Multidimensional LC for separation of protein digests – A technical description of the Agilent 1100 Series nanoflow LC/MS system

Technical Overview

Abstract

Today, multidimensional LC/MS is a well-established tool for proteome identification. For samples of lower complexity one-dimensional enrichment LC/MS is used. For medium complex samples on-line two-dimensional nano LC/MS is the method of choice, and for samples of highest complexity off-line two-dimensional LC/MS is best suited. In this publication we describe instrument configurations for on-line and off-line two-dimensional nano LC/MS, one-dimensional enrichment nano LC/MS and a system checkout. Furthermore, methods showing how to successfully start with the analysis of "real life" proteome digests are provided.

Agilent Technologies

Introduction

Multidimensional liquid chromatography (MDLC) with orthogonal separation mechanisms offers significant benefits for the separation of complex protein digests from biological samples[1]. MDLC can be easily automated and offers high reproducibility, since it is performed in liquid phase. In addition, it works well for hydrophobic, acidic, basic, very small, very large and low abundance proteins that may be difficult to analyze using more traditional separation techniques. Multidimensional LC can be implemented in a number of ways and can be tailored to the requirements of particular sample types.

On-line 2D nano LC/MS

On-line 2D nano LC/MS is well-suited for digests from gel spots or bands, which need further separation and for subcellular fractions of higher complexity[2,3]. It is also useful for identifying biomarkers in pre-enriched biological fluids, such as blood, spinal fluid or urine. Finally, it is the best choice for further separation of affinity-purified post-translationally modified (PTM) peptides or protein complexes for functional proteomics research.

Off-line 2D nano LC/MS

Off-line 2D nano LC/MS is well-suited for extremely complex proteome samples such as whole cell lysates or tissues[4]. It can also be applied for purification and analysis of low abundance proteins or biomarkers from highly complex biological matrices.

Equipment

Description

Recommended system for on-line 2D nano LC/MS and for one-dimensional enrichment nano LC/MS (figure 1):

- Nanoflow LC system for MS including nanoflow pump, micro vacuum degasser, micro well-plate autosampler and handheld controller (G2229A).
- Agilent 1100 Series 2-position/ 10-port micro valve (G1163A). Offers highest flexibility for different techniques for proteomics.
- As second pump, different pump types can be used, for example, isocratic pump (G1310A), quaternary pump (G1354A), binary pump (G1312A). For applications of higher complexity the capillary pump with micro vacuum degasser (G1382A) should be used because it delivers a robust and reliable second flow in the micro litre range.
- LC/MSD Trap XCT[5] (G2451AA) – This ion trap mass spectrometer offers the highest sensitivity with an extended capacity trap Ion trap software. ChemStation software and PC hardware is included.
- Orthogonal nanospray source (G1982B) – Especially designed ion source ensures reliable and robust nanospray conditions.
- Capillary kit (G2228-687000) including columns (for details see appendix A) – Contains optimized fittings and capillaries, which make system handling and system set-up easy.
- Agilent 1100 Series column and valve organizer (G1383A)
- Spectrum Mill MS Proteomics Workbench[6] (G2751AA) – This software allows the extraction of peptide MS/MS spectra, spectra validation, database search and display of results obtained in LC/MS analysis of proteome samples.
- Thermostat for micro well-plate autosampler (G1330B) – Valuable samples can be stored at 4 °C to prevent degradation of thermolabile samples.

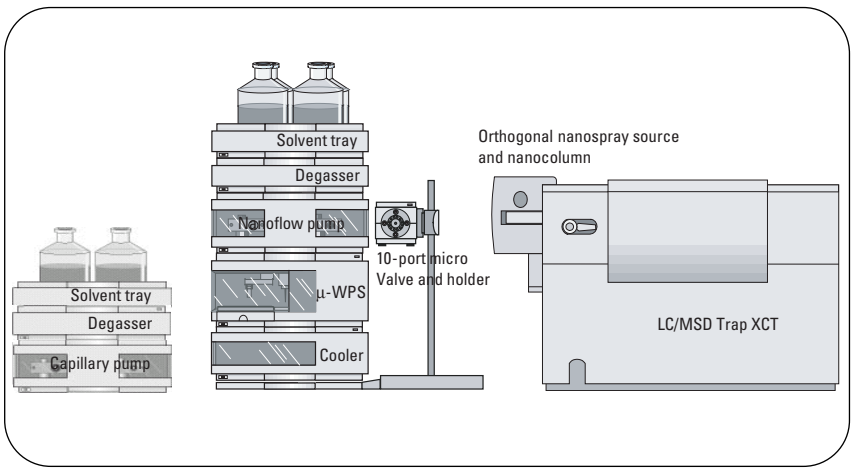


Figure 1
Nano LC/MS system for 2D nano LC/MS and 1D nano enrichment LC/MS proteomics applications

Optional:

- LC/MSD Trap Bioanalysis software for deconvolution of multiply charged ion series, and also software for protein and peptide data evaluation (G1981AA).
- The Nanoflow Proteomics XCT Solution bundle[7] (G2455AA) containing the following modules:
  - Nanoflow LC System for MS including nanoflow pump, micro vacuum degasser, micro well-plate autosampler and handheld controller (G2229A)
  - LC/MSD Trap XCT (G2451AA), which includes software and PC hardware, as well as the orthogonal nanospray source (G1982B)
  - Thermostatted column compartment with micro 2-position/6-port valve (G1316A056)

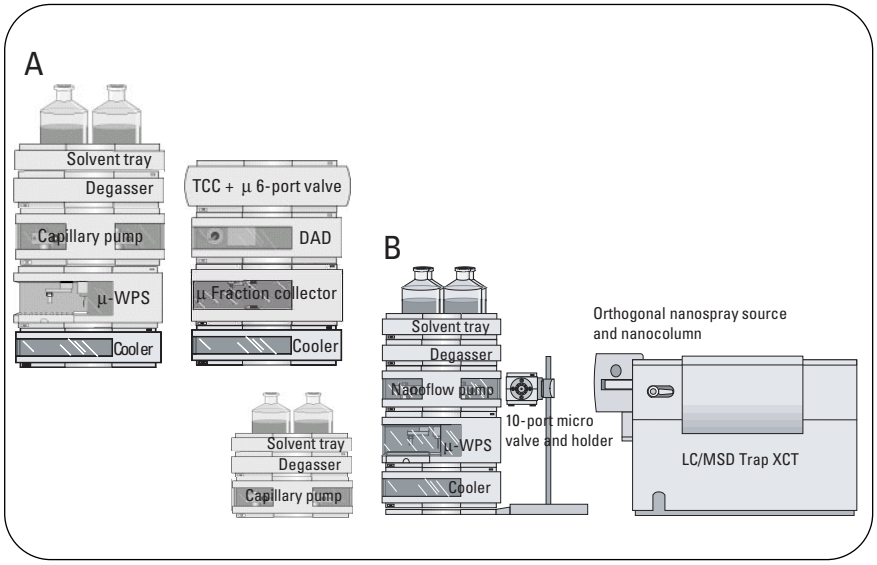


Figure 2
Agilent 1100 Series micro fraction collection system (A) and Agilent 1100 Series nanoflow LC system for MS (B) for off-line 2D LC/MS proteomics applications

Recommended system for off-line 2D nano LC/MS (figure 2)[8]:

The recommended system for off-line 2D nano LC/MS requires the same components as the on-line 2D nano LC/MS system, as well as the Agilent 1100 Series micro fraction collection system consisting of the following parts:

- Capillary pump with micro vacuum degasser (G1382A) – Delivers a reliable and robust flow rate necessary for high resolution chromatography.
- Agilent 1100 Series micro well-plate autosampler (G1377A) – Enables injections from the nL range to the µL range.
- Agilent 1100 Series diode-array detector (G1315B014) with 500-nL and 80-nL flow cell. With these flow cells high resolution chromatograms and spectra can be acquired.
- Agilent 1100 Series micro fraction collector (G1364D) Enables fractionation in the low µL range with exact liquid deposition in 96- and 384 well-plates. The module includes the special micro fraction collection capillaries (G1364-87304, 25-µm i.d., G1364-87305, 50-µm i.d., G1364-87306, 100-µm i.d. and the capillary (5065-9906, 1.12 m length, 50-µm i.d.) to connect the micro well-plate autosampler to the column located in the thermostatted colum compartment in the recommended LC stack.

