DIONEX

Thermo SCIENTIFIC

UltiMate 3000 RSLCnano

Standard Applications

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UHPLC+ focused

Table of Contents

1 Introduction

This document describes the standard setup, recommended experimental conditions and testing procedures to run standard applications on the Thermo Scientific Dionex UltiMate 3000 RSLCnano system.

Notes: This document is intended for Thermo Fisher Scientific (or authorized) service personnel as well as customers to assist in the installation and system test of UltiMate™ 3000 RSLCnano systems.

It is assumed that the individual using this manual has sufficient training in the installation and usage of analytical instrumentation and is aware of the potential hazards including (but not limited to) electrical hazards, chemical solvent hazards, exposure to UV radiation and the exposure to pressurized solvents.

At various points throughout the manual, messages of particular importance are indicated by the following symbols:

Tip: Indicates general information intended to optimize the installation and setup steps or the performance of the instrument.

Important: Indicates that failure to take note of the accompanying information may result in damage to the instrument.

Warning: Indicates that failure to take note of the accompanying information may result in personal injury.

This manual is provided "as is." Every effort has been made to supply complete and accurate information and all technical specifications have been developed with the utmost care. The information contained in this manual should not be construed as a commitment by Thermo Fisher Scientific. Thermo Fisher Scientific assumes no responsibility for any errors that may appear in this document. This document is believed to be complete and accurate at the time of publication. In no event shall Thermo Fisher Scientific be liable for incidental or consequential damages in connection with or arising from the use of this document.

The information contained in this document is subject to change without notice.

1.1 Additional Documents

In addition to the information provided in this manual, the following documents are available:

- Operating Instructions of the individual modules
- Installation Qualification manual for installation of the UltiMate 3000 RSLCnano
- Micro Fraction Collection Option for the Thermo Scientific Dionex UltiMate WPS-3000PL Nano/Cap Autosampler
- Application/technical notes to be published on the Dionex website

In addition to these documents, Chromeleon templates are available for these applications. Please contact your local Thermo Scientific sales or service representative to obtain the templates.

2 Application Setup

2.1 General Recommendations for Applications

The experimental conditions for each application are presented together with related information such as schematics, installation tips and examples, results and interpretation.

2.1.1 Connections

All high-pressure fittings used in the applications on the UltiMate 3000 RSLCnano system are made with nanoViper. nanoViper is a fingertight high-pressure fitting that is dead volume free by design and back pressure resistant up to 1000 bar. The fittings are assembled in the factory to ensure consistent fittings and prevent experimental failure due to bad connections.

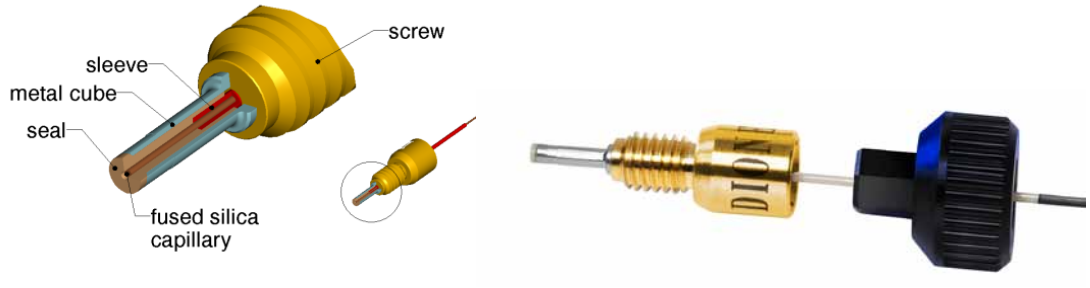


Figure 1: Example of a nanoViper fitting

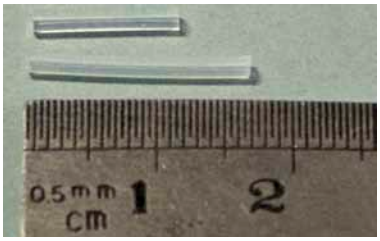
1. Install nanoViper as any fingertight fitting.
2. Do not over-tighten connections (general guide line: finger-tight + maximum an additional one eighth-turn).
3. Remove the black knurled screw.

The outlet of the nano columns is fitted with a nano connector. This is a dedicated connection designed to offer maximum flexibility in connecting fused silica capillaries and offering pressure stability up to 300 bars. The nano connector uses a special sleeve to ensure pressure tightness. The assembly of a nano connector is described step by step below.

Figure 2: nano connector layout

- Use a new nano connector sleeve (P/N 6720.0391) for each connection

⚠ **Important:** Do NOT use a PTFE sleeve. The size does not match the nano connector and the pressure resistance is much lower.



Nano connector sleeve (~1.1 cm)

PTFE (1.8 cm)

Figure 3: nano connector (top) and PTFE (bottom) sleeve comparison

1. Slide the golden nut and silver union onto one of the fused silica outlets; slide the second golden union on the other fused silica end. (→ Figure 4a and Figure 4b)
2. Slide the nano connector sleeve onto the fused silica, slide the other end of the fused silica into the nano connector sleeve and make sure the connection is dead volume free. Figure 4c
3. Tighten the golden nuts equally fingertight to ensure the nano connector sleeve is centered in the silver union. Figure 4d
4. For traditional fittings, for example, PTFE sleeves see Appendix – Traditional Capillary Connections (page 54)

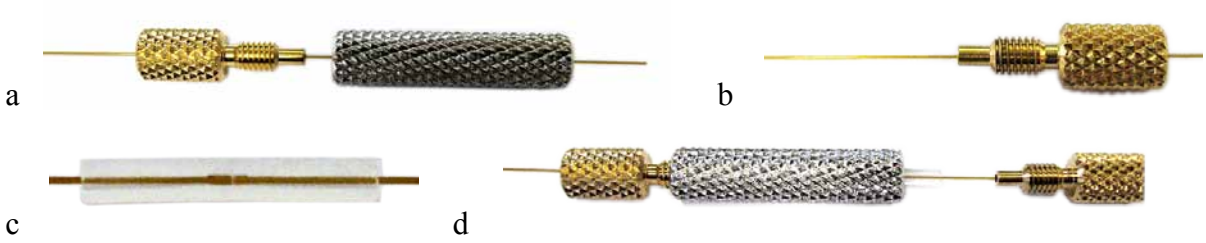


Figure 4: Using the nano connector

2.1.2 Sample Preparation

- Follow the instructions provided with the test sample and use the solvents as indicated.
- After re-dissolving the sample wait at least 15 min before further diluting it.
  - Use water with a minimum of 2 % ACN and appropriate ion-pairing agent to dissolve.
  - Dilution can be done in the same solvent or with water with appropriate ion-pairing agent.

Tip: To limit the risks of peptides or proteins adsorption on the walls of the vials, Thermo Fischer Scientific recommends using glass inserts (Polypropylene vials for WPS with glass insert, 250 µL, set of 25, P/N 6820.0027).

Tip: When using IEX columns, make sure that the sample solvent contains a very limited amount of salt and is at the right pH level (for example, adjust to pH 3 when using a SCX column to separate peptides).

2.1.3 Mobile Phases

- Always use fresh solvents.
- When running online multidimensional approaches, make sure that the mobile phase of the first dimension will allow for trapping the sample on the (trap) column used on the second dimension.
- For example, do not use phosphate buffers from SCX separations as loading solvent in RP separations.
- Thermo Fischer Scientific recommends replacing (aqueous) solvents at least once every two weeks.

Tip: Replace solvents completely; do not 'top up' to avoid unwanted components building up in the mobile phases.

2.2 Installing the UltiMate 3000 RSLCnano System

The general UltiMate 3000 RSLCnano system overview is shown in Figure 5. The modular nature of the Thermo Scientific Dionex UltiMate 3000 platform does allow modifications to this layout.

SRD-3400, SRD-3200 with degassing or
SR-3000 without degassing.
NCS-3500RS module featuring
- NC pump, up to 800 bar
- Loading pump, micro Titanium up to 500 bar
- Column compartment with up to two switching valves

Optional:
- NCP-3200RS
- PAEK valve

VWD-3400RS with flow cells for
- nano (3nL),
- capillary (45 nL)
- micro (180 nL) LC

WPS-3000TPL RS
- Temperature controlled autosampler equipped with a 1000 bar switching valve
- *Optional:*
  8-port valve (350 bar) for automated off-line applications

Figure 5: RSLCnano system overview

Figure 6 shows the interior of the NCS-3500RS module. The module consists of two pumps and an integrated column compartment. The most important elements required for system setup are indicated in the picture.
Figure 7 shows the interior of the NCP-3200RS module. This module contains only the NC_Pump, but is identical in capabilities and performance as the NC_Pump in the NCS-3500RS.

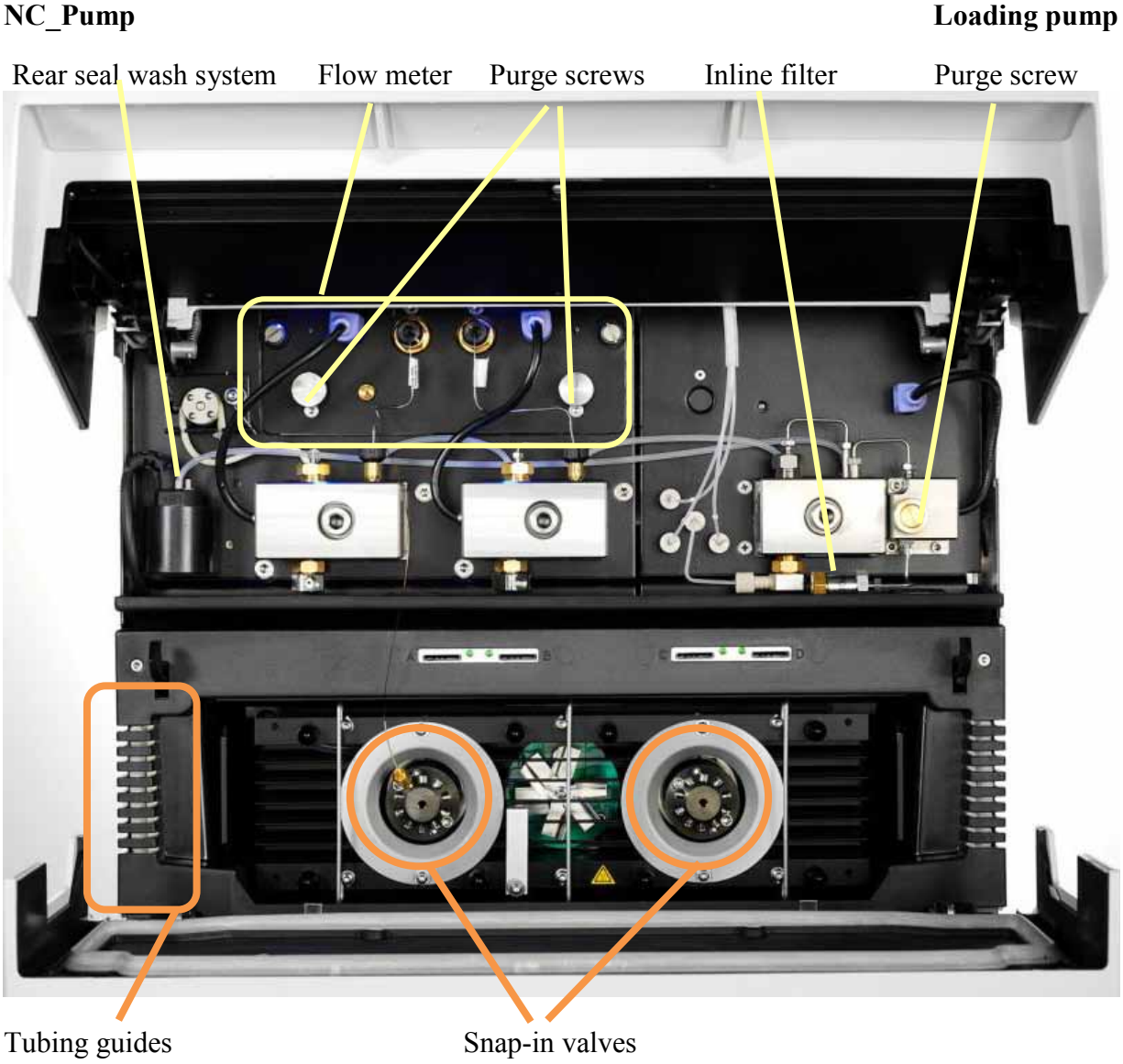


Figure 6: Detailed overview of the NCS-3500RS interior

Figure 7: Detailed overview of the NCP-3200RS interior, components are identical to the NC_Pump in the NCS-3500RE

Before the parts of the application kit are installed, the UltiMate 3000 RSLCnano system has to be prepared and primed. To prepare the system, the following steps are required. For more details, see the operating instructions for the respective module.

1 To control the module, Thermo Scientific Dionex Chromeleon 6.80 SR8 (or later) or DCMSLink version 2.8 (or later) and appropriate license are required.

2 Prepare electrical and USB connections, power up modules, and then prepare server configuration.

3 Prepare solvents and install them according to the application.
- NC pump channels A and B
- Loading pump channels A, B, and C (10-50% isopropanol in water for unused channels)
- Rear seal wash solvent (~10% isopropanol in water)
- Autosampler wash solvent (~10% isopropanol 0.1% FA in water).

Important Use the PEEK solvent inlet filter frits in both, the NC pump and loading pump solvent lines.

Tip Degassing of the loading pump solvents is required when the loading pump is used for gradient formation or the flow rate is above 20 µL/min.

4 Purge both blocks of the NC pump (min 30 min.), while assisting with a syringe until liquid exits the purge lines. Purge all channels of the loading pump (minimum 10 min).

Tip Make sure that the purge screws of the NC_Pump are entirely opened.

5 Purge flow meter for 30 min.

Tip The purge time with a nano flow selector is 30 minutes. For capillary and micro flow selectors purge times may be shorter. Please refer to the NCS-3500RS or NCP-3200RS manual for details.

6 Perform pressure sensor offset calibration, using Chromeleon Diagnostics. To do so, open the More options panel of the NC_Pump and click the related button.

7 Perform viscosity calibration using Chromeleon Diagnostics or select default viscosities from the available viscosity list.

Tip To see the solvent list for which default viscosities are available, use the Commands dialog (F8) and scroll to Pump module → NC_Pump → %A_Viscosity (%B_Viscosity).

8 Prepare all fluidics using the provided nanoViper connection tubing.

Tip Do not overtighten the fingertight connection!

