Agilent Nanospray and HPLC-Chip/MS Protein Identification Solutions

LC/MS Application Guide

Agilent Technologies

Notices

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In This Guide...

The *LC/MS Application Guide* presents the information you need to conduct the LC/MS steps for the Nanospray and HPLC-Chip/MS Protein Identification Solutions. In this guide you will learn:

- How to fractionate peptides by liquid chromatography
- How to perform MS/MS analyses of peptides
- How to analyze the resulting data with the Spectrum Mill MS Proteomics Workbench or the Mascot protein database search software

1 Overview of LC/MS Steps with the Protein ID Solutions

Get an overview of the entire process for peptide separation, MS/MS analysis, and translation of spectra to protein identifications.

2 Setup of Conventional Nanoflow LC/MS/MS System

Learn how to choose the appropriate 1D or 2D LC method, how to plumb the system, how to install the nanospray needle, and how to prepare the LC and MS just before an analysis.

3 Peptide Separation and Detection with Conventional Nanoflow LC/MS/MS

Follow the protocols to perform online 1D and 2D nanoflow LC/MS/MS analyses.

4 Setup of HPLC-Chip/MS Ion Trap System

Learn how to plumb the system, how to insert the HPLC-Chip, and how to prepare the LC and MS just before an analysis.

5 Peptide Separation and Detection with HPLC-Chip/MS

Follow the protocols to perform online 1D LC/MS/MS analyses with an enrichment column or 2D LC/MS/MS analyses.

Contents

1 Overview of LC/MS Steps with the Protein ID Solutions

Figure 1 gives an overview of the LC fractionation and MS analysis portion of the Agilent Nanospray and HPLC-Chip/MS Protein Identification Solutions. The applicable chapters from this *LC/MS Application Guide* are indicated adjacent to the workflow. You do the steps in this manual after you complete the sample preparation steps covered in the *Sample Preparation Guide*.

The fundamental difference between the Nanospray Protein Identification Solution and the HPLC-Chip/MS Protein Identification Solution is that the former uses a conventional nanoflow HPLC system, while the latter uses a microfluidic HPLC-Chip.

This guide describes how peptides are fractionated by liquid chromatography (LC) in one or more dimensions. An initial separation by strong cation exchange (SCX) chromatography may be done offline (Chapter 6), online (Chapter 3 and Chapter 5), or not at all. For the final reversed-phase separation, the LC or HPLC-Chip is interfaced to a nanospray ion trap mass spectrometer (MS) system for MS/MS analysis of the eluted peptides. The MS/MS spectra are then searched against protein databases using either Mascot or the Spectrum Mill MS Proteomics Workbench.

WARNING **Before you begin, be sure you understand all safety considerations and have read all applicable material data safety sheets.**