Optional:

- Thermostats (G1330B) for micro fraction collector and/or micro well-plate autosampler.
- Second micro fraction collector and 2-position/6-port valve (G1157A) to switch between the two fraction collectors.
- 100-µL flow sensor (G1376-60002) for flow rates up to 100 µL/min.

Chromatographic columns

Recommended ion exchange columns for the 1st dimension of on-line and off-line 2D LC:

- ZORBAX BioSCX Series II strong cation exchanger, 35 x 0.3 mm (Agilent part number 5065-9912)
- ZORBAX BioSCX Series II strong cation exchanger, 50 x 0.8 mm (Agilent part number 5065-9942)

Recommended reversed-phase columns for 2nd dimension of 2D LC:

- ZORBAX 300SB-C18, 150 mm x 75 µm, 3.5 µm particles (Agilent part number 5065-9911)
- ZORBAX 300SB-C18, 50 mm x 75 µm 3.5 mm particles (Agilent part number 5065-9924)
- ZORBAX 300SB-C18, Trap columns 5 x 0.3 mm, 5 µm particles, 5/pk (Agilent part number 5065-9913)
- Holder for 5-mm trap cartridges (Agilent part number 5065-9915)

Check-out sample

Dissolve the 500-pmol bovine serum albumin (BSA) digest (G1990-85000) by adding 500 µL of water/acetonitrile (85:15 v/v) containing 0.1 % formic acid. Vortex the vial for a couple of minutes, dilute 10 µL of the 1 pmol/µL digest stock solution 1/10 (v/v) with water in an Eppendorf vial (vortex this vial) and transfer the 100 fmol/µL solution to a glass vial (5182-0543 and 5181-1210) with low volume polypropylene insert (5182-0549) and place it in the micro well-plate autosampler. Inject 1 µL (100 fmol) for each experiment.

Technical principles

Principle of on-line 2D nano LC

The Nanoflow Proteomics Solution is a unique instrument configuration, which offers highest flexibility for proteomics applications (figure 1). The micro well-plate autosampler is connected directly to the capillary pump. From the pump the hydraulic flow passes through the micro 2-position/6-port valve of the micro well-plate autosampler and then the sample loop and needle. The SCX column is positioned after the injector seat capillary and the SCX column outlet flow is directed to the 6-port valve of the autosampler. This valve is connected with a capillary to the micro 2-position/10-port valve in the separate valve box (figure 3) or to the 2-position/6-port valve in the column compartment (figure 4). Sample peptides, which are eluted from the SCX column by injecting salt solution plugs of increasing concentration, are concentrated on a C18 enrichment column, which is mounted between two ports of this second valve, and the eluting flow goes to waste. The nanoflow pump is also connected to this second valve and the flow from this pump passes through the analytical reversed phase nanocolumn into the LC/MSD Trap XCT ion trap mass

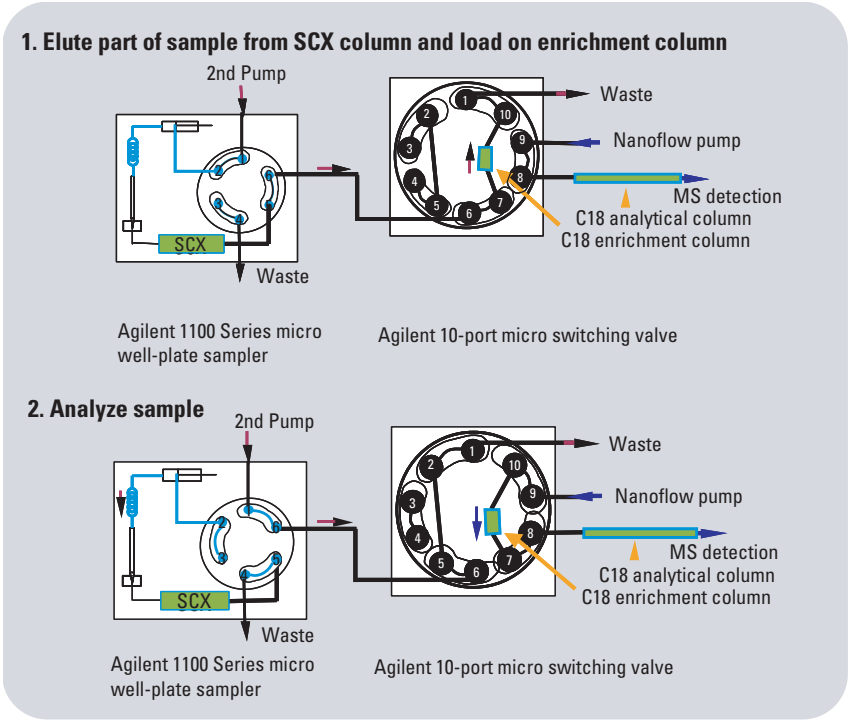


Figure 3
Flow diagram for on-line nano 2D LC using a 10-port valve

spectrometer. Then, the enrichment column containing the trapped tryptic peptides is switched into the nanoflow path. The nanoflow pump delivers the elution gradient and the peptides are separated on the reversed-phase nanocolumn. The principle of on-line two-dimensional LC/MS is outlined in figure 5.

Principle of off-line 2D LC

In the off-line two-dimensional LC methodology the first and the second dimension are separated. The first dimension in which the sample peptides are separated on a strong cation exchanger (SCX) column is performed with the Agilent 1100 Series micro fraction collection system (figure 2). This system consists of a standard capillary system enhanced with a micro fraction collector. This system is plumbed since it is standard for capillary LC systems with the micro fraction collector connected to the exit capillary of the DAD cell. The fractions collected in the well-plate are subsequently transferred to the nanoflow LC/MS system and analyzed in the second dimension with reversed phase chromatography. The principle of off-line two-dimensional LC/MS is outlined in figure 6.