2.3 Application Overview

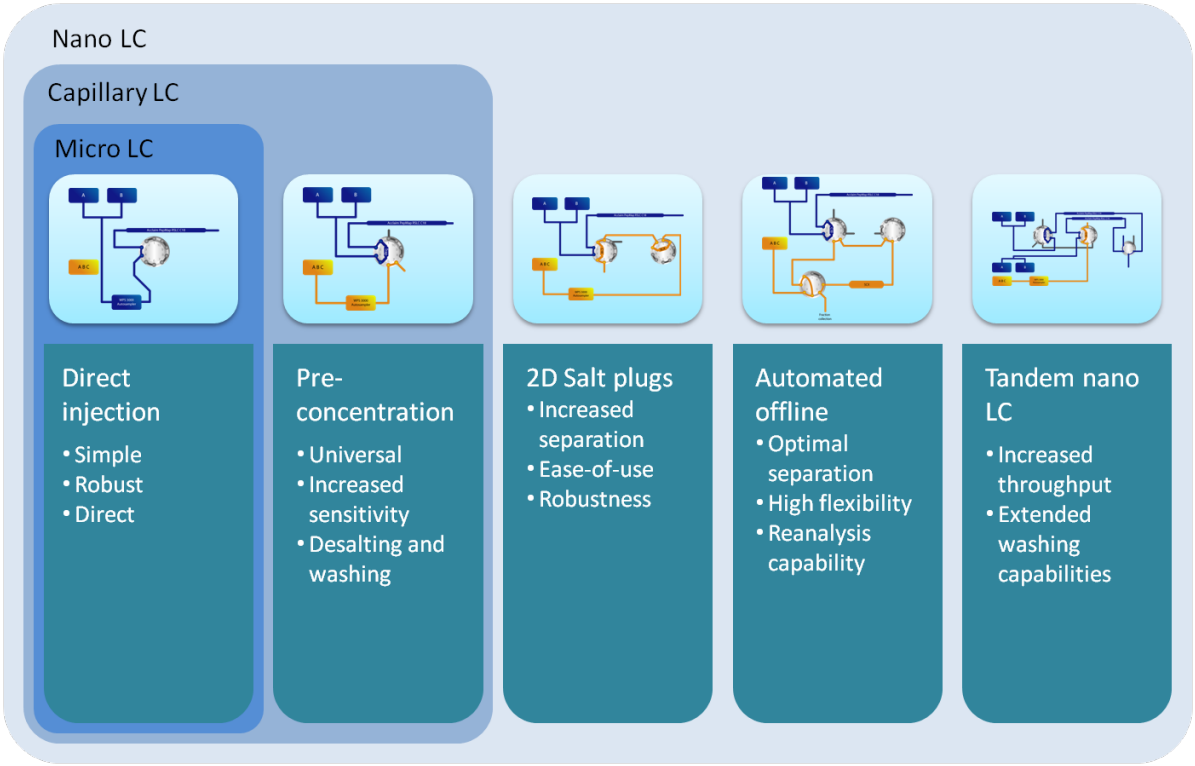


Table 1: Property overview of different flow selectors

Flow Selector Type	**I.D.**	**P/N**	**Total Flow Rate** *(Sum of channel A and B)*		
			Nominal	**Minimum**	**Maximum**
Nano	Nan	6041.0002	500 nL/min	50 nL/min*	1000 nL/min
Capillary	Cap	6041.0003	5 µL/min	500 nL/min	10 µL/min
Micro	Mic	6041.0014	25 µL/min	2.5 µL/min	50 µL/min

*Lower flow rates are available upon request

2.4 Direct Injection onto a Nano Column

2.4.1 Hardware Layout

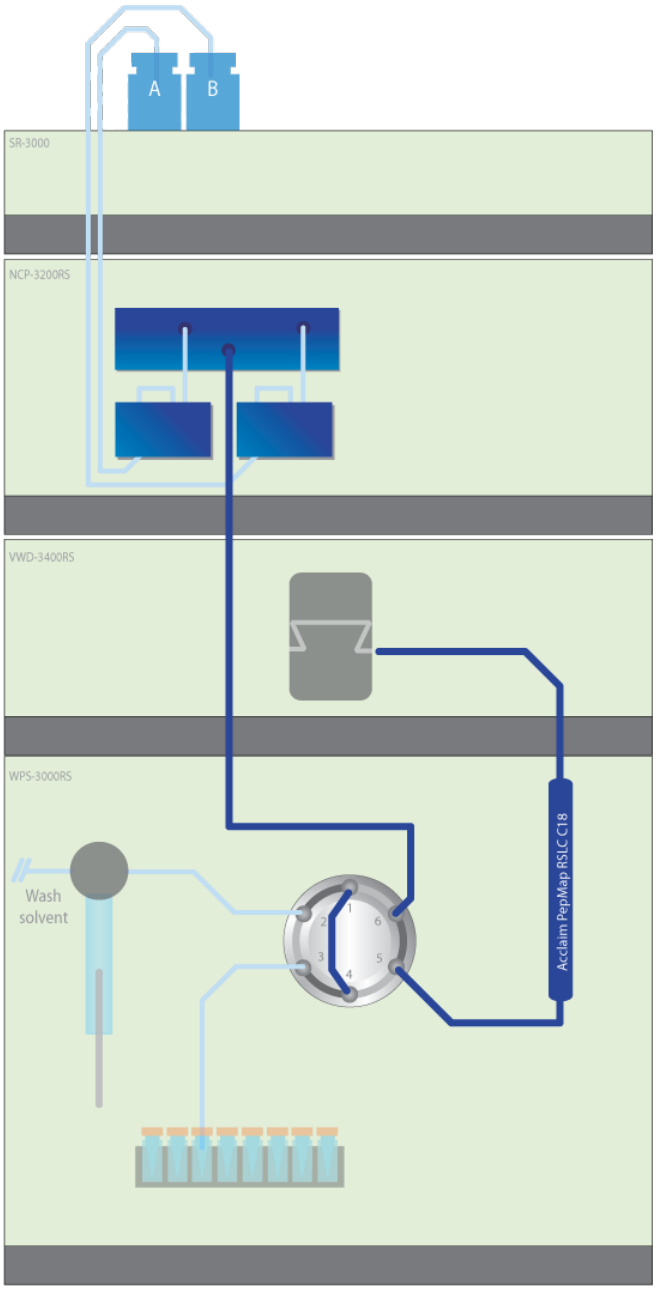


Figure 8: Setup for a Direct Injection experiment onto a nano column

The preferred setup is presented in Figure 8 and consists of:

SR-3000	5035.9200
NCP-3200RS	5041.0030
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0300

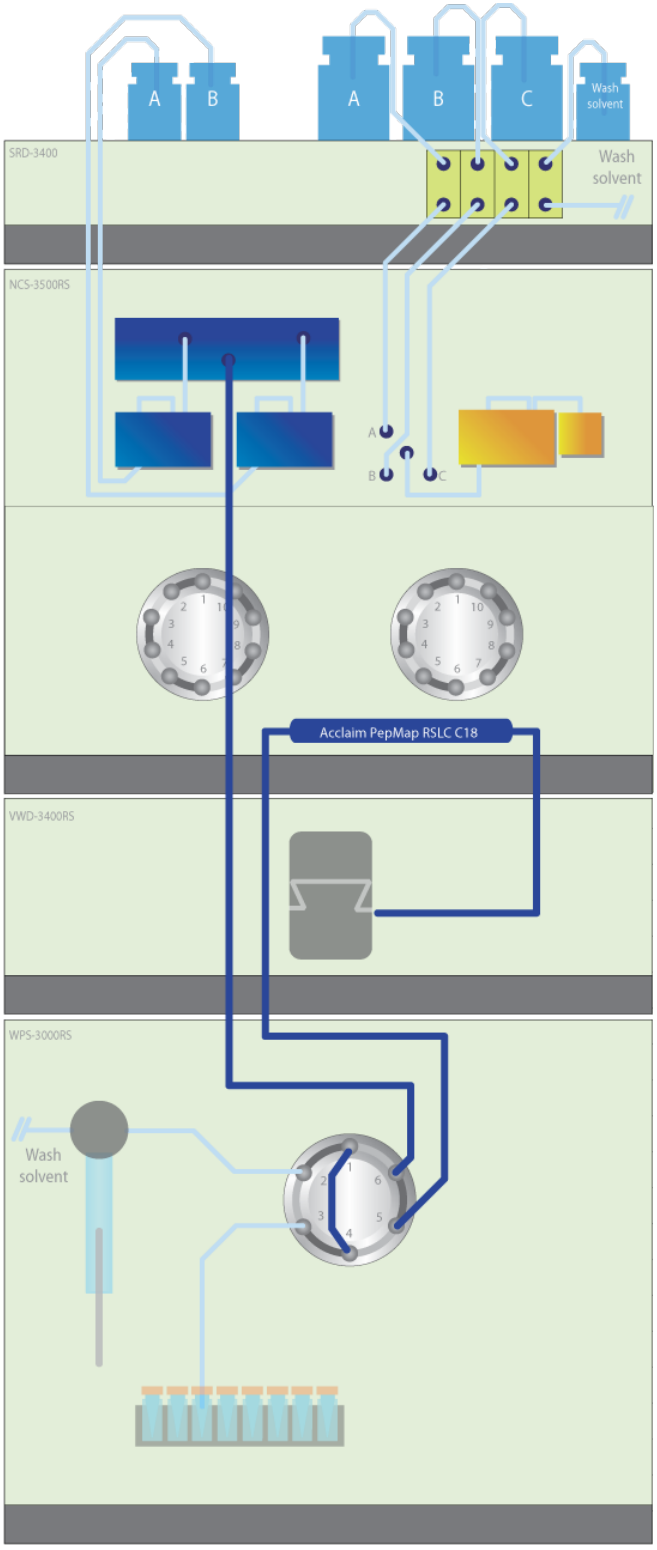


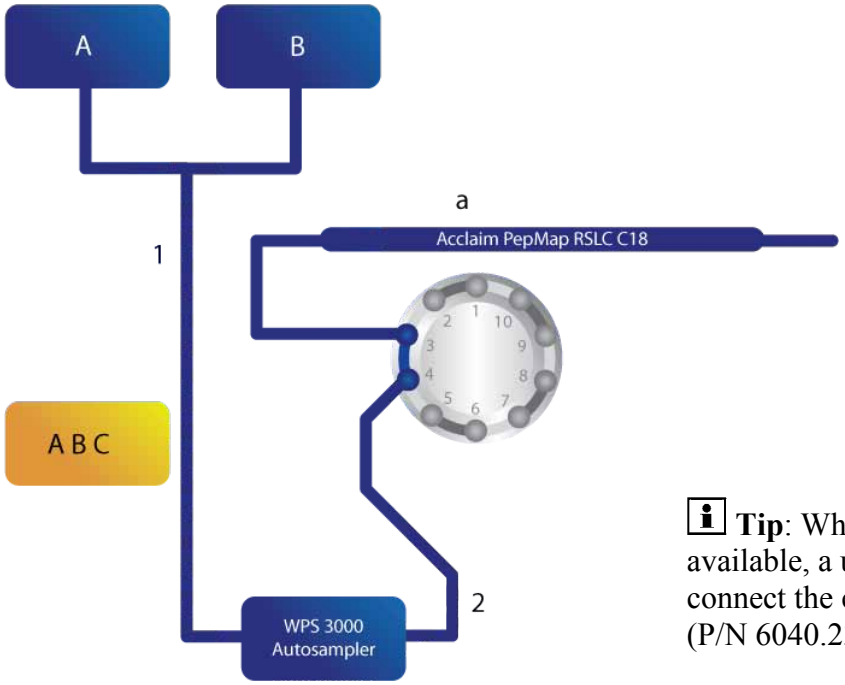
Figure 9: Setup for a Direct Injection experiment onto a nano column

The alternative setup is presented in Figure 9 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0300

2.4.2 Fluidic Setup

Figure 10 presents the setup using the parts of the Direct Injection application kit. Columns are marked with letters, tubing with digits, and the sample loop is installed in the WPS-3000PL Autosampler



Tip: When no valve is available, a union can be used to connect the capillary column (P/N 6040.2304)

Figure 10: Fluidic connections for a Direct Injection experiment onto a nano column

Table 2: UltiMate 3000 RSLCnano Direct Injection nano LC kit (P/N 6720.0300) contents

#	Item	Replacement P/N
a	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 550 mm	6041.5260
	nanoViper sample loop 1 µL, FS/PEEK sheathed I.D. x L 100 µm x 127 mm	6826.2401
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089

2.4.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- The impact of dwell (dead) volumes on reproducibility is very important. Improper connections of the different elements are the most likely cause of failure for this application.

2.4.4 Testing the Application

The direct injection can be tested using the following conditions:

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v) water/ACN + 0.04% TFA
Sample	Cytochrome C digest 1 pmol/µL, prepared according to the instruction sheet
Injection volume	1 µL
UV detection	214 nm
Gradient	4% to 55% B in 30 min 90% B for 5 min 25 min equilibration
WPS temperature	5°C (WPS-3000(B)T only)
Flow rate	300 nL/min (nano flow selector)

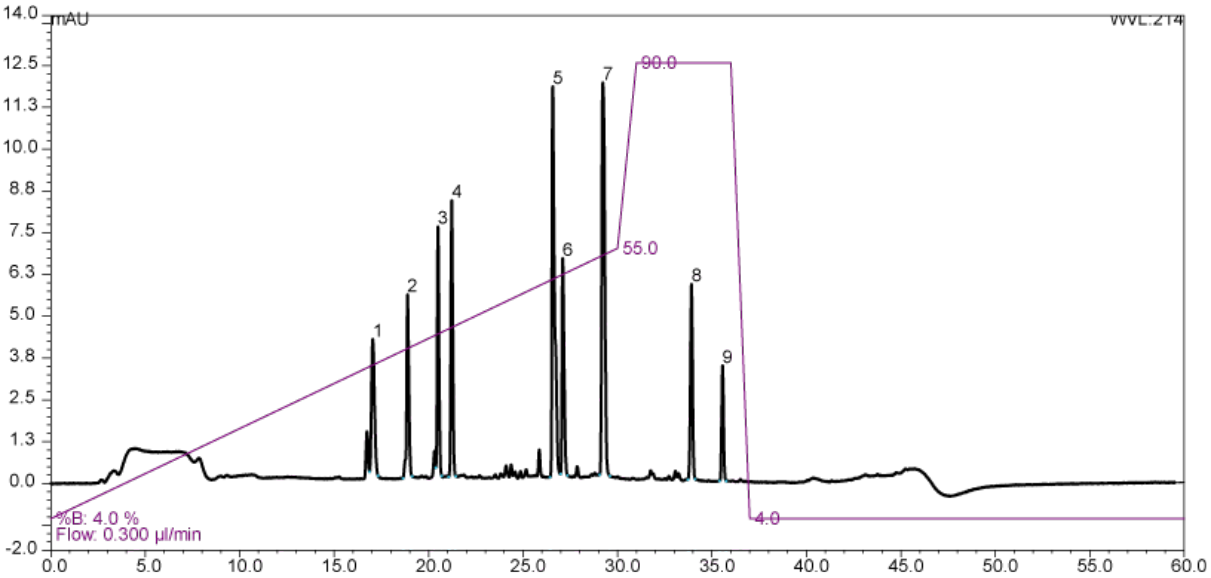


Figure 11: Typical chromatogram for a Direct Injection of 1 pmol Cyt C onto a nano column

For details on interpretation and troubleshooting of the Cytochrome C nano LC separation, see the FAQ section on page 50.

2.4.5 Large Volume Injections

Typically direct injections in nano LC are performed with 1 µL loop sizes to minimize the gradient delay. Larger volume injections are performed with a pre-concentration setup. The WPS-3000PL autosampler series allows for a custom injection program (UDP) to switch back the injection valve after sample loading to bypass the loop. This way a larger sample volume can be injected and pre-concentrated directly onto the nano column, without using a pre-concentration setup.

The advantage of such a setup is the ease of use and a minimum loss of peptides, especially hydrophilic ones. The prerequisites of this setup are desalted samples, since all that is injected will enter the MS, and an investment of extra analysis time to accommodate the loading of sample with nano flow.

