thermoscientific

UltiMate™3000

RSLCnano

Standard Applications Guide

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Contents

1 Using this Manual

This chapter provides information about this manual, the conventions used throughout the manual, and the reference documentation that is available in addition to this manual.

1.1 About this Manual

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

NOTICE This document is intended for Thermo Fisher Scientific (or authorized) service personnel as well as customers to assist in the installation and application testing of UltiMate 3000 RSLCnano systems. It does **not** replace the IQ or OQ procedures. It is assumed that the individual using this manual has had 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 hazards, exposure to UV radiation and exposure to pressurized solvents.

This manual contains important information about the correct care and use of the UltiMate 3000 RSLCnano. Please read this manual carefully before installing or running any of the applications described. Keep this manual close to the UltiMate 3000 RSLCnano for future reference and pass it on to any subsequent user.

1.2 Conventions

This section describes the conventions used throughout this manual

1.2.1 Special Notices and Informational Notes

Special notices and informational notes in this manual appear different from the main flow of text. They appear in boxes and a note label identifies them. The label text appears in uppercase letters and in bold type.

NOTICE Highlights information necessary to prevent damage to the instrument or invalid test results.

TIP Highlights information of general interest or helpful information that can make a task easier or optimize the performance of the instrument

1.2.2 Typographical Conventions

These typographical conventions apply to the descriptions in this manual:

References and Messages

References to figures and tables appear *italicized*.

Viewpoint

If not otherwise stated, the expressions *left* and *right* in this manual always refer to the viewpoint of a person that is facing the instrument from the front.

Particularly Important Words

Particularly important words in the main flow of text appear ***in bold***.

Electronic Manual Version (PDF)

The electronic version (PDF) of the manual contains numerous links that you can click to go to other locations within the manual. These include:

- Table of contents entries
- Index entries
- Cross-references (in blue text), for example, to sections, figures or online reference materials

1.3 Reference Documentation

Further information relating to the UltiMate 3000 RSLCnano systems and associated applications is available as follows:

- The UltiMate 3000 RSLCnano system webpage: https://www.thermofisher.com/order/catalog/product/ULTIM3000RSLCNANO
- Module operating instructions

  NCS-3500RS / NCP-3200RS pump modules

  VWD-3100 and VWD-3400RS variable wavelength detectors

  WPS-3000TPL RS and WPS-3000FC autosamplers

  Additionally, you can also find these operating instructions in the following installation folder (Chromeleon 7/ SII): "C:\Program Files (x86)\Thermo\Chromeleon\bin\Troubleshooting Guides" (or "C:\Chromel\Bin\Troubleshooting Guides" when using Chromeleon 6.80)
- Upgrading the UltiMate 3000 RSLCnano System with ProFlow Technology – Quick Installation Guide
- Viper™ and nanoViper™ EASY-Spray™ Column tips and tricks document
- Details on Viper and nanoViper capillaries and application kits

  Thermo Scientific Viper and nanoViper Fingertight Fitting System - brochure

  Viper and nanoViper Fingertight Fitting Systems - specifications
- The complete and easy guide to configuring your Thermo Scientific nano LC
- Nano, Capillary and Micro LC Columns detailed in the Chromatography Columns and Consumables catalogue

2 Application Setup

This chapter provides details on each of the application kits available for the UltiMate 3000 RSLCnano system.

2.1 General Recommendations for Applications

The experimental conditions for each application are described together with schematics, installation tips, examples and results.

2.1.1 nanoViper Connections

NanoViper (*Figure 1*) is a fingertight high-pressure fitting that is virtually dead volume free by design and rated for backpressures up to 1200 bar. All high-pressure fittings used in the applications on the UltiMate 3000 RSLCnano system use nanoViper. The fittings are factory assembled to ensure quality and prevent experimental failure due to bad connections.

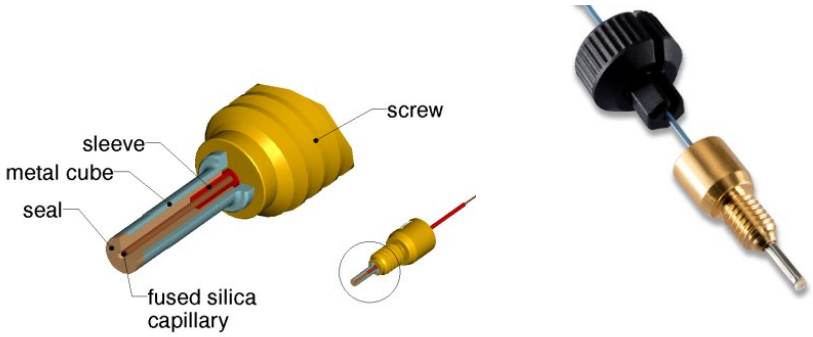


Figure 1: Internal and external view of a nanoViper fitting

1. Install nanoViper using the removable knurled black nut.
2. Do not overtighten connections (the general guideline is fingertight plus an additional one eighth of a turn).
3. Remove the knurled black nut once the fitting is tight.

2.1.2 Making Connections using the Nano Connector

The outlet of the linear nano columns are fitted with a nano connector. This zero dead volume connection is designed to interface the linear column outlet with 280 µm fused silica capillaries and is pressure stabile 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 in *Figure 2* below.

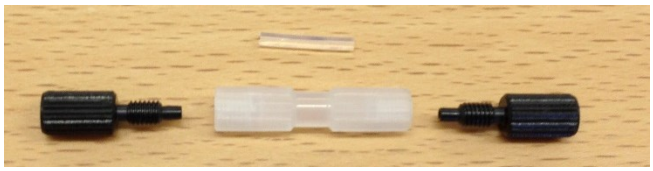
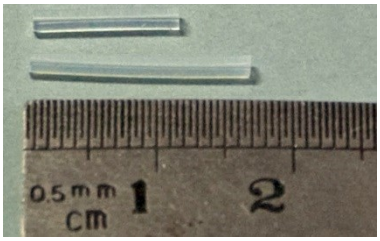


Figure 2: The components of the nano connector

- Use a new nano connector sleeve (P/N 6720.0391) each time the connection is made.

NOTICE: Do NOT use a PTFE sleeve (P/N 160486; supplied with the columns). The size does not match the nano connector union (*Figure 3*) and the pressure resistance is much lower.



Nano connector sleeve (1.1 cm)

PTFE sleeve (1.8 cm)

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

1. Slide the black nut and transparent union onto one of the ends of the fused silica, and the other black nut onto the other fused silica end →*Figure 4*a.

2. Slide the nano connector sleeve onto one end of the fused silica until it reaches the middle of the sleeve. Slide the other end of the fused silica into the connector sleeve. Make sure that the connection is dead volume free (*i.e.* that the ends meet in the middle →*Figure 4*b).

3. Tighten both sides of the black nut equally to ensure that the nano connector sleeve is in the center of the transparent union →Figure 4c.

a

b

c

Figure 4: Nano connector assembly. (a) The two black nuts and transparent union are mounted on the two outlets to be connected. (b) A dead volume free connection with the nano connector. (c) The complete fitting with the black nuts and union housing the nano connector.

2.1.3 Installing and Configuring the Application Fluidics

Each capillary must be installed sequentially starting from the pump outlet. Please flush each capillary using the respective pump and ensure that a droplet is visible at the capillary outlet in question before making the next connection. This will ensure that all air is removed from the capillaries and connections and that no air is passed through the column.

2.1.4 Interfacing the UltiMate 3000 RSLCnano with the Nanospray Flex™ Ion Source

LC–MS based applications using linear columns commonly use the Nanospray Flex Ion source (see *Figure 41*) to interface with Thermo Scientific Mass Spectrometers. As of January 2019, 1.5 m of fused silica (20 µm and 50 µm) and a tile for cutting fused silica capillaries have been included in the pump (NCS-3500RS and NCP-3200RS) accessory kits. This capillary should be used to connect the outlet of the column or UV flow cell, if included in the setup, with the emitter installed in the ion source. The connection between the capillary outlet and the emitter is realized using a 1/32” micro tight® union assembly included with the Nanospray Flex Ion Source. For capillaries and columns with 280 µm O.D. a black sleeve (P/N SC903) should be used with the microtight fitting.

For 360 µm O.D. capillaries and columns (*e.g.* the Acclaim PepMap RSLC C18 75 cm PepMap RSLC, P/N 164939) a beige sleeve (P/N SC603) should be used (both types are included with the ion source). For more details on connecting the UltiMate 3000 RSLCnano with a Thermo Scientific mass spectrometer, please refer to "The Complete and Easy Guide to Configuring Your Thermo Scientific Nano LC for Mass Spectrometric Analysis".

2.1.5 Sample Preparation for Reversed Phase LC Separation

The following are recommendations for Cytochrome C standard digest (P/N 161089) preparation. The glass vial contains 1.6 nmol lyophilized Cytochrome C digest. The sample preparation procedure depends on the system configuration and application in question (*e.g.* nano, capillary or micro).

NOTICE The sample dilution protocol described here differs from the product sheet and is designed to offer the user a starting point. The sample concentration required to run a particular application may deviate from the sample concentrations given below. Sample dilutions may also need to be prepared in a different buffer to that given below. Please **check the required sample concentration** and dilution conditions for the application and **prepare the sample accordingly!**

- **Reconstitution Solvent – (98% Water / 2% Acetonitrile containing 0.1% FA)**. Prepare by mixing 980 µL Water + 0.1% FA and 20 µL 100% Acetonitrile + 0.1% FA in a vial. (See section 2.3.4.4 for information about the individual solvents on page 32.). The use of 2% acetonitrile is recommended, to ensure complete dissolution of hydrophobic peptides.
- Reconstituted the sample in **200 µL** reconstitution solvent to prepare a stock solution of **8 pmol / µL for nano / cap applications.**
- Reconstituted the sample in **100 µL** reconstitution solvent to prepare a stock solution of **16 pmol / µL for micro applications.**
- **Vortex briefly and wait at least 10 minutes** to ensure reconstitution of all peptides prior to use / further dilution.

- **For nano flow applications, dilute the stock solution to 500 fmol / µL** using **mobile phase A** (direct injection) or **loading buffer** (pre-concentration) as follows:
  - Prepare 150 µL mobile phase A in an autosampler vial (with insert) and add 10 µL from the 8 pmol / µL Cytochrome C stock solution.
  - Mix (on Vortex or with pipette) briefly to homogenate the solution.
  - Ensure there are no air bubbles at the bottom of the vial.

TIP: To limit the risk of peptide or protein adsorption on the walls of the vials, Thermo Fisher Scientific recommends using vials containing glass inserts (Polypropylene vials for WPS with glass insert, 250 µL, set of 100, P/N 6820.0027).

2.1.6 Mobile Phases

- Always use fresh LC-MS grade solvents.
- Thermo Fisher Scientific recommends replacing your solvents at least once every two weeks.
- Avoid the use of detergents when cleaning glassware. All glassware used for LC-MS applications (including graduated cylinders) should be rinsed with LC-MS grade solvents prior to use and should be labelled and stored separately.

IMPORTANT: When installing fresh mobile phase on the LC system, replace the mobile phase solvent in the bottle completely. **DO NOT "top up" mobile phases** to avoid solvent composition changes or unwanted components building up in the mobile phase bottles.

2.2 Available Trapping Columns

2.2.1 Available Formats

Trap columns are available in two formats, which are a cartridge-based µ-precolumn (*Figure 5*) and a nano trap column (*Figure 6*). Both types of trapping columns are UHPLC compatible due to the nanoViper fittings employed. The choice between a µ-precolumn or a nano trap depends on application needs such as flexibility, sample loading flow rate and robustness as well as sample quality, desired loading capacity and personal preference.

Figure 5: µ-Precolumn (cartridge-based)

TIP P/N 164648 contains two 30 µm ID x 100 mm nanoViper capillaries and can be used to order replacement capillaries

µ-precolumns are small trap cartridges that are inserted into a cartridge holder, connected to the switching valve by two 30 µm ID x 100mm nanoViper capillaries. The stationary phase is retained by a frit at both ends of the cartridge allowing the mobile phase to flow through it in both directions without disrupting the column packing. Therefore, µ-precolumns can be used in both forward- and back-flush operation (see section 2.2.2 for details). The bed volume is large, but short, giving it higher absolute loadability compared to nano traps, but the short bed could result in earlier sample breakthrough for hydrophilic components. Backpressure is lower compared to nano trap columns and therefore µ-precolumns can accommodate higher loading flows and are often preferred when large sample volumes need to be injected.

Figure 6: Nano trap

Nano trap columns consist of a single 15-cm-long nanoViper capillary containing 1 or 2 cm of stationary phase at one end of the capillary. Nano traps must be operated exclusively in forward-flush mode. The chromatographic bed volume is lower than that of µ-precolumns, but the longer bed length minimizes sample breakthrough. Nano traps give a higher backpressure than µ-precolumns and are thus operated at lower flow rates.

> **TIP:** Note that for Acclaim PepMap RSLCnano columns, the difference between the pressures on the nano trap column and analytical column is smaller than with the combination of the µ-precolumn and the analytical column.

2.2.2 The Difference between Forward Flush and Back Flush

The terms forward-flush and back-flush are used to indicate whether the mobile phase from the NC pump during gradient elution flows in the same or opposite direction compared to the mobile phase flow during sample loading. *Figure 7* shows the different fluidic setups for a forward- and a back-flush fluidic pathway.

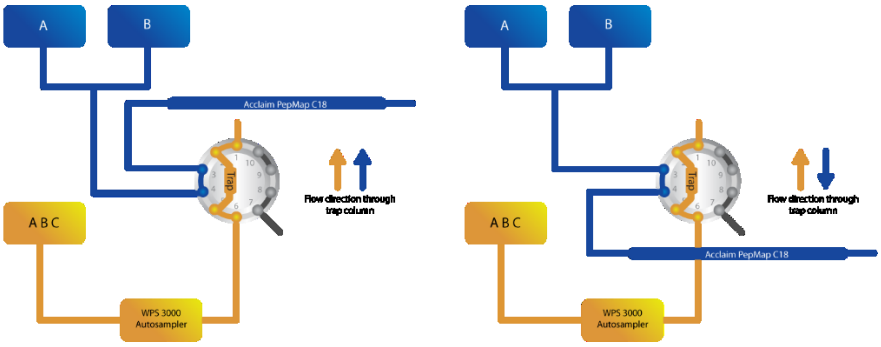


Figure 7: The different fluidic configurations for forward-flush (left) and back-flush (right)

For nano trap columns, the packing material is only retained by the frit at one end of the trap column. In order not to damage nano traps, only forward-flush can be used.

In the µ-precolumn design, the stationary phase is retained by a frit at both ends of the column packing. This means that the mobile phase can flow through the cartridge in either direction without disrupting the column packing, *i.e.* forward-flush and back-flush operation.

The choice between forward- and back-flush for the µ-precolumn design is made on the following criteria.

- In forward-flush, the trap column also acts as a guard column to protect the separation column.
- In back-flush, better separation is obtained, but any particulates or insoluble debris from the sample could end up on the separation column.

NOTICE For pre-concentration applications, better chromatographic resolution (narrower chromatographic peaks) are produced when the µ-precolumn is installed in back-flush mode.

2.3 Installing the UltiMate 3000 RSLCnano System

2.3.1 UltiMate 3000 RSLCnano System Components

SRD-3400, (*Optional):*
SRD-3200 with degassing
or SR-3000 without degassing.

NCS-3500RS module featuring
- NC pump, up to 900 bar
- Loading pump, micro Titanium up to 620 bar
- Column compartment with up to two 860 bar switching valves
Optional:- NCP-3200RS, - PAEK valve

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

WPS-3000TPL RS
- Temperature controlled autosampler equipped with a
  860 bar switching valve
- *Optional:*
  8-port valve (350 bar) for micro-fractionation applications

Figure 8: RSLCnano system overview

2.3.2 NC Pump Configurations

2.3.2.1 ProFlow™ and Classic Flow Meters

Flow meters are used to actively regulate NC pump flow on the instrument in order to deliver very precise low-flow gradients. There are two types of flow meter available:

- ProFlow flow meter

  The ProFlow flow meter controls pump flow using thermal flow sensors built into the flow meter. It is a unit dedicated to nano and low capillary flow rates (50 nL / min – 1500 nL / min) and allows a pump pressure rating of 900 bar at the full flow rate range for all common solvents used for reversed phased LC applications.

- Classic flow meter

  The classic flow meter determines the flow rate indirectly by measuring the pressure drop across a restriction capillary contained within the flow meter itself. The pressure rating of the pump using the classic flow meter is 800 bar for flow rates ≤ the nominal flow rate (see *Table 1*).

2.3.2.2 Flow Selectors for the Classic Flow Meter

Each classic flow meter contains a flow selector that defines the flow rate range of the flow meter. These flow selectors are interchangeable. The flow rate ranges of the respective flow selectors and the nominal flow rates are given in *Table 1* below.

Flow Selector Type	Total Flow Rate (Sum of Channels A and B)		
	Nominal	Minimum	Maximum
Nano (Nan)	500 nL/min	50 nL/min	1000 nL/min
Capillary (Cap)	5 µL/min	500 nL/min	10 µL/min
Micro (Mic)	25 µL/min	2.5 µL/min	50 µL/min

Table 1: Properties of the different flow selectors

2.3.2.3 NCS-3500RS with the ProFlow Flow Meter

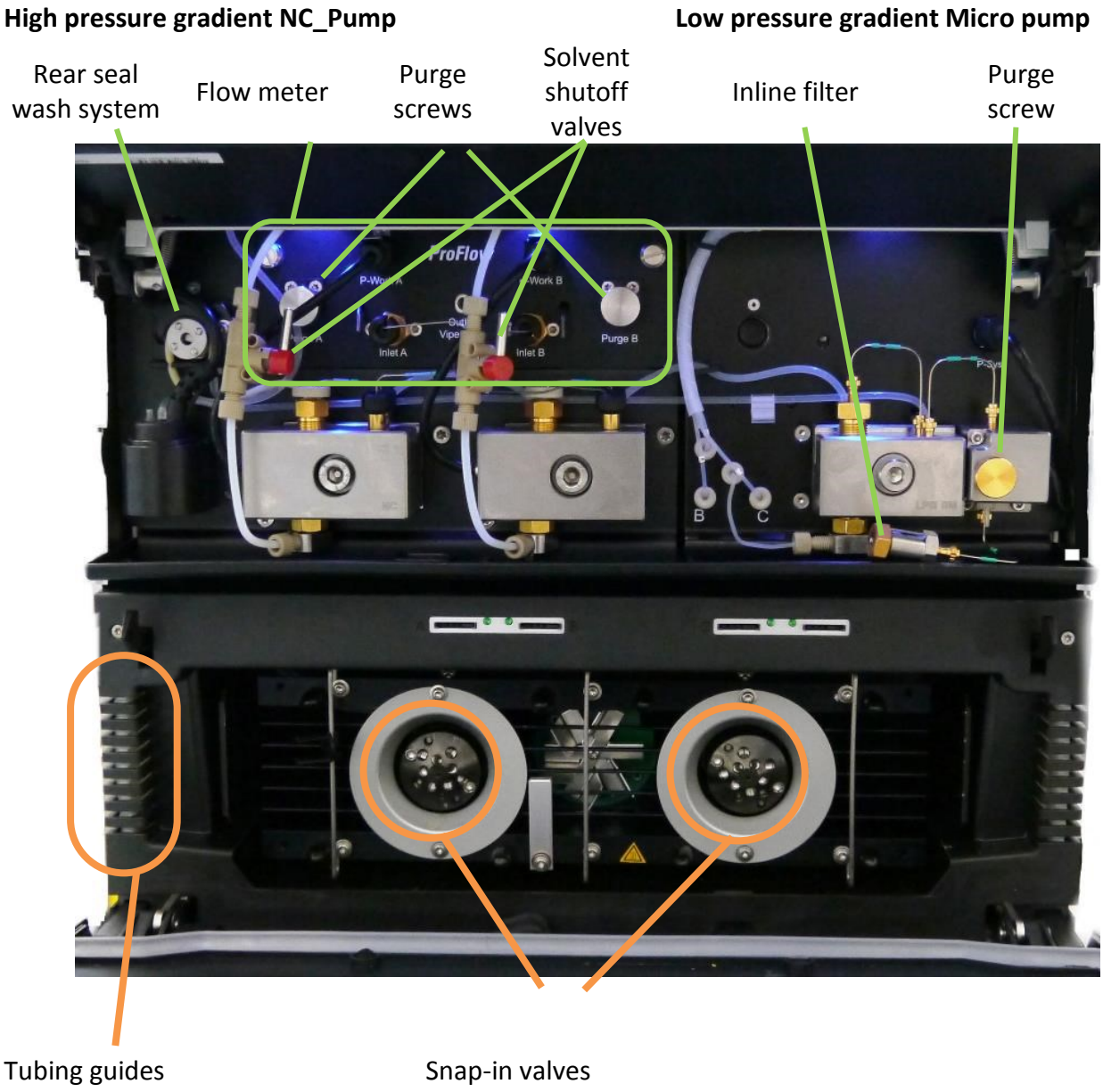


Figure 9: NCS-3500RS with ProFlow flow meter

NOTICE The maximum pump pressure available for the column is 900 bar if a ProFlow flow meter is installed.