In this methodology the second dimension itself is performed in a one-dimensional way including an enrichment column prior to the analytical nanocolumn. The collected peptide fractions eluted from the SCX column are injected onto a C18 enrichment column. On this column the peptides are trapped and desalted. The enrichment column is mounted between

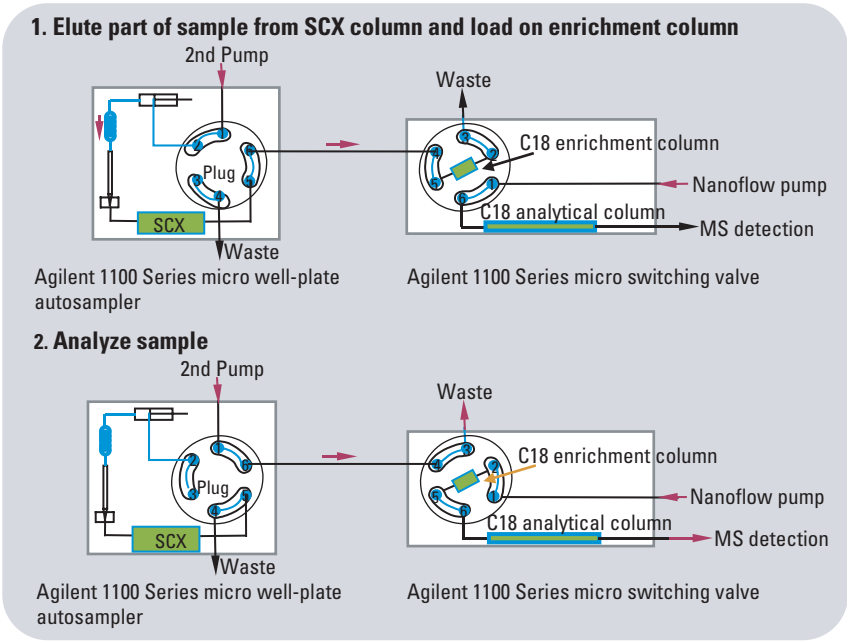


Figure 4
Flow diagram for on-line nano 2D LC using a 6-port valve

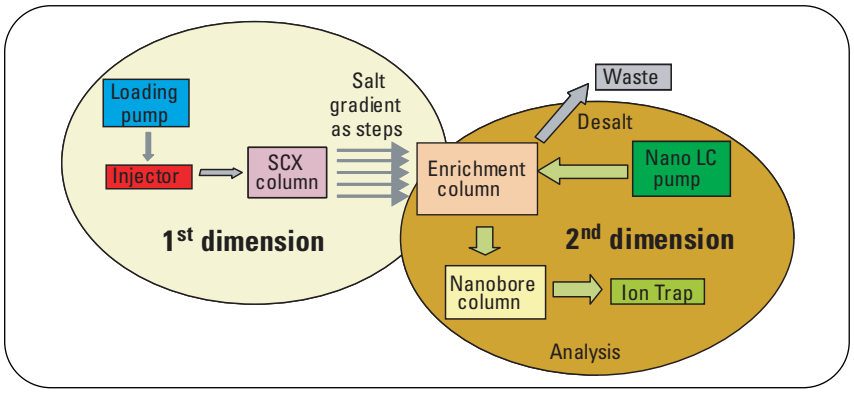


Figure 5
Principle of on-line two dimensional chromatography with mass spectrometry

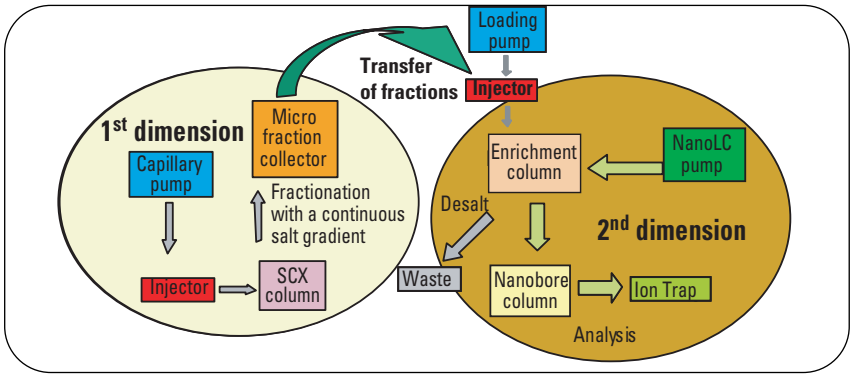


Figure 6
Principle of off-line two dimensional chromatography with mass spectrometry

two ports of the micro 10-port- or micro 6-port valve (figure 7) and during the desalting process the flow is directed to waste. After this desalting process the enrichment column is switched into the nanoflow path. The peptides are eluted from the enrichment column and separated on the analytical nanocolumn by the gradient delivered from the nanoflow pump.

Connecting the Agilent 1100 Series nano LC system to the mass spectrometer

The Agilent orthogonal nanospray source with a special column- and needle-holder (figure 8) offers several advantages. The column and needle are easy to assemble and disassemble without any tools (figure 8 A). Fragile springs for electrical grounding are also not needed. Grounding is achieved with an electrical conductive ferrule and conductive pads with less than 1 Ohm electrical contact resistance. To assemble the sprayer needle into the column fitting no separate reducing sleeves or additional connectors are necessary. The holder is a simple clam shell design for quick and easy column and needle change-out during the analysis without electrical danger. The clam shell can be removed and reinserted while maintaining the original needle position, even during acquisition and when the spray chamber is closed. The needle tip is automatically positioned between the high voltage MS inlet electrode and the special counter electrode. Adjustment is accomplished by turning one or both adjusters along the x- and y-axes (figure 8 B). The counter electrode is operated at

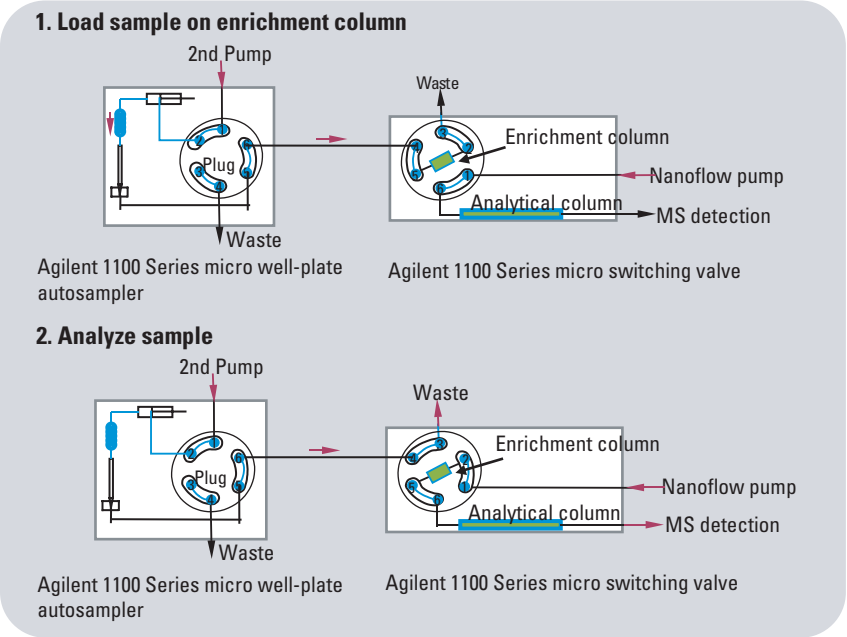


Figure 7
Flow diagram for nano LC/MS in one-dimensional enrichment mode and for the second dimension of offline 2D LC

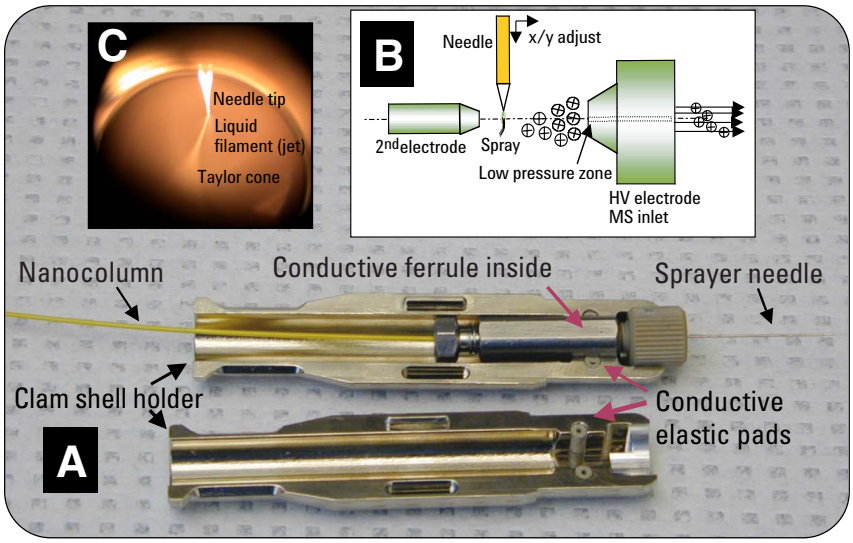


Figure 8
Agilent orthogonal nanospray source and nanospray needle and column holder

