cytiva

Optimal configuration of **ÄKTA** pure 25 for small-scale SEC

Cue Card

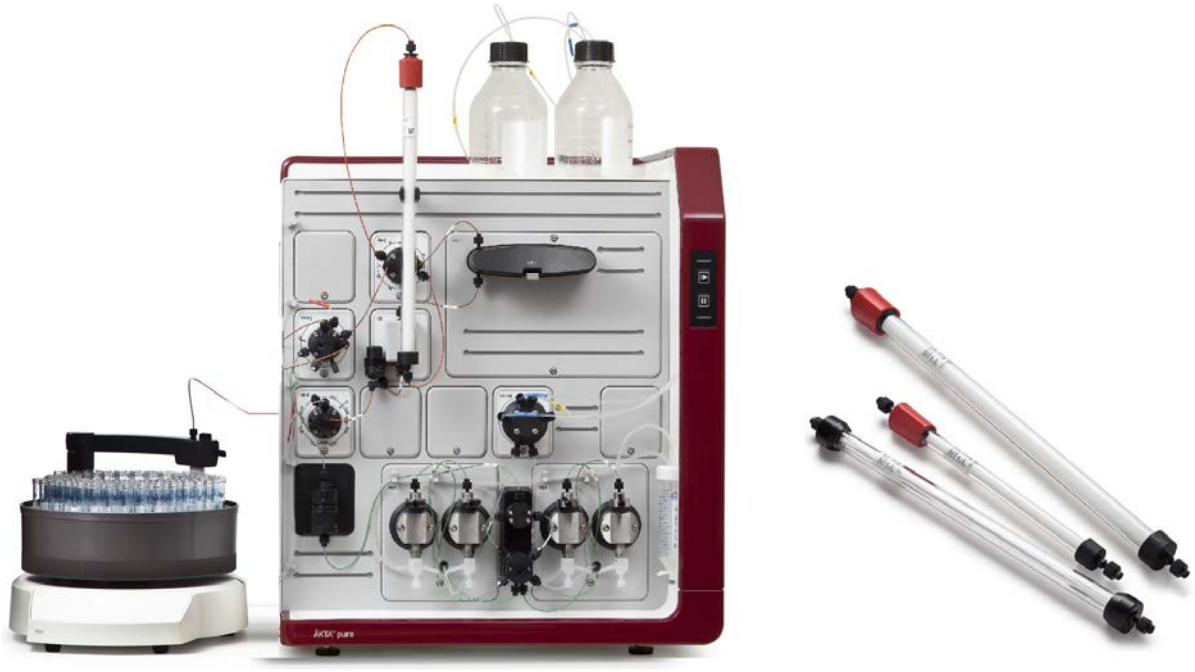

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Table of Contents

1 Introduction

This cue card describes how to configure ÄKTA™ pure 25 for optimal performance of size exclusion chromatography (SEC) columns for analysis or small-scale preparative purification with sample volumes from 10 µL to 500 µL. The configuration is optimal for the following Cytiva columns and their predecessors (including Superdex™ peptide and Superose™ 12):

- Superdex 200 Increase 10/300 GL
- Superdex 200 Increase 5/150 GL
- Superdex 200 Increase 3.2/300
- Superose 6 Increase 10/300 GL
- Superose 6 Increase 5/150 GL
- Superose 6 Increase 3.2/300
- Superdex 75 Increase 10/300 GL
- Superdex 75 Increase 5/150 GL
- Superdex 75 Increase 3.2/300

2 Principles

Impact of system and sample volume on resolution in SEC

It is important to have as small internal volumes as possible in the system, especially for the 3.2/300 column. The 10/300 column, that has larger bed volume, can be used on a standard ÄKTA pure system with 0.5 mm i.d. tubing achieving good resolution. However, the recommendations in this cue card will improve the results. If the 10/300 column is run on a standard system with fraction collection, we suggest to not use both the multi-column valve **V9-C** and the pH flow cell in line, since they will increase internal volume. Fraction collector **F9-R** would be favoured before **F9-C** due to smaller volumes. Fraction collector **F9-C** can be used in combination with the 10/300 column, but are not optimal together with the smaller 5/150 and 3.2/300 columns. For the best resolution in SEC it is important that:

- the sample is not diluted between injection valve and column inlet
- the separated proteins are not diluted between column outlet and UV flow cell or between UV flow cell and fraction collector

You can achieve this by following the recommendations in this Cue Card. For most applications the sample volume should not exceed 2% of total column volume in order to achieve maximum resolution. For analytical separations, start with a sample volume of approximately 0.5% of the total column volume.

The following table summarizes the sample volume recommendations by type of SEC column used.

Table 2.1:

Column size	Column volume (mL)	Recommended start volume (µL)	Recommended max sample volume (µL)
10/300	24	120	500
5/150	3	20	50
3.2/300	2.4	20	50

3 Setup for SEC analysis

In this chapter

3.1 System configuration from injection valve to UV-monitor without column valve

The optimal configuration for analytical SEC is to connect the column with the shortest possible flow path between the sample injection valve and the UV monitor flow cell. This gives a minimum of system volume before and after the column. For the 10/300 column, shortening the flow path has less impact and is thus not described in this section. This column is preferably mounted with column valve V9-Cs, see *Fig. 3.4, on page 10* and *Table 3.10, on page 11*.