2.3.2.4 NCS-3500RS with Classic Flow Meter

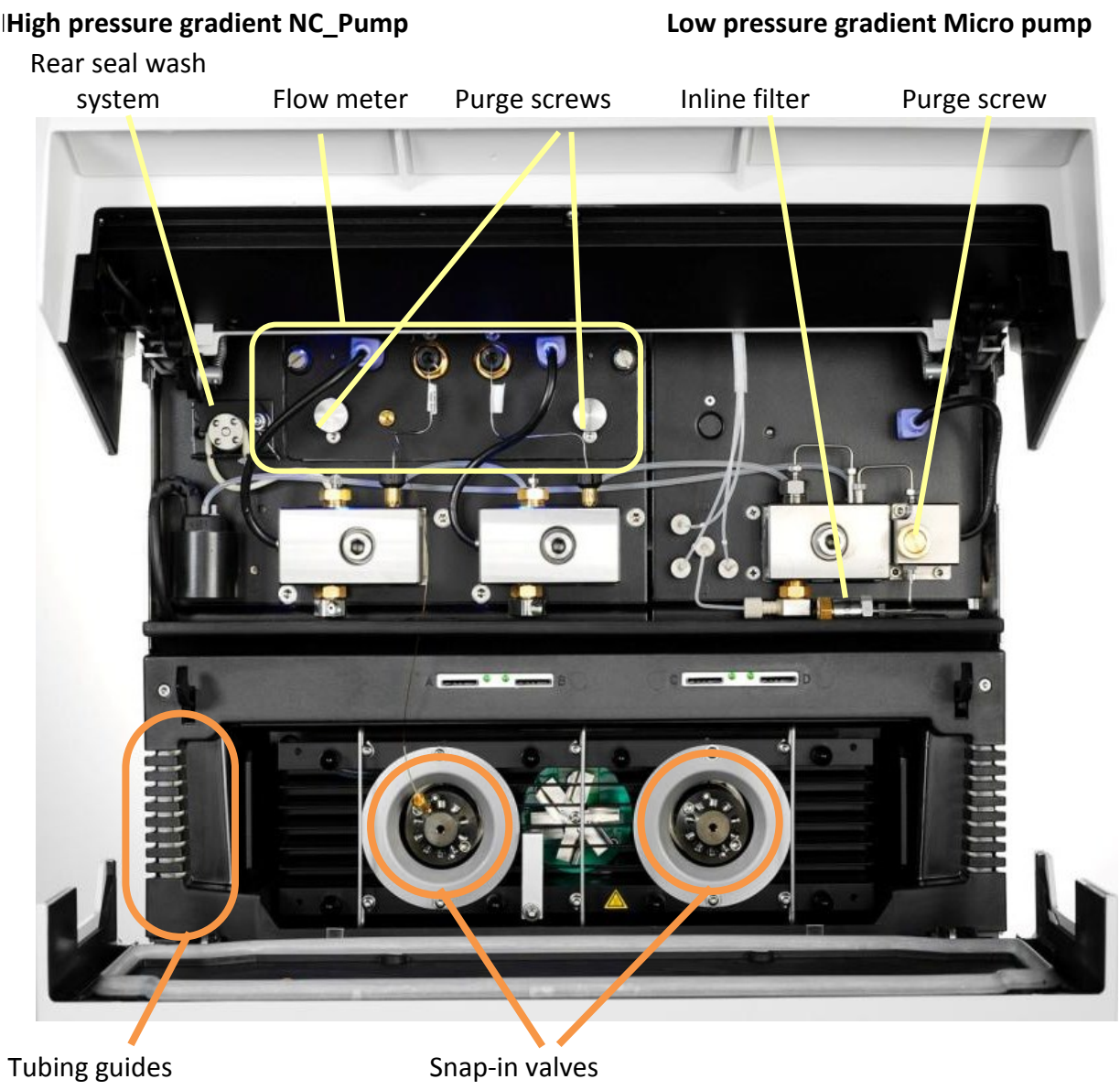


Figure 10: NCS-3500RS with classic flow meter

NOTICE The maximum pump pressure available for the column is 800 bar with a classic flow meter installed.

2.3.2.5 NCP-3200RS

NCP-3200RS module featuring
- NC pump

Figure 11: NCP 3200RS pump

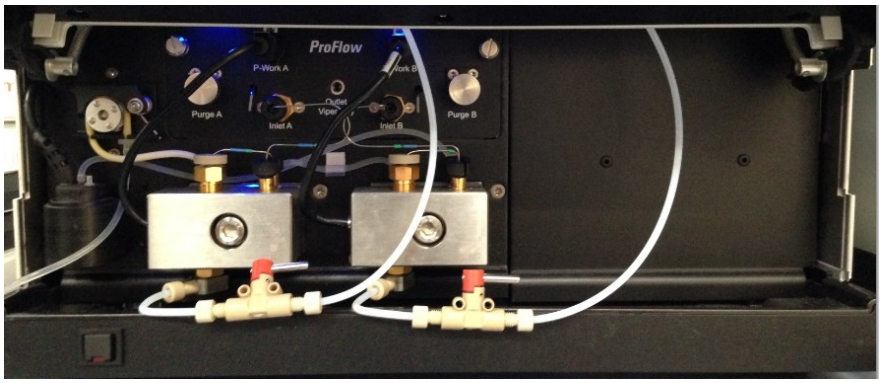


Figure 12: NCP 3200RS pump with ProFlow flow meter installed

Figure 13: NCP-3200RS with a classic flow meter installed

TIP The NCS-3500RS and NCP-3200RS are both compatible with the ProFlow and classic flow meters.

TIP An upgrade kit from classic nano to ProFlow is available for both NCS-3500RS and NCP-3200RS. Please order P/N 6041.7850 (ProFlow flow meter) and P/N 6041.3003 (Upgrade Kit for ProFlow flow meter). Please see the ProFlow quick installation guide for more details.

2.3.3 Software Compatibility for NCx-3x00RS Operation with ProFlow and Classic Flow Meters

- ProFlow technology is fully compatible with all previous NCx-3x00RS modules (mandatory firmware upgrade to version ≥ 1.40 required).
- For LC control via Xcalibur, ProFlow technology requires SII for Xcalibur ≥ 1.2 with Chromeleon 7.2 SR4 (or later) driver updates.
- ProFlow technology is **NOT supported** by DCMSLink (based on Chromeleon 6.8) for Xcalibur.
- For LC control using Chromeleon, ProFlow technology requires:

  ≥ SR 15b for Chromeleon 6.80

  ≥ SR4 for Chromeleon 7.2

NOTICE The Chromeleon and SII software versions show in *Figure 14* are the minimum requirements. Later versions are fully compatible.

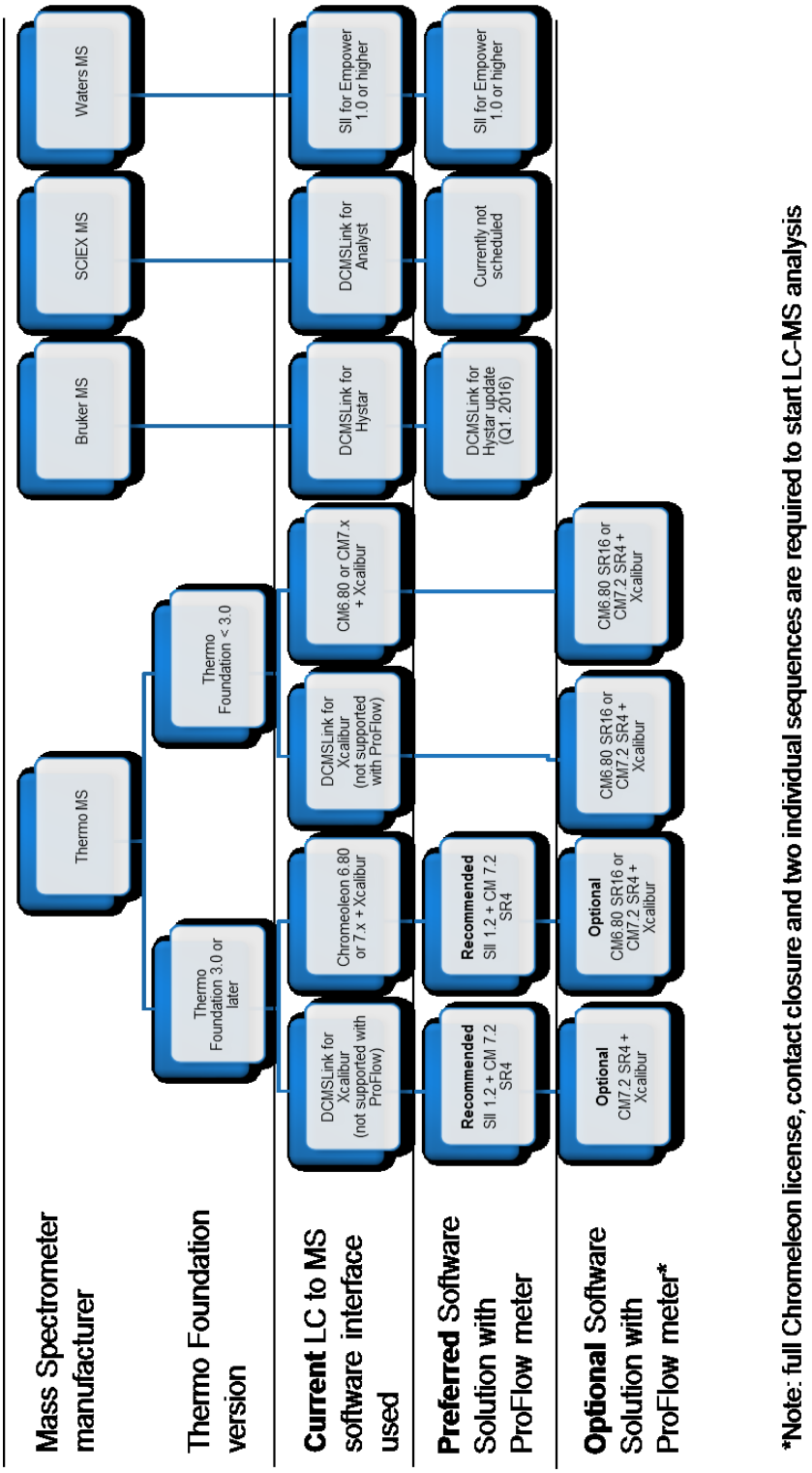


Figure 14: UltiMate 3000 RSLCnano with ProFlow flow meter – compatibility matrix

2.3.4 Preparing the RSLCnano for Use

The UltiMate 3000 RSLCnano system must be prepared and primed prior to use. A brief description of each step is given below. For detailed information on configuring the system, please refer to the respective operating instructions for each module.

2.3.4.1 Hardware Installation

- Install the power, SRD and USB cables but **do not connect the PC**.
- The real seal wash solvent should be installed and primed prior to powering up the modules (see section 2.3.4.5 for details on how to prepare the rear seal wash solvent).
- Use the PEEK solvent inlet filter frits for both the NC pump and the loading pump solvent lines. Do not use metal filters.
- The online degasser for the loading pump must be employed when:

  The loading pump is used for gradient formation.

  The loading pump flow rate is above 20 µL / min.
- Connect the contact closure cable between relay 4 of the autosampler and mass spectrometer I/O.
- The WPS-3000TPL RS as well as the WPS-3000 T(B)FC is delivered with the buffer tubing, needle and sample loop preconfigured on the 6-port injection valve. The positions of each of these components is essential to the normal operation of the sampler. The correct configuration is depicted in each of the applications respective hardware layout.
- Power up the modules.

Notice: 60 minutes are required after pump power up for the flow meter to reach a stable operating temperature. It is recommended to execute pump and flow meter purges during this time. Flow meter zero offset adjustments / calibration routines can only be started after the module has been powered on for 60 minutes.

2.3.4.2 Software Installation

- For LC-MS control using Xcalibur with SII, the order of software installation should be Foundation -> Xcalibur -> MS Driver -> SII.
- Once the software is installed, connect the USB cable(s) to the PC.

2.3.4.3 Instrument Configuration

- LC-(UV)-MS configuration is done through the instrument configuration panel accessed via Xcalibur Foundation Instrument Configuration.
- Verify that the correct flow meter type is displayed under the flow meter tab within the instrument configuration panel and that the valve(s) are correctly configured (oven/valves tab of the NC module).
- When configuring the WPS-3000TPL RS or FC autosampler, ensure that the settings in the instrument configuration match the fluidic components installed in the autosampler.

NOTICE The sample loop volume will vary according to the application. Ensure that the sample loop volume in the instrument configuration matches the hardware.

2.3.4.4 Solvent Preparation

- Only use fresh LC-MS grade solvents
- Degas (sonicate) 10 minutes before installing
- Refresh every 2 weeks to eliminate bacterial growth and/or changes in solvent composition
- For best results, use premixed Fisher Chemical Optima LC-MS grade solvents:

NC Pump solvent A - Water with 0.1% Formic Acid (FA) **P/N LS118-500**

NC Pump solvent B - 80/20 (v/v) Acetonitrile / Water with 0.1% FA **P/N LS122-500**

Alternative for NC Pump solvent B (ProFlow only) - Acetonitrile with 0.1% FA **P/N LS120-500**

2.3.4.5 Auxiliary Solvents:

The following solvents are recommendations to ensure robust operation

- Rear Seal Wash:
  10% methanol in water + 0.1% Formic Acid (FA)
- Loading Pump solvent:
  Water with 0.1% FA **P/N LS118-500**
- Autosampler needle wash:
  80/20 (v/v) Acetonitrile / Water with 0.1% FA **P/N LS122-500**
- Transport liquid for µLiter pick up injection:
  application dependent, should be the same as mobile phase A (direct injection) or the loading buffer (pre-concentration).

NOTICE The autosampler needle wash example is a typical strong wash solvent. Depending on the application, this may need to be adjusted to prevent carry-over *e.g.* to 100% Acetonitrile with 0.1% FA.

2.3.4.6 Purging the Pumps and Flow Meter

The NC pumps and flow meter (NCS-3500RS and NCP-3200RS) and the loading pump (NCS-3500RS only) require purging each time solvents are refreshed or changed.

- Purging the NC Pump blocks

The pump block purge time (*Table 2*) depends on the flow meter type and whether the solvents are being refreshed or changed:

Flow Meter Type	Refresh Solvent	Change Solvent
ProFlow	5 min	15 min
Classic	10 min	30 min

Table 2: NC Pump block purge times

- Purging the flow meter

The flow meter purge time (*Table 3)* depends on the flow meter type and the application scale (nano, capillary or micro):

Application	Recommended Purge Time
Nano LC (ProFlow)	10 min
Nano LC (classic)	30 min
Capillary LC (classic)	5 min
Micro LC (classic)	5 min

Table 3: Flow meter purge times

- Purging the loading pump

It is important that **all three** solvent lines are purged, irrespective of whether all three channels are used for an application or just one.

Unused solvent channels should be purged with 10 – 50% isopropanol in water.

Purge each channel for at least 5 minutes.

2.3.4.7 *Performing the Adjust Zero Balance Test / Pressure Transducer Test*

The type of test required will depend on the flow meter installed.

- ProFlow flow meter -> adjust zero balance test

The test “Adjust Zero Balance” is located under the “Wellness” button on the NC pump tab on the ePanel. A wizard will guide you through the procedure.

- Classic flow meter -> pressure transducer test

The test is located under the “NC_Pump_Diagnostics” tab of the ePanel.

TIP The descriptions above refer to the test locations in SII / CM 7.2. For information on where to find the test in CM 6.8 / DCMS^{Link} please refer to the NCx-3x00 operating instructions.

2.3.5 Flow meter calibration

Solvent calibration routines differ for the two types of flow meter.

2.3.5.1 ProFlow flow meter solvent calibration

The ProFlow flow meter uses thermal flow sensors for direct measurement of solvent flow. The ProFlow flow meter comes pre-calibrated with four solvent types: Water, 100% Acetonitrile, 80% Acetonitrile and 100% Methanol. The calibration values for these solvent types are stored locally on the flow meter and are valid for the *life* of the flow meter. They **are also valid** when the flow meter is transferred from one UltiMate 3000 RSLCnano system to another.

NOTICE The solvent types are **valid** for a variation in composition **≤ 2%** *i.e.* a solvent consisting of 98% Water / 2% Acetonitrile can be run using the pre-calibrated solvent type Water. This is also applicable if the added modifier changes >2%.

For all other solvents, a custom solvent calibration is required.

Custom solvent calibrations are only valid for the channel on which they are carried out. To perform a custom solvent calibration, click on the "Wellness" button on the NC_Pump panel and then"Calibrate Solvent". A wizard guides the user through the calibration procedure.

TIP Custom solvent calibrations are also stored locally on the flow meter hardware and are valid for the life of the flow meter.

The solvent type for the A and B channels should match the solvents used in the application. To select the solvent type, go to the PumpModule tab of the ePanel. Under NC Pump -> More Options select the desired solvent type for each channel from the drop down menu under 'solvents'.