500 V below the MS inlet electrode. The 2nd electrode stabilizes the spray and draws the liquid stream to the opposed direction of the MS inlet (figure 8 C), causing less background and chemical noise. Positioning the nanospray emitter in the ortho position and increas-

ing the spacing eliminates electron avalanches and high voltage discharges. This allows for successful ion polarity switching with a full MS scan each 1.25 seconds. The spray chamber is fully sealed and vented for operator safety, eliminating possible escape of aerosol particles.

Check-out methods for the Agilent 1100 Series nanoflow LC/MS system

Functionality check-out of the Agilent 1100 Series nanoflow pump and the Agilent 1100 Series micro well-plate autosampler with MS

For a functionality check of the nanoflow pump and the micro well-plate autosampler, the nanoflow pump is connected directly to the micro well-plate autosampler. The micro 6-port valve of the autosampler is connected directly to the nanobore column, which is located in the orthogonal nanospray source. The corresponding flow- and plumbing diagram with a detailed description of the capillaries is shown in figure 12 on page 13. To check the instrument performance 1 µL of the BSA test sample (vide supra) is injected. After the injection the autosampler loop is switched to bypass to avoid its dead volume and the resulting gradient delay time. The method for this performance check is described in appendix B.1. and the corresponding MS settings are described in appendix D. This check-out method is recommended only if a second pump is not available in the system or for troubleshooting purposes. The result obtained for the 100-fmol BSA check–out procedure after data analysis with Spectrum Mill is shown in tables 1A and 1B. Table 1A shows one example for each of the 29 identified peptides and table 1B summarizes the result showing that in some cases more than one spectra per peptide was acquired. The corresponding base peak chromatogram recorded with the direct injection method is shown

Score	SPI (%)	Spectrum Intensity	Sequenze	MH^+ Matched(Da)
6.70	66.00	4.08E+05	(K) AFDEK(L)	609.288
9.52	80.50	3.33E+05	(K) ATEEQLK(T)	818.426
10.86	85.60	1.11E+06	(R) LCVLHEK(T)	899.466
10.16	94.90	7.74E+06	(K) AEFVEVTK(L)	922.489
9.47	98.30	5.11E+06	(K) YLYEIAR(R)	927.494
8.94	78.70	2.20E+05	(R) EKVLTSSAR(Q)	990.558
14.39	78.70	5.95E+05	(K) SHCIAEVEK(D)	1073.494
11.00	87.40	1.04E+06	(K) EACFAVEGPK(L)	1108.498
16.67	96.60	2.06E+07	(K) LVNELTEFAK(T)	1163.631
6.52	86.00	2.36E+05	(R) CCTKPESER(M)	1168.461
13.26	84.20	8.74E+05	(R) FKDLGEEHFK(G)	1249.622
14.69	89.00	2.39E+06	(K) HLVDEPQNLIK(Q)	1305.717
14.85	86.00	1.29E+07	(K) SLHTLFGDELCK(V)	1420.678
19.22	94.00	5.43E+06	(R) RHPEYAVSVLLR(L)	1439.812
16.65	96.00	2.03E+07	(K) YICDNQDTISSK(L)	1444.627
17.48	91.90	1.54E+06	(K) TCVADESHAGCEK(S)	1465.557
13.73	80.10	7.19E+06	(K) LGEYGFQNALIVR(Y)	1479.796
9.21	92.80	1.65E+06	(R) ETYGDMADCCEK(Q)	1480.492
12.82	82.70	3.68E+06	(K) EYEATLEECCAK(D)	1504.582
14.63	86.80	1.30E+06	(K) LKECCDKPLLEK(S)	1534.75
12.78	75.80	1.36E+06	(R) LCVLHEKTPVSEK(V)	1540.805
14.26	91.60	1.62E+06	(K) LKPDPNTLCDEFK(A)	1577.752
15.12	92.10	4.96E+06	(R) KVPQVSTPTLVEVSR(S)	1639.938
6.00	100.00	2.89E+05	(R) MPCTEDYLSLILNR(L)	1725.819
14.01	77.50	2.57E+07	(R) RPCFSALTPDETYVPK(A)	1881.906
11.83	76.40	1.52E+06	(R) NECFLSHKDDSPDLPK(L)	1902.854
12.32	83.50	5.27E+05	(K) LKPDPNTLCDEFKADEK(K)	2020.954
14.13	79.50	1.42E+06	(K) VHKECCHGDLLECADDR(A)	2116.837
9.45	88.90	7.12E+05	(R) ETYGDMADCCEKQEPER(N)	2119.789
11.95	97.10	3.18E+06	(K) DAIPENLPPLTADFAEDKDVCK(N)	2459.165

Table 1A
Tryptic peptides identified from 100 fmol BSA analyzed with the direct injection method

Protein name	Spectra (#)	Distinct Peptides	Score	Coverage% AA	Spectral Intensity
Bovine Serum Albumin	52	29	366.64	51	4.72E+06

Table 1B
Summarized result of the analysis of 100 fmol BSA by direct injection method

in figure 9. With this method the peptides are eluted between 22 and 35 minutes from the column.

Functionality check-out of the Agilent 1100 Series nanoflow LC/MS system with valve switching for one-dimensional enrichment LC/MS

This check-out is recommended to check the functionality of the complete nanflow LC/MS system which does not only include the nanoflow pump, the autosampler and the mass spectrometer but also the micro 6- or 10-port valve located in the separate valve box, or the micro 6-port valve in the thermostatted column compartment with the enrichment column. This system configuration is necessary for online 1D enrichment LC/MS, for online 2D LC/MS and for the off-line 2D LC/MS methodology. The system plumbing for the 6-port and 10-port valves is described in detail in figures 13 and 14 on page 13. For the one-dimensional set-up the SCX column is removed and the seat capillary is connected directly to the 6-port valve in the autosampler. The checkout method is shown in appendix B.2 and the according MS methods are listed in appendix D. To check the instrument set-up 1 µL BSA sample (vide supra) is injected and analyzed. The result obtained for the 100 fmol BSA check–out procedure after data analysis with Spectrum Mill is shown in tables 2A and 2B. Table 2A shows one example for each of the 20 identified peptides and table 2B summarizes the whole result showing that in some cases more than one spectra per peptide was acquired. The base peak chro-