In this setup you can choose between UV monitor **U9-M** and **U9-L**, since they give similar good results.

Table 3.1: Total volume UV monitor flow cells

UV monitor	Flow cell path length (mm)	Total volume (µL)
U9-M	0.5	10
	2	11
	10	12
U9-L	2	30
	5[1]	20

[1] Recommended in U9-L

Note: *When the column is mounted directly between the injection valve and the UV flow cell, avoid using system wash since this might compress the packed column bed. For quick buffer exchange set the injection valve* ***V9-Inj*** *in* ***System pump waste*** *position.*

Configuration with UV-monitor U9-M

Two options for column mounting:

1. Horizontally with the shortest possible tubing between column outlet and UV flow cell inlet
2. Vertically with a rigid male/male union between column outlet and UV flow-cell inlet.

A. Horizontal configuration

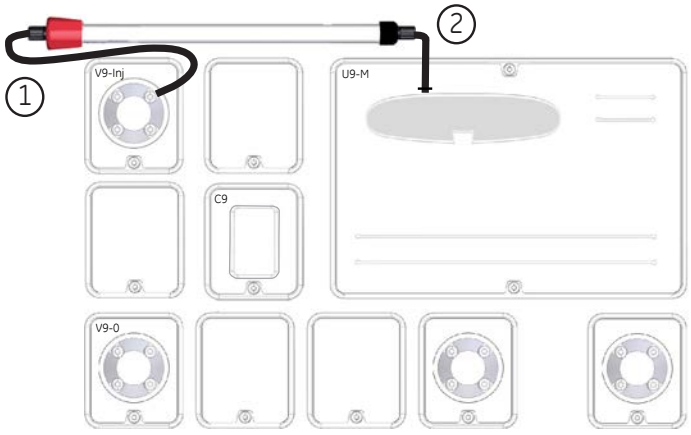


Figure 3.1:

Table 3.2: 3.2/300 column, horizontal configuration with U9-M

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**Column**	inlet	0.15	360
2	**Column**	outlet	**U9-M**	inlet	0.15	60

Table 3.3: 5/150 column, horizontal configuration with U9-M

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**Column**	inlet	0.25	220
2	**Column**	outlet	**U9-M**	inlet	0.25	60

The different PEEK tubing used in ÄKTA pure has different colors:

- id 0.15 mm violet
- id 0.25 mm blue
- (id 0.5 mm orange, id 0.75 mm green, though not used in this Cue Card)

B. Vertical configuration

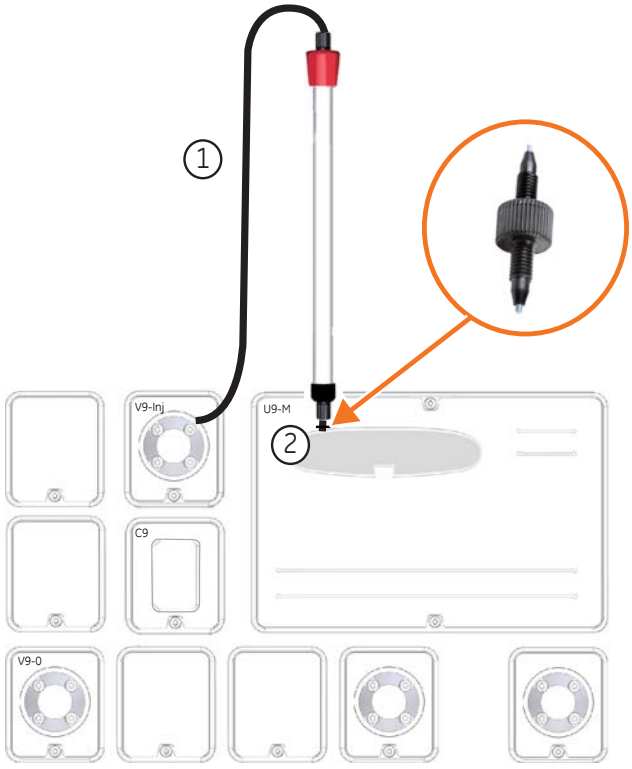


Figure 3.2:

Table 3.4: 3.2/300 column, vertical configuration with U9-M

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**Column**	inlet	0.15	490
2	**Column**	outlet	**U9-M**	inlet	Male-male 0.13	

Table 3.5: 5/150 column, vertical configuration with U9-M

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**Column**	inlet	0.25	360
2	**Column**	outlet	**U9-M**	inlet	Male-male 0.25	

Configuration with UV-monitor U9-L

With UV monitor **U9-L** it is possible to be more flexible in the module placement to shorten the column inlet tubing. **U9-L** is placed low in the system.