2.3.5.2 Viscosity Measurement Test using the Classic Flow Meter

The classic flow meter uses the pressure drop across a restriction capillary (integrated in the flow selector) to determine solvent flow rate. During the viscosity measurement calibration, the resistance of the solvent is measured relative to the factory calibration value for water

and is reported as a percentage of that value. The viscosity measurement test is located under the “NC_Pump_Diagnostics” tab in the ePanel. Typical viscosity values for a number of common solvents are given in *Table 4* below:

Solvent	Viscosity %
Water	100
80 / 20 (v/v) Acetonitrile / Water	66
50 % ACN / 50% Water	100
Acetonitrile	50
MeOH	75
IPA	220

Table 4: Viscosity values relative to water for common solvents

TIP The viscosity measurement test takes about 15 minutes. The user should remain present throughout the test, as prompt user intervention is required part way through. Once the test is finished, the measured viscosity values can be stored by selecting the ‘apply’ button for the respective channel. The results should be examined for plausibility (see *Table 4* for reference values).

2.4 Application Overview

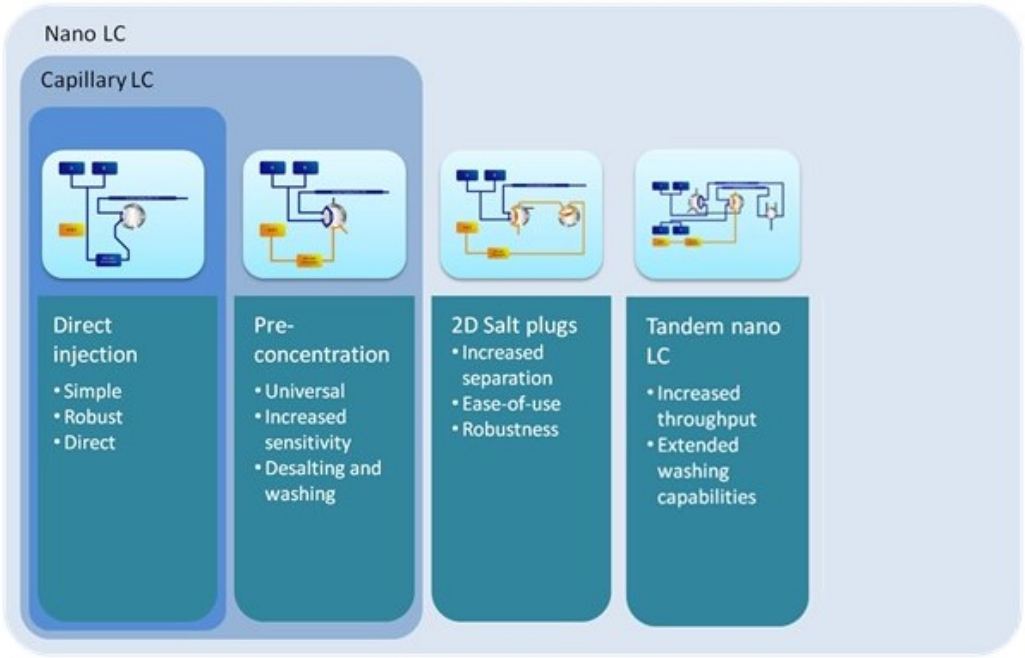


Figure 15: Overview of the Applications available for the RSLCnano

2.5 Direct Injection onto a Nano Column

2.5.1 Hardware Layout

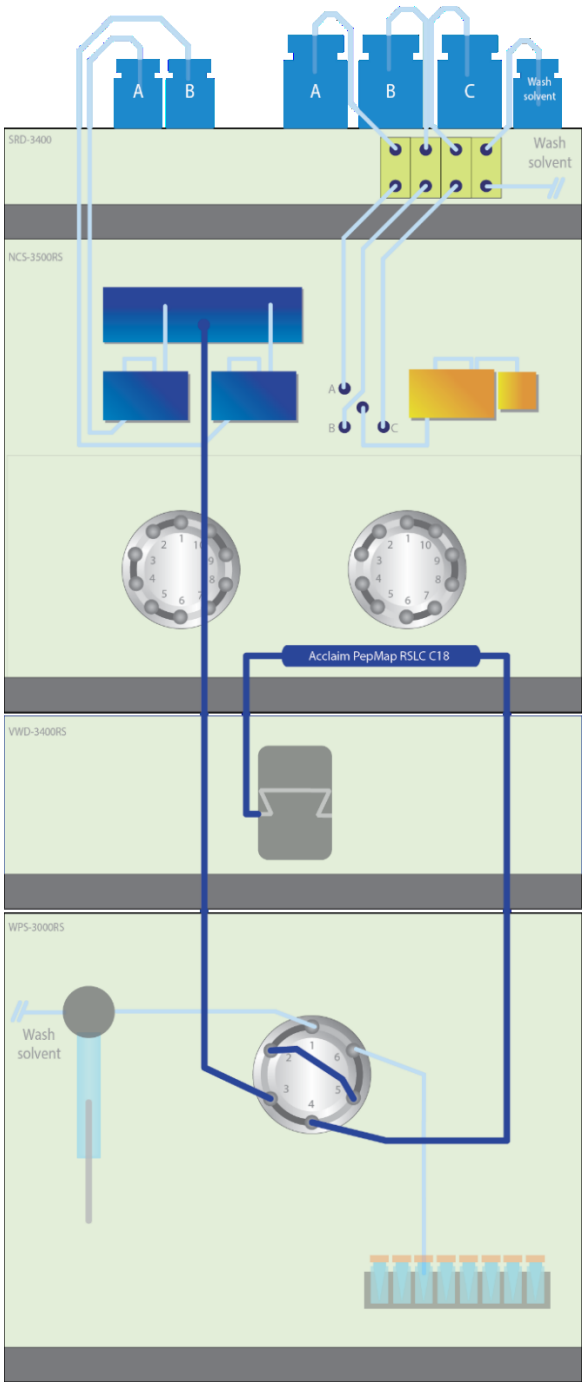


Figure 16: Setup for a Direct Injection experiment onto a nano column including the optional UV detector

The instrument setup presented in *Figure 16* consists of:

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

NOTICE The NCS-3500RS in this setup can be exchanged for an NCP-3200RS (P/N 5041.0030A)

TIP If no valve is available, a Viper union (P/N 6040.2304, ordered separately) can be used to connect the capillary from the sampler valve directly to the column positioned here in the column oven.

2.5.2 Fluidic Setup

Figure 16 and *Figure 17* show the setup using the parts provided in the Direct Injection nano application kit. Columns are marked with letters and the tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 16*.

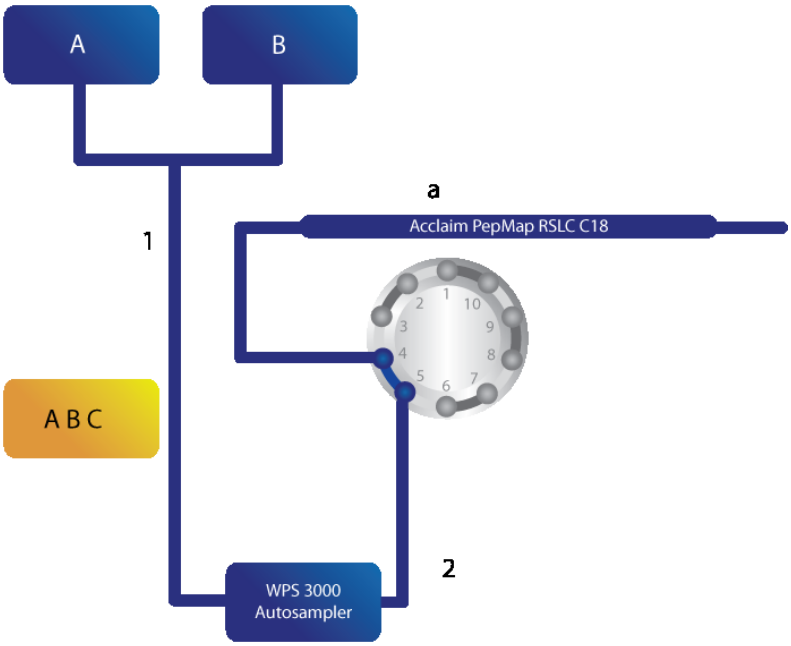


Figure 17 Fluidic connections for a direct injection experiment onto a nano column

TIP If no valve is available, a Viper union (P/N 6040.2304, ordered separately) can be used to connect the capillary (2) directly to the column (a).

#	Item	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

Table 5: RSLCnano Direct Injection nano LC kit (P/N 6720.0300) contents

2.5.3 Installation Tips

- Follow the “General Recommendations for Applications” (section 2.1 on page 15).
- The impact of dwell/dead volumes on reproducibility is highly significant. Improper connections of the different elements are the most likely cause of failure for this application.

2.5.4 Testing the Application

Test the direct injection application using the following conditions:

Property	Setting
Mobile Phase A	Water + 0.1% FA
Mobile Phase B	80/20 (v/v) Acetonitrile / Water with 0.1% FA
Sample	Cytochrome C digest, 500 fmol / µL
Injection Volume	1 µL (Full Loop)
UV detection (optional)	214 nm
Gradient	4% to 50% B in 30 minutes, 90% B for 5 minutes, 25 minutes equilibration
Oven temperature	35 °C
WPS temperature	5 °C
Flow Rate	300 nL / min

Table 6: Test conditions for direct injection of 500 fmol Cytochrome C onto a nano column

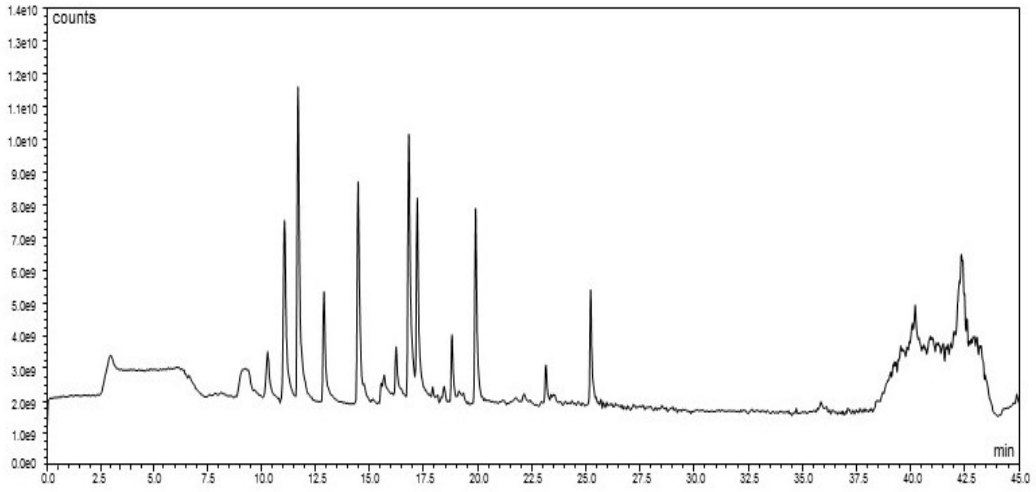


Figure 18: Typical MS TIC for a direct injection of 500fmol Cytochrome C onto a nano column

2.5.5 Large Volume Injections

Direct injection applications in nano LC are typically performed with 1 µL loops to minimize the gradient delay. Larger volume injections are most commonly performed using a pre-concentration setup. The WPS-3000TPL RS and FC autosamplers support custom injection programs (User-Defined Program (UDP)) which switch the injection valve offline after sample loading to bypass the loop and thereby reduce gradient delay (See TN 72277 for details). This way, a larger sample volume can be injected 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 i) desalted samples, since all the sample that is injected will enter the MS, and ii) an investment of extra analysis time to accommodate the complete loading of sample at low flow rates.

2.6 Direct Injection onto a Capillary Column

2.6.1 Hardware Layout

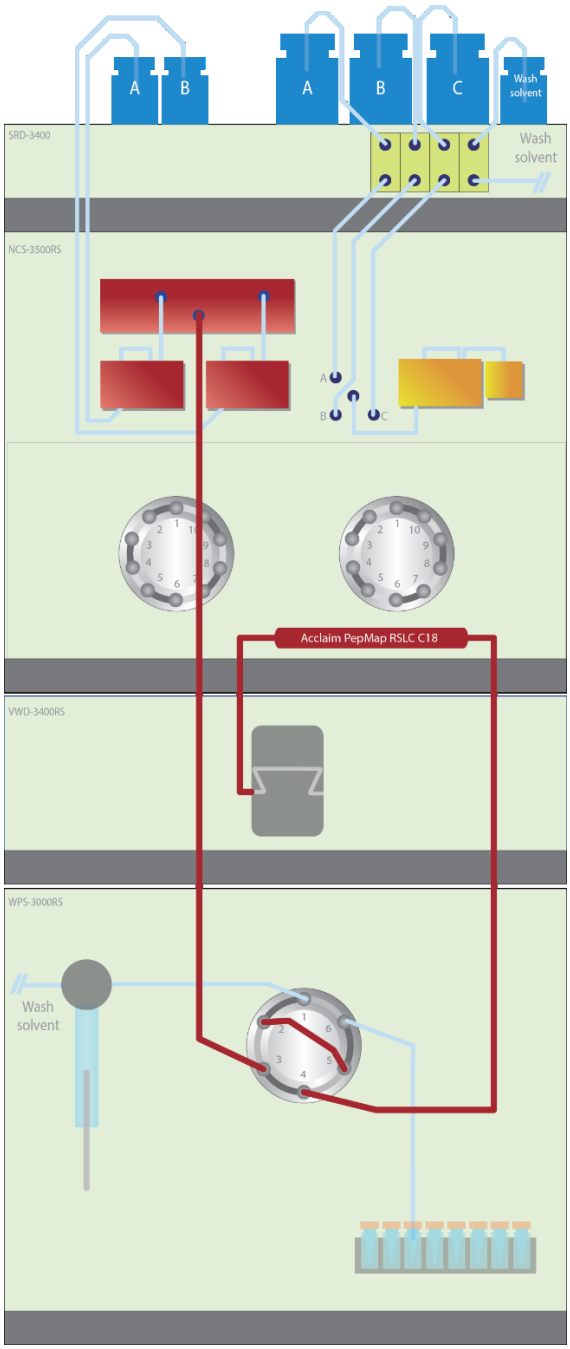


Figure 19: Setup for a Direct Injection experiment onto a capillary column including the optional UV detector

The instrument setup presented in *Figure 19* consists of:

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

NOTICE The NCS-3500RS in this setup can be exchanged for an NCP-3200RS

TIP If no valve is available, a Viper union (P/N 6040.2304, ordered separately) can be used to connect the capillary from the sampler valve directly to the column positioned here in the column oven.

2.6.2 Fluidic Setup

Figure 19 and *Figure 20* shows the setup using the parts provided in the Direct Injection capillary application kit. Columns are marked with letters and the tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 19*.

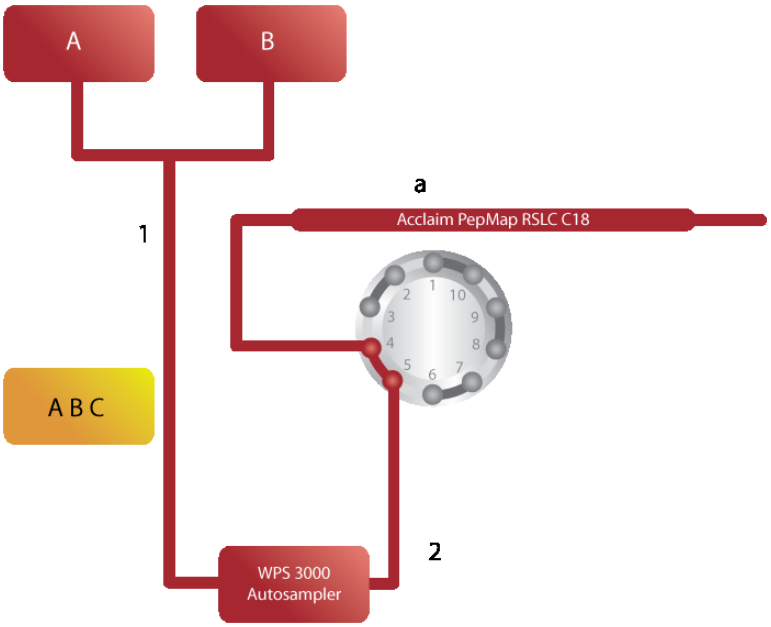


Figure 20: Fluidic connections for a direct injection onto a capillary column

TIP If no valve is available, a Viper union (P/N 6040.2304, ordered separately) can be used to connect the capillary (2) directly to the column (a).

#	Item	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

Table 7: RSLCnano Direct Injection capillary LC kit (P/N 6720.0305) contents

2.6.3 Installation Tips

- Follow the "General Recommendations for Applications" (section 2.1 on page 15).
- A detailed description of how to set up and run this application together with optimized measurement parameters for both LC and MS is also available in TN72277.