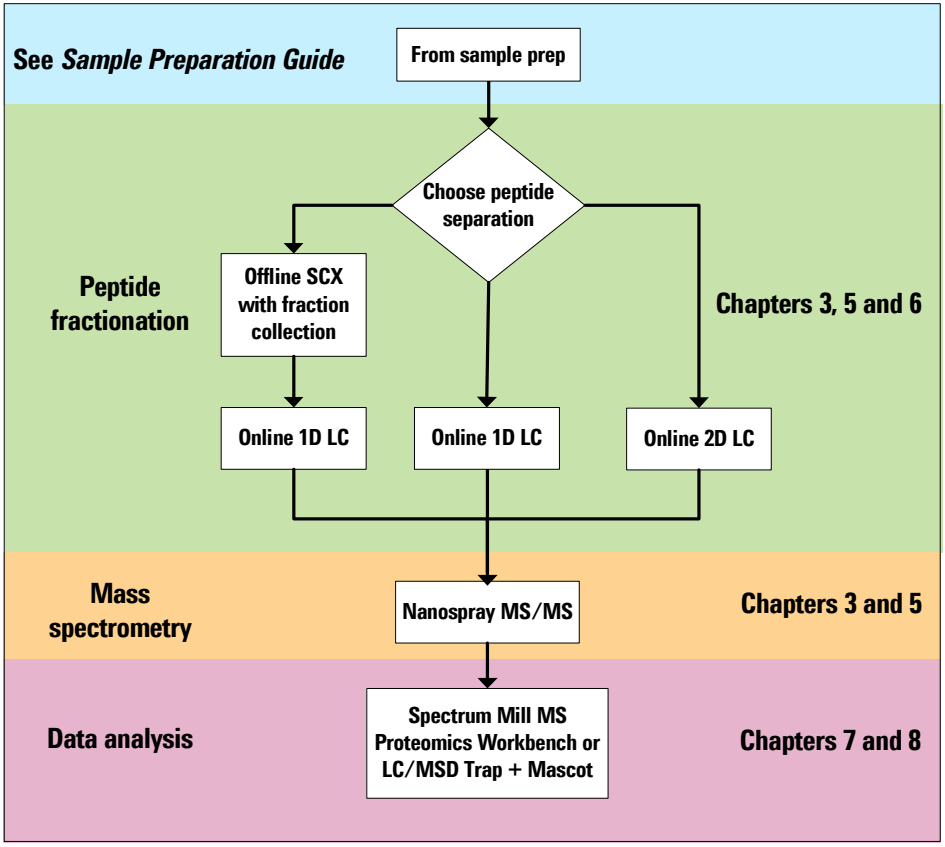


Figure 1 Workflow for LC/MS steps of the Nanospray and HPLC-Chip/MS Protein Identification Solutions

Peptide fractionation by LC

When MS/MS analyses are performed to identify peptides, the peptides are analyzed sequentially, i.e., one peptide at a time. To maximize the number of identified proteins, it is important that peptides be well-separated. Liquid chromatography (LC) is commonly used to fractionate peptides. Popular separation techniques include strong cation exchange (SCX) and reversed-phase (RP) LC.

RPLC can be performed alone or in combination with prior SCX. RPLC can be the sole separation technique for simpler mixtures of peptides, for samples where it is not as critical to identify as many proteins, or where time constraints apply.

For the Nanospray Protein Identification Solution, RPLC is done with a conventional nanoflow LC system. For the HPLC-Chip/MS Protein Identification Solution, RPLC is accomplished with a specialized HPLC-Chip, the Agilent Protein ID Chip.

The HPLC-Chip is a microfluidic device that integrates sample preparation and analysis on a single chip. The dedicated Protein ID Chip includes a sample enrichment column, an analytical column, a nanospray tip, and all connections between them. It inserts into the HPLC-Chip/MS interface, which includes the nanospray source, connections to the LC pumps and autosampler, and the microvalve for column switching.

When both SCX and RP separations are performed, they can be combined in a number of ways. Both separations can be done online (online 2D LC). In this case, the peptides are eluted from the SCX column by injection of salt plugs, are further separated by RPLC, and are then subjected to MS/MS analysis. All this is automated on a single LC/MS/MS system. Alternatively, the SCX can be done offline with a continuous salt gradient and fraction collection. Then those fractions are separated online with RPLC and analyzed by MS/MS. The offline approach requires two LC systems, one for SCX and one for RPLC. It often yields more protein identifications, but can take significantly more time, depending on the number of fractions collected and analyzed. An intermediate technique in terms of separating power and time commitment is online 2D separation with a semi-continuous salt gradient.

Mass spectrometry

Following LC separation, the peptides are analyzed by mass spectrometry (MS). With the Agilent system, the LC eluent enters the ion source of an ion trap mass spectrometer via an orthogonal nanospray source. The molecular weights of the peptides are determined by MS precursor ion scans. MS/MS scans are then triggered on selected precursor ions according to data-dependent acquisition rules that are established prior to the analysis. The MS spectra provide molecular weights of the peptides, while ion fragments in the MS/MS spectra provide amino acid sequence information.

Data analysis

The final step in the workflow is data analysis. A major challenge in proteomics research is analysis of the vast amounts of data that are generated. Often, thousands of MS/MS spectra must be converted to protein identifications. With the Nanospray and HPLC-Chip/MS Protein Identification Solutions, two different data analysis paths are possible.

One path uses the Agilent LC/MSD Trap DataAnalysis software to prepare and export the MS/MS spectra to Mascot generic format. The exported mass list files are then searched using the Mascot protein database search engine. The searches are conducted sample-by-sample over the internet, or are automated via licensed Mascot Daemon software. The results are then tabulated manually or via software developed in the laboratory.