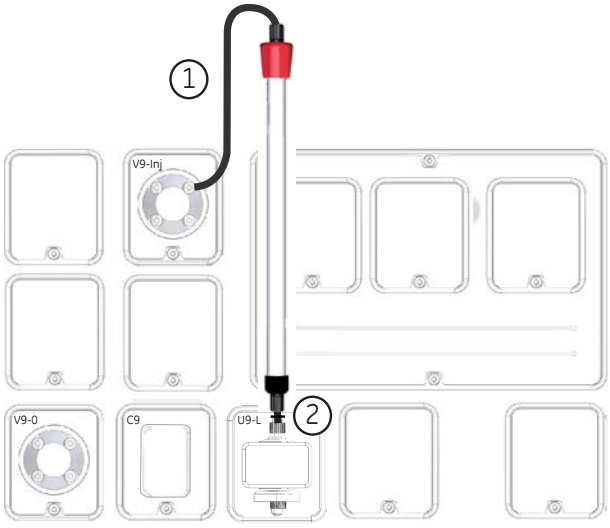


Figure 3.3:

Table 3.6: 3.2/300 column, vertical configuration U9-L

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**Column**	inlet	0.15	290
2	**Column**	outlet	**U9-M**	inlet	Male-male 0.13	

Table 3.7: 5/150 column, vertical configuration U9-L

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**Column**	inlet	0.25	160
2	**Column**	outlet	**U9-M**	inlet	Male-male 0.25	

See *Chapter 8 Ordering Information, on page 26*, regarding tubing and male-male union product codes.

3.2 Configuration from injection valve to UV-monitor with column valve V9-Cs

The flow path between the injection valve and the UV flow cell, with the column connected to column valve **V9-Cs**, is shown in the illustration below.

If the UV monitor **U9-L** is used in combination with column valve **V9-Cs** it should be placed to the left on the multi-module panel with the same tubing lengths described below. It is recommended that flow restrictor **FR-902** is in the flow path for 5/150 and 10/300 columns with 0.25 mm i.d. tubing.

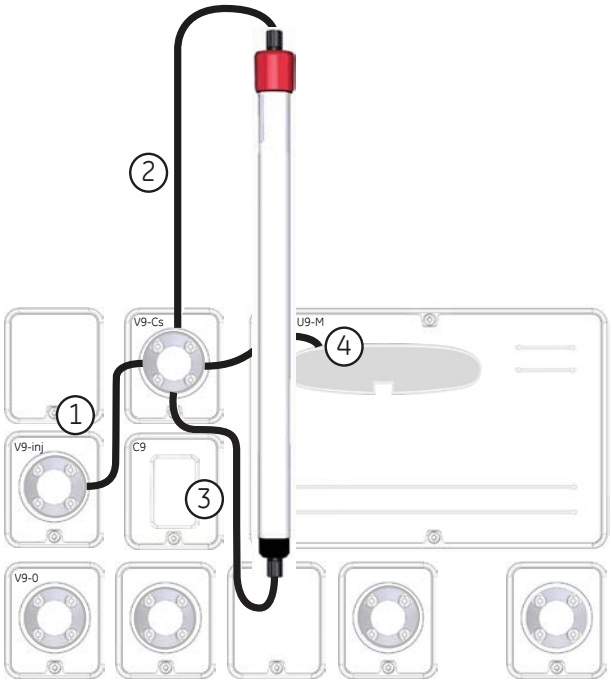


Figure 3.4:

Table 3.8: 3.2/300 column, injection valve to UV-monitor with column valve V9-Cs

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**V9-Cs**	In	0.15	160
2	**V9-Cs**	1A	Column	inlet	0.15	275
3	Column	outlet	**V9-Cs**	1B	0.15	200
4	**V9-Cs**	Out	**U9-M**	inlet	0.15	160

Table 3.9: 5/150 column, injection valve to UV-monitor with column valve V9-Cs

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**V9-Cs**	In	0.25	160
2	**V9-Cs**	1A	Column	inlet	0.25	200
3	Column	outlet	**V9-Cs**	1B	0.25	200
4	**V9-Cs**	Out	**U9-M**	inlet	0.25	160

Table 3.10: 10/300 column, injection valve to UV-monitor with column valve V9-Cs

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	Col	**V9-Cs**	In	0.25	160
2	**V9-Cs**	1A	Column	inlet	0.25	275
3	Column	outlet	**V9-Cs**	1B	0.25	200
4	**V9-Cs**	Out	**U9-M**	inlet	0.25	160

Tip: *When cutting i.d. 0.15 mm tubing, make sure that a perpendicular cut is made and that no edges can block the hole. Check that the tubing is completely inserted in the connection and that the fitting is not over-tightened.*

See *Chapter 8 Ordering Information, on page 26*, regarding tubing product codes.

4 Setup for small-scale preparative SEC with fraction collector

In this chapter

4.1 System configuration from UV-monitor U9-M to fraction collector

For configuration between injection valve and UV monitor, see *Chapter 3 Setup for SEC analysis, on page 5*. UV monitor **U9-M** would be favored before **U9-L** if more components is to be added in the flow path, due to smaller total volume. The outlet valve **V9-Os** can be used equally well as **V9-O**.