2.6.4 Testing the Application

Test the direct injection application using the following conditions:

Property	Setting
Mobile Phase A	Water + 0.1% FA
Mobile Phase B	80/20 (v/v) Acetonitrile / Water with 0.1% FA
Sample	Cytochrome C digest, 200 fmol / µL
Injection Volume	5 µL (Full Loop)
UV detection (Optional)	214 nm
Gradient	1 min 5% B, then 5% to 35% B in 10 minutes, then to 90% B in 1 min. Ramp to 10 µL / min in 0.1 min. Hold at 90% B for 1 min (10 µL / min), then to 5% B in 0.1 min (10 µL / min). Hold at 5% B for 2.4 min then ramp to 5µL/min in 0.1 min
Oven temperature	40 °C
WPS temperature	5 °C
Flow Rate	5 µL / min during gradient, 10 µL / min during wash and equilibration

Table 8: Test conditions for direct injection of 1 pmol Cytochrome C onto a capillary column

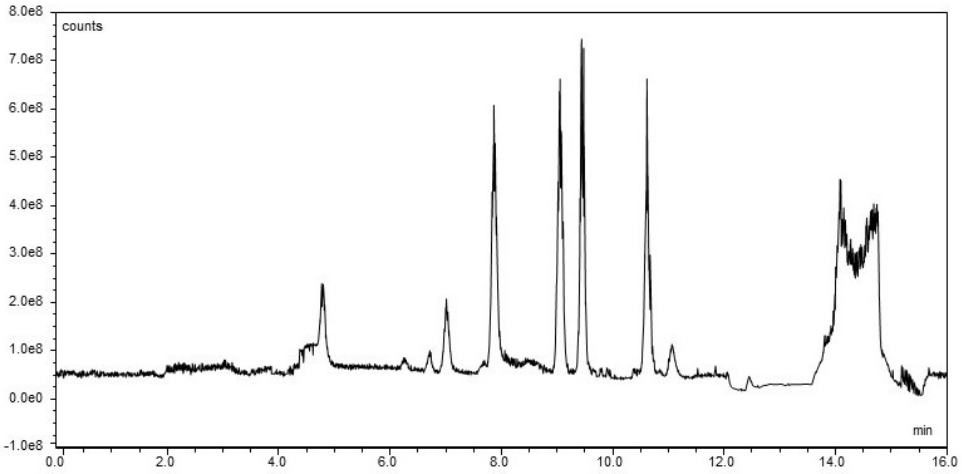


Figure 21: Typical chromatogram for a direct injection of 1pmol Cytochrome C onto a capillary column

2.7 Pre-concentration onto a Nano Column

2.7.1 Hardware Layout

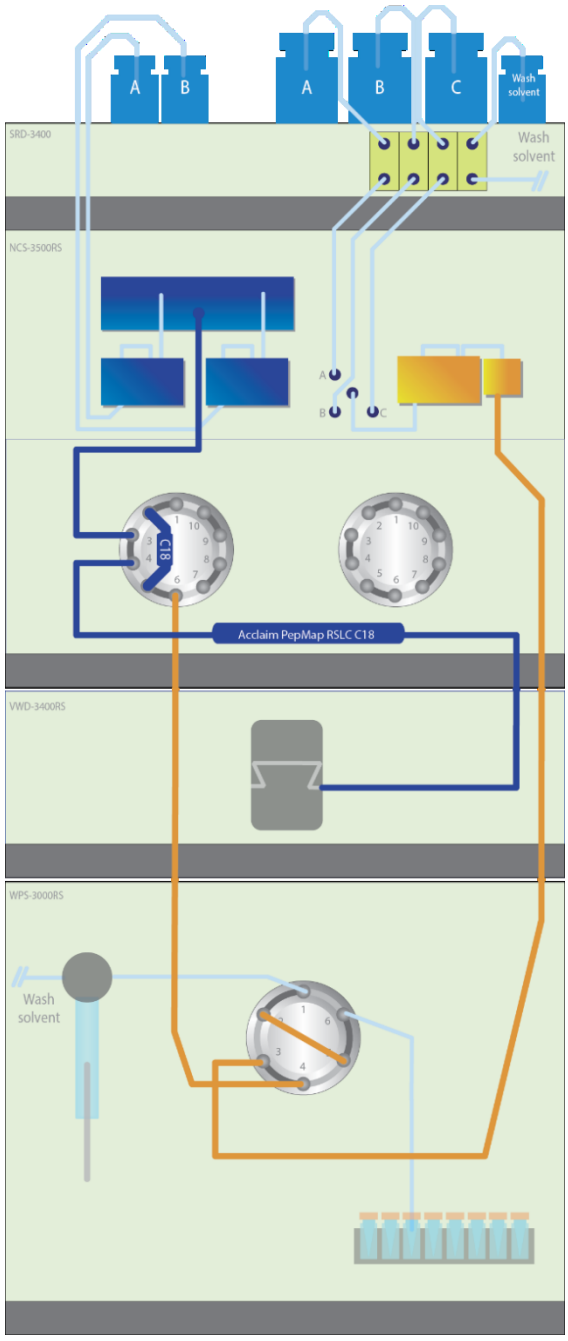


Figure 22: Setup for a pre-concentration experiment onto a nano column including the optional UV detector

The instrument setup presented in *Figure 22* consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010A
1x 10-port sw. valve	6041.0001A
VWD-3400RS (optional)	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0310

2.7.2 Fluidic Setup

Figure 22 and *Figure 23* shows the setup using the parts provided in the pre-concentration nano application kit. Columns are marked with letters and tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 23*.

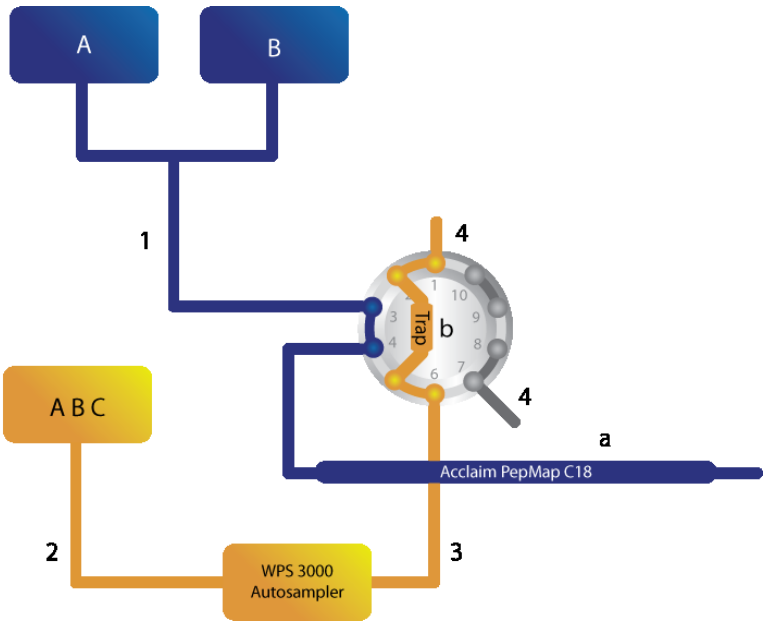


Figure 23: Fluidic connections for a pre-concentration experiment onto a nano column

TIP The schematic shows a 10-port switching valve. This application can also be performed using a 6-port valve.

#	Item	P/N
a	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
b	300 µm I.D. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 5 cartridges)	160454
	µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, nanoViper fittings	164649
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

#	Item	P/N
	nanoViper sample loop 20 µL, FS/PEEK sheathed	6826.2420
4	PTFE tubing, 500 µm I.D. 100 cm, used in waste tubing	6720.0077
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread (4 pieces)	6720.0015
	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

Table 9: RSLCnano pre-concentration nano LC kit (P/N 6720.0310) contents

2.7.3 Installation Tips

- Follow the “General Recommendations for Applications” (section 2.1 on page 15).
- For details on installing the trap column, refer to section 2.2– “available trapping columns” on page 21.
- If a loss of hydrophilic peptides is observed, adding a stronger ion-pairing agent such as Trifluoroacetic acid (TFA, up to 0.1%) or heptafluorobutyric acid (HFBA (0.05%) to the loading solvent can be considered.
- The 20 µm x 750 mm capillary (P/N 6041.5280) can be used to convert the pre concentration setup to a direct injection configuration (see capillary 1 in *Figure 17*). One further capillary is required (P/N 6041.5260, capillary 2 in *Figure 17*) which must be ordered separately.

2.7.4 Testing the Application

Test the pre-concentration setup using the following conditions:

Property	Setting
Mobile Phase A	Water + 0.1% FA
Mobile Phase B	80/20 (v/v) Acetonitrile / Water with 0.1% FA
Loading solvent	Water + 0.1% FA
Sample	Cytochrome C digest, 500 fmol / µL
Injection Volume	1 µL (partial loop fill or µL pickup)
UV detection (Optional)	214 nm
Loading time	0.5 minutes (may vary according to required injection volume / routine)
Gradient	4% to 55% B in 30 minutes, 90% B for 5 minutes, 8.5 minutes equilibration
Oven temperature	35 °C
WPS temperature	5 °C
Loading flow rate	30 µL / min
NC Flow Rate	300 nL / min (ProFlow or classic flow meter with nano flow selector)

Table 10 Test conditions for pre-concentration injection of 1 pmol Cytochrome C onto a nano column

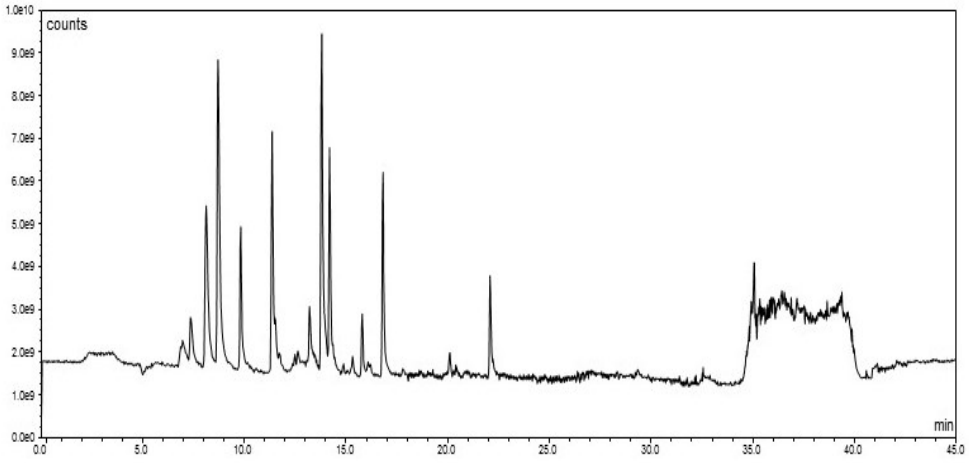


Figure 24: Typical chromatogram for a pre-concentration of 500 fmol Cytochrome C onto a nano column

2.8 Pre-concentration onto a 200 µm Monolithic Column

2.8.1 Hardware Layout

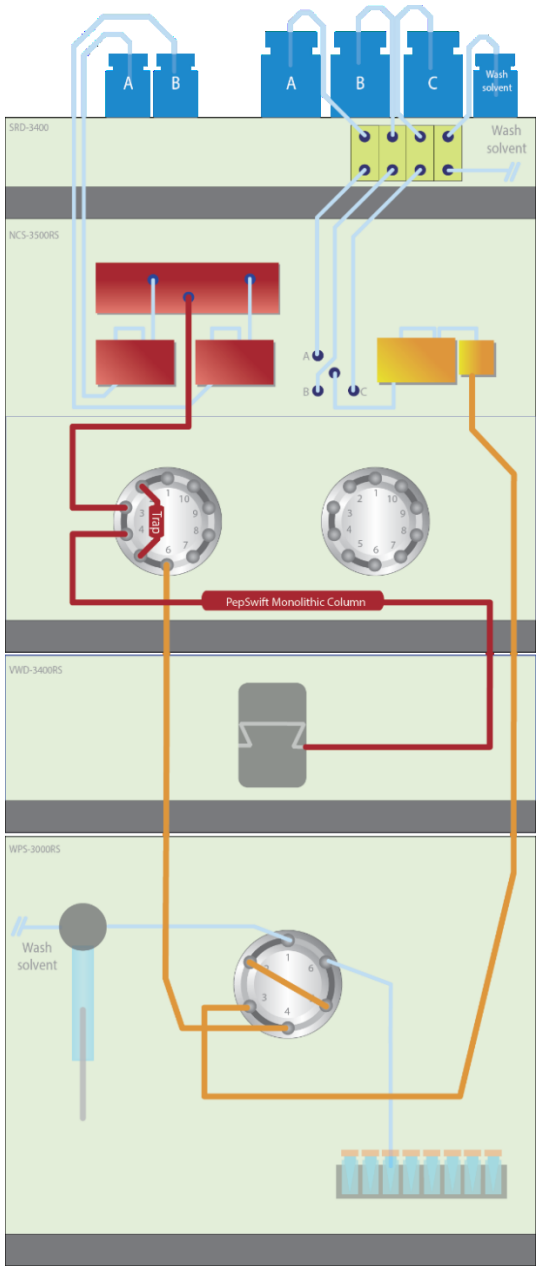


Figure 25: Setup for a Pre-concentration experiment onto a monolithic column including the optional UV detector

The instrument setup presented in *Figure 25* consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0020
1x.10 port sw valve	6041.0001A
VWD-3400RS *(optional)*	5074.0010
3 nL flow cell	6074.0270
WPS-3000TPL RS	5826.0020
Application kit:	6720.0320

2.8.2 Fluidic Setup

Figure 25 and *Figure 26* shows the setup using the parts provided in the pre-concentration monolithic application kit. Columns are marked with letters and tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 25*.

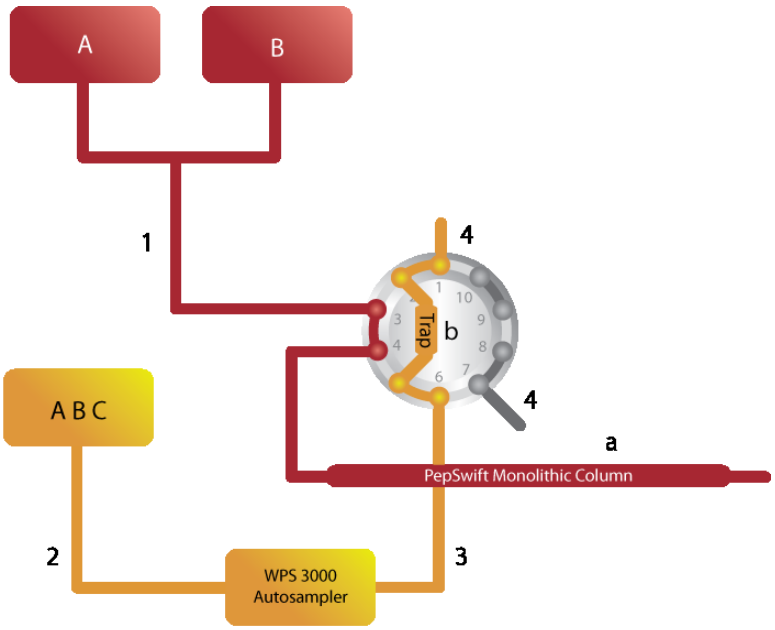


Figure 26: Fluidic connections for a pre-concentration onto a monolithic column

TIP The schematic shows a 10-port switching valve. This application can also be performed using a 6-port valve.

#	Item	P/N
a	PepSwift Monolithic Capillary Column 200 µm I.D. x 5 cm, (PS-DVB), nanoViper	164557
b	PepSwift Monolithic Trap Column	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	6826.2420
	PTFE tubing, 500 µm I.D. x 100 cm, used as waste tubing	6720.0077
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread, 4 pieces	6720.0015
	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

Table 11: RSLCnano Pre-concentration monolithic LC kit (P/N 6720.0320) contents

2.8.3 Installation Tips

- Follow the “General Recommendations for Applications” (section 2.1 on page 15).
- With column oven temperatures below 45°C, TFA can be used instead of HFBA as a loading solvent modifier.
- If a UV detector is used in the setup, the detectors time constant should be reduced to 0.1 seconds due to the speed of the separation.
- If a loss of hydrophilic components is observed, the HFBA solvent concentration in the loading solvent can be increased to 0.1%.

2.8.4 Testing the Application

Test the pre-concentration setup using the following conditions:

Property	Setting
Mobile Phase A	100% Water + 0.05% TFA
Mobile Phase B	50 %/ 50% (v/v) Acetonitrile / Water + 0.04% TFA
Loading Solvent	Water + 0.05% HFBA

Property	Setting
Sample	Cytochrome C digest, 1 pmol / µL, in 98% mobile phase A, 2% mobile phase B. **Note: The sample must be diluted in the loading solvent**
Injection Volume	0.5 µL (partial loop or µL pickup)
UV detection (Optional)	214 nm
Loading time	3 minutes (may vary depending on the required injection volume / routine)
Gradient	1% to 70% B in 8 minutes, 90% B for 2 minutes, 8.5 minutes equilibration
Oven temperature	60 °C
WPS temperature	5 °C
Loading flow	10 µL / min
Flow Rate	3 µL / min (capillary flow selector)

Table 12 Test conditions for pre-concentration injection of 500 fmol Cytochrome C onto a monolithic column

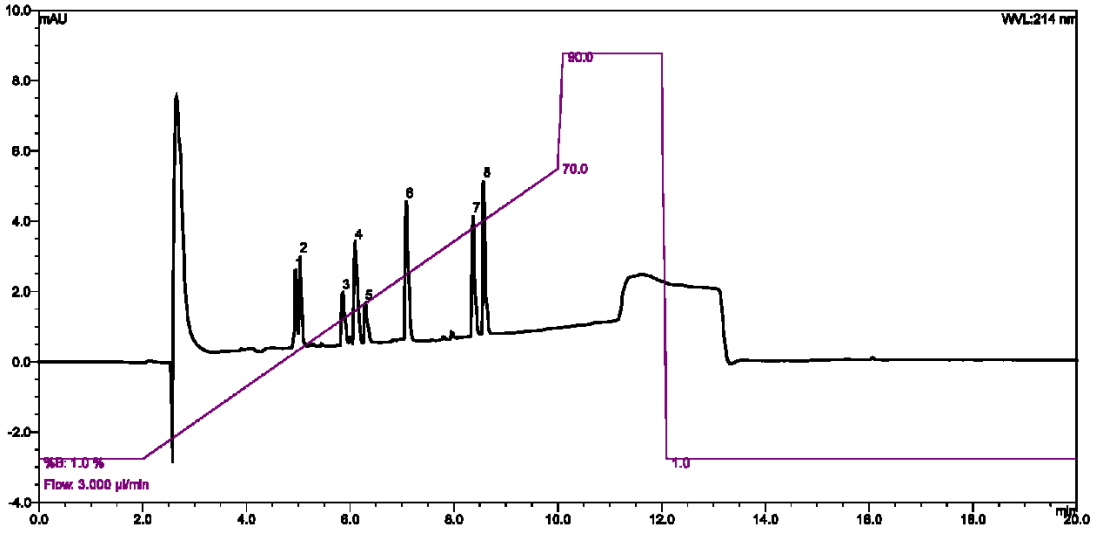


Figure 27: Typical chromatogram for a pre-concentration of 500 fmol of Cytochrome C onto a monolithic column

TIP When the trap column is switched in line with the analytical column, a large absorption (injection) peak is detected at 214 nm. This is due to the different UV absorbance of the ion-pairing agents (*e.g.* HFBA *vs.* TFA) in the loading and analytical solvent.

2.9 Pre-concentration onto a Capillary Column

2.9.1 Hardware Layout

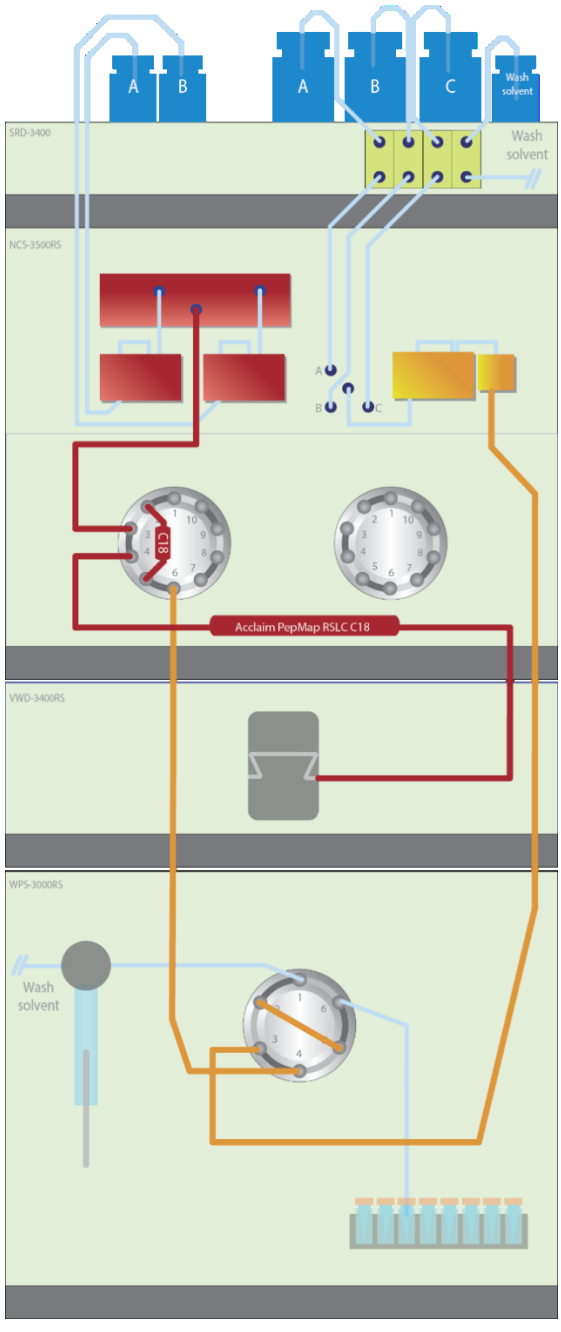


Figure 28: Setup for a pre-concentration experiment onto a capillary column including the optional UV detector

The instrument setup presented in *Figure 28* consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0020
1x.10 port sw. valve	6041.0001A
VWD-3400RS	5074.0010
45 nL flow cell	6074.0280
WPS-3000TPL RS	5826.0020
Application kit:	6720.0315

2.9.2 Fluidic Setup

Figure 28 and *Figure 29* presents the setup using the parts provided in the pre-concentration capillary application kit. Columns are marked with letters and tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 28*.