Another path uses the Spectrum Mill MS Proteomics Workbench to extract the MS/MS spectra from the raw data files, conduct the protein database searches, validate the results via a combination of automated and manual validation, and summarize the results in biologist-friendly format. The Spectrum Mill workbench allows data from the various LC fractions to be automatically consolidated, and permits ready sample comparison.

2 Setup of Conventional Nanoflow LC/MS/MS System

The conventional nanoflow LC/MS system used for the Nanospray Protein Identification Solution includes the Agilent nanoflow LC modules and nanoflow LC columns coupled with Agilent's orthogonal nanospray source to the LC/MSD Trap XCT Plus or XCT Ultra. The LC/MSD Trap software is used for complete system control and automation.

The system includes everything you need to run successful 1D and 2D nanoflow LC/MS/MS analyses, including the enrichment column, analytical column, connection capillaries, and low-dispersion fittings.

This chapter describes how to set up this system and how to prepare it for an analysis.

If you wish to use the HPLC-Chip/MS Protein Identification Solution, skip to Chapter 4.

System components

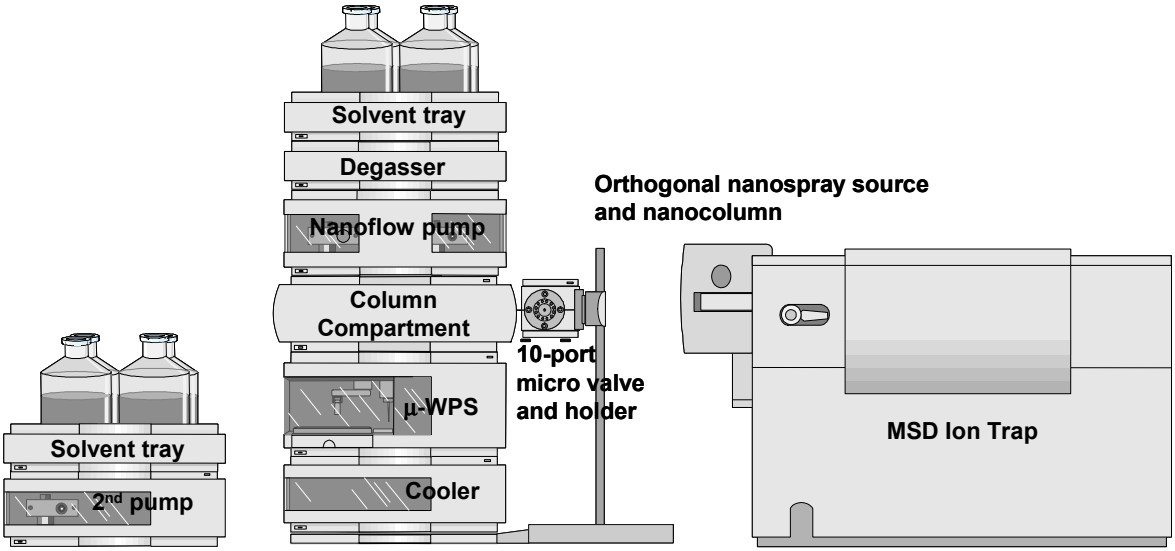


Figure 2 Nanoflow LC/MS/MS system

The nanoflow LC/MS/MS system (Figure 2) consists of:

- Agilent 1100 Series nanoflow pump with micro vacuum degasser
- Agilent 1100 Series micro well-plate autosampler with optional thermostat
- Agilent 1100 Series thermostatted column compartment with 2-position/ 6-port micro valve or the optional Agilent 10-port micro valve and holder (required only for online SCX with semi-continuous salt gradient)
- Agilent 1100 Series LC/MSD Trap XCT Plus or XCT Ultra
- Agilent orthogonal nanospray ion source
- Agilent ChemStation B.01.03 and LC/MSD Trap software 6.0
- Optional second pump: Agilent 1100 Series quaternary, binary, isocratic or capillary pump

Safety

WARNING **Always take proper precautions for handling and disposing of solvents and other chemicals. Consult the material data safety sheets supplied by the vendors.**

For additional safety precautions, see the manuals you received with the individual system components.