3.2/300 column

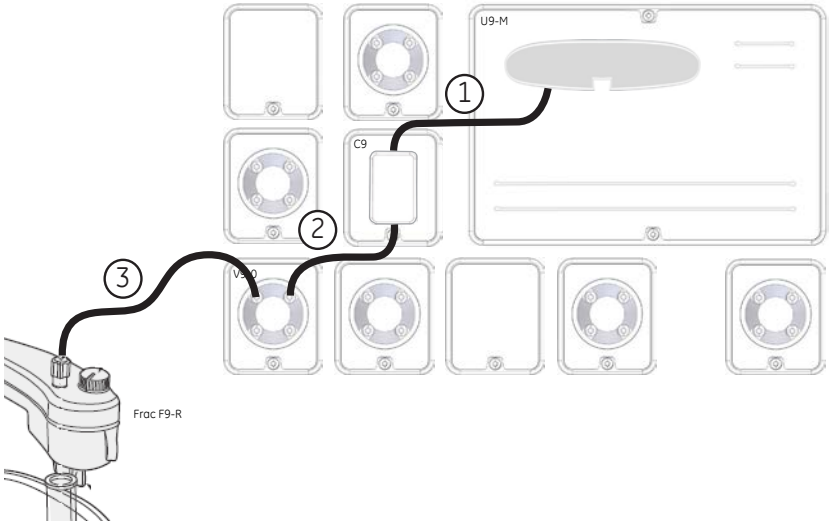


Figure 4.1:

Note: *This is also applicable when using UV monitor **U9-L** placed to the left on the multimodule panel.*

Table 4.1: 3.2/300 column, UV-monitor U9-M to fraction collector

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**U9-M**	outlet	**C9n**	Inlet	0.15	170
2	**C9n**	outlet	**V9-O**	In	0.15	190
3	**V9-O**	Frac	**F9-R**	tubing connector	0.15	400

Note: *Flow restrictor **FR-902** is not needed, since the 0.15 mm tubing creates sufficient backpressure.*

5/150 and 10/300 columns

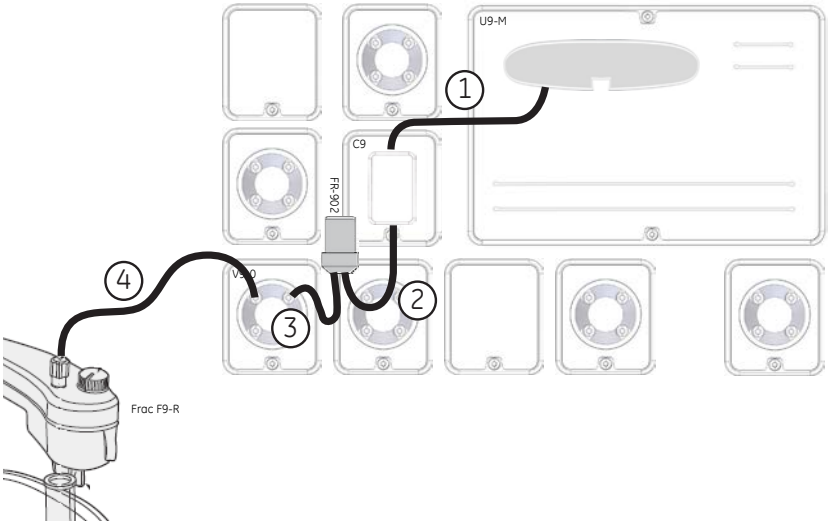


Figure 4.2:

Note: *This is also applicable when using UV monitor **U9-L** placed to the left on the multi-module panel.*

Table 4.2: 5/150 and 10/300 columns, UV-monitor U9-M to fraction collector

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**U9-M**	outlet	**C9n**	Inlet	0.25	170
2	**C9n**	outlet	**FR-902**	Inlet	0.25	95
3	**FR-902**	outlet	**V9-O**	In	0.25	135
4	**V9-O**	frac	**F9-R**	tubing connector	0.25	400

10/300 column and pH valve

When collecting samples using the 10/300 column for purification, it is possible to have the **V9-pH** valve in the flow path. If resolution during fraction collection is critical, the pH flow cell should not be inline.

You can keep the flow restrictor **FR-902**, attached to the **V9-pH** valve, inline since the effect on the resolution will be limited .

Table 4.3: 10/300 column and pH valve

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**U9-M**	outlet	**C9n**	Inlet	0.25	170
2	**C9n**	outlet	**V9-pH**	In	0.25	180
3	**V9-pH**	Out	**V9-O**	In	0.25	170
4	**V9-O**	Frac	**F9-R**	tubing connector	0.25	400

See *ÄKTA pure User Manual, article number 29119969*, for placement of the pH valve module.

See *Chapter 8 Ordering Information, on page 26*, regarding tubing product codes.

4.2 System configuration from UV-monitor U9-L to fraction collector

This describes the flow path between UV-monitor **U9-L** and fraction collector **F9-R** when the column is attached directly between the injection valve and the UV monitor flow cell, see *Configuration with UV-monitor U9-L, on page 9*. If a column valve **V9-Cs** is used in combination with UV monitor **U9-L**, the UV monitor should be connected in the same position as the **U9-M**, see *Section 4.1 System configuration from UV-monitor U9-M to fraction collector, on page 13*, and the tubing arrangement to the fraction collector will be the same as for the **U9-M** solution.