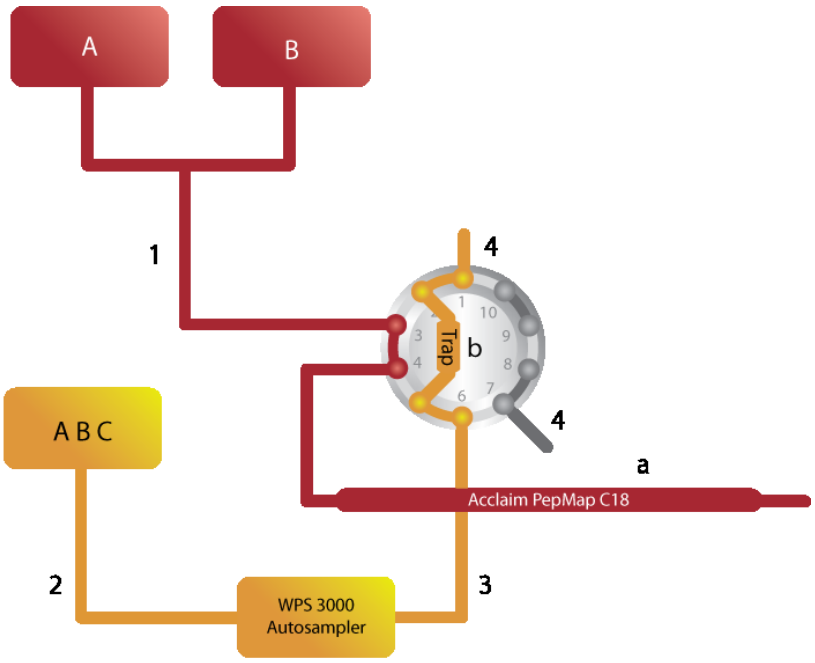


Figure 29: Fluidic connections for a pre-concentration onto a capillary column

TIP The schematic shows a 10-port switching valve. This application can also be performed using a 6-port valve.

#	Item	P/N
a	300 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164537
b	300 µm I.D. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 5 cartridges)	160454
	µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, nanoViper fittings	164649
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

#	Item	P/N
	nanoViper sample loop 20 µL, FS/PEEK sheathed	6826.2420
4	PTFE tubing, 500 µm I.D. 100 cm, used in waste tubing	6720.0077
	1/16" Universal Fingertight Fitting, one-piece design, extra long thread (4 pieces)	6720.0015
	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

Table 13: RSLCnano pre-concentration capillary LC kit (P/N 6720.0315) contents

2.9.3 Installation Tips

- Follow the “General Recommendations for Applications” (section 2.1 on page 15).
- A detailed description of how to set up this application for LC-MS is available in TN72277.
- For details on trap column selection and installation, please refer to section 2.2 - available trapping columns on page 21.
- If a loss of hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be reduced to ‘1’ % or be completely removed. The ion paring agent concentration can also be raised or a strong ion-pairing agent such as trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA) can considered.
- The 50 µm x 750 mm capillary (P/N 6041.5580) can be used to convert the pre concentration setup to a direct injection configuration (see capillary 1 in *Figure 20*). One further capillary is required (P/N 6041.5560, capillary 2 in *Figure 20*) which must be ordered separately.

2.9.4 Testing the Application

Test the pre-concentration application using the following conditions:

Property	Setting
Mobile Phase A	Water + 0.1% FA
Mobile Phase B	80/20 (v/v) Acetonitrile / Water with 0.1% FA
Loading solvent	Water + 0.1% FA
Sample	Cytochrome C digest, 200 fmol / µL
Injection Volume	1 µL (partial loop fill or µL pickup)
UV detection (Optional)	214 nm
Loading time	3 min (may vary according to required injection volume / routine)
Gradient	5% for 1 minute, 5 to 35% B in 10 minutes, 35% B to 90% B in 1 minute, for further details see TN-72277
Oven temperature	35 °C
WPS temperature	5 °C
Loading flow rate	20 µL / min
NC Flow Rate	4 µL / min (capillary flow selector)

Table 14: Test conditions for pre-concentration injection of 500 fmol Cytochrome C onto a capillary column

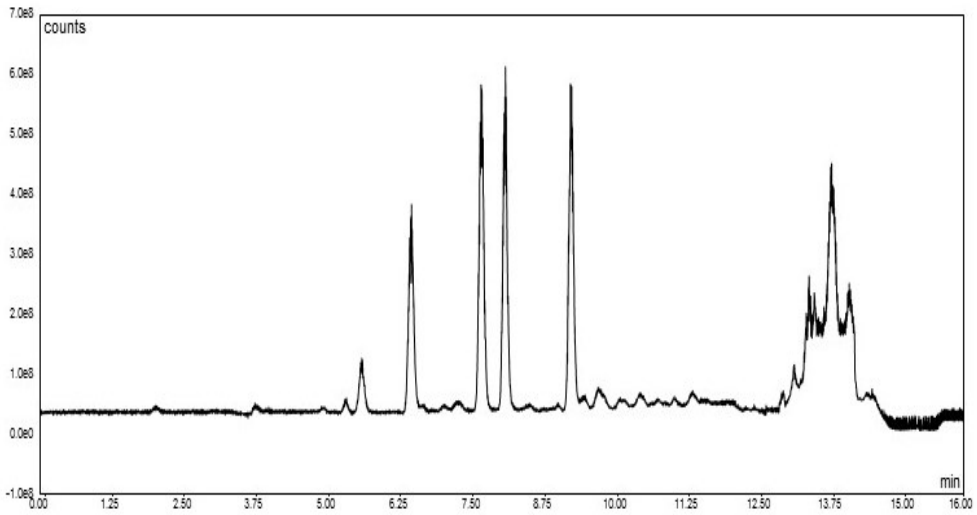


Figure 30: Typical chromatogram for a pre-concentration of 1 pmol Cytochrome C onto a capillary column

2.10 EASY-Spray Columns with the RSLCnano

2.10.1 EASY-Spray Concept

EASY-Spray is an integrated separation column and emitter concept designed for robust plug and play low-flow LC-MS analysis. EASY-Spray columns consist of an integrated separation column and emitter connected via a zero dead volume union, minimizing post column volumes and dispensing of the need for tricky post column emitter connections.

Figure 31 shows a schematic of the EASY-Spray column hardware. The integrated emitter is protected by a spring-loaded cover, which shields it when it is not installed. The cover is retracted when the EASY-Spray column is inserted into the EASY-Spray source.

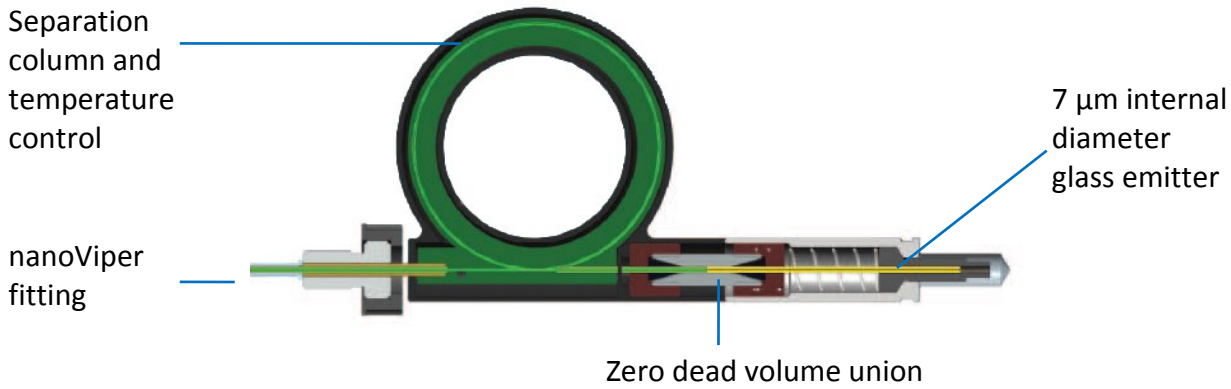


Figure 31: Schematic overview of an EASY-Spray column

The EASY-Spray column simply slots into the EASY-Spray source and is connected to the LC outlet using a viper union. In-built column temperature control ensures optimal retention stability and consistent chromatographic performance. *Figure 32* shows the EASY-Spray source with an EASY-Spray column installed.

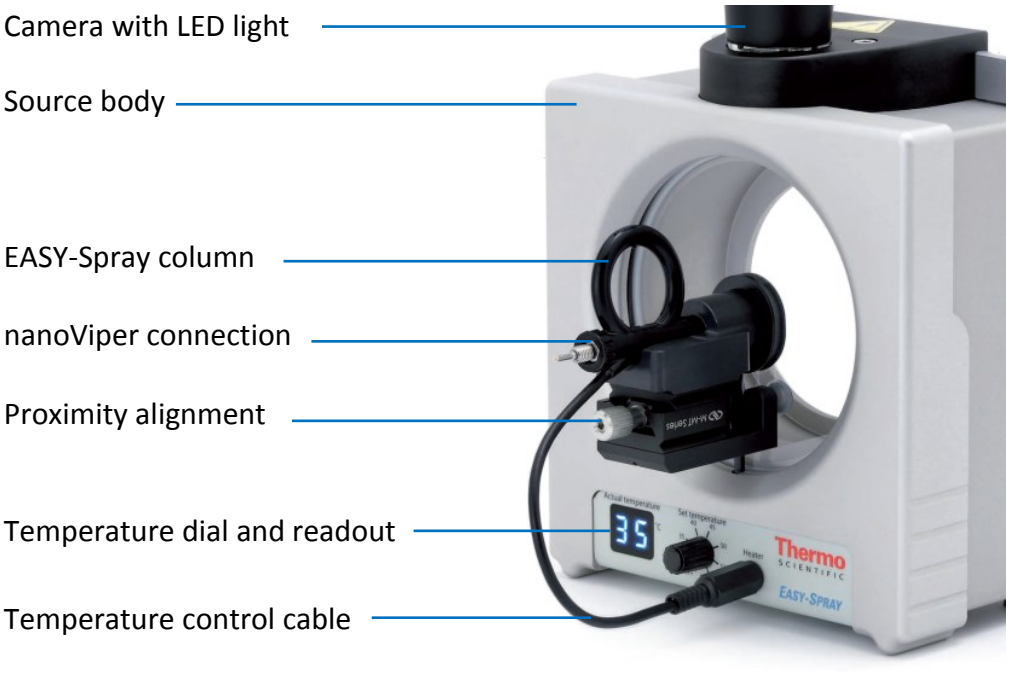


Figure 32: EASY-Spray source with EASY-Spray column installed

Alignment of the EASY-Spray column is only required when the source is installed for the very first time on the Mass Spectrometer.

NOTICE The EASY-Spray Ion Source interface is available in two formats, the EASY-Spray Ion Source (P/N ES081) and EASY-Spray Ion Source NG (P/N ES082). The source required will depend on the mass spectrometer type (see *Table 15 below*).

Ion Source Model	Thermo Scientific Mass Spectrometer
EASY Spray NG (ES082)	TSQ Series Orbitrap Fusion™ Series Endura MD™
EASY-Spray (ES081)	Exactive™ Series Orbitrap™ Series LTQ™ Series LCQ™ Dec XP Max

Table 15: EASY-Spray ion sources and compatible mass spectrometers

2.10.2 Example Separation Performance of an EASY-Spray Column

An example base peak Chromatogram for a BSA digest run on an Easy-Spray (ES801) column is shown in *Figure 33*. The direct connection between the column outlet and the column emitter affords highly resolved peaks with virtually zero post column band broadening (*Figure 33 A and B)*.

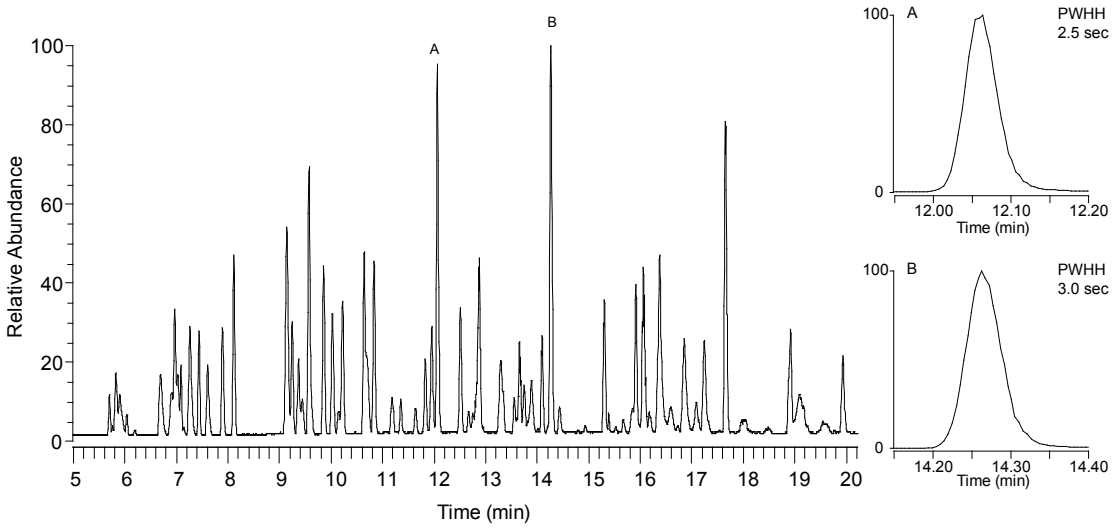


Figure 33: Base Peak Chromatogram of an EASY-Spray column in pre-concentration mode

2.10.3 Hardware Layout Direct Injection

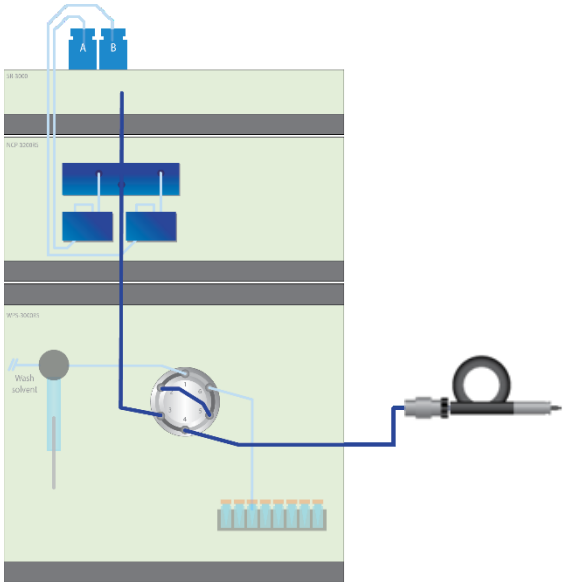


Figure 34: Setup for direct injection with EASY-Spray

The instrument setup for EASY-Spray direct injection presented in *Figure 34* consists of:

SR-3000	5035.9200
NCP-3200RS	5041.0030A
WPS-3000TPL RS	5826.0020
Application kit:	6720.0395

NOTICE The NCP-3200RS in this setup can be exchanged for an NCS-3500RS.

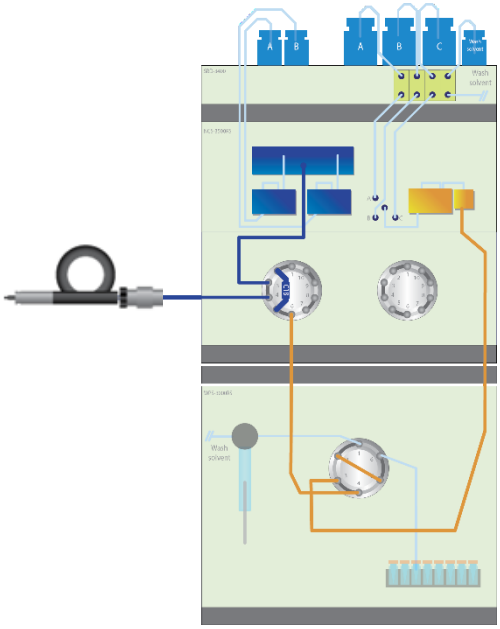


Figure 35: Setup for pre-concentration with EASY-Spray

The instrument setup for pre-concentration with EASY-Spray presented in *Figure 35* consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010A
1x 10-port sw.valve	6041.0001A
WPS-3000TPL RS	5826.0020
Application kit:	6720.0395

2.10.4 Fluidic Setup using EASY-Spray Columns

The fluidic setups for EASY-Spray with an UltiMate 3000 RSLCnano are essentially identical to those used for the direct injection and pre-concentration applications already described.

The only difference is that instead of connecting the separation column to the valve (injection or 10-port) directly, it is connected using a Viper union and nanoViper capillary running from the valve to the EASY-Spray column. Because the connection tubing is placed before the separation column, it has no negative impact on separation performance. (*i.e.* band dispersion).

TIP The gradient delay resulting from the connecting capillaries varies according to the length and inner diameter of the tubing. As a rule of thumb, every 10 cm of 20 µm I.D. tubing contributes 30 nL or 6 seconds delay at 300 nL/ minute.

Various lengths of connecting capillaries are provided in the UltiMate 3000 RSLCnano EASY-Spray connection kit (see *Table 16* below) to offer maximum user flexibility when connecting the UltiMate 3000 RSLCnano to the EASY-Spray column. To minimize gradient delay volumes, the shortest possible connecting capillary is recommended.

Item	P/N>
300 µm I.D. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 5 cartridges)	160454
µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, nanoViper fittings	164649
nanoViper Capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240
nanoViper Capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 550 mm	6041.5260
nanoViper Capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 650 mm	6041.5275
nanoViper Capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 750 mm	6041.5280
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 75 µm x 650 mm	6041.5775
nanoViper Sample Loop 20 µL, FS/PEEK sheathed	6826.2420
Union Viper	2261.5061
PTFE tubing, 500 µm I.D. x 100 cm, used as waste tubing	6720.0077

Item	P/N>
1/16" Universal Fingertight Fitting, one-piece design, with extra long thread, 4 pieces	6720.0015
Polypropylene vials for WPS with glass insert, 250 µL, 25 pieces	6820.0027
Polypropylene caps for WPS vials, 25 pieces	6820.0028
Cytochrome C digest, 1.6 nmol, Lyophilized	161089

Table 16: UltiMate 3000 RSLCnano EASY-Spray connection kit (P/N 6720.0395) contents

For a list of available EASY-Spray columns, see – *Table 28: EASY-Spray columns* in the Appendix.