Additional materials required

In addition to the nanoflow LC/MS/MS system, you need the following solvents:

- Water, HPLC grade (18 megohm) - Agilent p/n 8500-2236 or equivalent
- Acetonitrile, HPLC grade - Agilent p/n G2453-85050 or equivalent
- Formic acid, analytical grade - Agilent p/n G2453-85060 or equivalent

To prepare solvents

1 To avoid bacterial growth, use fresh, clean solvent bottles. If you plan to analyze a lot of samples, use a 2-L bottle (Agilent p/n 9301-6342) for the aqueous solvent.

NOTE The solvent channels used for the nanoflow pump are A1 and B1.

2 Prepare 0.1% formic acid in water for solvent A1.

3 Prepare 90% acetonitrile + 0.1% formic acid in water for solvent B1.

CAUTION Replace the solvent for the B1 channel at least weekly. The A1 solvent is stable longer.

Remember to check your waste bottle frequently. The bottle fills more quickly because you split your flow.

To plumb the system

1. Decide which LC configuration is most appropriate for your samples. If you are unsure, Read "Choosing the LC method" below.
2. Read and follow all instructions in the *Agilent Nanoflow LC System for Mass Spectrometry (MS) G2229 Quick Start Guide*. (Skip this step if you did it when the system was installed.)
3. Arrange your LC modules as shown in "Stacking diagrams" on page 10.
4. Read "Plumbing overview" on page 11.
5. Plumb the system.
   - For the capillaries to use, refer to the diagram and table in the *Agilent Nanoflow Proteomics Solution Quick Reference Guide*. These provide an overview of all the capillaries needed for the installation.
   - Be sure to select only the plumbing components you need for your mode of operation (direct injection, enrichment column, 2D LC). For more details, refer to diagrams in "Direct injection mode (1D LC)" on page 98.

Choosing the LC method

Before you can plumb the system per instructions in this chapter, you need to decide which LC configuration is most appropriate for your samples:

- For very complex samples (e.g., whole cell lysates without prior protein or peptide separation), choose 2D LC. Within this category, select one of the following:
  - If you want the maximum number of proteins identified (and are willing to devote the longest analysis time), choose offline SCX. See "Optional Peptide Fractionation with Offline SCX" on page 65. Plumb the nanoflow LC/MS/MS for 1D LC with enrichment column.
  - If you want a moderate number of proteins identified (and are willing to devote a moderate analysis time), choose online SCX with semi-continuous salt gradient. To plumb and run this method, refer to the Agilent application note, "Improved 2D Nano LC/MS for Proteomics Applications – a Comparison on Yeast," Agilent publication number 5989-0212EN. You must have the optional 10-port valve and additional tubing and enrichment column, as described in the publication.
  - For a sample of only moderate complexity (such as a gel band with less than 20-30 proteins), choose online SCX with salt steps. Plumb the nanoflow LC/MS/MS for 2D LC.

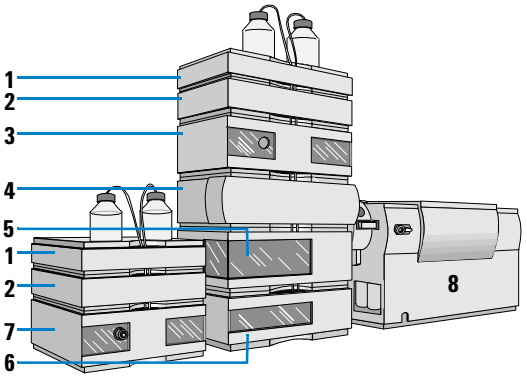
- For less complex samples, choose 1D LC.
  - If samples contain salts or require concentration (i.e., you want to do large-volume injections), choose enrichment column mode.
  - If samples are clean and sufficiently concentrated so that a 1 µL injection is adequate for sensitivity, choose direct injection mode.

For more details on 1D and 2D LC configurations, see Chapter 9, “Reference: Sample Analysis Strategy,” starting on page 97.

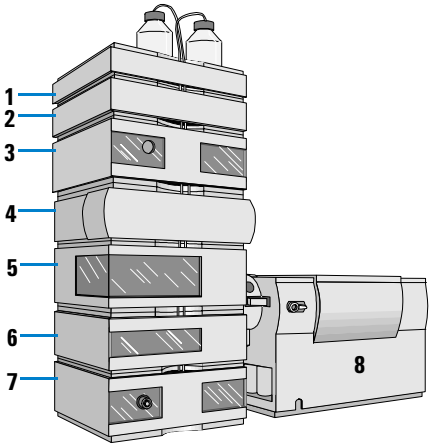
NOTE For the direct injection mode, the column compartment and associated six-port valve are unused. The analytical column is housed in the nanospray needle holder.