Note: *Exclude the flow restrictor when using 3.2/300 column. The flow restrictor adds additional volume and the smaller tubing diameter will anyhow produce sufficient backpressure.*

Note: *The 10/300 column is not included in this description as the benefit from a direct configuration is limited.*

3.2/300 column

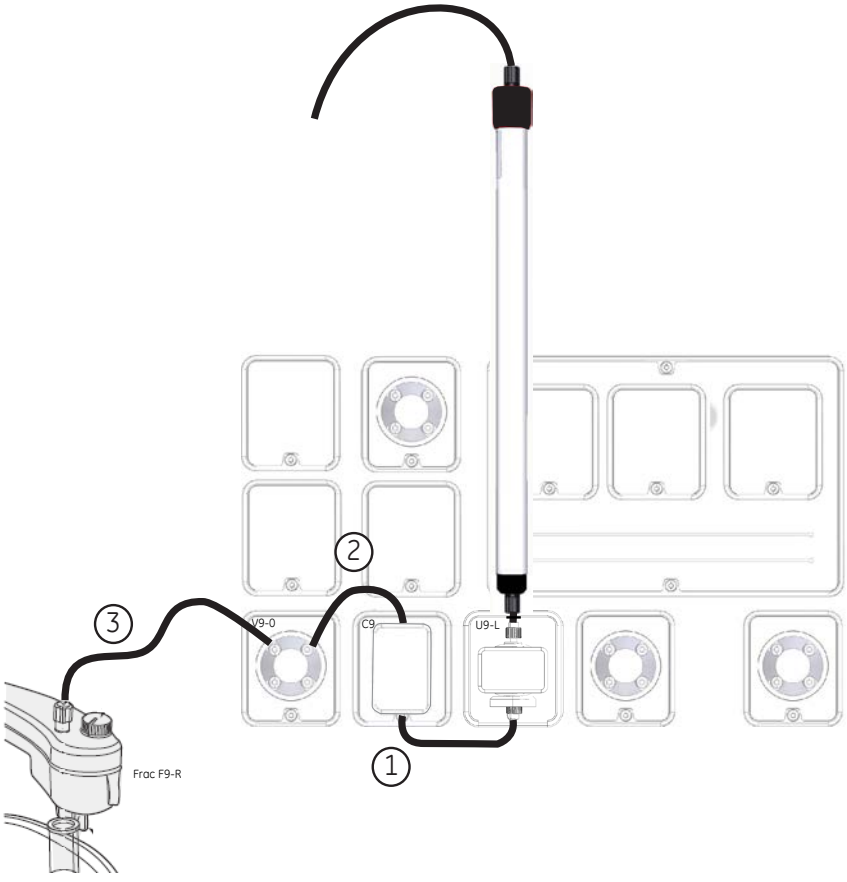


Figure 4.3:

Table 4.4: 3.2/300 column, UV-monitor U9-L to fraction collector

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**U9-L**	outlet	**C9n**	Inlet (lower)	0.15	150
2	**C9n**	outlet (upper)	**V9-O**	In	0.15	170
3	**V9-O**	frac	**F9-R**	Tubing connector	0.15	400

5/150 column

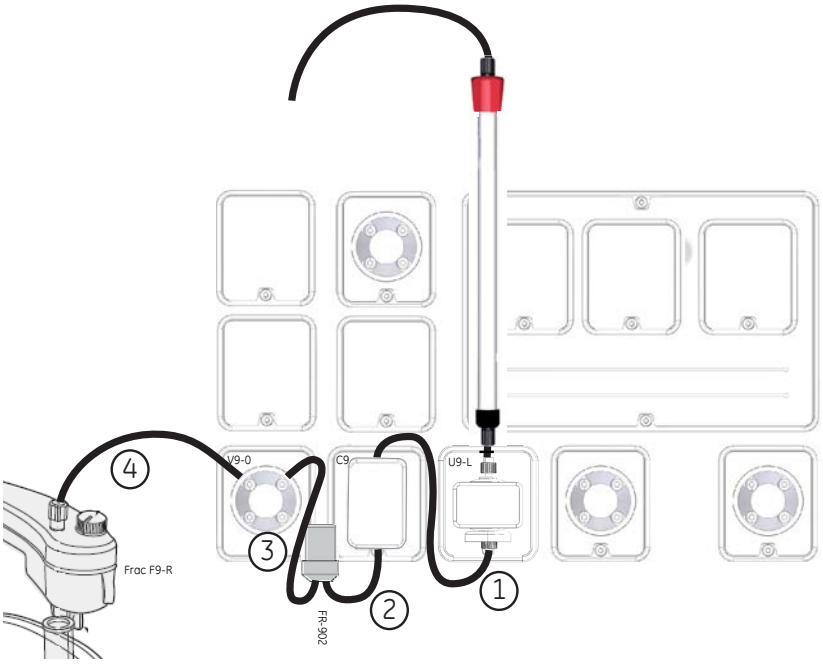


Figure 4.4:

Table 4.5: 5/150 columns, UV-monitor U9-L to fraction collector

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**U9-L**	outlet	**C9n**	Inlet (upper)	0.25	210
2	**C9n**	outlet (lower)	**FR-902**	Inlet	0.25	95
3	**FR-902**	outlet	**V9-O**	In	0.25	190

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
4	**V9-O**	frac	**F9-R**	Tubing connector	0.25	400

Delay volumes

Delay volumes from the UV flow cell to the outlet valve or the fraction collector **F9-R** with the different tubing arrangements are described here:

