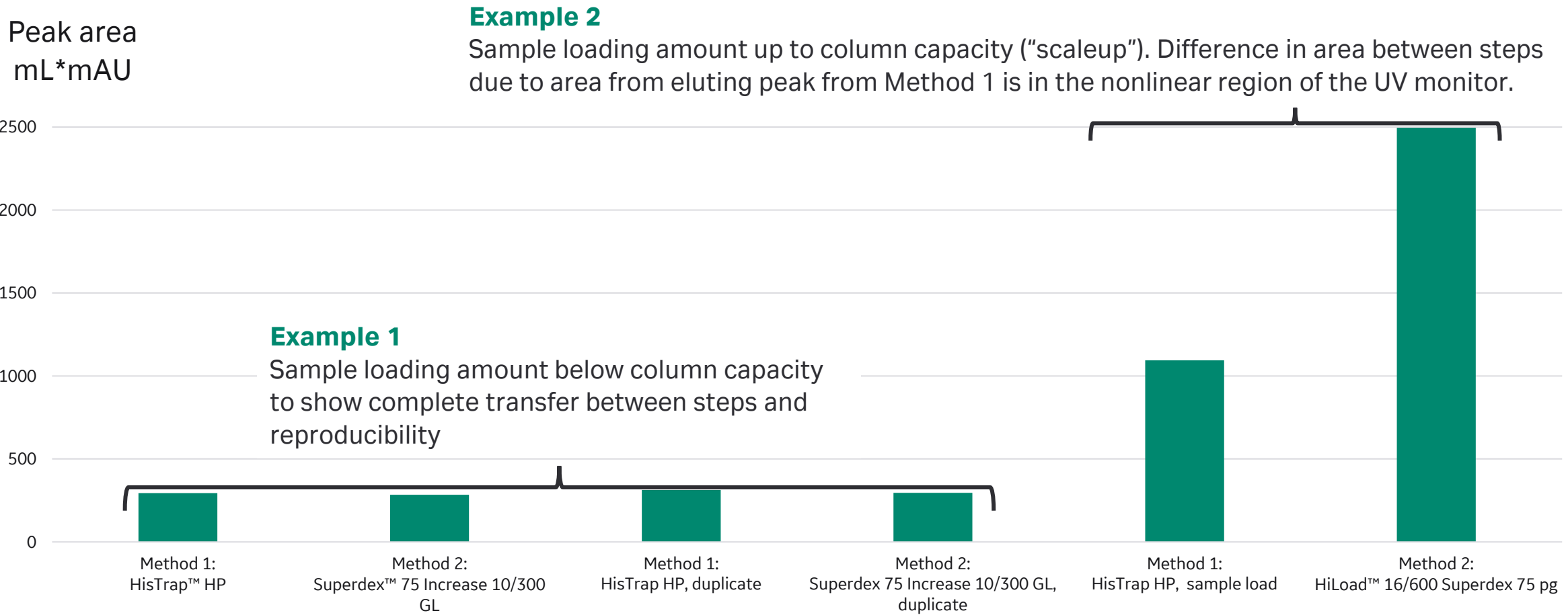
Method	Peak	Retention (mL)	Area (mL*mAU)	Volume (mL)
AC	Peak A	31.246	1094	2.076
SEC	Peak B	65.158	2496	22.000

In the capture step UV is measured outside of the linear range of the monitor, therefore peak areas cannot be compared.

Sample: GFP-His (clarified homogenate, 5.3 mg GFP-His/g *E. coli* cell pellet)
*Size exclusion chromatography, also called gel filtration (GF)

7. Evaluation

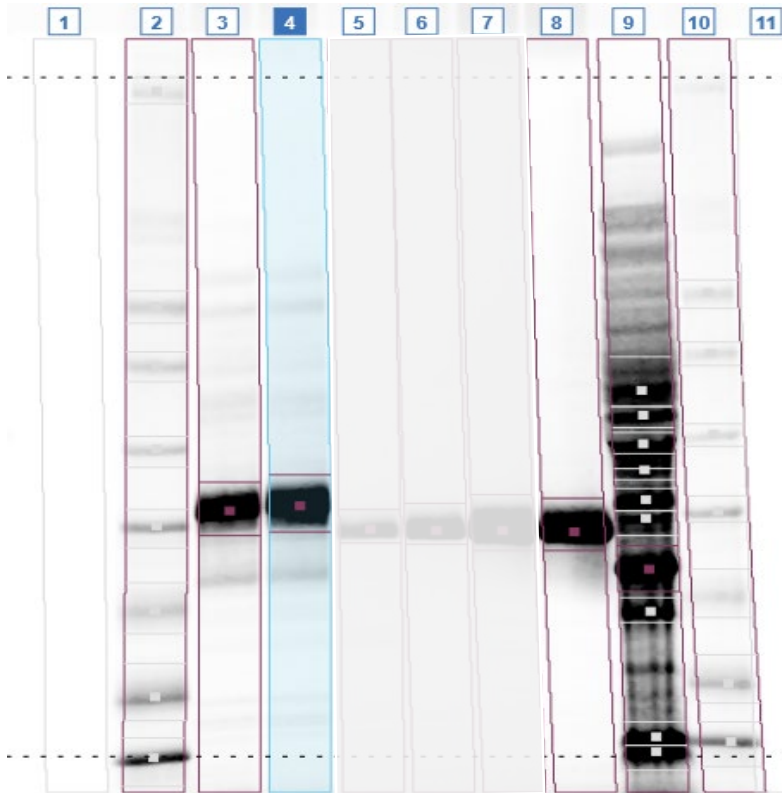
Integration results from example 1 and 2



7. Verification

Reference analysis with electrophoresis

Results form electrophoresis



Reference analysis to verify identified peaks

Sample ID:

1. Blank
2. Amersham™ WB molecular weight markers
3. Two-step purification protocol AC-GF HisTrap™ 1 mL + Superdex™ 75 Increase 10/300 GL
4. Two-step purification protocol AC-GF HisTrap 1 mL + Superdex 75 Increase 10/300 GL, duplicate
- 5.–7. Blanks
8. Two-step purification protocol AC-GF HisTrap 1 mL + HiLoad™ 16/60 Superdex 75 pg, scaleup
9. Start material His-GFP, *E. coli*
10. Amersham WB molecular weight markers

Utilize standard hardware and predefined UNICORN methods for automated two-step purification

ÄKTA™ systems in automated multistep purification



Combine different chromatography steps in automated multistep purification to save time and add consistency.

Set up automated, two-step purification using predefined methods and **Method queue** function to quickly get started.

Store peak from step one in loop before automatically continuing to second step.

ÄKTA pure was used in the example application shown. ÄKTA avant can also be used in the same way.

Thank you



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