Stacking diagrams

version I **Recommended for enrichment column mode, direct injection mode, and 2D LC**

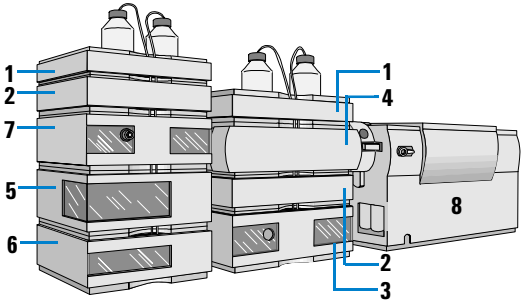


version II **Recommended for enrichment column mode, direct injection mode, and 2D LC**



1	Solvent compartment	**2**	Micro degasser
3	Nanoflow pump	**4**	Thermostatted column compartment
5	Micro well-plate sampler	**6**	Cooler (optional)
7	Second pump	**8**	Ion trap

version III **Recommended for enrichment column mode and 2D LC only**



Plumbing overview

With traditional LC plumbing, you make all the system connections before you apply flow. With the nanoflow system, you apply flow after the addition of each capillary. This allows you to systematically remove small particles before they migrate through the system and cause blockages.

1 Connect capillaries. Start installing capillaries at the pump. Follow this procedure:

   a Before connecting each capillary tube, set the LC flow to zero.

   b Use a wash bottle of isopropyl alcohol or acetone to lightly rinse the outside of the capillary and fitting. This will remove any particles before you insert the capillary into the fitting or valve.

   c Install the capillary. Tighten the fitting (at the flow sensor if this is the first capillary) and set the LC to pump 50/50 A1/B1 at 0.5 µL/min.

   d Monitor flow and pressure at the LC control panel. Make sure these stabilize before you make the next connection. If they do not stabilize, there are likely particles at the front end of the capillary. Backflush the capillary to remove them.

   e After you observe liquid at the outlet of the capillary, and the flow and pressure have stabilized, proceed to the next step.

   f Repeat step a through step e until all the capillaries have been installed.

2 Connect columns. Depending on your configuration, you will need to connect one to three columns.

   - Connect columns in the same manner as the capillaries.
   - Allow each column to purge to waste before you connect the outlet.
   - Note that you may need to activate the column switching valve to establish flow to the various columns.

NOTE If you have not yet calibrated the MS, do not connect the outlet of the analytical column to the nanospray needle. First follow the instructions under "To calibrate the MS" on page 17.

To prepare and install the nanospray needle

Insertion of C18 particles to prevent needle blockage

1 Assemble the following materials:
   - 30 µm C18 particles, Agilent p/n 79903-85031, included with the Nanospray Protein Identification Solution.
   - 1 mL methanol or acetonitrile
   - Microcentrifuge tube, 1.5 mL capacity

2 Place a very small amount (less than 1 mg) of the C18 particles into the vial. There should be only enough to see at the bottom of the vial.

3 Add 1 mL of the organic solvent, close lid, and shake rapidly for about five seconds.

4 Dip the blunt end of a nanospray needle (Agilent 9301-6378, or New Objective FS360-50-8-D) into the suspension of C18 particles for only one second. Do this step quickly because it is easy to get too many of the C18 particles into the needle, which could result in unwanted chromatography.

5 Recap the vial and store.

Needle installation

1 Insert the blunt end of the needle through the nanospray needle nut and conductive ferrule.

NOTE It is not necessary to rinse the blunt end prior to installation.

2 Connect the fitting directly to the outlet of the LC column, as shown in Figure 3.

3 Tighten the needle *very gently*. It should seem as if the needle is not tightened enough.

CAUTION Over-tightening may break off glass particles that plug the needle.

If you suspect you have glass particles, use a can of pressurized air to blow out the fitting and ferrule. If you fail to do this, the particles may become lodged in subsequent replacement needles.