Table 4.6: Delay volumes

UV monitor	Figure number	Tubing configuration	Suitable for columns	Delay volume monitor to outlet valve (µL)	Delay volume monitor to frac F9-R (µL)
U9-M, 2 mm flow cell	5	0.15	3.2/300	43	50
	6	0.25	5/150, 10/300	66	85
	-	0.25 with pH valve[1]	10/300	77	97
U9-L, left multimodule panel, 5 mm flow cell	5[2]	0.15	3.2/300	47	54
	6[2]	0.25	5/150, 10/300	71	90
	-	0.25 with pH valve[1]	10/300	82	101
U9-L, mounted low, 5 mm flow cell	7	0.15	3.2/300	47	54
	8	0.25	5/150	75	95

[1] If resolution is critical, we suggest to not have the pH flow cell inline, even if the pH valve is connected.
[2] Note that U9-L should be placed in the same position as U9-M in *Fig. 4.1, on page 13* and *Fig. 4.2, on page 14* for this configuration.

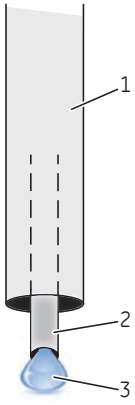
The value for "Delay volume monitor to frac F9-R" in the table above should be entered into UNICORN, see *Chapter 6 UNICORN settings, on page 22*. For the 10/300 column it is possible to use fraction collector **F9-C** instead of **F9-R**. In that case you should use the value from "Delay volume monitor to outlet valve" in the table above and add 310 µL (with **F9-C** internal tubing 0.5 mm id) to get the value that should be entered into UNICORN."

For more information about module volumes see *Delay volumes and Component volumes, in ÄKTA pure User Manual, article number 29119969.*

Collecting small volumes in F9-R

With a standard PEEK tubing in the fraction collector **F9-R** the drop size is normally 25 to 50 µl. The drop size can be reduced to approximately 5 to 10 µl by using silica tubing with smaller outer diameter and tubing sleeve in the connecting ends.

Make sure that the silica tubing protrudes a few millimeters from the sleeve tubing in the fraction collector connection (see the figure below), to create small drop volumes. How to handle silica tubing with PEEK sleeves is also described in the *Instruction Micro fraction collection, article number 28948234*.



1. PEEK sleeve
2. Silica tubing
3. Drop size reduced

Figure 4.5:

Tip: *Eppendorf™ tubes can be inserted into the collection vials for sample collection.*

See *Chapter 8 Ordering Information, on page 26*, regarding tubing product codes.

5 Injection techniques

Manual sample injection

For good chromatographic resolution on SEC columns in analytical or small-scale preparative work, the sample volume should not exceed 2% of the column volume. The ÄKTA pure injection valve **V9-Inj** is a multi-functional injection valve. It has features that are important to take into consideration when injecting small volumes. When connecting a small loop and working with complete loop filling techniques, the total sample volume is the sum of the loop volume and the loop connecting channels in the valve. Remember not to remove the syringe before the valve has turned.

When loading small sample volumes into **V9-Inj**:

- Use a PEEK (Polyetheretherketone) sleeve around the needle and a standard fingertight connector 1/16" M in the syringe connection position of the valve.
    1. Make sure that the needle of the syringe has an o.d. of 0.70 mm and is blunt ended.
    2. Cut a PEEK tubing o.d. 1/16" i.d. 0.75 mm (green) 5 mm shorter than the needle.
    3. Attach the connector lightly and insert the syringe needle with PEEK sleeve around it in the connection and make sure that both the PEEK sleeve and the needle reaches the bottom.
    4. Turn the connector firmly so that no backward leakage can occur, inject the sample.
- Alternatively, use the Fill port INV-907 made for syringes with needle o.d. 0.7 mm. Make sure that the needle is blunt ended.
- Do not use Luer connections since that is designed for larger volumes.

Figure 5.1:

Manual complete filling

A suitable sample volume for the 3.2/300 column is 20 µL.

- The sample injection valve **V9-Inj** adds 10 µL in channel contribution to the loop volume.
- Attach a 10 µL loop, preferably a 204 mm long tubing i.d. 0.25 mm, to get a total sample volume of 20 µL.

For filling the loop and injection valve channels completely:

- Due to laminar flow, it is recommended to fill with an excess volume of sample that corresponds to 3 to 5 times the loop- and valve-volume. This will be 60-100 µL with a 10 µL loop in the 20 µL case above. This recommendation is a general recommendation for all small SEC columns irrespective of the loop size chosen.

- Correspondingly, when injecting the sample into the column, empty the loop and the injection valve channels with an excess of buffer, see *Chapter 6 UNICORN settings, on page 22*.

Manual partial filling

It is possible to load a small sample volume in a larger loop with manual partial filling using the sandwich technique. With partial filling it is important to:

- Never load more than 50% of the loop volume due to the laminar flow profile.
- Make sure that the sample enters the loop, "the bottom" of the syringe should contain 10 µL of buffer which is the volume of the connecting channel (fill port channel + loop channel).
- Make sure the loop is filled with buffer before partial sample loading. Make sure that the waste tubing outlet is on the same level as the fill port to avoid siphoning of the loop when the syringe is not connected.
- Empty the loop with an excess of buffer, 3 to 5 times the loop volume.

Sample injection using Alias autosampler

Manual partial filling requires experience with liquid chromatography systems and a more reliable method is to use an Alias™ autosampler (Spark Holland).