2.10.5 EASY-Spray Transfer lines

The EASY-Spray source can also be used in conjunction with linear columns by adopting an EASY-Spray transfer line (*Figure 36*: EASY-Spray Transfer Lines) consist of an emitter with a form factor compatible with the EASY-Spray source, attached to a nanoViper fitting via a fused silica capillary.

Figure 36: EASY-Spray Transfer Lines

EASY-Spray transfer lines are available in both nano flow (P/N ES791A) and capillary /micro flow (P/N ES792A) compatible formats.

2.11 2D Salt Plugs with Nano Column

2.11.1 Hardware-Layout

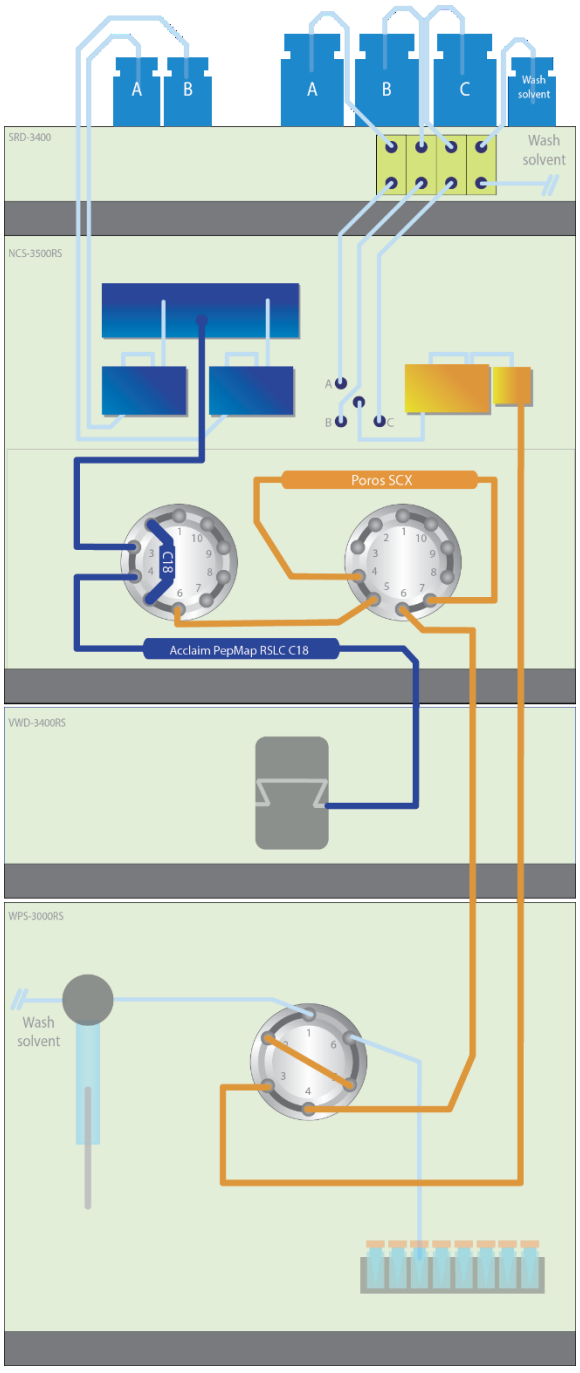


Figure 37: Setup for a 2D Salt Plugs experiment including the optional UV detector

The Instrument setup presented in *Figure 37* consists of:

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

2.11.2 Fluidic Setup

Figure 37 and *Figure 38* presents the setup using the parts provided in the 2D-LC Salt Plugs application Kit. Columns are marked with letters and tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 37*.

TIP The schematic shows 10-port switching valves. This application can also be performed on 6-port valves. Ensure that the relative positions on the connections are correct and update the valve switching in the instrument setup and method as necessary.

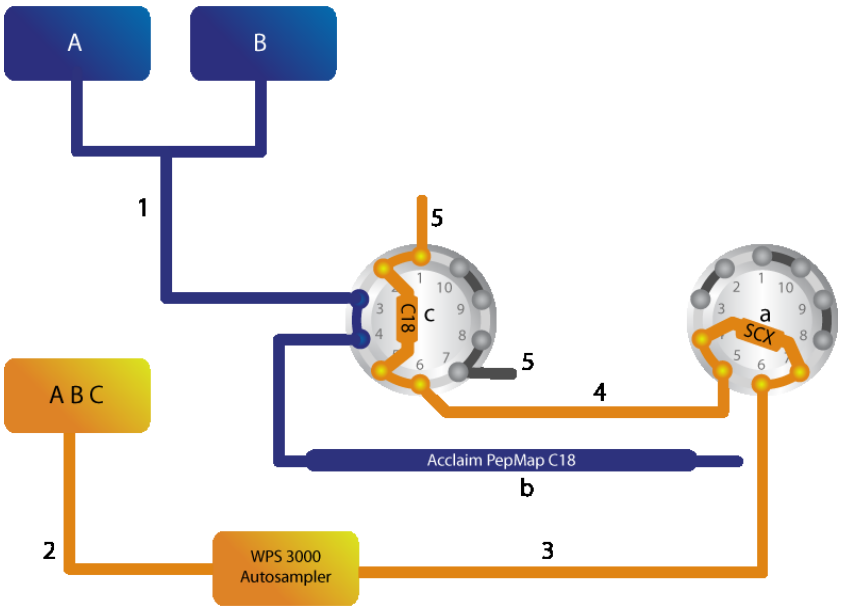


Figure 38: Fluidic connections for a 2D-LC Salt-Plugs experiment

#	Item	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	300 µm I.D. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 5 cartridges)	160454
	µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, nanoViper fittings	164649
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. x L 20 µm x 350 mm	6041.5240

#	Item	P/N
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	6826.2420
5	PTFE tubing, 500 µm I.D. 100 cm, used in waste tubing	6720.0077
	1/16” Universal Fingertight Fitting, one piece design, long thread, 4 pieces	6720.0015
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs	6820.0027
	Polypropylene caps for WPS vials, 25 pcs	6820.0028
	6 Protein Digest Standard, 100 pmol, Lyophilized	88342
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089

Table 17: RSLCnano 2D salt plug Kit NCS-3x00 (P/N 6720.0325) contents

2.11.3 Installation Tips

- Follow the “General Recommendations for Applications” (section 2.1 on page 15).
- If a loss in hydrophilic peptides is observed, the concentration of acetonitrile in the loading solvent can be decreased down to 99/1 water/acetonitrile + 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 RP 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 (maximum of 0.025%). Alternatively, FA (~ 0.5%) can be used.
- The salt plugs listed here have been chosen 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.

- After each series of injections, it is useful to wash the column with consecutive 2 M 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.

2.11.4 Testing the Application

Test the 2D salt plug setup using the following conditions.

Property	Setting
Mobile Phase A	Water + 0.1% FA
Mobile Phase B	80/20 (v/v) Acetonitrile / Water with 0.1% FA
Loading solvent	95%/2% (v/v) water/ acetonitrile + **0.025% TFA**
Sample	Protein mix digest 100 pmol, lyophilized, prepared according to 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 1000 mmol NaCl 2000 mmol NaCl
Injection Volume	Sample: 10 µL (partial loop fill or µL pickup) Salt plugs 20 µL
UV detection (Optional)	214 nm
Loading time	5 min (may vary according to required injection volume / routine)
Desalting time	7 min (started after the loading time has passed)
Gradient	Isocratic 4% for 10 minutes 4% to 55% B in 30 minutes, 90% B for 5 minutes, 18 minutes equilibration
Oven Temperature	35 °C
WPS temperature	5 °C
Loading flow rate	10 µL / min
NC Flow Rate	300 nL / min (capillary flow selector)

Table 18: Test conditions for a protein digest separation using 2D Salt Plugs

The successful installation of this application is based on the following attributes:

- The injection profile should be reproducible.
- The peptides should be equally distributed over and within the different fractions (orthogonal separation).

2.11.5 Salt Solution Preparation

The following protocol can be used to prepare the salt plugs

- Prepare two stock solutions using the loading solvent:

1. 2000 mM NaCl (*e.g.*, 467.5 mg of NaCl in 4 ml loading solvent
2. 100 mM NaCl (*e.g.* prepare the 100 mM solution of the first table two times)

- Dilute the stock according to *Table 19* and *Table 20* below: Use standard 1.5 ml vials (do not use vials with inserts).

Concentration of NaCl	Volume of 2000mM 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	1250 µL	750 µL	1000 µL
200 mM	100 µL	900 µL	1000 µL
100 mM	50 µL	950 µL	1000 µL

Table 19: Guide for the preparation of the Salt Plugs (dilutions from 2000 mM NaCl)

Concentration of NaCl	Volume of 100mM NaCl stock solution	Volume of loading solvent	Total Volume
50 mM	500 µL	500 µL	1000 µL
20 mM	200 µL	800 µL	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

Table 20: Guide for the preparation of the Salt Plugs (dilutions from 100mM NaCl)

2.12 Tandem Nano LC

2.12.1 Hardware Layout

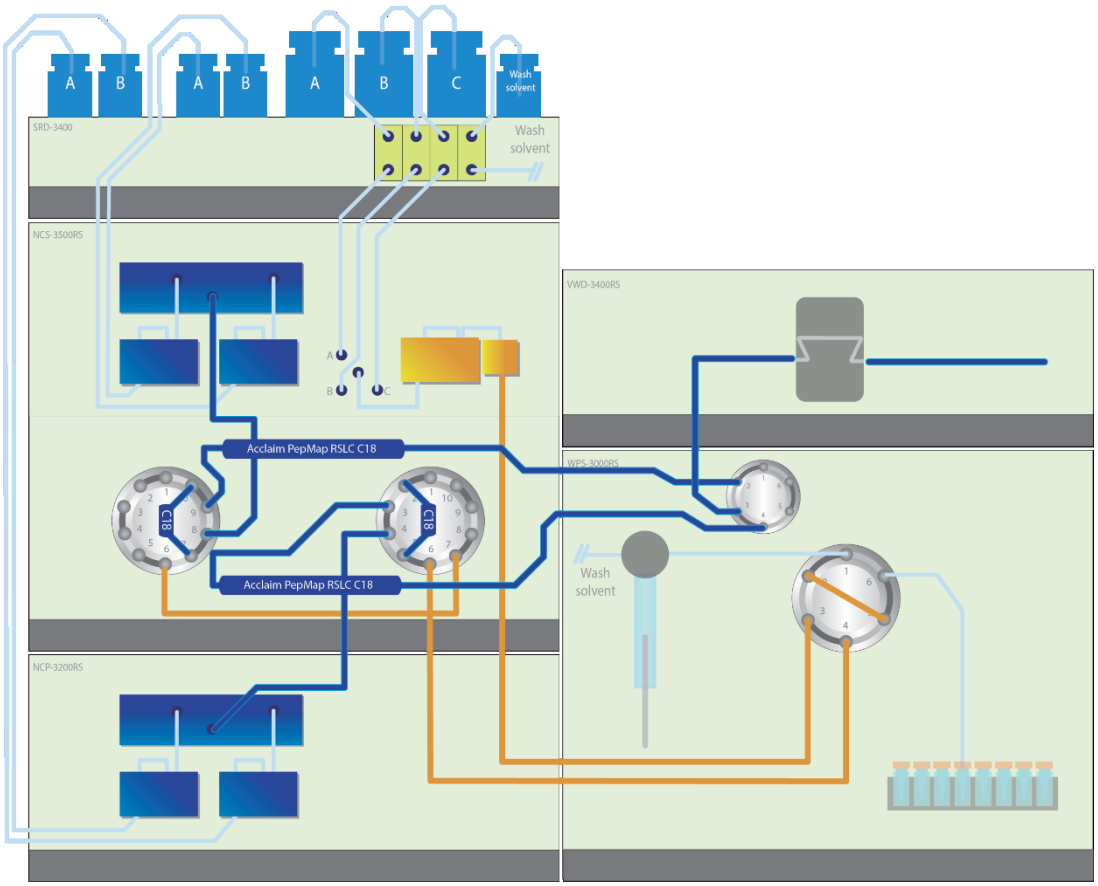


Figure 39: Setup for a Tandem nano LC experiment including the optional UV detector

The instrument setup presented in *Figure 39* consists of:

SRD-3400	5035.9245
NCS-3500RS	5041.0010A
NCP-3200RS	5041.0030A
2x 10-port sw.valve	6041.0001A
VWD-3400RS	5074.0010
3 nL flow cell	6074.0270
WPS-3000FC	5824.0020
Injection valve	6826.0011A
Application kit:	6720.0335

TIP All the components required to convert the WPS-3000FC for tandem nano LC use are included in the application kit, except the WPS injection valve (P/N 6826.0011A) which must be ordered separately.

The NCP 3200RS accessory kit has two 130 cm long solvent inlet tubings, which supply solvent from the bottles placed on the solvent rack to the NCP at the bottom of the stack.

2.12.2 Fluidic Setup

Figure 39 and *Figure 40* shows the setup using the parts provided in the Tandem nano LC application kit. Columns are marked with letters and tubing with digits. The sample loop is installed in the WPS-3000TPL RS autosampler. The correct configuration for the sample loop, needle and buffer tubing is shown in *Figure 39*.

TIP The schematic below shows 10-port switching valves. This application can also be performed using 6-port switching valves. Ensure that the relative positions of the connections are correct and update the valve switching in the instrument setup and method as necessary.

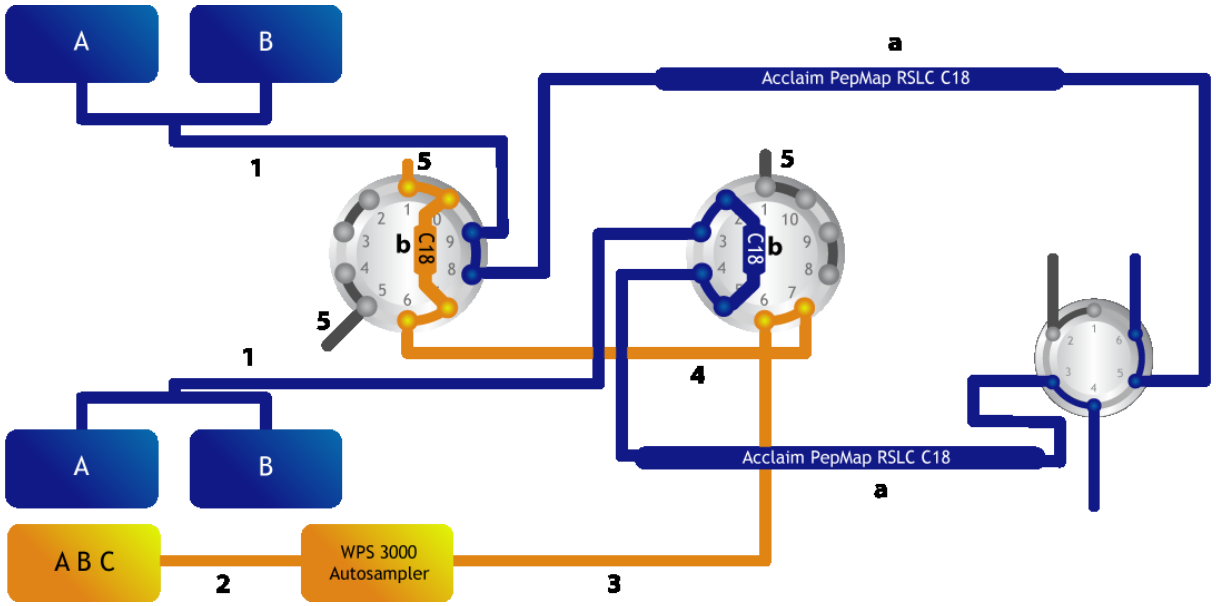


Figure 40: Fluidic connections for a Tandem nano experiment

NOTICE Control of the post column nano valve can either be performed using the WPS-3000FC autosampler or by using an external USB controlled universal electric actuator (P/N EUHB) and corresponding mounting hardware (P/N CMH12H) from VICI® Valco Instruments, to control the valve. If the WPS-3000FC sampler is used, the nano injection kit (P/N 6824.0030) should be installed and the lower valve on the WPS-3000FC replaced with the 1/32” nano switching valve (P/N 6820.6232). All necessary parts are included in the kit. A detailed description of how to set-up, configure and control both hardware variants is given in TN72899.

#	Item	P/N
a	75 µm I.D. x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper	164534
b	300 µm I.D. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100Å (set of 5 cartridges)	160454
	µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, nanoViper fittings	164649
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
	nanoViper sample loop 20 µL, FS/PEEK sheathed	6826.2420
5	PTFE tubing, 500 µm I.D. 100 cm, used in waste tubing	6720.0077
	1/16” Universal Fingertight Fitting, one piece design, long thread, 4 pieces	6720.0015
	Fused silica tubing I.D. 20µm O.D. 280µm, 5 meters for nano LC connections	160475
	Cutter for fused silica tubing (cleavage stone)	6720.0016
	Upgrade kit nano/cap WPS-3000TFC	6824.0030
	1/32” 2 pos 6 port nano switching valve (C2N series)	6820.6232
	Fittings 1/32” for C2N series nano valve	6820.1320
	1/32" PEEK sleeve, 3 cm, 300 µm I.D. (6 pieces)	6720.0079
	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

Table 21: RSLCnano Tandem nano LC kit (P/N 6720.0335)

2.12.3 Installation Tips

- Follow the “General Recommendations for Applications” (section 2.1 on page 15).
- The columns are shipped with I.D. x L. 20 µm x 30 cm attached. Fused silica capillary tubing is provided in the kit to extend the column outlets in order to reach the nano valve if necessary. Replacing the attached fused silica by the appropriate length using the nano connector on the column will give the best result.
- If a loss in hydrophilic peptides is observed, the acetonitrile in the loading solvent can be removed and / or the 0.1 % FA can be replaced with 0.05% TFA or 0.1% TFA as required.

- The WPS-3000FC is typically used for fraction collection. By replacing the divert valve with the nano valve, the autosampler is compatible with tandem nano LC. Controlling the divert valve position to switch between the two analytical columns is performed with the commands **Sampler.Collect** and **Sampler.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 section 2.7 Pre-concentration onto a Nano Column shown on page 47.

TIP Testing both pre-concentration setups individually is recommended before setting them in combination.

Property>	Setting
Mobile Phase A	Water + 0.1% FA
Mobile Phase B	80/20 (v/v) Acetonitrile / Water with 0.1% FA
Loading Pump A	Water + 0.1% FA
Sample	Cytochrome C digest, 500 fmol / µL
Injection Volume	1 µL (Full Loop)
UV detection (Optional)	214 nm
Loading Time	3 min
Gradient NC Pump	4% to 55% B in 30 minutes, 90% B for 5 minutes, 15 minutes equilibration
Oven Temperature	35 °C
WPS temperature	5 °C
Loading flow Rate	5 µL / min
NC Pump flow rate	300 nL / min

Table 22 Test conditions for running the tandem nanoLC application

2.12.5 Tandem NanoLC-MS

Tandem nanoLC can also be coupled directly to the MS detector (*i.e.* without employing an optical UV detector). For this configuration, the modules are mounted together in a single stack.

A deep proteomics application which employs long shallow gradients on 50 cm columns together with full instructions on how to connect, configure and program the system is detailed in TN72899. The customized LC methods describe how the system can be used to constantly deliver sample to the mass spectrometer, thus enabling the equivalent sample throughput provided by two single nano LC-MS systems.