2.5 Direct Injection onto a Capillary Column

2.5.1 Hardware Layout

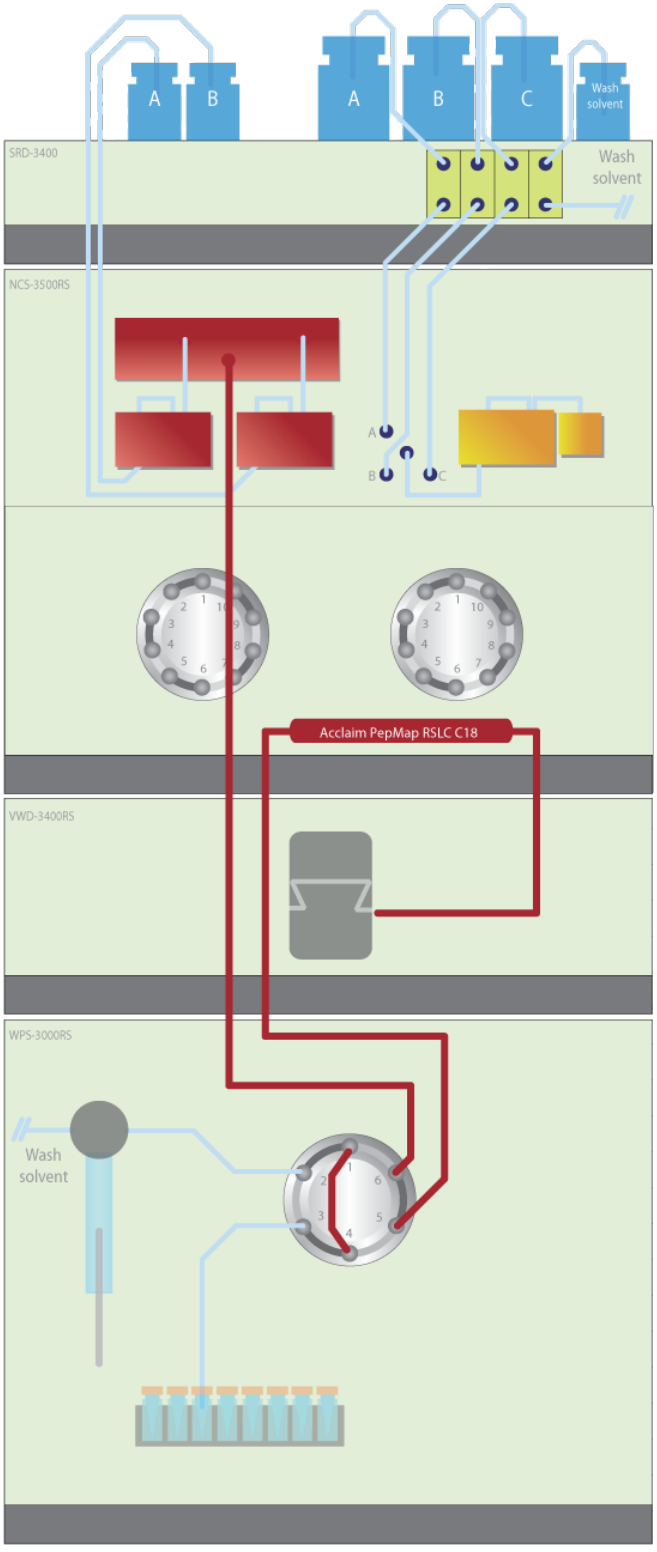


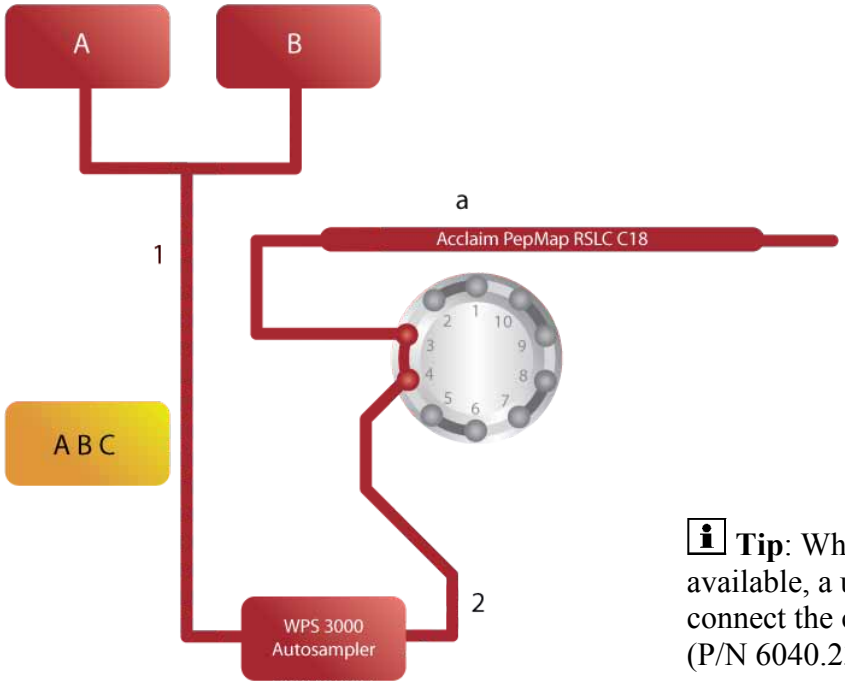
Figure 12: Setup for a Direct Injection experiment onto a capillary column

The recommended setup is presented in Figure 12 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0020
VWD-3400RS	5074.0010
45 nL flow cell	6074.0280
WPS-3000TPL RS	5826.0020
Application kit:	6720.0305

2.5.2 Fluidic Setup

Figure 13 presents the setup using the parts of the Direct Injection application kit. Columns are marked with letters, tubing with digits, and the sample loop is installed in the WPS-3000PL Autosampler.



Tip: When no valve is available, a union can be used to connect the capillary column (P/N 6040.2304)

Figure 13: Fluidic connections for a Direct Injection experiment onto a capillary column

Table 3: UltiMate 3000 RSLCnano Direct Injection capillary LC Kit (P/N 6720.0305) contents

#	Item	Replacement P/N
a	300 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164537
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 750 mm	6041.5580
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 550 mm	6041.5560
	nanoViper sample loop 5 µL, FS/PEEK sheathed I.D. x L 200 µm x 159 mm	6826.2405
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089

2.5.3 Installation Tips

Follow the General Recommendations for Applications (→ page 6).

2.5.4 Testing the Application

The direct injection can be tested using the following conditions:

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v) water/ACN + 0.04% TFA
Sample	Cytochrome C digest 8 pmol/µL, prepared according to the instruction sheet
Injection volume	1 µL
UV detection	214 nm
Gradient	4% to 55% B in 30 min 90% B for 5 min 25 min equilibration
WPS temperature	5°C (WPS-3000(B)T only)
Flow rate	4 µL/min (capillary flow selector)

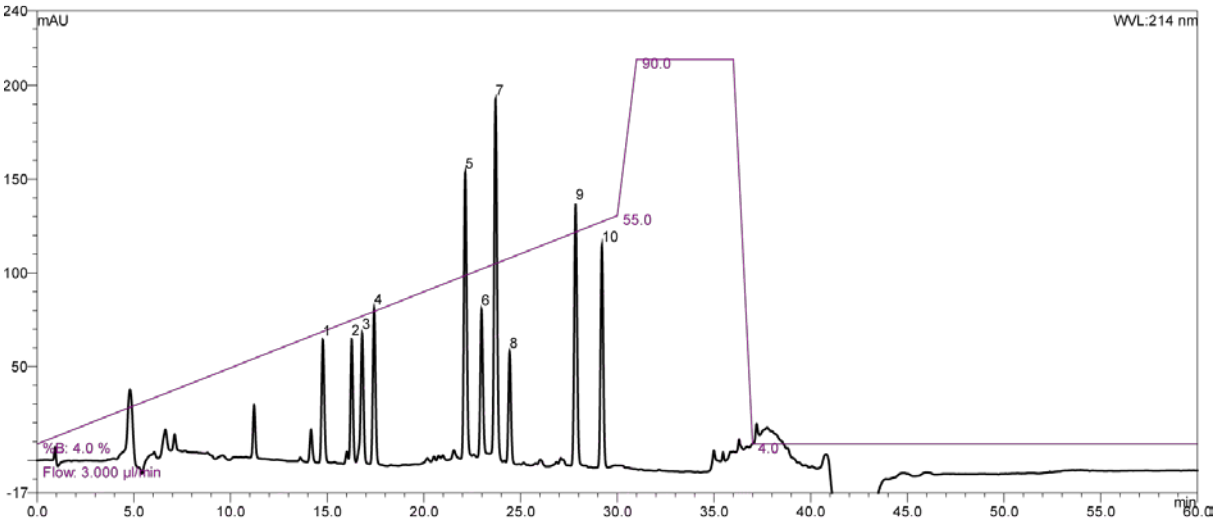


Figure 14: Typical chromatogram for a Direct Injection of 8 pmol Cyt C onto a capillary column

2.6 Pre-concentration onto a Nano Column

2.6.1 Hardware Layout

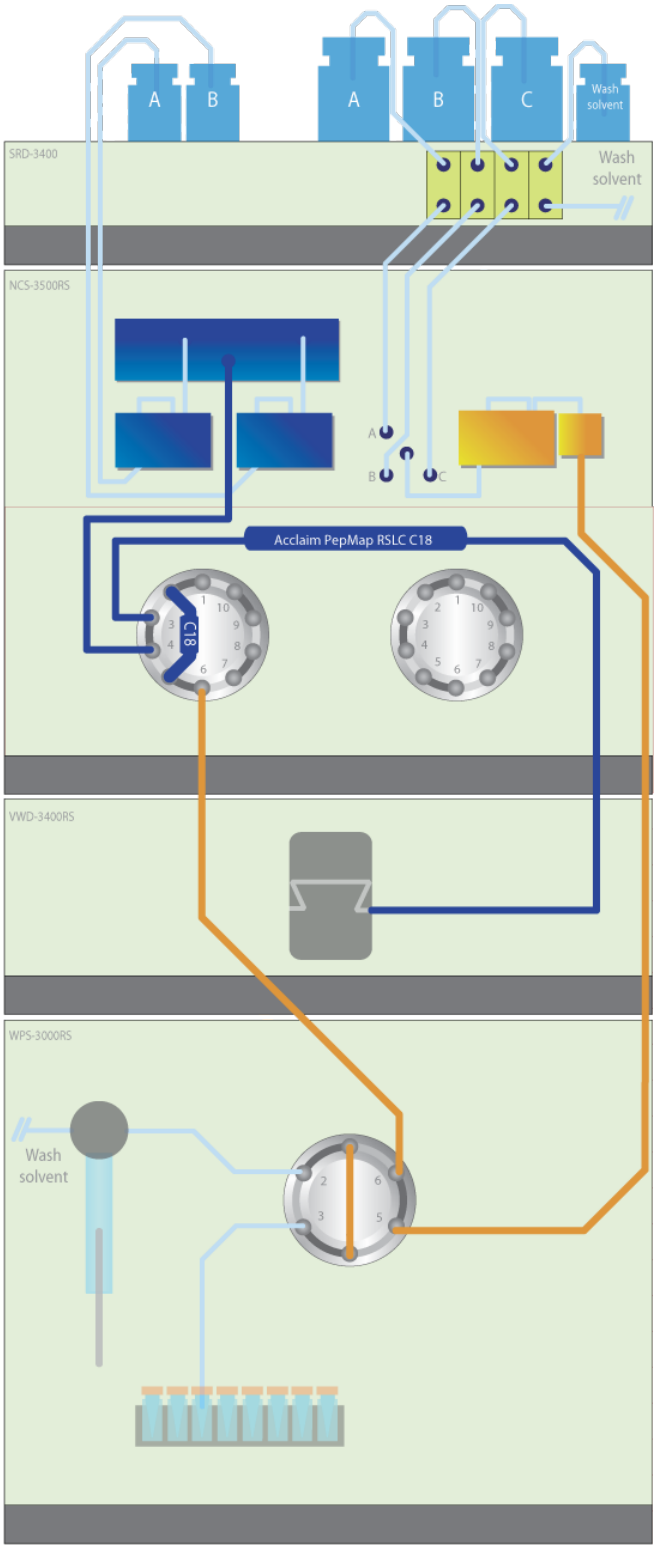


Figure 15: Setup for a Pre-concentration experiment onto a nano column

The recommended setup is presented in Figure 15 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
10-port sw. valve	6041.0001
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0310

2.6.2 Fluidic Setup

Figure 16 presents the setup using the parts of the Pre-concentration application kit. Columns are marked with letters, tubing with digits, and the sample loop is installed in the WPS-3000PL Autosampler

Tip: The schematic shows a 10-port switching valve, but this application can be performed on a 6-port valve. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

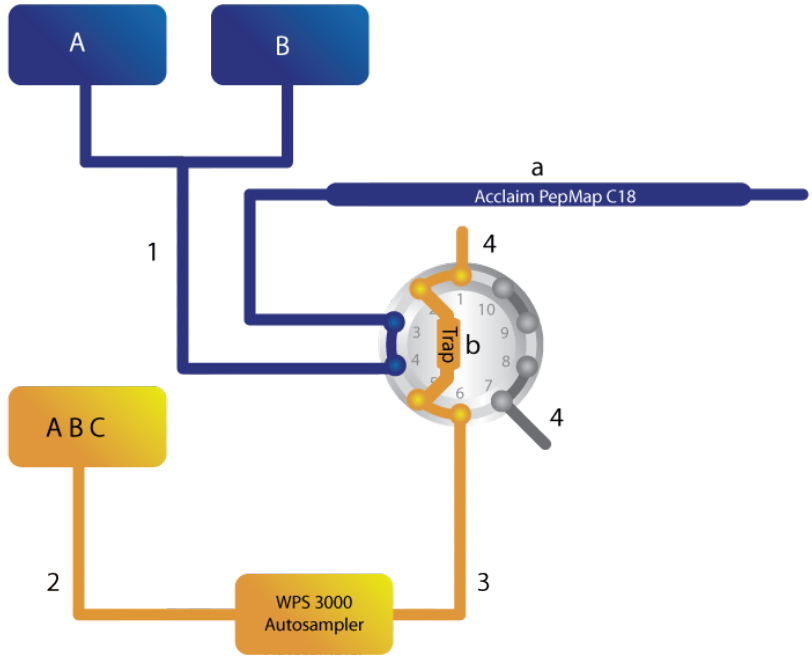


Figure 16: Fluidic connections for a Pre-concentration experiment onto a nano column

Table 4: UltiMate 3000 RSLCnano Pre-concentration nano LC kit (P/N 6720.0310) contents

#	Item	Replacement P/N
a	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
b	Nano Trap Column, 75 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 3 µm, 100Å (set of 2) nanoViper	164535
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 650 mm	6041.5775
3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 550 mm	6041.5760
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
	nanoViper sample loop 20 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2420
4	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015

2.6.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- The design of the nano trap column provides the easiest connections, but must only be used in forward flush operation. Please check the indicated flow direction when installing a nano trap column.
- If the loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 Water/ACN + 0.05% TFA or the use of a stronger ion-pairing agent such as heptafluorobutyric acid (HFBA) can be considered.

2.6.4 Testing the Application

The pre-concentration setup can be tested using the following conditions:

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v) water/ACN + 0.04% TFA
Loading solvent	98%/2% (v/v) water/ACN + 0.05% TFA
Sample	Cytochrome C digest 1 pmol/µL, prepared according to the instruction sheet
Injection volume	1 µL (partial loop fill of a 20µL loop)
UV detection	214 nm
Loading time	3 min (may vary with different injection volume/routine)
Gradient	4% to 55% B in 30 min 90% B for 5 min 25min equilibration
WPS temperature	5°C (WPS-3000(B)T only)
Loading flow	5 µL/min
Flow rate	300 nL/min (nano flow selector)

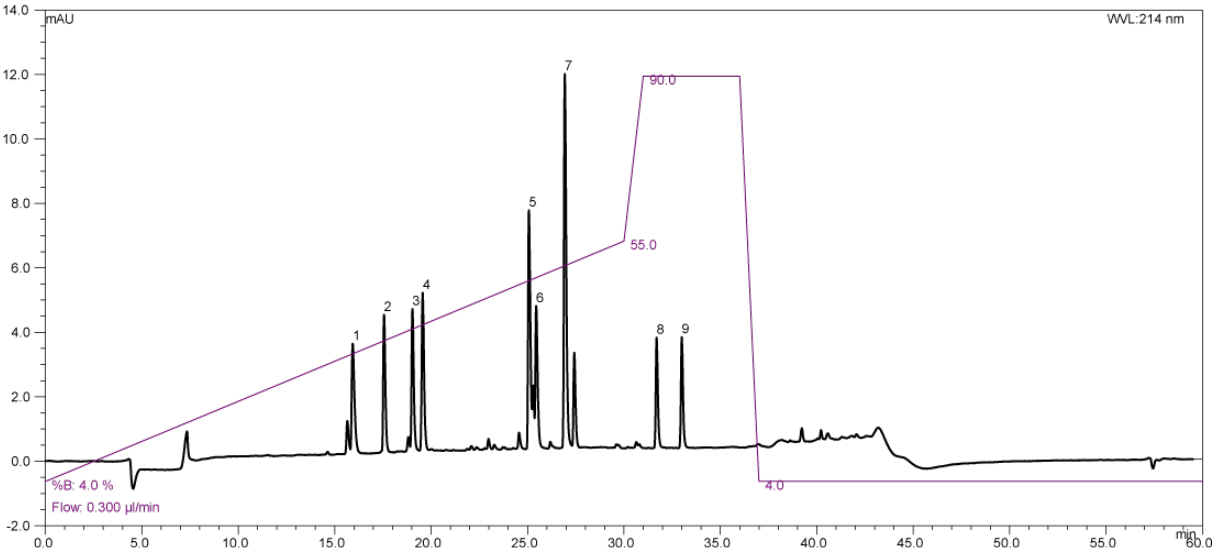


Figure 17: Typical chromatogram for Pre-concentration of 1 pmol Cyt C onto a nano column

To evaluate the result of the experiment, the chromatogram should look very similar to the one obtained with direct injection.