The recommended volumes for manual loop filling also applies when using the Alias autosampler, both for complete and partial filling. Partial filling of a small loop, for example 1 µL in a 10 µL loop, is a way to further increase the resolution.

The table below shows recommended tubing for connection of the autosampler when running 3.2/300 column.

Table 5.1: 3.2/300 column system connected to autosampler

No.	From	port	To	port	Tubing i.d. (mm)	Tubing length (mm)
1	**V9-Inj**	E	Autosampler valve	1	0.15	500
2	**V9-Inj**	F	Autosampler valve	6	0.15	450

When running 5/150 and 10/300 columns, use the same lengths as above but with tubing i.d. 0.25 mm.

For more information about installing and operating the autosampler see, *Connect Alias autosampler to ÄKTA pure, article number 29040427*.

6 UNICORN settings

Sample loading with autosampler

For UNICORN programming with the Alias autosampler, see the instruction *Connect Alias autosampler to ÄKTA pure, article number 29040427*.

Sample loading with injection valve

When a small sample volume is loaded into the injection valve **V9-Inj**, flush the injection valve and the loop with a volume 3 to 5 times larger than the sample volume. Change the value for ***Empty loop*** with in the ***Phase Propertie***s for ***Sample Application***, as shown in *Fig. 6.1, on page 22*. Example: With 20 µL sample volume, enter 0.06 mL in ***Empty loop***.

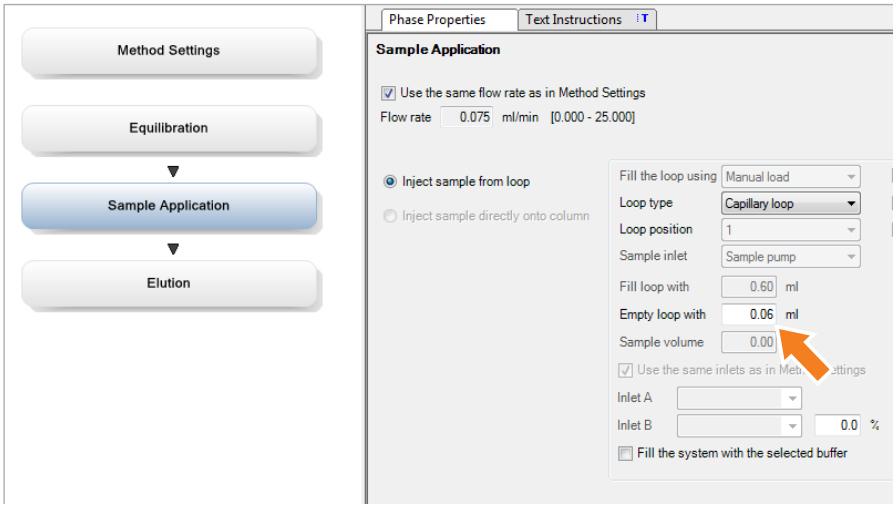


Figure 6.1:

Programmning new delay volume

When collecting fractions, insert the correct delay volume between UV flow cell and fraction collector into UNICORN, see *Delay volumes, on page 18*.

Follow the instructions below to insert delay volume in UNICORN.

1. Select ***System Settings*** in the ***System Control*** module.
2. Select ***Tubing and Delay Volumes*** and select ***Delay volume: Monitor to frac***.
3. Enter the delay volume.

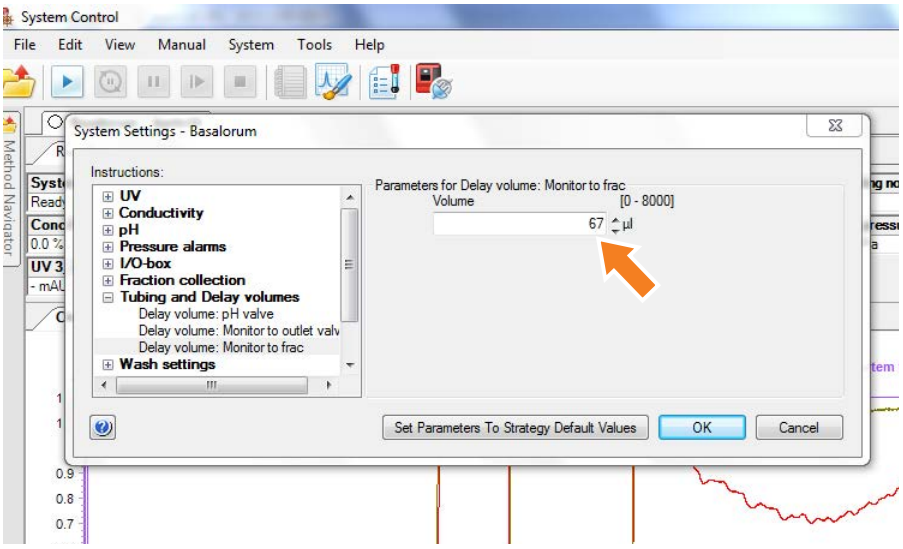


Figure 6.2:

UNICORN column lists

Make sure that you have the latest version of the column lists in UNICORN. See *UNICORN 6.1 (or later versions) Pre Defined Columns file*.

The lists are available on each system's product side on the web (under 'Related documents' and 'Software'), see *cytiva.com*.