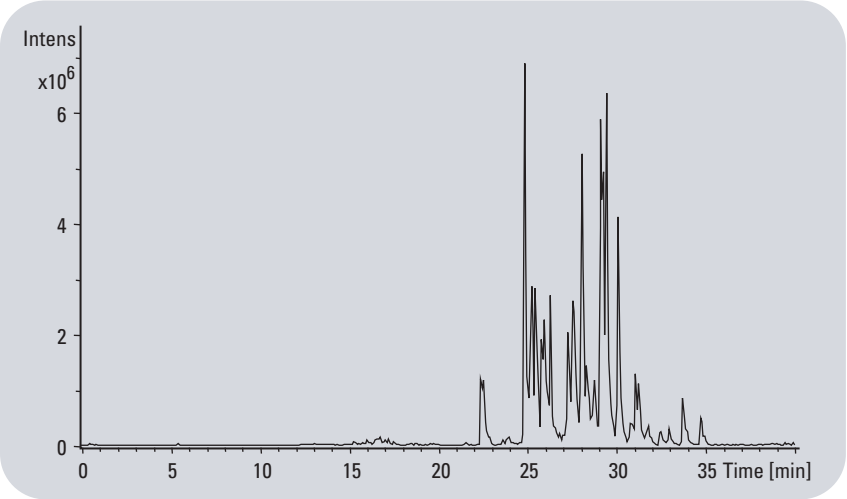


Figure 9
Base peak chromatogram from 100 fmol BSA digest analyzed with the direct injection method

Score	SPI (%)	Spectrum Intensity	Sequenze	MH^+ Matched(Da)
10.81	89.80	1.21E+07	(K) AEFVEVTK(L)	922.489
8.44	95.90	1.97E+06	(K) YLYEIAR(R)	927.494
9.72	78.60	1.32E+06	(K) QTALVELLK(H)	1014.620
11.50	88.40	2.02E+06	(K) EACFAVEGPK(L)	1108.498
16.11	94.60	5.93E+07	(K) LVNELTEFAK(T)	1163.631
12.33	85.60	1.31E+06	(R) FKDLGEEHFK(G)	1249.622
15.77	91.40	4.51E+06	(K) HLVDEPQNLIK(Q)	1305.717
13.26	76.80	4.37E+06	(K) SLHTLFGDELCK(V)	1420.678
18.53	91.70	6.68E+06	(R) RHPEYAVSVLLR(L)	1439.812
12.18	97.10	1.28E+07	(K) YICDNQDTISSK(L)	1444.627
8.57	87.00	3.74E+06	(K) LGEYGFQNALIVR(Y)	1479.796
8.90	96.60	2.85E+06	(R) ETYGDMADCCEK(Q)	1480.492
13.30	81.90	5.25E+06	(K) EYEATLEECCAK(D)	1504.582
14.48	70.30	3.06E+06	(K) LKECCDKPLLEK(S)	1534.750
9.37	60.60	3.98E+06	(R) LCVLHEKTPVSEK(V)	1540.805
15.00	83.30	4.72E+06	(K) LKPDPNTLCDEFK(A)	1577.752
15.53	90.40	7.13E+06	(R) KVPQVSTPTLVEVSR(S)	1639.938
16.96	91.70	2.10E+07	(R) RPCFSALTPDETYVPK(A)	1881.906
13.95	80.70	3.29E+06	(R) NECFLSHKDDSPDLPK(L)	1902.854
6.33	79.80	2.26E+06	(K) VHKECCHGDLLECADDR(A)	2116.837

Table 2A
Tryptic peptides identified from 100 fmol BSA analyzed with the enrichment method

Protein name	Spectra (#)	Distinct Peptides	Score	Coverage% AA	Spectral Intensity
Bovine Serum Albumin	60	20	256.22	39	7.14E+06

Table 2B
Summarized result of the analysis of 100 fmol BSA with the enrichment method.

matogram recorded with the enrichment method is shown in figure 10. With this method the peptides are eluted between 24 and 36 minutes from the column.

Functionality check-out of the nanoflow system for online two-dimensional LC/MS

For two-dimensional LC/MS experiments it is essential that the performance of the LC/MS system is checked in the one-dimensional enrichment mode as described previously. With this special check-out procedure for two-dimensional LC/MS experiments the performance of the SCX column and the salt plug injections are tested. The SCX column is connected to the system as shown in figures 3 and 4. 1-µl BSA sample (vide supra) is injected onto the SCX column. In the first run no BSA peptides should be detected by the Spectrum Mill data analysis. This proves the optimum performance of the SCX column – all tryptic BSA peptides are retained. In the second analysis step the tryptic BSA peptides are eluted with a salt solution plug of a concentration of 150 mM NaCl and analyzed by MS/MS after separation on the C18 reversed phase nanocolumn. Finally, the SCX column is cleaned from the remaining peptides by an injection of a 500-mM NaCl salt solution plug. The method and the injection sequence list for the check-out of the 2D LC methodology is described in appendix B.3. The distribution of the detected tryptic BSA peptides over the fractions is shown in table 3A and the complete result of the 2D LC/MS analysis of BSA is shown in table 3B. No BSA peptides break through the SCX column in the first run. All peptides are eluted with the injected salt solution plug of 150 mM NaCl concentration. Only one peptide is eluted in the 500 mM NaCl salt solution plug.

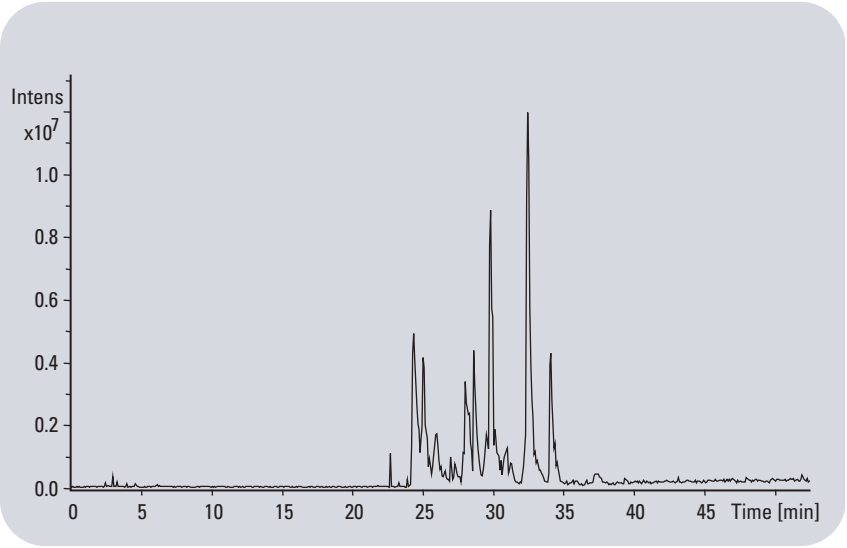


Figure 10
Base peak chromatogram from 100 fmol BSA digest analyzed with the enrichment method

#	Sequence	0 mM NaCl Score SPI(%)	150 mM NaC Score SPI(%)	500 mM NaCl Score SPI(%)
1	(K)AEFVEVTK(L)		1,05E+06	
2	(K)EYEATLEECCAK(D)		2,57E+05	
3	(K)HLVDEPQNLIK(Q)		1,93E+06	1,23E+06
4	(R)KVPQVSTPTLVEVSR(S)		2,24E+06	
5	(K)LGEYGFQNALIVR(Y)		6,52E+05	
6	(K)LVNELTEFAK(T)		4,22E+06	
7	(K)YICDNQDTISSK(L)		2,06E+06	
8	(K)YLYEIAR(R)		1,10E+06	

Table 3B
Tryptic peptides identified from 100 fmol BSA analyzed with the online 2D LC method

Protein name	Spectra (#)	Distinct peptides	Score	Coverage% AA	Spectral intensity
Bovine Serum Albumin	20	8	80.77	14	7.37E+05

Table 3B
Summarized result of the analysis of 100 fmol BSA with the online 2D LC method

Functionality check-out of the Agilent 1100 series nanoflow pump and the Agilent 1100 Series micro well-plate autosampler without mass spectrometer

To check the functionality and the performance of the nanoflow pump/micro well-plate autosampler combination without mass spectrometer a special procedure is required. For this purpose a restriction capillary is connected to port 6 of the micro valve of the micro well-plate autosampler instead of a nanobore column. To check the flow rate precision a special flow program is used, which switches the flow rate between 600 nL/min and 300 nL/min. During the flow rate check-out test the micro well-plate autosampler loop is switched to bypass. The check-out procedure and the method are described in appendix B.4. Figure 11 shows the pressure and flow diagram obtained with the check-out procedure for the standalone nanoflow pump and well-plate autosampler without mass spectrometer. The typical result is a pressure of 100 bar at 600 nL/min and 50 bar at 300 nL/min. The average pressure of the different plateaus at the same flow rate is in a range of ± 2 bar and typically ± 1 %.