Complete LC method parameters for this set-up are also available for download from the AppsLab library.

2.13 High throughput tandem capillary-flow LC-MS

Tandem capillary-flow LC workflows combine high sensitivity with high throughput, permitting the analysis of up to 200 samples per day whilst yielding 100% MS utilization.

Details on how to connect and configure the UltiMate 3000 RSLCnano in order to run this application are provided in TN72827 together with complete LC-MS method parameters.

Example data files containing full LC and MS parameters are also available for download from the AppsLab library.

TIP There is no tandem capillary-flow application kit available to support this workflow. However, a full list of all the fluidic components required together with part numbers and set-up instructions necessary for a successful installation of the application are provided in TN72827.

2.14 Micro LC Applications

Micro LC applications typically employ flow rates from between 10 and 50 µL / minute using columns with internal diameters between 500 µm and 1 mm.

For such applications, Thermo Fisher Scientific recommends the use of 50 µm I.D. nanoViper capillaries. A list of the available nanoViper capillaries is available in *Table 31* on page 99.

If UV detection is required, the cap flow cell (45 nL; P/N 6074.0280) is recommended for all micro LC applications.

2.15 MS Connection Kit

The UltiMate 3000 RSLCnano MS connection kit (P/N 6720.0345; *Table 23*) contains a variety of common tubings, unions and connectors necessary to facilitate coupling of the LC column to a mass spectrometer interface.

Item	P/N
Nano LC column to MS tubing I.D. x O.D. x L 20 µm x 280 µm x 1m	6041.5292
Nano LC column to MS tubing I.D. x O.D. x L 20 µm x 360 µm x 1 m	6041.5293
Capillary LC column to MS tubing I.D. x O.D. x L 50 µm x 280 µm x 1 m	6041.5294
Capillary LC column to MS tubing I.D. x O.D. x L 50 µm x 360 µm x 1 m	6041.5295
Fused silica tubing I.D. 20 µm ±3 µm/O.D. 280 µm ±10 µm, 5 meters	160475
Fused silica tubing I.D. 50 µm ±3 µm/O.D. 280 µm ±10 µm, 5 meters	160477
Cutter for fused silica tubing (cleavage stone)	6720.0016
PTFE tubing, 250 µm I.D., low pressure connection of 280 µm O.D. fused silica capillaries, 5 pieces	6720.0030
1/16" Valco Ferrule and Nut, stainless steel, 10 pc. (for 10-port valve)	161103
PEEK sleeves, precision cut and polished for connections with fused silica tubing (280 µm O.D.), 5 pieces	6720.0064
PEEK sleeves, precision cut and polished for connections with fused silica tubing (360 µm O.D.), 5 pieces	6720.0078
Microtight Union inclusive 2 fittings and 1 gauge plug	6720.0074
PEEK sleeves, precision cut and polished for connections with fused silica tubing (280 µm O.D.), 10 pieces	6720.0075
PEEK sleeves, precision cut and polished for connections with Microtight Union (360 µm O.D.), 10 pieces	6720.0076
Nano connector incl. sleeves, dead-volume free, up to 300 bar	6720.0390
Sleeves for nano connector, 5 pieces	6720.0391

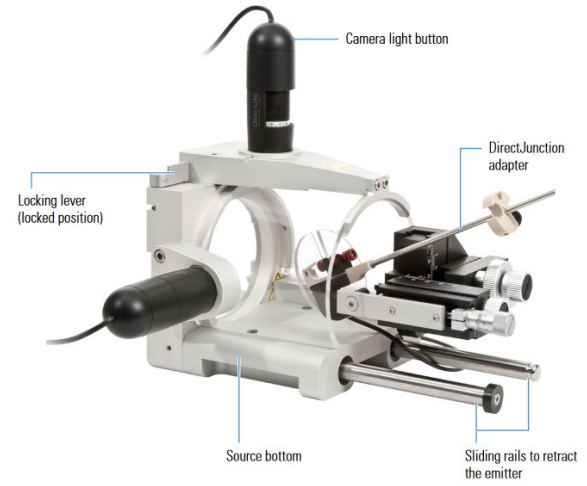
Table 23: RSLCnano MS Connection Kit (P/N 6720.0345)

TIP The connection between the LC separation and the MS should have the lowest volume possible to minimize dispersion. Keep in mind that **internal diameter** has a much bigger effect on dispersion then the **length** of the connecting tubing. Ensure that tubing with the correct diameter is used at all times.

2.15.1 Mass Spectrometry Interfaces for Linear columns and / or interfacing the UV detector with the MS.

The mass spectrometry interface connects the LC outlet to the mass spectrometer inlet. It is here that the column effluent is ionized and introduced into the mass spectrometer. The type of interface required is dictated by the application flow rate and type of MS instrument. The common interfaces applicable to linear columns and / or the VWD 3400RS UV detector available for Thermo Scientific mass spectrometers are shown below. For details on the connections, also see section 2.1.4. The MS interface for EASY-Spray is discussed in section 2.10.

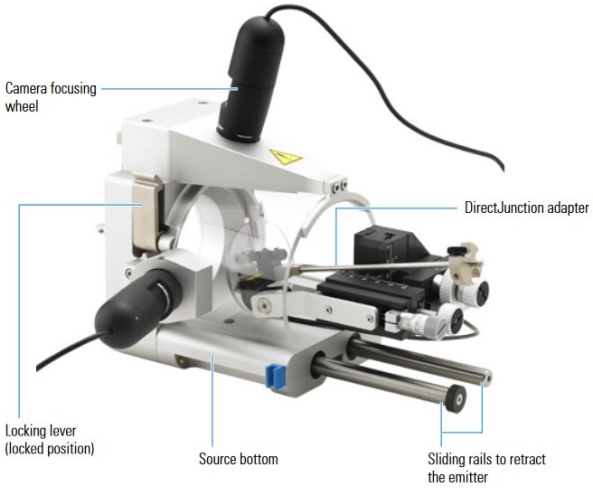
2.15.1.1 Nanospray Flex Series Ion Sources



Nanospray Flex™ Ion Source
P/N: ES071

Nano Flow: 20 -1000 nl/min*
*depending on spray needle

MS compatibility
LTQ™ and Velos™ Series
Orbitrap™ Series
Exactive™ series
Legacy TSQ™ series (Quantum Access Max, Vantage, Ultra etc.)



Nanospray Flex NG™ Ion Source
P/N: ES072

Nano Flow: 20 -1000 nl/min*
*depending on spray needle

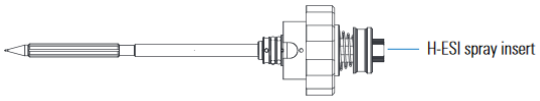
MS compatibility

Orbitrap Fusion™ series
TSQ™ Quantis, Altis, Endura and Quantiva

Figure 41: Nanospray Flex Series ion sources

2.15.1.2 *Heated Electrospray Ionization (HESI-II) Probe and H-ESI Spray Insert*

For micro- and capillary-flow applications with columns ≥ 300 µm i.d. and flow rates ≥ 5 µL/min, the Ion Max Source and accompanying ionization probe can be used when adapted for low flow rates. Two electrospray ionization source housing types are available for the Thermo Fisher Mass Spectrometers, the Ion Max and Ion Max NG source. The source type depends on the mass spectrometer type. Each has their own electrospray ionization probe (see *Figure 42*).



Heated Electrospray Ionization (HESI-II) Probe for the Ion MaX source
P/N OPTON-20037 Kit

MS Compatibility
LTQ™ and Velos™ Series
Orbitrap™ Series
Exactive™ series
Legacy TSQ™ series (Quantum Access Max, Vantage, Ultra *etc*.)
H-ESI Spray Insert for Ion Max NG Source
P/N 80000-60321

Figure 42: Ionization probes for the Ion Max and Ion Max NG sources.

A low-flow (50 µm I.D.) metal needle is required for low-flow experiments to give the best chromatographic performance. Both PEEK and fused silica capillaries are available to interface the source with the column outlet. A compatibility matrix for the different low-flow options is shown in *Figure 43*.

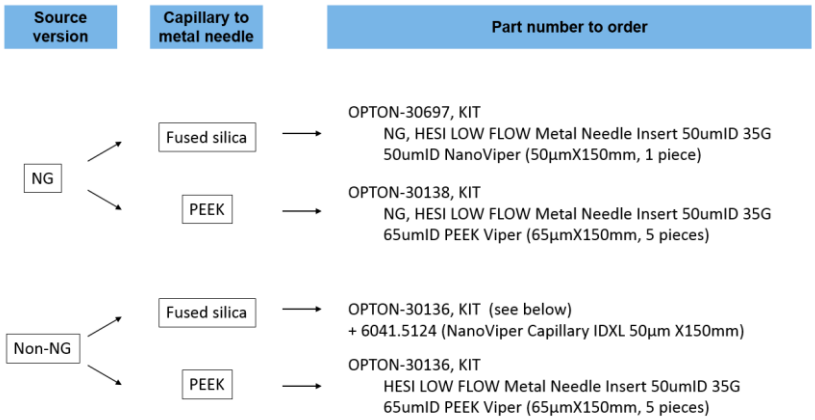


Figure 43: Ionization probes for the Ion Max and Ion Max NG sources

3 FAQs

3.1 NC_Pump Solvent Recalibration – Best Practice

The main reason for performing a solvent calibration is to improve retention time stability or to correct a retention time drift. The frequency and type of recalibration differs between the ProFlow and the classic flow meter. Recommendations for re-calibration routines for the two types of flow meter are provided below.

NOTICE The pump blocks and flow meter should be purged regularly with fresh solvents. Purging is especially important, before any calibration routines are carried out. Please see section 2.3.4.6 for details.

3.1.1 ProFlow Flow Meter

3.1.1.1 Adjust Zero Balance Test

Once every 3 months or when a retention time drift is observed.

3.1.1.2 Solvent Calibration

One time only per solvent per channel, for **custom solvents ONLY.** No recalibration required.

3.1.2 Classic Flow Meter

3.1.2.1 Pressure Transducer Test

Every time solvents are refreshed or when a retention time drift is observed.

3.1.2.2 Viscosity Measurement Test

Every time solvents are exchanged or refreshed.

TIP The test also acts as a diagnostic tool.

3.2 Interpreting a Chromatogram

An LC-UV example Cytochrome C separation using TFA as ion pairing agent is shown in *Figure 44*. The different areas of a chromatographic separation are marked inside the figure.

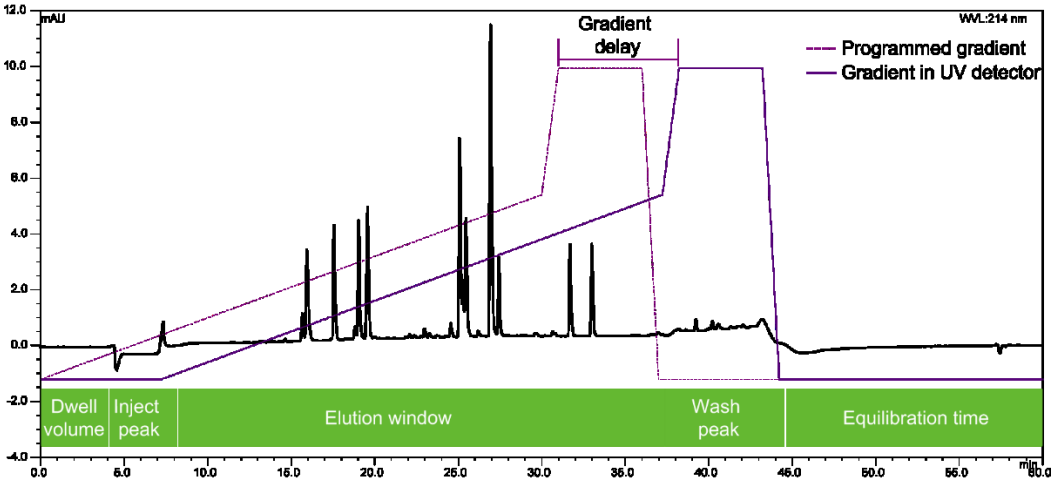


Figure 44: Example Cytochrome C separation with different parts of the run identified

The finite volume of an HPLC system results in time offset between the formation of a gradient, its delivery onto the column and the detection of the gradient change by the (UV and / or MS) detector. This so-called gradient delay can be visualized by comparing the programmed gradient to the UV signal. *Figure 44* shows the gradient delay between pump and UV detector.

The inject peak corresponds to the injection peak in direct injection setups; in pre-concentration setups this part of the baseline, corresponds to the switching of the trapping column 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, introducing additional capillaries and connections, the dwell volume and gradient delay are not the same volume in direct injection and pre-concentration setups.

3.3 Troubleshooting Nano LC Peptide Applications

The above LC-UV chromatogram *Figure 44* shows the separation of a Cytochrome C digest on a nano column. The Cytochrome C standard is simple compared to a typical proteomics sample and is, therefore, ideal for troubleshooting nano LC setups.

TIP When troubleshooting a pre-concentration setup, Thermo Fisher Scientific recommends switching back to a direct injection setup if the tips below do not provide the remedy. An important and often overlooked step in troubleshooting is simplifying the setup to isolate the problem.

In assessing the separation performance of a system, several factors are evaluated, which are organized in the flow chart below (*Figure 45*). The values in the flowchart are based on a Cytochrome C digest separation; when working with a different standard use a trusted reference chromatogram for the expected values for number of peaks, intensity and elution window.

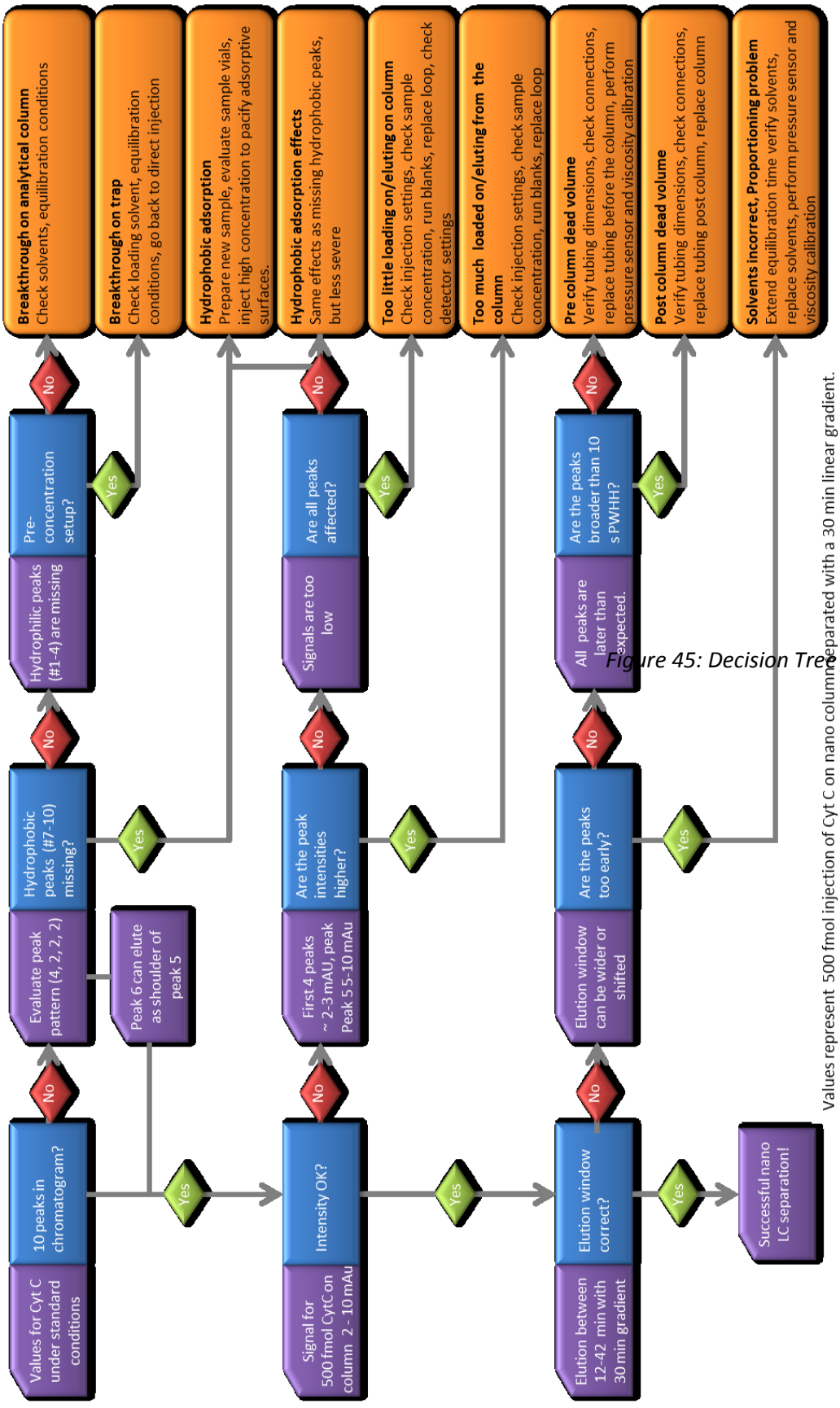


Figure 45: Decision Tree for troubleshooting a low-flow

3.4 The Use of TFA and FA

The separation of peptides by reversed phase is carried out in the presence of an ion-pairing agent, which serves a double function. First, these (typically) weak acids bring the pH of the solvents down to pH 2-3, causing most peptides to have an overall positive charge. Secondly, the negative counter-ion of the acid will serve as an ion-pairing agent for the positively charges peptides to create an overall neutral analyte that is more efficiently separated on the RP column. The double function of the ion-pairing agent results in an efficient separation with minimal additives added to the solvents.

Most commonly, trifluoro acetic acid (TFA) and formic acid (FA) are used. In this manual and in most LC-MS applications, FA is preferred as its use minimizes ion-suppression effects. TFA is a stronger ion-pairing agent and results in better chromatography, but can result in ionization suppression. The use of TFA is generally restricted to the loading buffer or when increased retention (compared to FA) is necessary. When performing the applications described in this manual with TFA instead of FA, use the amounts given in *Table 24*. *Figure 46* demonstrates the effect of ion pairing agent choice on the chromatographic separation of Cytochrome C.