For details on interpretation and troubleshooting of the Cytochrome C nano LC separation, see the FAQ section on page 50.

Tip: The example chromatogram provided (Figure 17) has been obtained under optimal conditions. When having difficulties to obtain a similar result, focus on the delay of the 'injection peak' and 'wash peak'; for example, check for any dwell (dead) volume.

2.7 Pre-concentration onto a 200 µm Monolithic Column

2.7.1 Hardware Layout

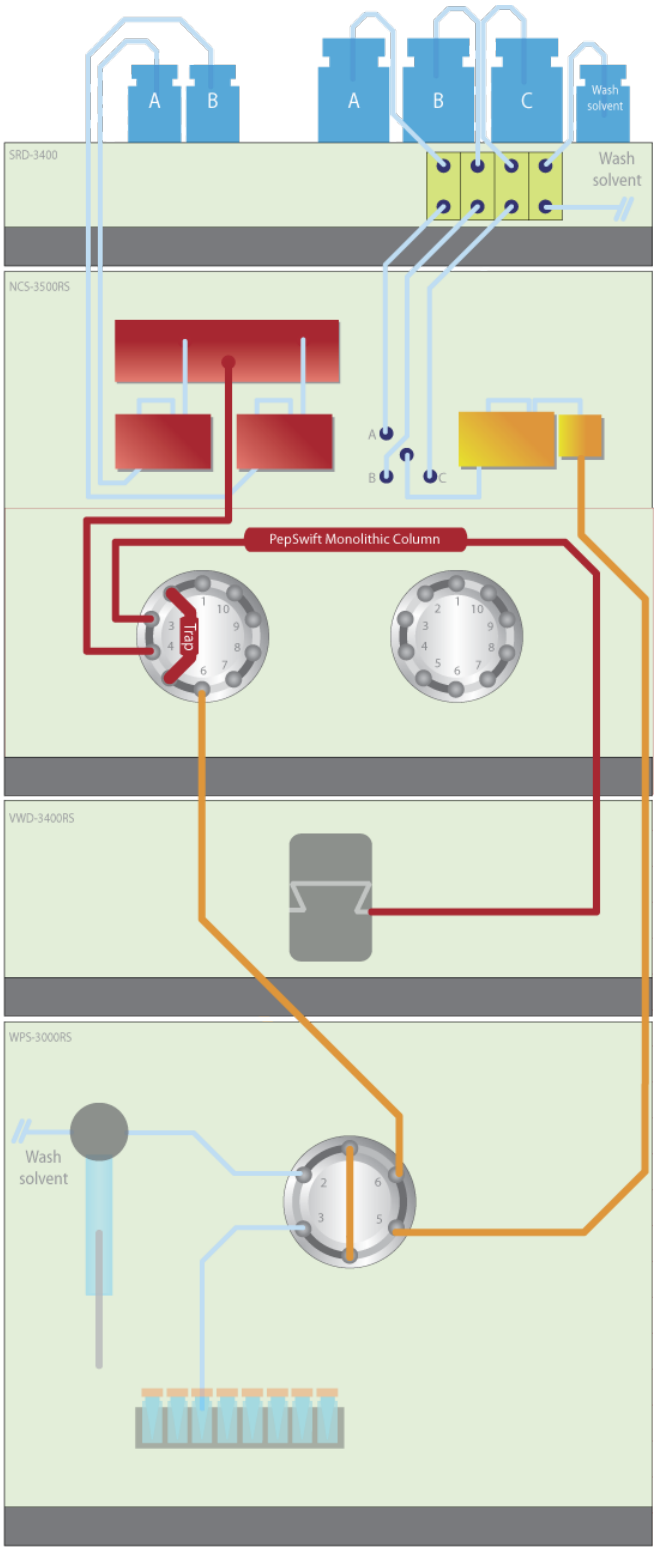


Figure 18: Setup for a Pre-concentration experiment onto a monolithic column

The recommended setup is presented in Figure 18 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0020
10-port sw. valve	6041.0001
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0320

2.7.2 Fluidic Setup

Figure 19 presents the setup using the parts of the Pre-concentration application kit. Columns are marked with letters, tubing with digits, and the sample loop is installed in the WPS-3000PL Autosampler.

Tip: The schematic shows a 10-port switching valve, but this application can be performed on a 6-port valve. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

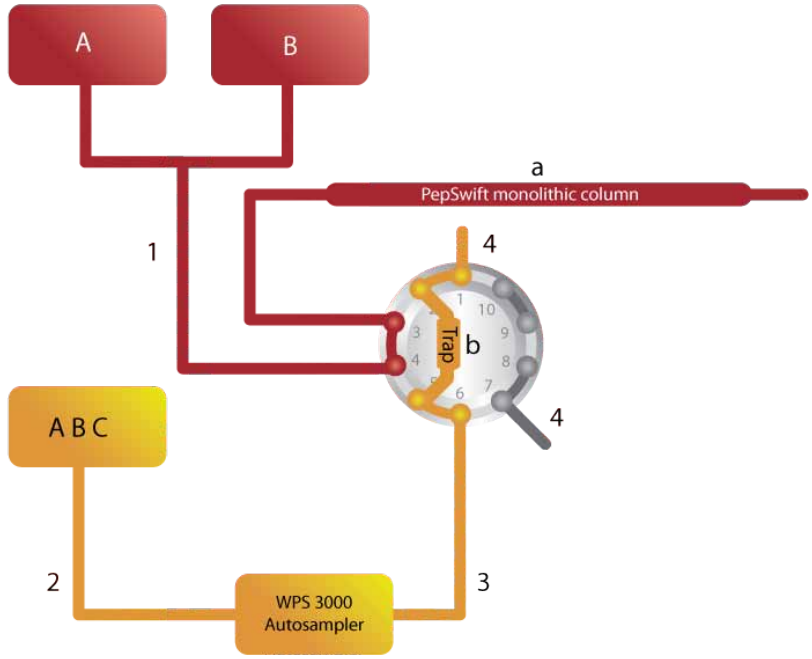


Figure 19: Fluidic connections for a Pre-concentration experiment onto a monolithic column

Table 5: UltiMate 3000 RSLCnano Pre-concentration monolithic LC kit (P/N 6720.0320) contents

#	Item	Replacement P/N
a	PepSwift Monolithic Capillary Column, 200 µm I.D. x 5 cm (PS-DVB), nanoViper	164557
b	PepSwift Monolithic Trap Column, 200 µm x 5 mm (PS-DVB), set of 2, nanoViper	164558
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 350 mm	6041.5540
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 650 mm	6041.5775
3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 550 mm	6041.5760
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 750 mm	6041.5580
	nanoViper sample loop 20 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2420
4	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015

2.7.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- Using a stainless steel loop (on a standard system) instead of a PEEK loop is recommended in case of injecting highly diluted samples.
- With column oven temperatures below 45°C, TFA can be used instead of HFBA in the loading solvent to load the sample onto the trap column.
- Due to the speed of the separation, the value for the detector's time constant (response) must be reduced to 0.1s.
- Concentration of HFBA in the loading solvent can be increased to 0.1% in case trapping problems are observed.
- The loss of hydrophilic peptides (for example, peaks 1 and 2 are lower than expected) may (partially) be decreased by lowering the oven temperature.

2.7.4 Testing the Application

The pre-concentration setup can be tested using the following conditions:

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	50%/50% water/ACN + 0.04% TFA
Loading solvent	100% water + 0.05% HFBA
Sample	Cytochrome C digest 1 pmol/µL, prepared according to the instruction sheet **Note: The sample must be diluted in the loading solvent.**
Injection volume	0.5 µL (partial loop)
UV detection	214 nm
Loading time	3 min (may vary with different injection volume/routine)
Gradient	1% to 70% B in 8 min 90% B for 2 min 8.5 min equilibration
Oven temperature	60°C
WPS temperature	5°C (WPS-3000(B)T only)
Loading flow	10 µL/min
Flow rate	3.0 µL/min (capillary flow selector)

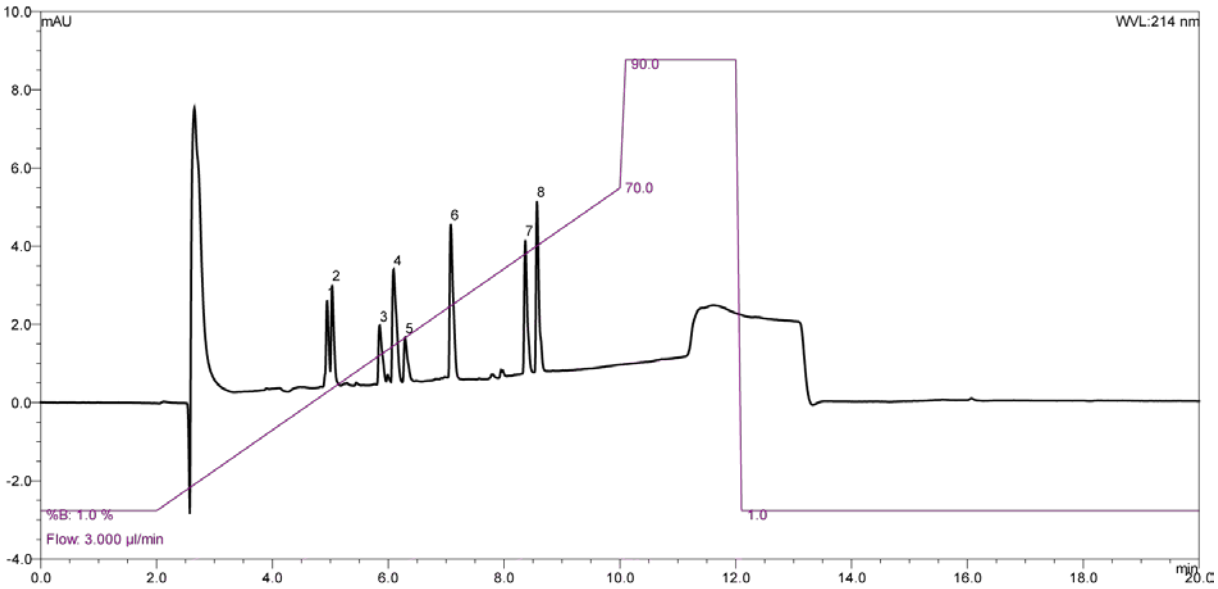


Figure 20: Typical chromatogram for Pre-concentration of 0.5 pmol Cyt C onto a monolithic column

Tip: When the trap column is switched in line with the analytical column, a large positive peak is detected at 214 nm. This is due to the different UV absorbance of the ion-pairing agents used in the loading and analytical solvent.

2.8 Pre-concentration on a Capillary Column

2.8.1 Hardware Layout

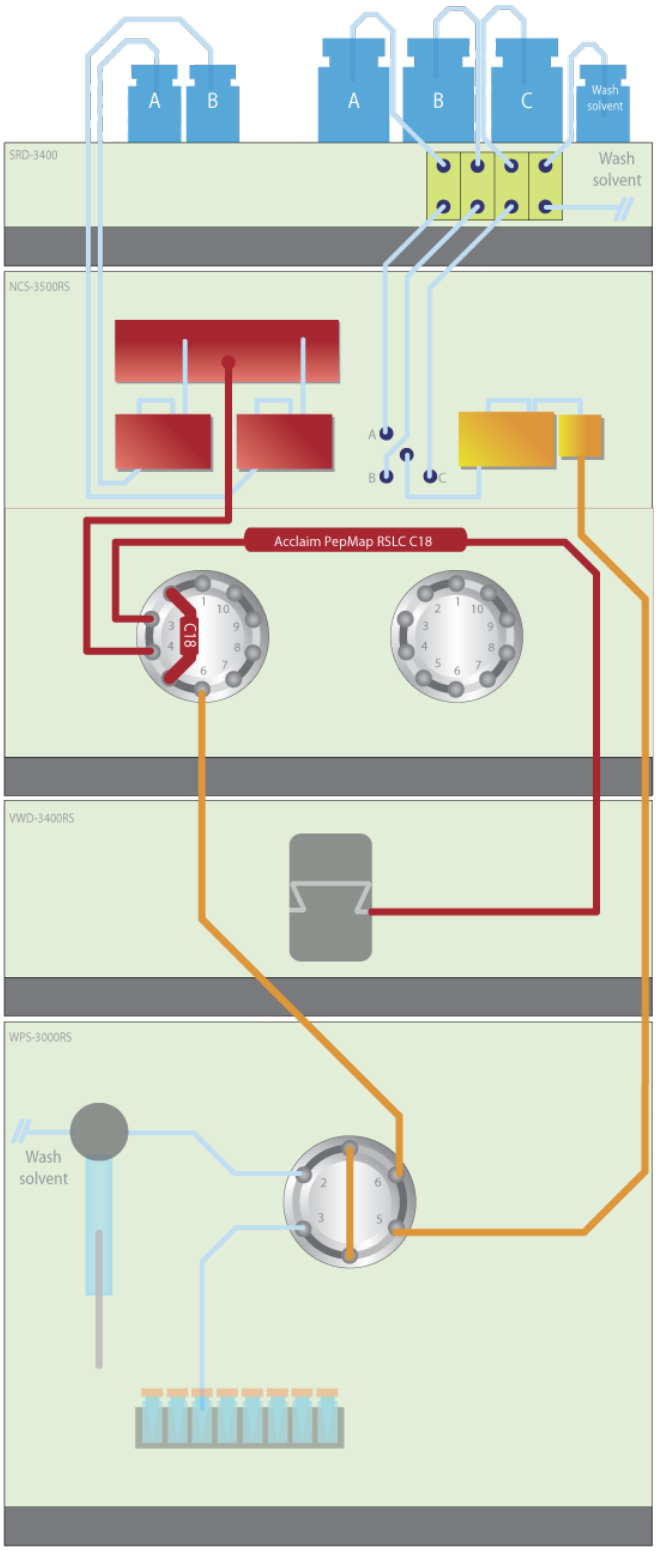


Figure 21: Setup for a Pre-concentration experiment onto a capillary column

The recommended setup is presented in Figure 21 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0020
10-port sw. valve	6041.0001
VWD-3400RS	5074.0010
45 nL flow cell	6074.0280
WPS-3000TPL RS	5826.0020
Application kit:	6720.0315

2.8.2 Fluidic Setup

Figure 22 presents the setup using the parts of the pre-concentration application kit. Columns are marked with letters, tubing with digits, and the sample loop is installed in the WPS-3000PL Autosampler.