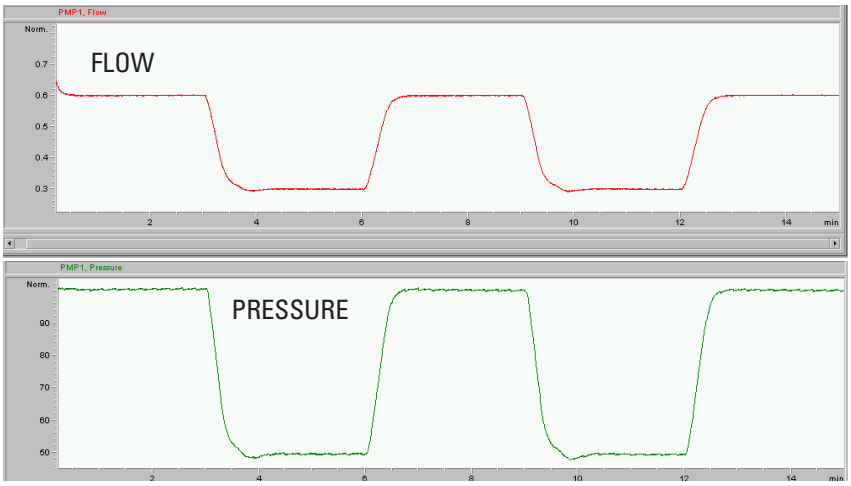


Figure 11
Pressure and flow diagrams obtained with the check-out procedure for the nanoflow pump and micro well-plate autosampler without mass spectrometer

Methods for real life

For less complex samples it is sufficient to work with a one-dimensional enrichment method. This method concentrates diluted sample peptides and cleans the sample from contaminants such as salts and detergents prior to RP separation and MS analysis. For samples of less complexity such as digested proteins from a gel spot which normally contains between one and three proteins it is sufficient to work with the method described in appendix C.1. For samples of increasing complexity, for example, digested proteins from a gel band containing up to 10 proteins the longer 1D enrichment method of appendix C.2 should be used. For samples of high complexity which are obtained, for example, from a subproteome the 2D LC/MS method shown in appendix C.3 should be applied. Samples of highest complexity containing several hundred proteins, normally obtained from a whole cell digest, should be analyzed with the off-line 2D LC/MS method described in appendix C.4. The corresponding MS methods for the Ion Trap SL and the Ion Trap XCT fitting to all LC methods are given in appendix D.

Conclusion

In this publication we described the instrument set-up for the nanoflow LC/MS system and the Agilent 1100 Series micro fraction collection system. Detailed plumbing diagrams for the different one- and two-dimensional LC approaches are given. For each set-up a check-out method is described and the obtained results are documented. Furthermore, approved methods for a successful start with the analysis of real life samples of different complexity are also provided.

Appendix – Method description

A. Nanoflow LC start up kit (G2228-687000)

0100-1700	Ferrule 1/8	
0100-1708	Nut 1/8 PPS	8
0890-1760	Flexible tubing meter	6
5022-2184	ZDV union, stainless steel, no fitting	3
5022-6536	FT fitting, long, with ferrule	1
5065-9911	ZORBAX 300SB-C18, 150 x 0.075 mm, 3.5 µm	1
5065-9913	ZORBAX 300SB-C18, 5 x 0.3 mm, 5 µm, 5/pk	1
5065-9915	Holder for 5 mm trap cartridges	1
G1375-87312	Fused silica/PEEK capillary, 100 µm, 20 cm	2
G1375-87316	Seat capillary, 75 mm, 150 mm	1
G1375-87320	Fused silica/PEEK capillary, 25 µm, 10 cm	2
G1375-87322	Fused silica/PEEK capillary 25 µm, 35 cm	3
G1375-87323	Fused silica/PEEK capillary, 25 µm, 55 cm	2
G1375-87324	Fused silica/PEEK capillary, 25 µm, 70 cm	1
G1375-87326	Waste tube, FEP, 1.6 mm o.d., 08 µm i.d.	2
G1375-87327	Fused silica/PEEK capillary, 75 µm, 65 cm	2

B. Methods for check-out of the Agilent 1100 Series nanoflow LC/MS system

B.1. 1D Direct LC method (figure 12)

Column:	ZORBAX 300SB-C18, 150 mm x 75 µm, 3.5-µm particles
Injection volume:	1 µL (maximum volume with this method)
Nanoflow pump:	
Mobile phase:	A = 0.1 % formic acid in water
Mobile phase:	B = 0.1 % formic acid in acetonitrile
Gradient:	3 % B at 0 min, 3 % B at 10 min, 45 % at 30 min. 65 % at 33 min. 3 % at 33.01 min
Stop time:	40 minutes
Post time:	5 minutes
Flow rate:	300 nL/min

Injector program:
1 Draw def. amount from sample, 10 µL/min speed, def. offset
2 NEEDLE wash in flush port, 5.0 sec.
3 INJECT
4 WAIT 10.00 min
5 VALVE bypass

Note:	Use 20 % methanol + 0.1 % formic acid in water for needle wash solvent Set bottom-sensing on with a 0.9-mm offset for the plastic micro insert vials

B.2. 1D Enrichment LC method (figures 13 and 14)

Columns:	ZORBAX 300SB-C18, 150 mm x 75 µm, 3.5 µm particles ZORBAX 300SB-C18, 5 x 0.3 mm, 5 µm particles
Injection volume:	up to 8 µl
Enrichment column	switch: position 1 at 0 min, position 2 at 5 min, then return to position 1 at 61 minutes
Nanoflow pump:	
Mobile phase:	A = 0.1 % formic acid in water
Mobile phase:	B = 0.1 % formic acid in acetonitrile
Gradient:	3 % B at 0 min, 3 % B at 5 min, 15 % at 8 min, 45 % at 50 min, 90 % at 55 min, 90 % at 60 min, 3 % at 61 min.
Stop time:	75 minutes
Post time:	5 minutes
Flow rate:	300 nL/min
Second pump:	isocratic, quaternary or binary: use flow gradient below. For capillary pump no flow gradient is needed, just use 15 µL/min
Mobile phase:	0.1 % formic acid in 3 % acetonitrile/water
Flow:	0.01 mL/min
Flow gradient:	0.1 mL/min at 0 min, 0.05 mL/min at 0.5 min, 0.01 mL/min at 0.51 min, 0.01 mL/min at 8 min, 0.005 mL/min at 8.01 min, 0.005 mL/min at 9.9 min

Injector program:
1 Draw def. amount from sample, 10 µL/min speed, def. offset
2 NEEDLE wash in flush port, 5.0 sec.
3 INJECT

Note:	Use 20 % methanol +0.1 % formic acid in water for needle wash solvent. Set bottom-sensing on with a 0.9-mm offset for the plastic micro insert vials.