How to handle pressure limits

The UNICORN column list must be updated with the columns listed on page 2. Make sure that the right column is chosen in the ***Method Settings*** phase when editing the method. This will automatically give a default value for the pressure limit.

The column valve **V9-C** has a built-in pressure sensor, while **V9-Cs** uses the system pressure sensor. UNICORN performs an automatic calculation to compensate for tubing backpressure between the injection valve and the column. This calculation is by default using a short 0.5 mm i.d. tubing, which gives a small compensation for tubing backpressure before the column.

UNICORN can be used to get a more valid compensation and to extend the pressure range available for the column. Follow the instructions below to set i.d. and length for the tubing between the injection valve and the column in UNICORN.

1. Select ***System Settings*** in the ***System Control*** module.
2. Select ***Tubing and Delay Volumes*** and select ***Tubing: Injection valve to column***.
3. Select ***I.D.*** for the tubing from the list.
4. Enter the tubing length.

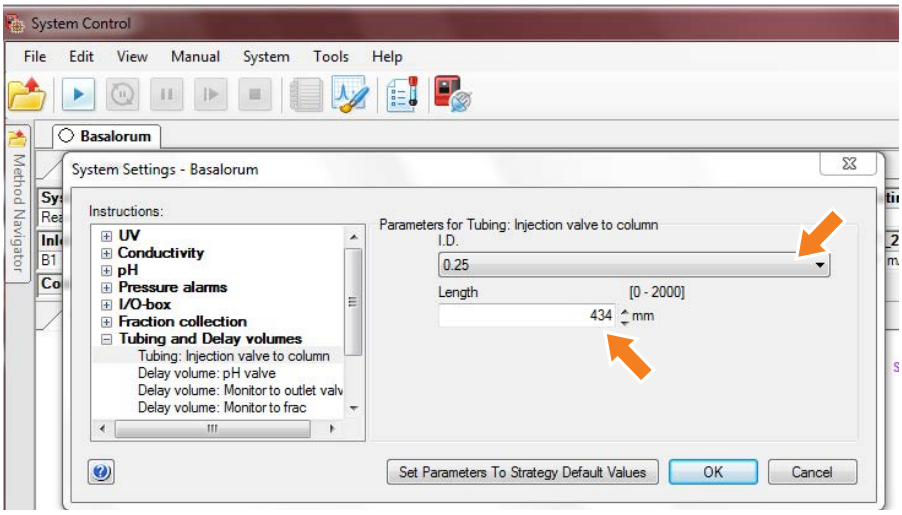


Figure 6.3:

If the used i.d. is not available in the list, use the nearest value.

To determine the pressure limit for each individual column, see the respective instruction that follows each column

7 Recommendations for other columns

System configured for 3.2/300 column

The 0.15 mm i.d. tubing gives high backpressure at flow rates around 1 mL/min and higher. To use HiTrap™ columns on the same system requires re-plumbing with 0.5 mm i.d. tubing.

System configured for 5/150 and 10/300 columns

The 0.25 mm i.d. tubing gives moderate backpressure at approximately 1 mL/min. A typical contribution from the tubing in the system is 0.2 to 0.3 MPa backpressure. This permits a wider array of columns to be used in the same system. Columns with pressure limits of 0.3 to 0.5 MPa (such as HiTrap columns) might require re-plumbing of the system with 0.5 mm i.d. tubing.

8 Ordering Information

System

ÄKTA pure 25 L	29018224
ÄKTA pure 25 M	29018226
ÄKTA pure 25 L1 (V9-IAB, V9-Os)	29018225
ÄKTA pure 25 M1 (V9-IAB, V9-Os)	29018227
ÄKTA pure 25 M2 (V9-IA, V9-IB V9-C V9-O)	29018228

Fraction collector

Fraction collector F9-R	29011362

Accessories

Fill port INV-907	18112766
Injection kit INV-907 (includes fill port, needles and syringe holder)	18111089
Injection needles, o.d. 0.70 mm blunt ended	18180142
Union 1/16″ Male - 1/16″ Male, i.d. 0.25 mm	18112092
Union 1/16″ Male - 1/16″ Male, i.d. 0.13 mm	18112090

Columns

Superdex 200 Increase 10/300 GL	28990944
Superdex 200 Increase 5/150 GL	28990945
Superdex 200 Increase 3.2/300	28990946
Superose 6 Increase 10/300 GL	29091596
Superose 6 Increase 5/150 GL	29091597
Superose 6 Increase 3.2/300	29091598

Tubing

PEEK Tubing, 2 m, i.d. 0.15 mm, o.d.1/16"	18115659
Tubing i.d. 0.25 mm, o.d. 1/16" (2 m)	18112095
Tubing kit i.d. 0.25 mm (precut tubing)	29011328

Silica tubing and accessories (from the company IDEX)

IDEX tubing sleeve for silica tubing o.d. 0.360 mm, NanoTight™ Sleeve Green	F-242X
IDEX silica tubing o.d. 0.360 mm i.d. 0.150 mm, 2m	FS-115
IDEX fused silica tubing cutter	FS-315

Related literature

ÄKTA pure Operating Instructions	29022997
ÄKTA pure User Manual	29119969
Connect Alias autosampler to ÄKTA pure	29040427

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