Tip: The schematic shows a 10-port switching valve, but this application can be performed on a 6-port valve. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

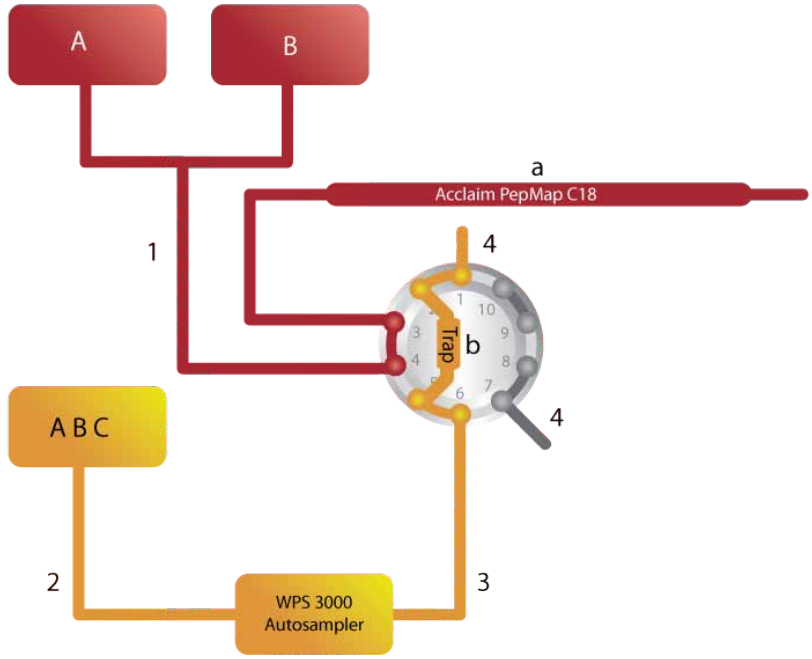


Figure 22: Fluidic connections for a Pre-concentration experiment onto a capillary column

Table 6: UltiMate 3000 RSLCnano Pre-concentration capillary LC kit (P/N 6720.0315) contents

#	Item	Replacement P/N
a	300 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164537
b	Nano Trap Column, 100 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 2) nanoViper	164564
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 350 mm	6041.5540
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 650 mm	6041.5775
3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 550 mm	6041.5760
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 750 mm	6041.5580
	nanoViper sample loop 20 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2420
4	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015

2.8.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- The design of the nano trap column provides the easiest connections, but must only be used in forward flush operation. Please check the indicated flow direction when installing a nano trap column.
- If the loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 water/ACN + 0.05% TFA

2.8.4 Testing the Application

The pre-concentration setup can be tested using the following conditions:

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v)water/ACN + 0.04% TFA
Loading solvent	98%/2% (v/v) water/ACN + 0.05% TFA
Sample	Cytochrome C digest 8 pmol, prepared according to instruction sheet
Injection volume	1 µL (partial loop fill of a 20 µL loop)
UV detection	214 nm
Loading time	3 min (may vary with different injection volume/routine)
Gradient	4% to 55% B in 30 min 90% B for 5 min 25min equilibration
WPS temperature	5°C (WPS-3000(B)T only)
Loading flow	20 µL/min
Flow rate	4 µL/min (capillary flow selector)

2.9 2D Salt Steps with Nano Column

2.9.1 Hardware Layout

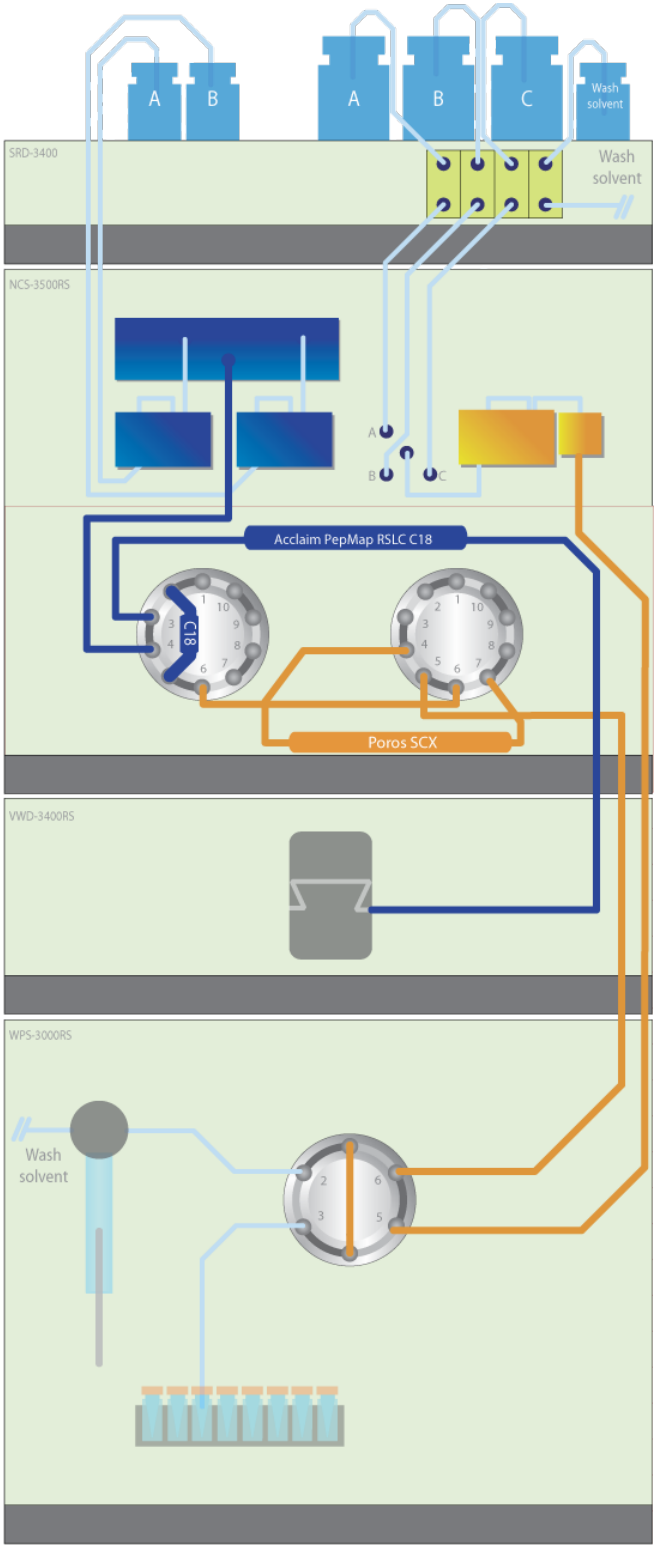


Figure 23: Setup for a 2D Salt Plugs experiment

The recommended setup is presented in Figure 23 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
2x 10-port sw.valve	6041.0001
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0325

2.9.2 Fluidic Setup

Figure 24 presents the setup using the parts of the 2D-LC Salt Plugs application kit. Columns are marked with letters, tubing with digits, and the sample loop is installed in the WPS-3000PL Autosampler.

Tip: The schematic shows 10-port switching valves, but this application can be performed on 6-port valves. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

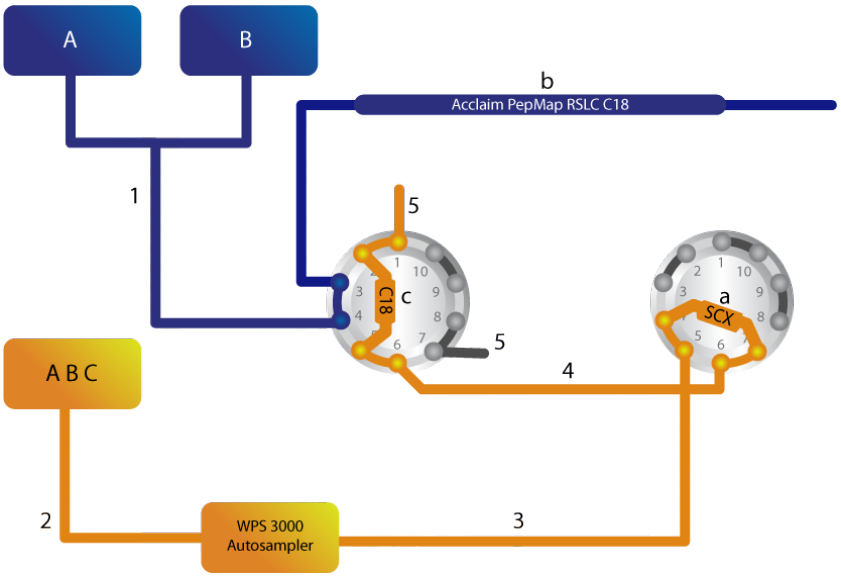


Figure 24: Fluidic connections for a 2D LC Salt Plugs experiment

Table 7: UltiMate 3000 RSLCnano 2D salt plugs kit (P/N 6720.0325) contents

#	Item	Replacement P/N
a	300 µm I.D. x 10 cm, packed with Poros 10 S with connections, 130 µm I.D. FS sheathed inlet and outlet, nanoViper	164565
b	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
c	Nano Trap Column, 100 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 2) nanoViper	164564
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 650 mm	6041.5775
3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 550 mm	6041.5760
4	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 250 mm	6041.5730
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
	nanoViper sample loop 20 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2420
5	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Protein mixture digest, 100 pmol, Lyophilized	161088
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015

2.9.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- The design of the nano trap column provides the easiest connections, but must only be used in forward flush operation. Please check the indicated flow direction when installing a nano trap column.
- If the loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 water/ACN + 0.025% TFA.
- If too much hydrophobic secondary interaction is observed on the IEX column, the amount of ACN can be increased up to 5% or 10%. This will be at the expense of the loading efficiency for hydrophilic peptides on the trap column.
- The loading time and desalting time are highly dependent on the sample quantity and purity. They can be adjusted to meet customer needs. However the desalting step must be kept long enough to avoid the formation of adducts between salt and sample.
- To limit the breakthrough on the SCX column, the loading solvent must contain as little TFA as possible (for example, max. 0.025%) or FA should be used.
- The salt plugs listed here have been chosen to check the system and work fine for the separation of the protein mix digest. The best sequence of plugs will highly depend on the affinity of the peptides present in the sample with the IEX column.
- It is useful to inject several times the last salt plug or to prepare one with a higher salt concentration in order to make sure that the column is clean before repeating an experiment.
- After each series of injection, it is useful to wash the column with consecutive 2M salt injections. When the salt is washed out from the column, a 60/40 water/ACN solution can also be used to wash out peptides which might be bound to the column due to hydrophobic interactions.
- SCX column regeneration can be performed by flushing the column overnight with a 10 mM phosphate buffer pH 3, 20% ACN and 600 mM NaCl salt solution. Please ensure that the column is conditioned with loading solvent before using it in the 2D salt plug application.

Tips: The combination of salts, buffers and organic solvents can result in precipitation. When preparing the solution described above, carefully evaluate if salts precipitate. Dilute or remove precipitation before using the wash solvent, to prevent system damage.

2.9.4 Testing the Application

The 2D salt plug setup can be tested using the following conditions:

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v) water/ACN + 0.04% TFA
Loading solvent	95%/5% (v/v) water/ACN + **0.025%** TFA
Sample	Protein mix digest, prepared according to the included instruction sheet
Salt plugs concentration (mol/L)	1 mmol NaCl 2 mmol NaCl 5 mmol NaCl 10 mmol NaCl 20 mmol NaCl 50 mmol NaCl 100 mmol NaCl 200 mmol NaCl 500 mmol NaCl 1000 mmol NaCl 2000 mmol NaCl
Injection volume	Sample: 10 µL Salt plugs: 20 µL
UV detection	214 nm
Loading time	5 min (may vary with different injection volume/routine)
Desalting time	7 min (started after loading time has passed)
Gradient	Isocratic 4% for 10min 4% to 55% B in 30 min 90% B for 5 min 18 min equilibration
WPS temperature	5°C
Loading flow	10 µL/min
Flow rate	300 nL/min (nano flow selector)

To evaluate the result of the experiment, focus on the following points:

- Injection profile should be reproducible.
- The peptides should be well distributed over the different fractions and among the fractions (orthogonal separation).

2.9.5 Salt Solutions Preparation

The following protocol can be used to prepare the salt plugs:

- Prepare two stock solutions using the loading solvent:
  1) 2000 mM NaCl (for example, 467.5 mg of NaCl in 4 ml of loading solvent)
  2) 100 mM NaCl (for example, prepare the 100 mM solution of the first table two times)
- Dilute the stock according to the tables below: Use standard 1.5 mL vials (for example, do not use inserts).

Concentration of NaCl	**Volume of 2000 mM NaCl stock solution**	**Volume of loading solvent**	**Total volume**
2000 mM	1000 µL	0 µL	1000 µL
1000 mM	500 µL	500 µL	1000 µL
500 mM	250 µL	750 µL	1000 µL
200 mM	100 µL	900 µL	1000 µL
100 mM	50 µL	950 µL	1000 µL

Concentration of NaCl	**Volume of 100 mM NaCl stock solution**	**Volume of loading solvent**	**Total volume**
50 mM	500 µL	500 µL	1000 µL
20 mM	200 µL	800µL	1000 µL
10 mM	100 µL	900 µL	1000 µL
5 mM	50 µL	950 µL	1000 µL
2 mM	20 µL	980 µL	1000 µL
1 mM	10 µL	990 µL	1000 µL

2.10 Automated off-line 2D LC of Peptides, micro SCX *x* nano RP

2.10.1 Hardware Layout

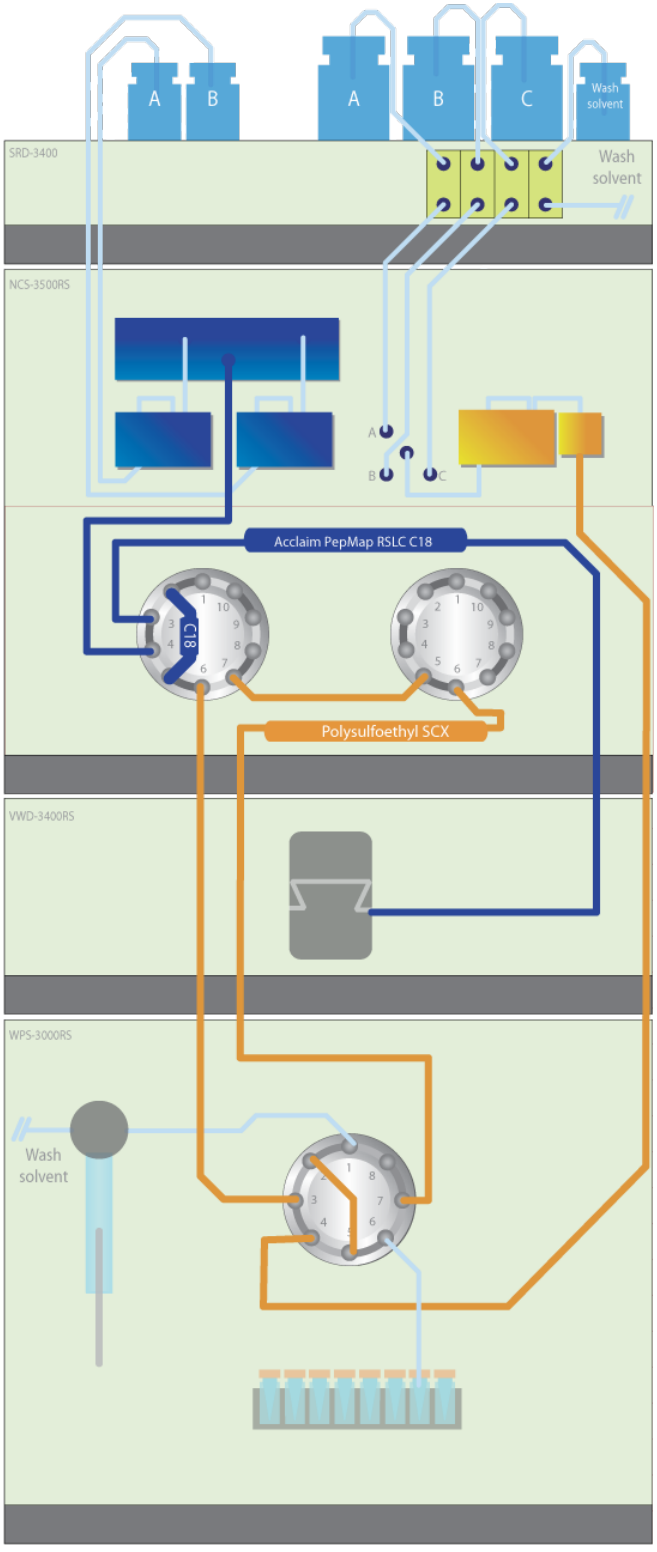


Figure 25: Setup for an Automated Off-line 2D Mic Nan experiment

The recommended setup with one detector is presented in Figure 25, when using two detectors a dual stack, as shown in Figure 26, is recommended. The single detector setup consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
2x 10-port sw.valve	6041.0001
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
or	
180 nL flow cell	6074.0290
WPS-3000TPL RS	5820.0010
µFC option	6820.0051
Application kit:	6720.0330

Tip: The µFC option limits the upper pressure of the first dimension to 350 bar, due to the applied 8-port valve.

A second UV detector is used when the nano column is not directly interfaced with the mass spectrometer.