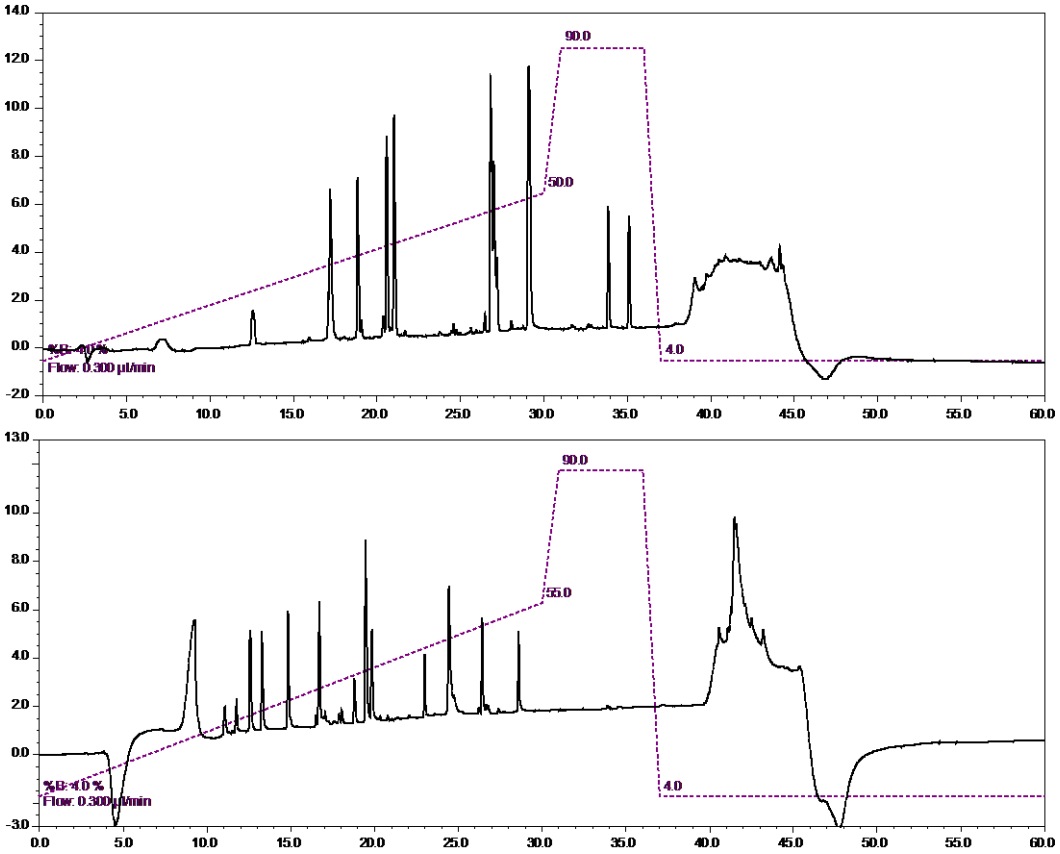


Figure 46: Comparison of Cytochrome C separation with different ion pairing agents. Top uses 0.05% TFA and bottom 0.1% FA

3.5 Minimizing Baseline Effects

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 detrimental post column band broadening.

Typically, peptide UV detection is performed at a wavelength of 214 nm, at which most organic compounds absorb quite strongly. A number of actions can be taken to minimize baseline drift and noise for optimal use of the UV detection.

3.5.1 Drift

Ensure that the UV lamp has been switched on for sufficient time in order that the lamp temperature can stabilize. Chromeleon can detect this and will give a warning during the 'Ready Check' whenever the UV lamp temperature is not stable. In such cases, the UV detector can be used but may not perform optimally.

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. Varying the ion-pairing agent concentration (typically FA or TFA) in the A and B solvent can be used to compensate the baseline rise. As a rule of thumb, the ion-pairing agent concentrations indicated in *Table 24* can be used to obtain a straight baseline.

Solvent A	Solvent B
0.1%	0.08%
0.05%	0.04%

Table 24: Ion pairing agent addition

The age of both lamp and flow cell can have a significant influence on baseline drift. New lamps and flow cells may show some drift during the so-called 'burn in' period. Allowing sufficient equilibration time for the lamp is necessary for obtaining a stable baseline.

Lamps should be replaced after approximately 2000 hours. Older flow cells can be cleaned by flushing overnight with organic solvent or for a

short period with a strong acidic solution; see the Variable Wavelength Detector's Operating Instructions for more details.

3.5.2 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 generally 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.

3.6 Typical WPS-3000TPL RS Autosampler Settings for Standard Injection Routines.

The Chromeleon software gives the user the freedom to define multiple settings for the standard injection routines including sample height, draw speed and puncture depth. Below are typical WPS-3000TPL RS settings, which can be adopted for common low-flow workflows.

Parameter	Description	Typical Value
DispSpeed	Sets the speed of the syringe used for dispensing the sample%	2 µL / sec
DrawSpeed	Sets the speed of the syringe used for drawing the sample.	0.2 µL / sec
DrawDelay	Sets the time that the needle remains in the vial after drawing the sample	5 sec
WashSpeed	Sets the speed of the syringe for the wash cycle.	4 µL / sec
WasteSpeed	Sets the speed of the syringe used for expelling liquid to the waste.	4 µL / sec
DispDelay	Time needle remains in vial after dispense	2 sec
DrawDelay	Time needle remains in vial before liquid is drawn	5 sec
Sample Height	The height at which sample is drawn	2 mm
TransLiquidHeight	The height at which transport liquid is drawn	3 mm
PunctureDepth	Depth of puncture needle beyond pusher trigger point for sample vial / well	8 mm
TransVialPunctureDepth	Depth of puncture needle beyond pusher trigger point for transport vial	8 mm
WashVolume	Volume used in wash operation	50 µL
FlushVolume	Flush volume used in full loop, partial loop and µL pick up injections	5 µL
FlushVolume2	Flush volume used in full loop and partial loop injections from the same vial.	3 µL
Loop Overfill	Loop overfill factor used in full loop only	2.0

Table 25: Typical WPS-3000TPL RS autosampler settings

4 Appendix

4.1 Customized Sample Injection Routines

4.1.1 Introduction to User Defined Program (UDP) Injection Routines

WPS-3000TPL RS autosamplers are equipped with standard injection routines for Full-loop, Partial-loop and µL pickup, which are suitable for the majority of applications. However, for applications requiring special injection routines, or for users wishing to customize one of the standard injection methods, a UDP should be used.

UDPs provide the user with full control over every aspect of the injection routine and enable every type of injection, from simply filling the loop and injecting the sample to complete liquid handling, such as in-well digestion.

4.1.2 Important Considerations when Writing a UDP

Although a UDP is flexible, it is also unforgiving: every step of the injection routine must be programmed manually and missing steps will lead to erroneous injections. The Chromeleon / SII software will execute the steps sequentially until it encounters an error after which it will either continue to the next sample or interrupt the analysis.

4.1.3 The UDP Commands

The general layout of a UDP can be broken down into five different steps:

1. Sample preparation (only when applying liquid handling)
2. Preflush of the injection needle
3. Filling the sample loop
4. Injecting the sample and starting the acquisition run
5. Washing the syringe and preparing for the next injection

Step ‘1’ is often omitted, as samples are usually ready to inject when placed in the autosampler.

4.1.4 Example µL pickup UDP for Maximum Sample Pickup.

This example details the steps required to increase the amount of sample that can be injected in a µL pickup experiment. This type of injection routine can be used in a pre-concentration experiment for example.

NOTICE The standard µL pickup injection routine included in the method editor contains built in volume restrictions, which limit the volume of sample that can be taken up into the loop, but are designed to ensure excellent quantitative properties; therefore they should be used in preference to the following UDP for absolute quantitation experiments.

In the following example, 10 µL of sample is injected using a 20 µL loop using transport liquid placed in vial R1.

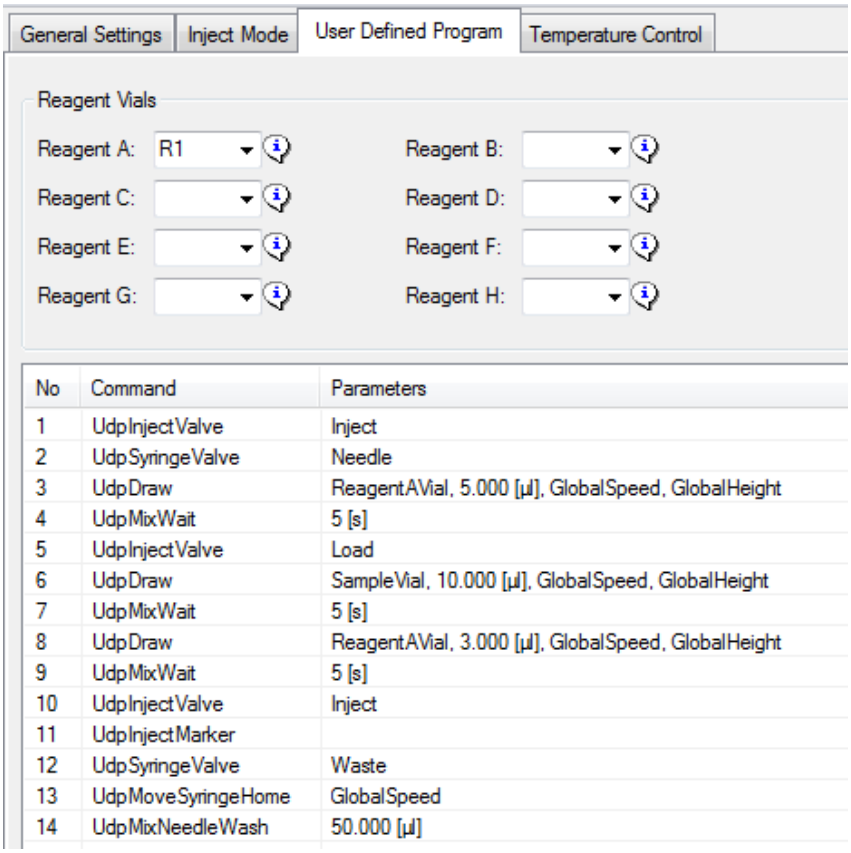


Figure 47: Example UDP injection routine for maximizing µL pickup volume

This injection routine can be considered in the steps (2 to 5) described above.

Step 2 – Preflushing the injection needle (commands 1 to 4)

Step 3 – Filling the sample loop (commands 5 – 9)

Step 4 – Injecting the sample and starting the run (commands 10 – 11)

Step 5 – Washing the syringe and needle and preparing for the next sample (commands 12-14)

4.1.5 Variable Injection Volumes

In UDPs such as that described in section 4.1.4 above, the injection volume is pre-defined within the UDP method itself. As such, the injection volume will remain the same for all analyses run using such a method, irrespective of the volume entered in the sample sequence table.

If flexibility in the injection volume is required, the UPD method can be altered such that the sample volume is defined by the injection volume as defined in the sample table.

The following steps describe how to adapt the method given in section 4.1.4 to permit injection volumes according to those defined in the sample table.

Step 1 – Select the “script Editor” tab in the method file

Step 2 - Navigate to the line where the **sample vial** volume is defined

Step 3 – Delete the text ‘Volume= 10 [µL]’

Step 4 - Replace with ‘Volume=system.Injection._Volume’ (make to place a comma at the end of the line)

Step 5 – Save the method with an appropriate name

4.2 Common Application Related Consumables

The tables below list consumables associated with the applications outlined in this handbook.

4.2.1 Columns

The most common analytical columns used with the UltiMate 3000 RSLCnano system are listed below. For a comprehensive guide of all available column formats please refer to the Thermo Scientific Chromatography columns and consumables catalogue.

Item	P/N
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, 50 µm I.D. x 25 cm, nanoViper	164709
Acclaim PepMap RSLC C18, 2 µm, 100 Å, 50 µm I.D. x 50 cm, nanoViper	164710
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, 75 µm I.D. x 75 cm, nanoViper	164939

Table 26 Acclaim PepMap C18 RSLC 2 µm particle size columns

Item	P/N
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
Acclaim PepMap, C18 3 µm, 100 Å, 75 µm I.D. x 15 cm (20 mm bed length), nanoViper	164535

Table 27 Acclaim PepMap C18 trap columns

Item	P/N
EASY-Spray column, 15 cm x 75 µm I.D., PepMap100 C18, 3 µm, 100Å	ES800A
EASY-Spray column, 15 cm x 50 µm I.D., PepMap RSLC C18, 2 µm, 100Å	ES801A
EASY-Spray column, 25 cm x 75 µm I.D., PepMap RSLC C18, 2 µm, 100Å	ES802A
EASY-Spray column, 50 cm x 75 µm I.D., PepMap RSLC C18, 2 µm, 100Å	ES803A
EASY-Spray column, 15 cm x 75 µm I.D., PepMap100 C18, 2 µm, 100Å	ES804A
EASY-Spray column, 75 cm x 75 µm I.D., PepMap RSLC C18, 2 µm, 100Å	ES805A
EASY-Spray column, 75 cm x 150 µm I.D., PepMap RSLC C18, 2 µm, 100Å	ES806A
EASY-Spray column, 25 cm x 200 µm I.D., PepMap Pepswift monolith	ES810A
EASY-Spray column, 15 cm x 75 µm I.D., Acclaim PepMap C4, 300Å	ES811A
EASY-Spray column, 15 cm x 75 µm I.D., Acclaim PepMap C18, 300Å	ES812A
EASY-Spray emitter, nano-flow (20µm I.D. transfer line, 7µm I.D. emitter)	ES791A
EASY-Spray emitter, micro-flow (75µm I.D. transfer line, 20µm I.D. emitter)	ES792A

Table 28: EASY-Spray columns

Item	P/N
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

Table 29: PepSwift Monolithic linear analytical columns

Item	P/N
PepSwift Monolithic Trap Column, 200 µm x 5 mm, set of 2, nanoViper	164558

Table 30: Pepswift Monolithic trap columns

4.2.2 nanoViper Capillaries, Sample Loops and Connectors

Length (mm)	I.D. [Colour Code]					
	10 µm [Green]	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	6041.5118 (L 180 mm)	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
850						
950		6041.5122	6041.5125	6041.5128		
1100				6041.5711		6041.5828

Table 31: Matrix for connection tubing for the UltiMate 3000 RSLCnano system

Description	P/N
nanoViper, ID x L, 20 µm x custom length	6041.5299
nanoViper, ID x L, 50 µm x custom length	6041.5599A
nanoViper, ID x L, 75 µm x custom length	6041.5799
nanoViper, thermally insulated, ID x L, 75 µm x300 mm	6083.2415
nanoViper ID x OD x L 75 µm x 360 µm x 550 mm	6041.5289
Liquid junction capillary interface ID x OD x L 20 µm x 360 µm x 550 mm	6041.5290
Nano LC column to MS tubing ID x OD x L 20 µm x 280 µm x 1 m	6041.5292
Nano LC column to MS tubing ID x OD x L 20 µm x 360 µm x 1 m	6041.5293
Nano LC column to MS tubing ID x OD x L 50 µm x 280 µm x 1 m	6041.5294
Nano LC column to MS tubing ID x OD x L 50 µm x 360 µm x 1 m	6041.5295

Table 32: Custom / One side nanoViper capillaries

Item	P/N
Sample loop 1 µL with nanoViper fittings connections	6826.2401
Sample loop 5 µL with nanoViper fittings connections	6826.2405
Sample loop 10 µL with nanoViper fittings connections	6826.2410
Sample loop 20 µL with nanoViper fittings connections	6826.2420
Sample loop 50 µL with nanoViper fittings connections	6826.2450
Sample loop 125 µL with nanoViper fittings connections	6826.2412

Table 33: Sample loops with nanoViper fittings

4.3 UltiMate 3000 RSLCnano convenience bundles, list of contents

Description	U3000 Nano RSLCnano with VWD		U3000 Nano RSLCnano, no detector		U3000 Cap flow RSLCnano, no detector		U3000 Nano RSLCnano with EASY-Spray	
Item	P/N: 5200.0350		P/N: 5200.0355		P/N: 5200.0356		P/N: 5200.0357	
Name	P/N	Module	P/N	Module	P/N	Module	P/N	Module
Solvent Rack	5035.9245	SRD-3400 with degasser	5035.9245	SRD-3400 with degasser	5035.9245	SRD-3400 with degasser	5035.9245	SRD-3400 with degasser
Pump	5041.0010A	NCS-3500RS **nano** pump with integrated column compt.	5041.0010A	NCS-3500RS **nano** pump with integrated column compt.	5041.0020	NCS-3500RS **Cap** pump with integrated column compt.	5041.0010A	NCS-3500RS **nano** pump with integrated column compt.
Autosampler	5826.0020	WPS-3000RPL RS nano/cap	5826.0020	WPS-3000RPL RS nano/cap	5826.0020	WPS-3000RPL RS nano/cap	5826.0020	WPS-3000RPL RS nano/cap
Detector	5074.0010	VWD-3400RS 4 channels without flow cell	-	-	-	-	-	-
Accessory 1	6074.0270	UZ-View™ flow cell 3nL(nano)	-	-	-	-	-	-
Accessory 2	6041.0001A	2P-10P valve for NCS-3500	6041.0001A	2P-10P valve for NCS-3500	6041.0001A	2P-10P valve for NCS-3500	6041.0001A	2P-10P valve for NCS-3500
Accessory 3	6720.0310	Preconcentration **nano** kit	6720.0310	Preconcentration **nano** kit	6720.0315	Preconcentration **cap** kit	6720.0395	**Easy-Spray** connection kit
Accessory 4	6000.1004	Signal cable, 6pol.5m	6000.1004	Signal cable, 6pol.5m	6000.1004	Signal cable, 6pol.5m	6000.1004	Signal cable, 6pol.5m
Accessory 5	6041.5292	Column to MS tubing ID x OD x L, 20µm x 280µm x 1m	6041.5292	Column to MS tubing ID x OD x L, 20µm x 280µm x 1m	6041.5294	Column to MS tubing ID x OD x L, 50µm x 280µm x 1m	-	-
Accessory 6	6041.5293	Column to MS tubing ID x OD x L, 20µm x 360µm x 1m	6041.5293	Column to MS tubing ID x OD x L, 20µm x 360µm x 1m	6041.5295	Column to MS tubing ID x OD x L, 50µm x 360µm x 1m	-	-
Accessory 7	6041.5294	Column to MS tubing ID x OD x L, 50µm x 280µm x 1m	6041.5294	Column to MS tubing ID x OD x L, 50µm x 280µm x 1m	6041.5560	Column to MS tubing ID x OD x L, 50µm x 360µm x 550mm	-	-
Accessory 8	6041.5295	Column to MS tubing ID x OD x L, 50µm x 360µm x 1m	6041.5295	Column to MS tubing ID x OD x L, 50µm x 360µm x 1m	6826.2405	nanoViper sampler loop for WPS-TPL RS, 5 µL	-	-
Accessory 9	161089	Cytochrome C digest sample	161089	Cytochrome C digest sample	161089	Cytochrome C digest sample	-	-

Table 34: Convenience Bundles Bill of Material (BOM) list

4.4 Hardware Accessories

The tables below list the common hardware accessories required for the applications described in this manual.

Item	P/N
Low-dispersion 2 pos 10 port valve high pressure for NCS-3500RS	6041.0001A
Low-dispersion 2 pos 6 port valve high pressure for NCS-3500RS	6041.0004A
Low-dispersion 2 pos 10 port valve, PAEK, bio, NCS-3500RS	6041.0012
Viper blind plug	6040.2303
Purge capillary	6040.2385

Table 35: Valves and accessories

Item	P/N
ProFlow flow meter	6041.7850
Upgrade Kit for ProFlow flow meter	6041.3003
Classic flow meter with nano flow selector	6041.7901A
Classic flow meter with capillary flow selector	6041.7902A
Classic flow meter with micro flow selector	6041.7903A
Flow selector, Nano LC (recommended: 50–1,000 nL/min)	6041.0002
Flow selector, Capillary LC (recommended: 0.5–10 µL/min)	6041.0003
Flow selector, Micro LC (recommended: 5–50 µL/min)	6041.0014

Table 36: NC Pump Flow Control

www.thermofisher.com

Thermo Fisher Scientific Inc.
168 Third Avenue
Waltham
Massachusetts 02451
USA