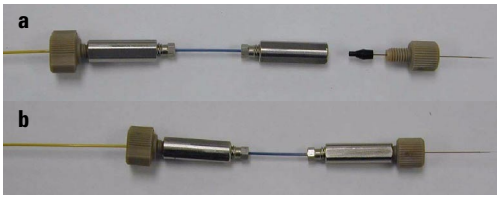


Figure 3 Nanospray needle installation

4 Verify that the LC solvent continues to flow out of the needle. A very small drop will form within 10 to 20 seconds. Hold the needle assembly under the nanospray lamp for best illumination.

CAUTION Do not wipe this drop away. Do not touch the tip of the needle to anything because it is very easily damaged.

Installation of assembly into nanospray source

1 Make sure the capillary voltage is set to 1400 V.

2 Insert the needle/column assembly into the needle holder assembly mount on the nanospray source. See Figure 4.

Figure 4 Needle holder in needle holder assembly

Needle positioning and starting of spray

1 With the needle assembly installed, use the microscope to position the needle within 3 mm of the flat electrode (modified capillary cap).

- The microscope field-of-view is 3.5 mm so if the needle tip is just seen at one end, the capillary cap will be just seen at the opposite end.
- For more information on proper alignment of the needle tip, see the *Orthogonal Nanospray Ion Source User's Guide*.

2 Start the spray with caution:

   a Verify that the flow rate is 300 nL/min and the solvent composition is 97:3 A1:B1 (the starting composition for the methods).

   b Be sure that a droplet has formed at the needle tip or that a stream has started.

   c Verify that the dry gas setting is 300 °C.

   d Gradually increase the capillary voltage to achieve good spray. View the spray through the ocular and, if necessary, slightly rotate the needle/column assembly so the spray bends away from the entrance to the MS. See Figure 5. This will reduce droplet noise.

   e Adjust the capillary voltage until a stable current is reached (typically 80 to 100 nA). The maximum voltage for the G1982B orthogonal nanospray source is 2200 V, and a typical voltage with a new needle is 1600 to 1700 V.

3 Save the method to store the new **Vcap** setting.

NOTE

Increase the **Vcap** only to the point where stable spray (without spiking) is achieved, as the lifetime of the needle is usually longer at lower **Vcap**. Look for both a stable and reasonable MS spectrum as well as several minutes of stable chromatographic signal (no spiking).

The capillary current should be around 80 to 100 nA at the starting composition of 97:3 A1/B1, and will drop as the percentage of B1 increases.

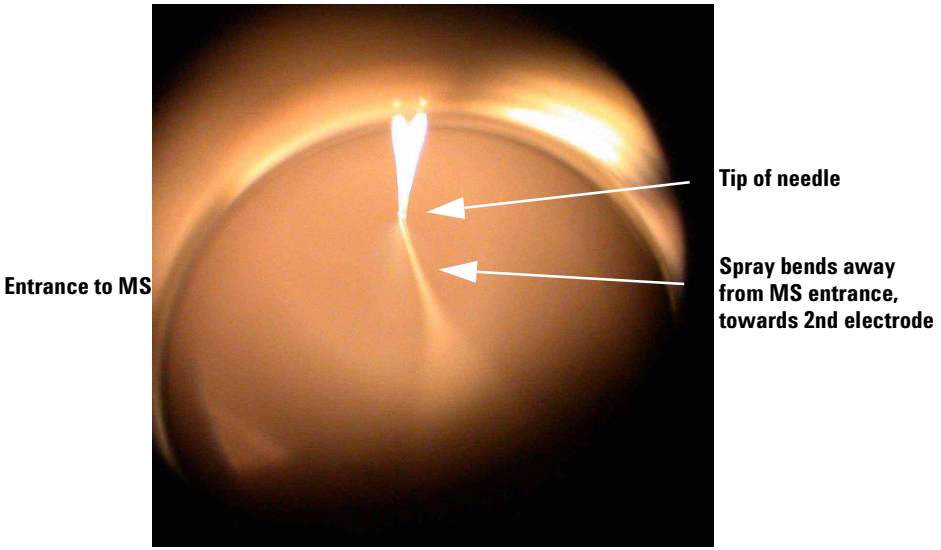


Figure 5 Bending of spray away from entrance to MS

To prepare the LC and MS just before an analysis

After you have plumbed your nanoflow LC/MS system, you prepare it for stable analyses. You purge the LC, calibrate the MS (if not already done), and connect the two. Repeat the LC purge any time the system has been shut down for more than a day.