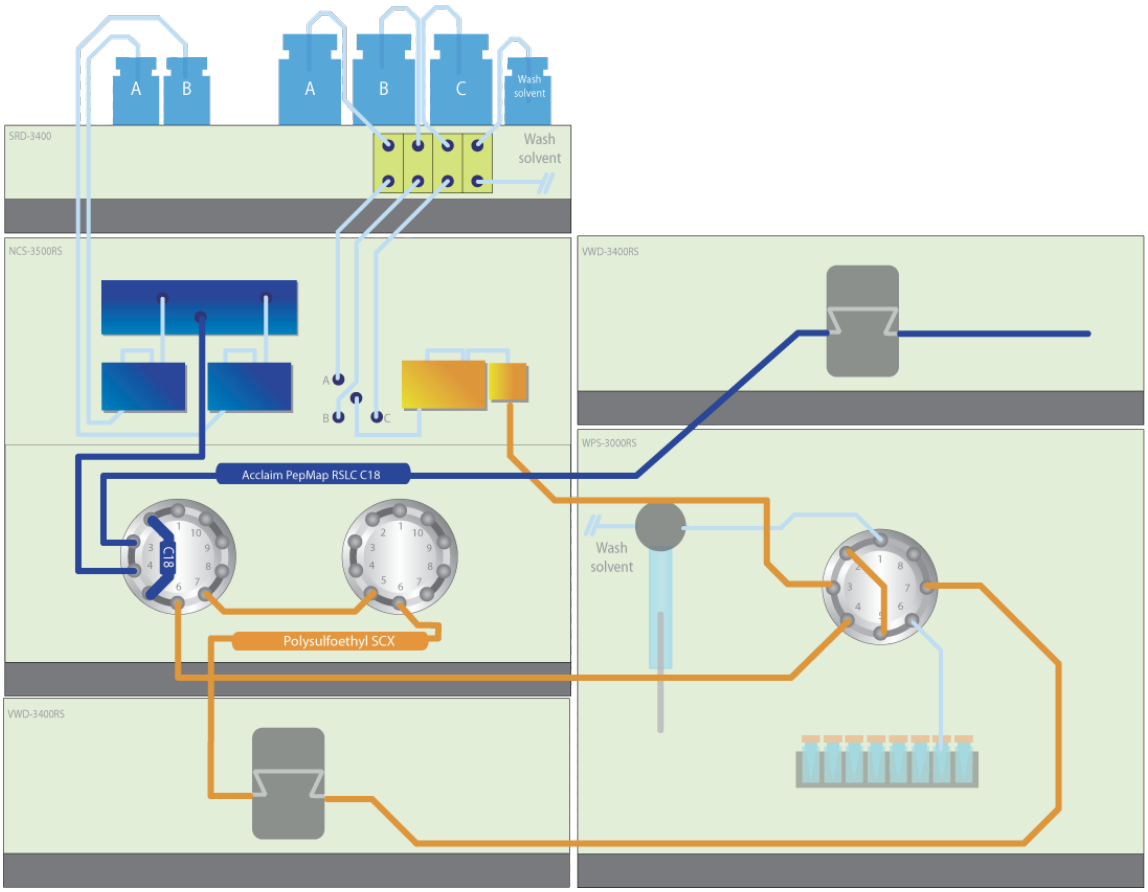


Figure 26: Setup for an Automated Off-line 2D Mic Nan experiment using two detectors

The recommended setup using 2 detectors is presented in Figure 26 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
2x 10-port sw.valve	6041.0001
2x VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
180 nL flow cell	6074.0290
WPS-3000PL	5820.0010
µFC option	6820.0051
Application kit:	6720.0330

Tip: The µFC option limits the upper pressure of the first dimension to 350 bar, due to the applied 8-port valve.

2.10.2 Fluidic Setup

Figure 27 presents the setup using the parts of the Automated Off-line Mic Nan SCX RP application kit. Columns are marked with letters, tubing with digits.

Tip: The schematic shows 10-port switching valves, but this application can be performed on 6-port valves. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

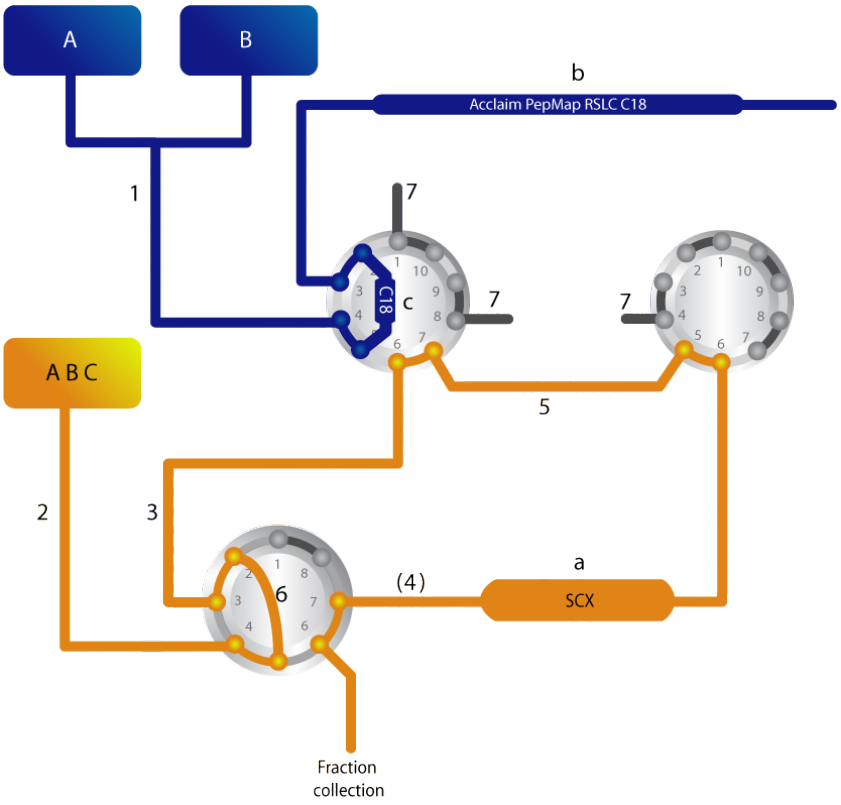


Figure 27: Fluidic connections for an Automated Off-line 2D experiment

Table 8: UltiMate 3000 RSLCnano Automated Off-line SCX-RP peptides kit (P/N 6720.0330) contents

#	Item	Replacement P/N
a	1.0 mm I.D. x 15 cm, packed with Polysulfoethyl ASP, 5 µm, 300Å, nanoViper	164566
b	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
c	Nano Trap column, 75 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 3 µm, 100Å (set of 2) nanoViper	164535
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 650 mm	6041.5775
3 (4)	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 550 mm	6041.5760
5	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 250 mm	6041.5730

Table 8: UltiMate 3000 RSLCnano Automated Off-line SCX-RP peptides kit (P/N 6720.0330) contents - Continued

#	Item	Replacement P/N
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
6	nanoViper sample loop 50 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2450
7	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	Protein mixture digest, 100 pmol, Lyophilized	161088
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	96 Well Microplate, PP, V-Bottom	6820.4113
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015
	Buffer tubing 500 µL, WPS-3000PL	6820.0020
	LCi Solutions Library CD	6830.0400
	Operating Instructions for automated off-line 2D-LC of peptides and proteins	164208

2.10.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- The design of the nano trap column provides the easiest connections, but must only be used in forward flush operation. Please check the indicated flow direction when installing a nano trap column.
- If the loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 water/ACN + 0.05% TFA
- If too much hydrophobic secondary interaction is observed on the IEX column, the amount of ACN can be increased up to 5% or 10%.
- The loading time and desalting time are highly dependent on the sample quantity and purity. They can be adjusted to meet customer needs. However, the desalting step must be kept long enough to avoid the formation of adducts between salt and sample.
- Optimal trapping in the second dimension is achieved by adding a strong ion-pairing agent to the fractions, prior to reinjection.

2.10.4 Testing the Application

The Mic Nan SCX – RP application setup can be tested using the following conditions:

Property	**Setting**
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v) water/ACN + 0.04% TFA
Loading pump A	5 mM HxPO4 pH 3 + 5% ACN
Loading pump B	Loading pump A with 1 M NaCl
Loading pump C	98%/2% (v/v) water/ACN + **0.05%** TFA
Sample	Protein mix digest, prepared according to the included instruction sheet
Injection volume	Sample: 10 µL Fractions: 20 µL
Gradient SCX	0-50% B in 20 min 90%B for 5 min 10 min equilibration
SCX flow rate	50 µL/min
UV detection	214 nm
Fractions	Every minute for 20 minutes
Loading time	7 min (may vary with different injection volume/routine)
Desalting time	5 min (counted after loading time has passed)
Gradient RP	Isocratic 4% for 10min 4% to 55% B in 30 min 90% B for 5 min 15 min equilibration
WPS temperature	5°C
Loading flow	5 µL/min
Flow rate	300 nL/min (nano flow selector)

The automated off-line application allows, as any off-line application, optimization of the individual separation dimensions. Thermo Fisher Scientific recommends testing and optimizing the performance of each separation dimension (SCX and RP) individually in a one-dimensional application.
This means that, for a nano RP second dimension, the test criteria are identical as described in the part of **Pre-concentration onto a Nano Column** on page 21.

2.11 Automated off-line 2D LC, Cap RP (basic) x nan RP (acidic)

2.11.1 Hardware Layout

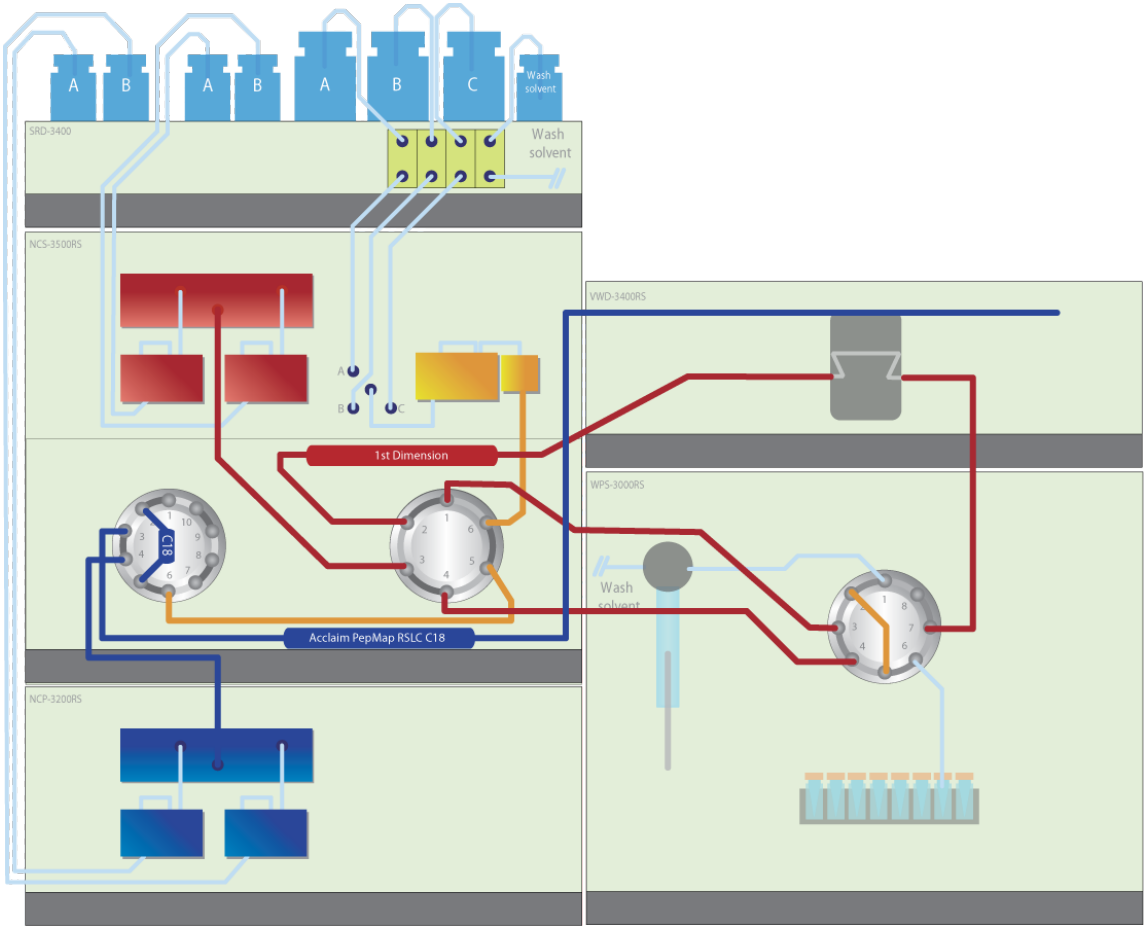


Figure 28: Setup for an Automated Off-line 2D Cap Nan experiment

The recommend setup is presented in Figure 28 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
NCP-3200RS	5041.0030
2x 10-port sw. valve	6041.0001
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
45 nL flow cell	6074.0280
WPS-3000PL	5820.0010
µFC option	6820.0051
Application kit:	6720.0340

Tip: The µFC option limits the upper pressure of the first dimension to 350 bar, due to the applied 8-port valve.

The NCP-3200RS spare parts kit has two 130 cm long solvent inlet tubing to place the bottles on top of the system.

2.11.2 Fluidic Setup

Figure 29 presents the setup using the parts of the Automated Off-line Cap Nan application kit. Columns are marked with letters, tubing with digits.

Tip: The schematic shows 10-port switching valves, but this application can be performed on 6-port valves. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

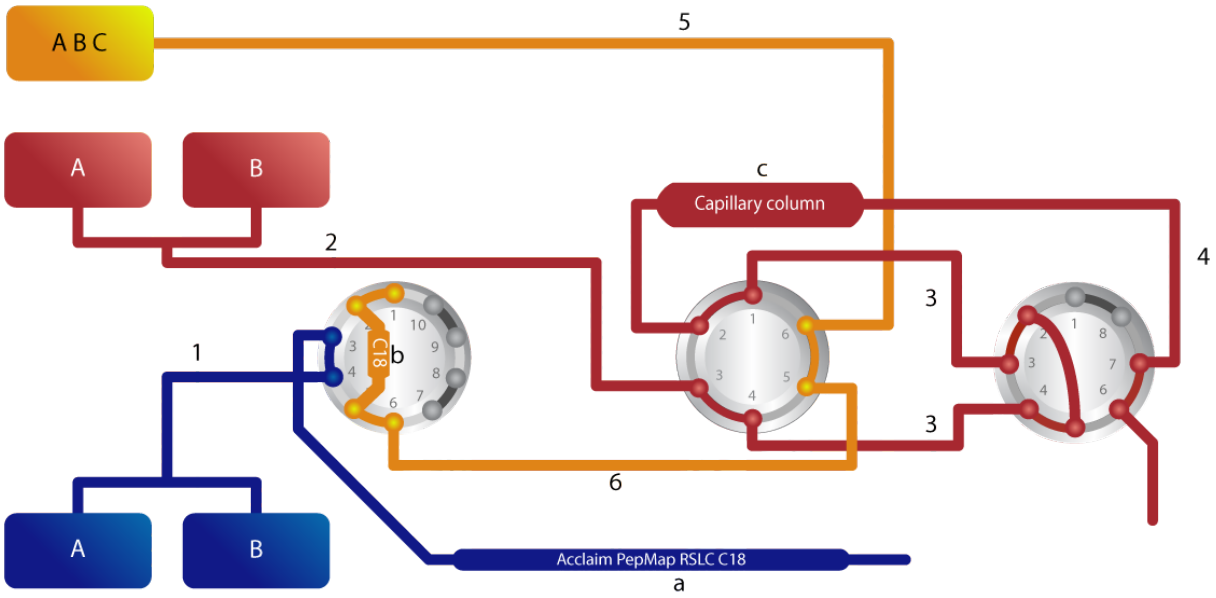


Figure 29: Fluidic connections for an Automated Off-line 2D experiment

Table 9: UltiMate 3000 RSLCnano Automated Off-line RP-RP peptides kit (P/N 6720.340) contents

#	Item	Replacement P/N
a	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
b	Nano Trap Column, 75 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 3 µm, 100Å (set of 2) nanoViper	164535
c	0.3 mm I.D. x 15 cm, packed with Acclaim PA2, nanoViper	164592
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 350 mm	6041.5540
3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 50 µm x 650 mm	6041.5575
4	nanoViper outlet tubing 50 um I.D. x 30 cm	6041.4573
	Nano connector including connection sleeves	6720.0390
5	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 350 mm	6041.5735
6	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 250 mm	6041.5730
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
	nanoViper sample loop 20 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2420

Table 9: UltiMate 3000 RSLCnano Automated Off-line RP-RP peptides kit (P/N 6720.340) contents - Continued

#	Item	Replacement P/N
	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015
	Protein mixture digest, 100 pmol, Lyophilized	161088
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	96 Well Microplate, PP, V-Bottom	6820.4113
	LCi Solutions Library CD	6830.0400
	Operating Instructions for automated off-line 2D-LC of peptides and proteins	164208

2.11.3 Installation tips

- Follow the General Recommendations for Applications (→ page 6).
- The design of the nano trap column provides the easiest connections, but must only be used in forward flush operation. Please check the indicated flow direction when installing a nano trap column.
- If the loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 water/ACN + 0.05% TFA.
- Optimal trapping in the second dimension is achieved by adding a strong, acidic ion-pairing agent to the fractions and a combination of evaporating and diluting the ACN from the first dimension.
- When two VWD-3400RS detectors are available, they should be placed on top of each other.
- Depending on the location of the MS outlet and the application of UV detection (1st or 2nd Dimension), the WPS can be placed on the left side of the system.