B.3. 2D LC method (figures 13 and 14)

For the reversed phase separation of the tryptic peptides in the second dimension the method described in B.2 is used. For the elution of the peptides from the SCX column with salt solution plugs of increasing concentration, the injection sequence in table 4 is used.

SCX column: ZORBAX BioSCX Series II strong cation exchanger 35 mm x 0.3mm.
Salt steps: 0, 150, and 500 mM NaCl (prepared in 3 % acetonitrile in 0.1 % formic acid).

B.4. Checkout procedure for an Agilent 1100 Series nanoflow LC system without MS (G2229A)

A) System preparation:

1. Connect the nanoflow pump and the micro well-plate autosampler with the capillary G1375-87322 (25 µm i.d., 35-cm length).
2. Check the system tightness by executing a micro pressure test, with the plug on port 6 of the injection valve in the micro well-plate autosampler.
3. Purge channel A1 with 100 % water at 2.5 mL/min during 2 minutes.
4. Purge channel B1 with 100 % acetonitrile at 2.5 mL/min during 2 minutes.
5. Pump 10 µL/min, normal mode, 100 % A (water). Pump at least 5 minutes before continuing.
6. Pump 10 µL/min, normal mode, 100 % B (acetonitrile). Pump at least 5 minutes before continuing.
7. Install the restriction capillary (G2226-67300) on port 6 of the injection valve in the micro well-plate autosampler.
8. Pump 1.5 µL/min, micro mode, 70 % A (water)/30 % B (acetonitrile). Pump as long as it takes for the pressure to become stable. Pump at least 5 minutes before continuing.
9. Pump 0.6 µL/min, micro mode, 70 % A (water)/30 % B (acetonitrile). Pump as long as it takes for the pressure to become stable. Pump at least 5 minutes before continuing.

B) Flow stability check:

Method parameters:

1. Nano pump

- Column flow: 0.6 µL/min
- Stoptime: 15 minutes
- Solvent A: 70 % (water)
- Solvent B: 30 % (acetonitrile)
- Calibrated as: H_2O/ACN
- Primary flow: 200-500 µL/min
- Compressibility A: 50*10^-6/bar
- Compressibility B: 115*10^-6/bar
- Min stroke A and B: Auto
- Fast composition change: ON
- Timetable

Time (min)	0.00	3.00	3.01	6.00	6.01	9.00	9.01	12.00	12.01	15.00
Flow (nL/min)	600	600	300	300	600	600	300	300	600	600

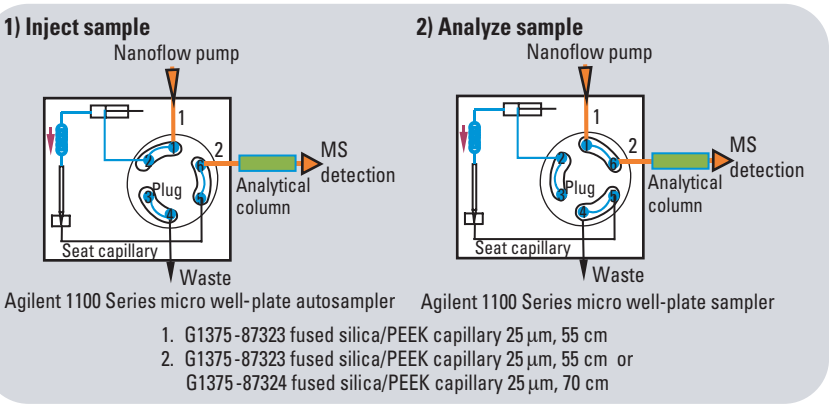
2. Micro well-plate autosampler

- Injection volume: 0.000 ml
- Injection mode: Edit injector program ➠ Inject ➠ Bypass

Line	Location	Sample name	Method name*	Injection location	Sample type	Data file	Injection volume
1	P1-A-01	Sample	2DLC.M	1	Sample	0 mM	1
2	Vial 1	150 mM NaCl	2DLC.M	1	Sample	100 mM	20
3	Vial 2	500 mM NaCl	2DLC.M	1	Sample	500 mM	20

* use the short 1D enrichment method here (B2.2)

Table 4
Default sequence for 2D LC check-out

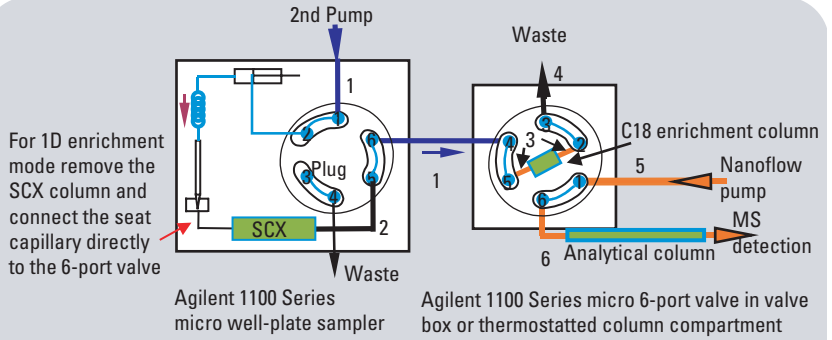


Agilent 1100 Series micro well-plate autosampler

Agilent 1100 Series micro well-plate sampler

1. G1375-87323 fused silica/PEEK capillary 25 µm, 55 cm
2. G1375-87323 fused silica/PEEK capillary 25 µm, 55 cm or G1375-87324 fused silica/PEEK capillary 25 µm, 70 cm

Figure 12
Plumbing diagram for nano LC in 1D direct injection mode

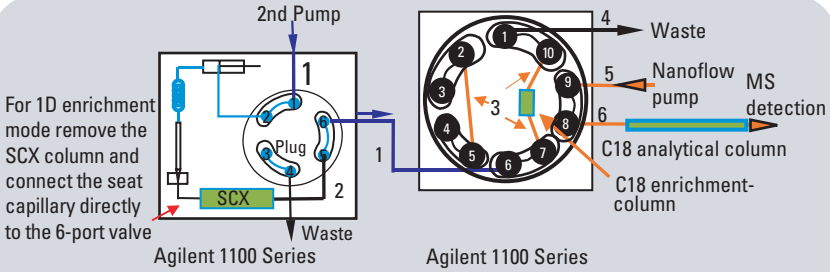


Agilent 1100 Series micro well-plate sampler

Agilent 1100 Series micro 6-port valve in valve box or thermostatted column compartment

1. G1375-87327 fused silica/PEEK capillary 75 µm, 65 cm
2. G1375-87316 fused silica /PEEK capillary 100 µm, 20 cm
3. G1375-87320 fused silica /PEEK capillary 25 µm, 10 cm
4. G1375-87326 waste tube, FEP, 1.6 mm o.d., 0.8 mm i.d., 2m
5. G1375-87322 fused silica /PEEK capillary 25 µm, 35 cm or G1375-87323 fused silica /PEEK capillary 25 µm, 55 cm
6. G1375-87324 fused silica /PEEK capillary 25 µm, 75 cm

Figure 13
Plumbing diagram for nano LC in 2D mode or 1D enrichment mode with 6-port valve



Agilent 1100 Series micro well-plate autosampler

Agilent 1100 Series micro 10-port switching valve

1. G1375-87327 fused silica/PEEK capillary 75 µm, 65 cm
2. G1375-87316 fused silica /PEEK capillary 100 µm, 20 cm
3. G1375-87320 fused silica /PEEK capillary 25 µm, 10cm
4. G1375-87326 waste tube, FEP, 1.6 mm o.d., 0.8 mm i.d., 2m
5. G1375-87322 fused silica /PEEK capillary 25 µm, 35 cm or G1375-87323 fused silica /PEEK capillary 25 µm, 55 cm
6. G1375-87324 fused silica /PEEK capillary 25 µm, 75 cm

Figure 14
Plumbing diagram for nano LC in 2D mode or 1D enrichment mode with 10-port valve

C) 1D and 2D example methods for easy start-up with the Nanoflow Proteomics Solution

C.1. 1D Enrichment LC method – Short method for less complex samples
Use the method described for the 1D enrichment LC/MS system check-out (see B.2).