2.11.4 Testing the application

The automated off-line application allows, as any off-line application, optimization of the individual separation dimensions. Thermo Fisher Scientific recommends testing and optimizing the performance of each separation dimension (RP basic and RP acidic) individually in a one-dimensional application.
This means that the test criteria for a nano RP second dimension are identical as described in the part of **Pre-concentration onto a Nano Column** on page 21.

Property	Setting
NCS NC_Pump A	100% water, 1% (72 mM) triethylamine (TEA) titrated to pH = 9.6 with acetic acid
NCS NC_Pump B	19%/80% (v/v) water/ACN, 1% (72 mM) triethylamine (TEA), titrated to pH = 9.6 with acetic acid
Loading pump A	98%/2% (v/v) water/ACN + 0.05% TFA
NCP NC_Pump A	100% water + 0.05% TFA
NCP NC_Pump B	20%/80% (v/v) water/ACN + 0.04% TFA

Property	**Setting**
Sample	Protein mix digest, prepared according to the included instruction sheet
Injection volume	Sample: 10 µL Fractions: 20 µL
Gradient RP basic	4-60% B in 15 min, 90% B for 5 min, 25 min equilibration
UV detection	214 nm
Fractions	Every minute for 20 minutes
Loading time	7 min (may vary with different injection volume/routine)
Gradient RP acidic	Isocratic 4% for 10min 4% to 55% B in 30 min 90% B for 5 min 15 min equilibration
WPS temperature	5°C
Flow rate cap	6 µL/min
Loading flow	5 µL/min
Flow rate nano	300 nL/min

2.12 Tandem nano LC

2.12.1 Hardware Layout

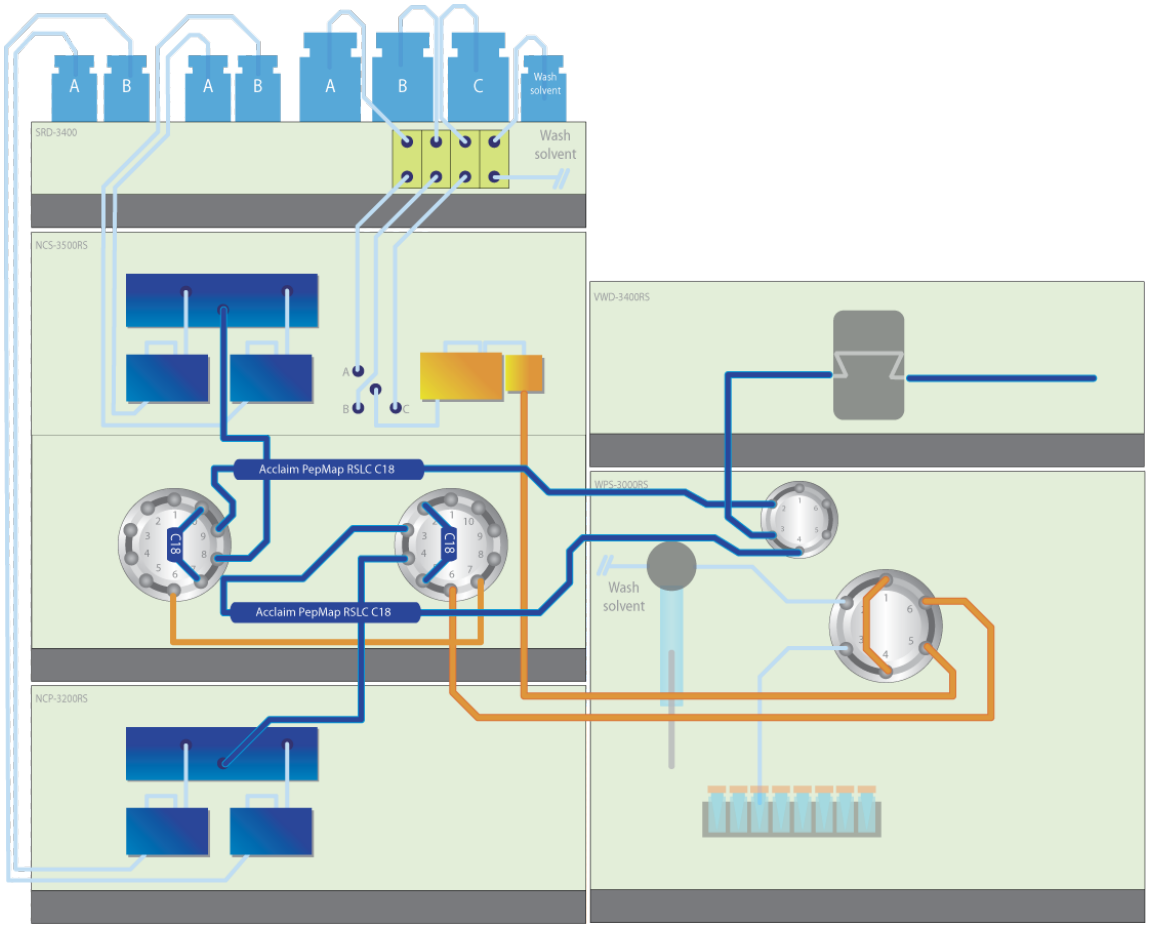


Figure 30: Setup for a Tandem nano LC experiment

The recommended setup is presented in Figure 30 and consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010
NCP-3200RS	5041.0030
2x 10-port sw.valve	6041.0001
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000FC	5824.0020
Application kit:	6720.0335

Tip: The components to convert the WPS-3000FC are included in the application kit.

The NCP-3200RS spare parts kit has two 130 cm long solvent inlet tubing to place the bottles on top of the system.

2.12.2 Fluidic Setup

Figure 31 presents the setup using the parts of the Tandem nano LC application kit. Columns are marked with letters, tubing with digits.

Tip: The schematic shows 10-port switching valves, but this application can be performed on 6-port valves. Ensure that the relative positions of the connections are correct, and update the valve switching in the Chromeleon templates if necessary.

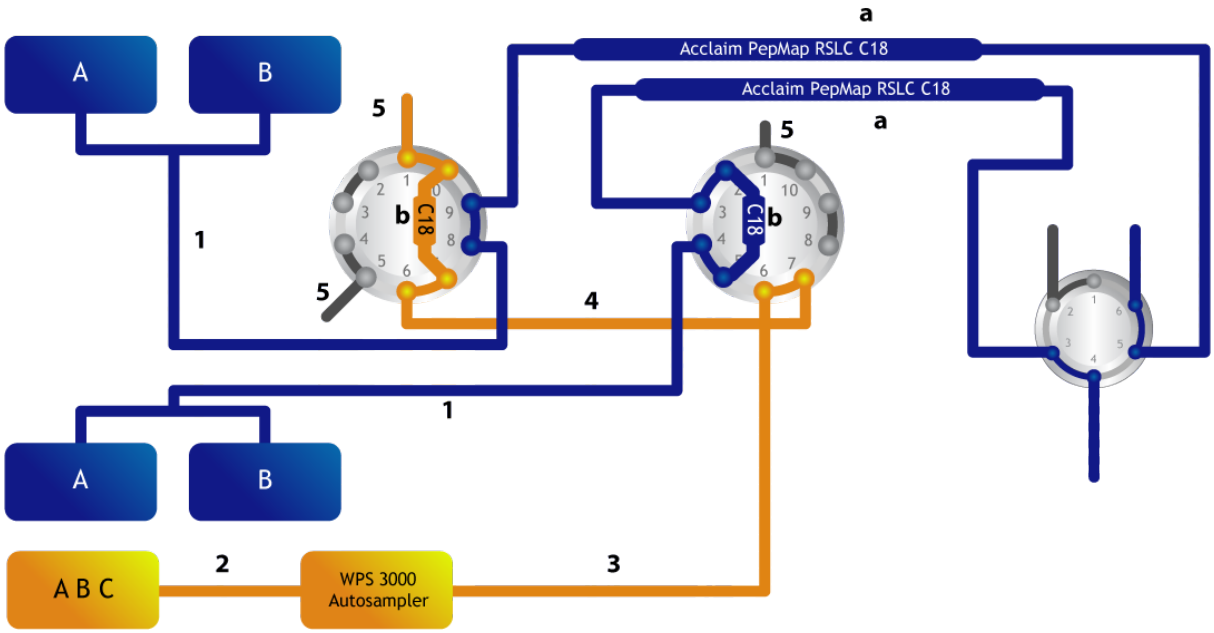


Figure 31: Fluidic connections for a Tandem nano LC experiment

A field upgrade to the WPS-3000FC needs to be performed to make the autosampler suitable for this application. First, the nano injection kit (P/N 6824.0030) should be installed and secondly the lower valve must be replaced with the nano switching valve (P/N 6825.0020). All necessary parts are included in the kit. For further information, refer to the operating instructions for the module.

Table 10: UltiMate 3000 RSLCnano Tandem nano LC kit (P/N 6720.0335) contents

#	Item	Replacement P/N
a	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
b	Nano Trap Column, 75 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 3 µm, 100Å (set of 2) nanoViper	164535
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240
2,3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 650 mm	6041.5775
4	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 75 µm x 250 mm	6041.5730
	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
5	PTFE tubing, 500 µm I.D. 100 cm, used as waste tubing	6720.0077
	nanoViper sample loop 20 µL, FS/PEEK sheathed I.D. x L 250 µm x 408 mm	6826.2420
	Fused silica tubing I.D. 20µm O.D. 280µm, 5 meters for nano LC connections	160475

Table 10: UltiMate 3000 RSLCnano Tandem nano LC kit (P/N 6720.0335) contents - Continued

#	Item	Replacement P/N
	Cleaving stone	160483
	Upgrade kit nano/cap WPS-3000TFC	6824.0030
	1/32” 2 pos 6 port nano switching valve	6825.0020
	Fittings for nano valve	6720.0080
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pcs.	6720.0015

2.12.3 Installation Tips

- Follow the General Recommendations for Applications (→ page 6).
- The standard column outlets are 30 cm fused silica capillaries. 20 µm I.D. fused silica tubing is provided in the kit to extend the column outlets if necessary. Replacing the attached fused silica by the appropriate length using the nano connector on the column will give the best result.
- The design of the nano trap column provides the easiest connections, but must only be used in forward flush operation. Please check the indicated flow direction when installing a nano trap column.
- If the loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 water/ACN + 0.05% TFA
- The WPS-3000FC is normally used for fraction collection. By replacing the divert valve by a nano valve, the autosampler is fitted for tandem nano LC. Controlling the divert valve position is performed with the commands **Collect** and **Drain**.

2.12.4 Testing the Application

The tandem nano LC setup consists of two pre-concentration nano setups that can be operated individually; therefore, the system can be tested and evaluated using the conditions in the table below, as also described in the part of **Pre-concentration onto a Nano Column** on page 21.

Property	Setting
Mobile phase A	100% water + 0.05% TFA
Mobile phase B	20%/80% (v/v) water/ACN + 0.04% TFA
Loading pump A	98%/2% (v/v) water/ACN + 0.05% TFA
Sample	Cytochrome C 1 pmol/µL, prepared according to instruction sheet
Injection volume	Sample: 1 µL
UV detection	214 nm
Loading time	3 min (may vary with different injection volume/routine)

Property	**Setting**
Gradient RP	4% to 55% B in 30 min 90% B for 5 min 15 min equilibration
WPS temperature	5°C
Loading flow	5 µL/min
Flow rate	300 nL/min (nano flow selector)

3 FAQ

3.1 Interpreting a Chromatogram

A typical Cytochrome C separation is shown in Figure 32. The different areas of a chromatographic separation are marked inside the figure.

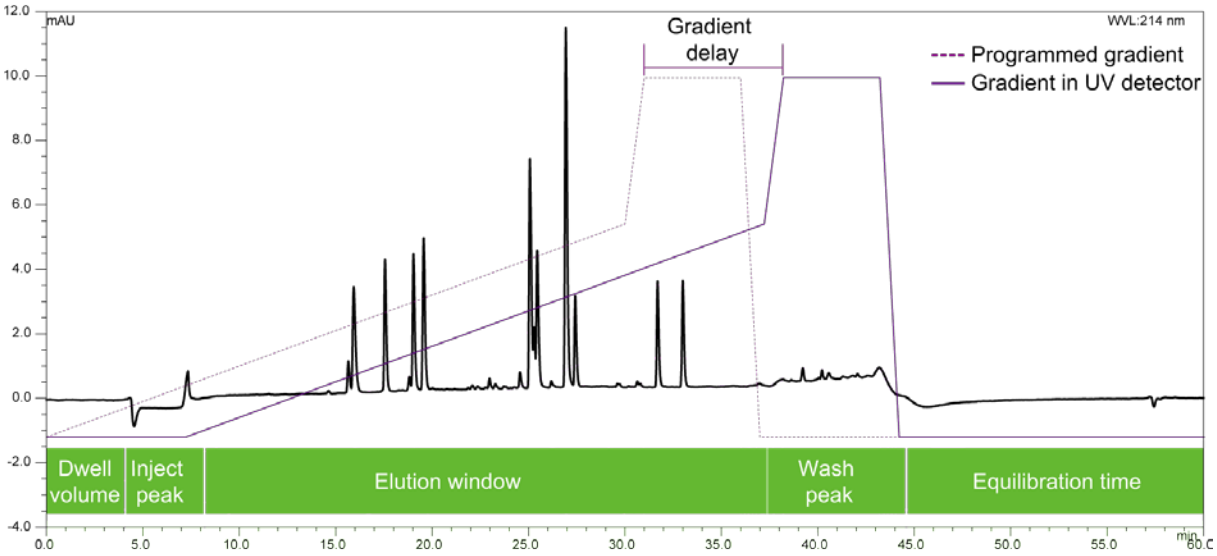


Figure 32: Example Cytochrome C separation with different parts of the run identified.

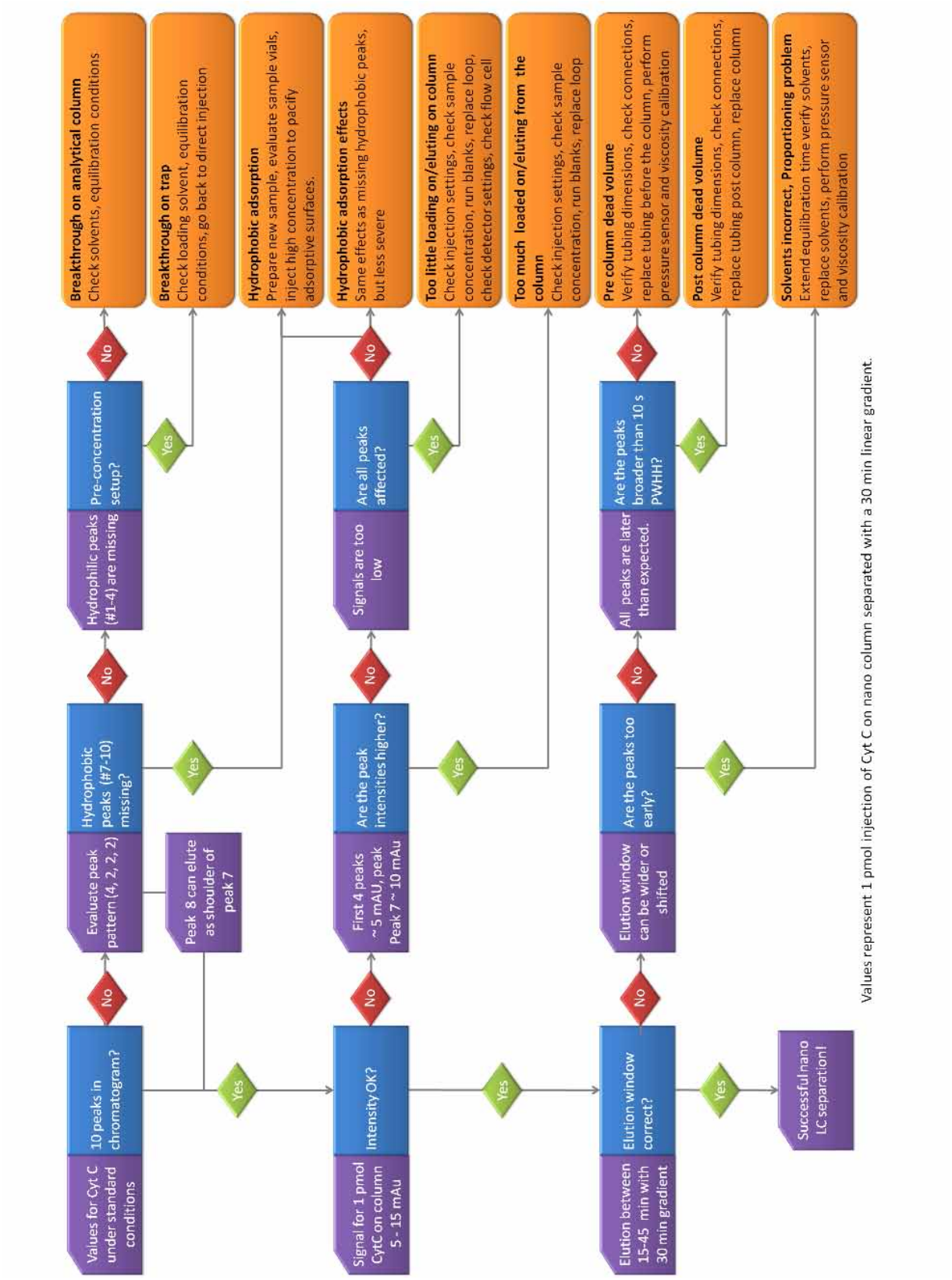
The finite volume of an HPLC system requires a certain time between the formation of a gradient and detecting the gradient change. This so-called gradient delay can be seen by comparing the programmed gradient with the detected signal. Figure 32 shows the gradient delay between pump and UV detector.
The 'inject peak' really corresponds to the inject peak in direct injection setups, in pre-concentration setups a similar baseline can be observed, but then the area marked as inject peak resembles the trapping column being placed in line with the nano column.
The dwell volume represents the volume between the autosampler and the nano columns, since there are usually one (direct injection) or two (pre-concentration) valve switches involved in the application, which introduce addition fluidics, the dwell volume and gradient delay are not the same volume.

3.2 Troubleshooting nano LC peptide Applications

The above chromatogram Figure 32 shows the separation of a Cytochrome C digest on a nano column. The Cytochrome C standard appears relatively simple compared to a typical proteomics sample, but is ideal for troubleshooting a direct injection and pre-concentration setup.

Tip: When troubleshooting a pre-concentration setup, Thermo Fisher Scientific recommends switching back to direct injection if the tips below do not provide remedy.

In assessing the separation performance of a system several points are evaluated, which are organized in the flow chart below. The values in the flowchart are based on a Cytochrome C digest separation; when working with a different standard use a reference chromatogram for the expected values for number of peaks, intensity and elution window.


Values for Cyt C under standard conditions
10 peaks in chromatogram?
No
Evaluate peak pattern (4, 2, 2, 2)
Hydrophobic peaks (#7-10) missing?
No
Hydrophilic peaks (#1-4) are missing
Pre-concentration setup?
No
Breakthrough on analytical column
Check solvents, equilibration conditions
Yes
Breakthrough on trap
Check loading solvent, equilibration conditions, go back to direct injection
Yes
Hydrophobic adsorption
Prepare new sample, evaluate sample vials, inject high concentration to pacify adsorptive surfaces.
Yes
Peak 8 can elute as shoulder of peak 7
Yes
Signal for 1 pmol CytC on column 5 - 15 mAu
Intensity OK?
No
First 4 peaks ~ 5 mAU, peak Peak 7 ~ 10 mAu
Are the peak intensities higher?
No
Signals are too low
Are all peaks affected?
No
Hydrophobic adsorption effects
Same effects as missing hydrophobic peaks, but less severe
Yes
Too little loading on/eluting on column
Check injection settings, check sample concentration, run blanks, replace loop, check detector settings, check flow cell
Yes
Elution between 15-45 min with 30 min gradient
Elution window correct?
No
Elution window can be wider or shifted
Are the peaks too early?
No
All peaks are later than expected.
Are the peaks broader than 10 s PWHH?
No
Pre column dead volume
Verify tubing dimensions, check connections, replace tubing before the column, perform pressure sensor and viscosity calibration
Yes
Post column dead volume
Verify tubing dimensions, check connections, replace tubing post column, replace column
Yes
Too much loaded on/eluting from the column
Check injection settings, check sample concentration, run blanks, replace loop
Yes
Successful nano LC separation!
Solvents incorrect, Proportioning problem
Extend equilibration time verify solvents, replace solvents, perform pressure sensor and viscosity calibration
Values represent 1 pmol injection of Cyt C on nano column separated with a 30 min linear gradient.

3.3 The use of TFA and FA

The separation of peptides by reversed phase is typically done in the presence of an ion pairing agent. The typical ion pairing agents serve a double function. First, these weak acids bring the pH of the solvents down to pH 2-3, causing almost all peptides to have an overall positive charge. Secondly, the negative counterion of the acid will serve as the ion pairing agent with the peptides to create an overall neutral analyte that is separates on the RP column. The double function of the ion pairing agent allows having an efficient separation with minimal additives added to the solvents. Nonetheless, there is a choice of ion pairing agents, where the most common choice is between Trifluoro Acetic Acid (TFA) and Formic Acid (FA). In this manual, TFA is used in the application as this is the stronger ion pairing agent and results in better chromatography. However, in LC-MS applications, often FA is preferred to minimize the effects of ion suppression. When performing the applications mentioned in this manual with FA, replace the volume (%) of TFA by double the volume (%) of FA; e.g. 0.05% TFA becomes 0.1% FA.

3.4 Minimizing Baseline Noise

The 3 nL flow cell (P/N 6074.0270) and 45 nL flow cell (P/N 6074.0280) are designed to function in the same way as transfer tubing normally used to connect a column outlet to a mass spectrometer. This allows UV detection in nano and capillary LC without introducing post column band broadening.
Typically peptide UV detection is performed at a wavelength of 214 nm, in which most organic compounds absorb quite strongly. There are some actions that can be taken to minimize baseline drift and noise for optimal use of the UV detection.

3.4.1 Drift

Ensure that the UV lamp has been switched on for sufficient time in order to have it running at a stable temperature. Chromeleon can detect this and will give a warning during the ‘Ready Check‘ if the UV lamp temperature is not stable yet. The UV detector can be used, but it is not at its optimal performance.

Gradient RP nano LC typically involves a significant change in solvent composition. The higher absorption from the organic modifier in the B solvent will result in a rise of the baseline. The ion pairing agent (typically FA or TFA) in the A and B solvent can be used to compensate the baseline rise. As a rule of thumb, the compositions as indicated in Table 11 can be used to obtain a straight baseline.

Table 11: Ion pairing agent addition

	A	B
FA	0.1%	0.08%
TFA	0.05%	0.04%

Lamp and flow cell age can have a significant influence on baseline drift. New lamps and flow cells may show some drift during the so-called ‘burn in’ period.
Lamps should be replaced after approximately 2000 hours and older flow cells can be cleaned by flushing overnight with organic solvent or for a shorter period with a strong acidic solution; see operating instructions for details.

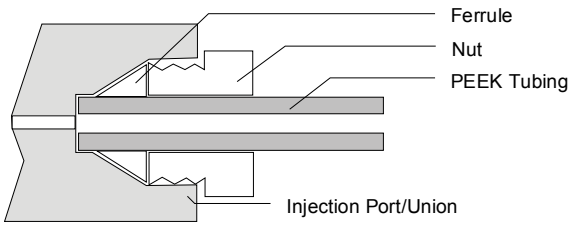
3.4.2 Unstable Baseline

Unstable baselines can have various causes. The UltiMate 3000 RSLCnano pumps are designed to provide the best gradient precision, but solvent miscibility can present a problem. Therefore, Thermo Fisher Scientific recommends using a minimum of 5% water in the organic mobile phase.

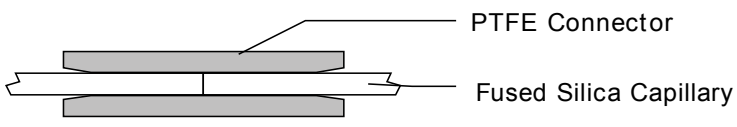
Baseline artifacts in pre-concentration applications using low loading flows (< 10 µL/min) may occur. These artifacts are only observed in the UV signal and have no effect on the performance of the analysis. If artifacts in the baseline are observed, Thermo Fisher Scientific recommends bypassing the degasser in pre-concentration applications where no gradient formation is required and loading flows are below 20 µL/min. If bypassing the degasser is undesired or impossible an alternative is to maintain degassing, but to increase the loading flow during the elution phase to values between 30 and 100 µL/min.

4 Appendix – Traditional Capillary Connections

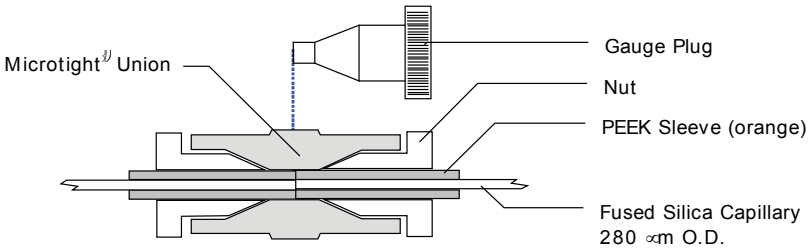
- When installing fused silica tubing (for example, the capillary column):
  - Insert the PEEK sleeve with the stainless steel nut (standard) or PEEK nut (biocompatible system) and the appropriate ferrule in a port (or the preassembly tool, when working on a biocompatible system!).
  - Push the sleeve all the way into the port. It is essential for a zero dead volume connection that the assembly seats firmly.
  - Tighten the nut by two or three turns to make certain that the ferrule grabs the sleeve.
  - Remove the pre-assembled fitting, and then slide the capillary (column) into the sleeve.
  - When re-installing the fitting together with the capillary (column), make certain that the tip of the tubing does not extend the tip of the sleeve. Tighten the nut finger-tight.
  - Push the capillary (column) all the way into the sleeve (port) to minimize dead volume. It is essential for a zero dead volume connection that the assembly seats firmly.



- To prepare connections with PTFE sleeves:
  - Start flow delivery.
  - Slide the fused silica capillary (for example, the column) approximately 1- 2 mm into the sleeve.
  - Wait until a droplet is formed at the outlet. Remove the droplet (together with potential dirt in the tubing).
  - Now push the capillary (column) half-way into the sleeve. Wait again until a droplet is formed and remove it.
  - Connect the second capillary (for example, the inlet capillary of the flow cell).



- When using a micro tight union, always use the gauge plug provided with the union to make sure that the connection is well centered.



5 Appendix – Common Spare Parts in nano LC

The three tables below list the most common parts to be used with the UltiMate 3000 RSLCnano system. They are divided by columns (Table 12), tubing (Table 13, Table 14), and hardware (Table 15).

Table 12: List of columns available for the UltiMate 3000 RSLCnano system

Item	P/N
Acclaim PepMap Columns	
Acclaim PepMap100, C18, 3 µm, 100 Å, 75 µm I.D. × 5 cm, nanoViper	164567
Acclaim PepMap100, C18, 3 µm, 100 Å, 75 µm I.D. × 15 cm, nanoViper	164568
Acclaim PepMap100, C18, 3 µm, 100 Å, 75 µm I.D. × 25 cm, nanoViper	164569
Acclaim PepMap100, C18, 3 µm, 100 Å, 75 µm I.D. × 50 cm, nanoViper	164570
Acclaim PepMap100, C18, 3 µm, 100 Å, 300 µm I.D. × 15 cm, nanoViper	164571
Acclaim PepMap100, C18, 3 µm, 100 Å, 1 mm I.D. × 15 cm, nanoViper	164572
Acclaim PepMap RSLC Columns	
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 50 µm I.D. x 5 cm, nanoViper	164561
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 50 µm I.D. x 15 cm, nanoViper	164562
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm I.D. x 5 cm, nanoViper	164563
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm I.D. x 15 cm, nanoViper	164534
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm I.D. x 25 cm, nanoViper	164536
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm I.D. x 50 cm, nanoViper	164540
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 300 µm I.D. x 5 cm, nanoViper	164560
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 300 µm I.D. x 15 cm, nanoViper	164537
Nano Trap Columns	
Nano Trap Column, 100 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 5 µm	164564
Nano Trap Column, 75 µm I.D. x 2 cm, packed with Acclaim PepMap100 C18, 3 µm	164535
Cartridge based trap columns.	
µ-Precolumn™ holder, 5 mm, with 30 µm i.d. connecting tubing, nanoViper fittings	164649
300 µm i.d. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 5 cartridges)	160454
PepSwift and ProSwift Monolithic Columns	
PepSwift Monolithic Nano Column, 100 µm I.D. × 5 cm, nanoViper	164584
PepSwift Monolithic Nano Column, 100 µm I.D. x 25 cm, nanoViper	164543
PepSwift Monolithic Capillary Column, 200 µm I.D. x 5 cm, nanoViper	164557
PepSwift Monolithic Capillary Column, 200 µm I.D. x 25 cm, nanoViper	164542
PepSwift Monolithic Capillary Column, 500 µm I.D. × 5 cm, nanoViper	164585
ProSwift RP-10R Monolithic Capillary Column, 1 mm I.D. × 5 cm, nanoViper	164586
PepSwift Trap Columns	
PepSwift Monolithic Trap Column, 200 µm x 5 mm, set of 2, nanoViper	164558

Table 13: Matrix for connection tubing for the UltiMate 3000 RSLCnano system

Length (mm)	ID [Color code]				
	20 µm [Orange]	**50 µm [Brown]**	**75 µm [Black]**	**100 µm [Red]**	**150 µm [Purple]**
70	6041.5120	6041.5123	6041.5126	6041.5810	6041.5817
150	6041.5121	6041.5124	6041.5127	6041.5811	6041.5818
250	-	-	6041.5730	6041.5812	6041.5819
350	6041.5240	6041.5540	6041.5735	6041.5813	6041.5820
450	-	-	-	6041.5814	6041.5821
550	6041.5260	6041.5560	6041.5760	6041.5815	6041.5822
650	6041.5275	6041.5575	6041.5775	-	-
750	6041.5280	6041.5580	6041.5780	6041.5816	6041.5823
950	6041.5122	6041.5125	6041.5128	-	-

Table 14: List of connection tubing for the UltiMate 3000 RSLCnano system

Item	P/N
Sample loops	
Sample loop 1 µL with nanoViper fittings connections	6826.2401
Sample loop 5 µL with nanoViper fittings connections	6826.2405
Sample loop 20 µL with nanoViper fittings connections	6826.2420
Sample loop 50 µL with nanoViper fittings connections	6826.2450
Connectors	
Nano connector including connection sleeves	6720.0390
Sleeve for Nano Connector, 5 pcs.	6720.0391
PTFE tubing, 250 µm I.D., for low pressure connection of 280 µm O.D. FS. capillaries, 5 pcs.	160486
Microtight Union including 2 fittings and 1 gauge plug	161497
PEEK sleeves, precision cut and polished for connections with Microtight Union (280 µm O.D.), 10 pieces	161498
PEEK sleeves, precision cut and polished for connections with Microtight Union (380 µm O.D.), 10 pieces	161405

Table 15: List of hardware components for the UltiMate 3000 RSLCnano system

Item	**P/N**
Low dispersion 2 pos 10 port valve high pressure for NCS-3x00	6041.0001
Low dispersion 2 pos 6 port valve high pressure for NCS-3x00	6041.0004
Low-dispersion 2 pos 10 port valve, PAEK, bio, NCS-3x00	6041.0012
Viper blind plug	6040.2303
Flow selector nano LC	6041.0002
Flow selector capillary LC	6041.0003
Flow selector micro LC	6041.0014
Mixer kit 8 µL NCS/NCP	6041.7130
NCS/NCP purge capillary	6040.2385