C.2. 1D Enrichment LC method – Long method for more complex samples

Columns:	ZORBAX 300SB-C18, 150 mm x 75 µm, 3.5 µm particles ZORBAX 300SB-C18, Trap columns 5 x 0.3 mm, 5 µm, particles, 5/pk
Injection volume:	up to 8 µL
Enrichment column	switch: position 1 at 0 min, position 2 at 5 min, then return to position 1 at 146 minutes.
Nanoflow pump:	
Mobile phase:	A = 0.1 % formic acid in water
Mobile phase:	B = 0.1 % formic acid in acetonitrile
Gradient:	3 % B at 0 min, 3 % B at 5 min, 15 % B at 11 min, 45 % B at 131 min (0.3 µL/min), 90 % B at 138 min (0.4 µL/min), 90 % B at 143 min (0.6 µL/min), 3 % B at 146 min (0.4 µL/min), 3 % at B at 160 min (0.3 µL/min)
Stop time:	160 minutes
Post time:	5 minutes
Flow rate:	300 nL/min
Second pump:	isocratic, quaternary or binary: use flow gradient below – for the capillary pump, no flow gradient is needed, just use 15µL/min)
Mobile phase:	0.1% formic acid in 3 % acetonitrile/water
Flow:	0.01 mL/min
Flow gradient:	0.1 mL/min at 0 min, 0.05 mL/min at 0.5 min, 0.01 mL/min at 0.51 min, 0.01 mL/min at 8 min, 0.005 mL/min at 8.01 min, 0.005 mL/min at 9.9 min

Injector program:
1 Draw def. amount from sample, 10 µL/min speed, def. offset
2 NEEDLE wash in flush port, 5.0 sec.
3 INJECT

Note:	Use 20 % methanol +0.1 % formic acid in water for needle wash solvent. Set bottom-sensing on with a 0.9-mm offset for the plastic micro insert vials.

C.3. On-line 2D LC method:

Columns:	ZORBAX BioSCX Series II strong cation exchanger 35 mm x 0.3 mm. ZORBAX 300SB-C18, 150 mm x 75 µm, 3.5 µm particles. ZORBAX 300SB-C18, Trap columns 5 x 0.3 mm, 5 µm, particles, 5/pk.
Injection volume:	20 µl for salt steps

Recommended salt steps for Poly LC or Agilent BioSCX columns: 0, 15, 30, 45, 60, 75, 90, 120, 300, and 500 mM NaCl (prepared in 3% acetonitrile in 0.1% formic acid).
Enrichment column switch: position 1 at 0 min, position 2 at 5 min
Nanoflow pump:
Use either the short or long enrichment methods shown above. Choose the appropriate method based on the complexity of the sample.
Second pump (isocratic, quaternary or binary pump use flow gradient below – if capillary pump no flow gradient is needed, just use 15µL/min)

Mobile phase:	0.1% formic acid in 3% acetonitrile/water
Flow:	0.01 mL/min
Flow Gradient:	0.1 mL/min at 0 min. 0.05 mL/min at 0.5 min, 0.01 mL/min at 0.51 min, 0.01 mL/min at 8 min, 0.005 mL/min at 8.01 min, 0.005 mL/min at 9.9 min, 0 mL/min at 10 min, 0 mL/min at 74 min (159 min on long method), 0.005 mL/min at 74.01 min (159.01 on long method)

Line	Location	Sample name	Method name*	Injection location	Sample type	Data file	Injection volume
1	P1-A-01	Sample	2DLC.M	1	Sample	0 mM	5
2	Vial 1	15 mM NaCl	2DLC.M	1	Sample	15 mM	20
3	Vial 2	30 mM NaCl	2DLC.M	1	Sample	30 mM	20
4	Vial 3	45 mM NaCl	2DLC.M	1	Sample	45 mM	20
5	Vial 4	60 mM NaCl	2DLC.M	1	Sample	60 mM	20
6	Vial 5	75 mM NaCl	2DLC.M	1	Sample	75 mM	20
7	Vial 6	90 mM NaCl	2DLC.M	1	Sample	90 mM	20
8	Vial 7	120 mM NaCl	2DLC.M	1	Sample	120 mM	20
9	Vial 8	150 mM NaCl	2DLC.M	1	Sample	150 mM	20
10	Vial 9	300 mM NaCl	2DLC.M	1	Sample	300 mM	20
11	Vial 10	500 mM NaCl	2DLC.M	1	Sample	500 mM	20

* use the long or short 1D enrichment method here (C.1. or C.2.)

Table 5
Default sequence for 2D LC

C.4. Off-line 2D LC method:

1st dimension (SCX chromatography): Agilent 1100 Series micro fraction collection system
Solvents: A: 5 % AcN +0,03 % formic acid, B: 500 mM NaCl, + 5 % AcN + 0.03 % formic acid.
Gradient: 0 min 0 % B, 5 min 0 % B, 35 min 20 % B, 38 min 100 % B, 41 min 100 % B
Flow: 5 µL/min
Column: Agilent BioSCX Series II, 0.30 x 35 mm, 3.5 µm particles
Autosampler: 5 µL sample injection volume
Time-based fraction collection mode: 1st fraction 0 - 5 min, 2nd to 13th fraction each 3 min, liquid contact control mode, fraction size 15 µL, cooling 4 °C

2nd dimension (RP chromatography): Agilent Nanoflow Proteomics Solution
For the second dimension use the methods described in appendix C.1. or C.2.

D) MS conditions:

LC/MSD Trap SL
Ionization mode: Positive nanoelectrospray (G1982A – original source)
Drying gas flow: 3 L/min
Drying gas temperature: 235 °C
Vcap: typical starting point is approx. 1200 V with an 8 µm needle
Skim 1: 30 V
Capillary exit offset: 75 V
Trap drive: 90 V
Averages: 2-4 (depending on sample level)
ICC: On
Maximum accumulation time: 50 ms
Target: 30000
Automatic MS/MS:
Number of parents: 3 or 4
Averages: 5
Fragmentation amplitude: 1.15 V
SmartFrag: On, 30-200 %
Active exclusion: On, 2 spectra, 1 min
Prefer +2: on

LC /MSD Trap XCT
Ionization mode: Positive nanoelectrospray with Agilent orthogonal source (G1982B)
Drying gas flow: 5 L/min
Drying gas temperature: 300 °C
Vcap: typically 1800-2000 V (current should not be much above 100 nA)
Skim 1: 30 V
Capillary exit offset: 75 V
Trap drive: 85 V
Averages: 1 or 2
ICC: On
Maximum accumulation time: 150 ms
Smart Target: 125,000
MS scan range: 300-2200
Automatic MS/MS:
Peptide scan mode (standard-enhanced for MS and ultra scan for MS/MS)
Number of parents: 3 or 4
Averages: 2
Fragmentation amplitude: 1.3 V
SmartFrag: On, 30-200 %
Active exclusion: On, 2 spectra, 1 min
Prefer +2: on
MS/MS scan range: 100-1800
Ultra scan on
ICC target: 125,000

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