To prepare the nanoflow pump

1 Activate the purge mode by selecting **Instrument > More Pump1 > Purge**. Purge the nanoflow pump A1 and B1 solvent channels at 2.5 mL/min for two minutes each. If the system has been shut down for more than a few days, extend the purge time to five to ten minutes.

2 Switch to 97:3 A1:B1 (the starting composition for the methods) and purge for one additional minute.

NOTE In the purge mode, the flow goes to waste rather than through the analytical system. You will not damage your system by using the purge mode at 2.5 mL/min.

3 Switch to the starting composition for your analysis. Pump solvent through the analytical flow path at 0.3 µL/min.

4 Continue pumping at 0.3 µL/min. and monitor both the flow and pressure. For stable nanospray conditions, it is very important that the flow be stable. Ripple should be less than 3%. The flow and pressure plots should look similar to those in Figure 6 and Figure 7.

5 While you wait for the system to stabilize, prepare the rest of the LC and the MS.

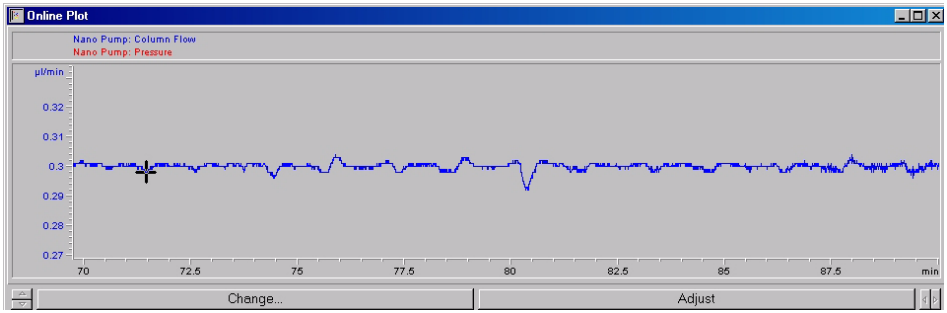


Figure 6 Desired stability of flow

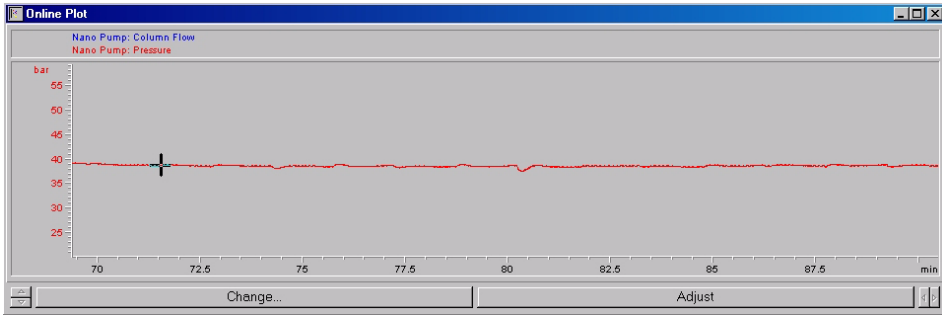


Figure 7 Desired stability of pressure

To prepare the second pump

If you are using a second pump for the enrichment column mode or 2D LC mode, prepare it as follows:

1. Purge the second (capillary- or standard-flow) pump. Open the manual purge valve and purge the solvent channel(s) you will use at 2.5 mL/min for four minutes each.
2. Switch to your composition and flow rate for sample loading.
3. Close the manual purge valve.

To prepare the well-plate sampler

If the well-plate sampler has not been used for two days, prime the needle wash for 120 seconds. Go to **Instrument > More Injector > Prime/Flush Pump**.

To prepare the LC columns

The first time you use a new SCX, enrichment, or nanoflow LC column, condition it as follows:

1. Make several injections of a high-level digest sample (e.g., 500 fmol BSA digest).
2. Inject several solvent blanks.

To calibrate the MS

NOTE A separate needle holder is provided so you can keep the tuning mix infusion assembly intact and easily interchange it with the column needle holder assembly.

Calibrate the ion trap mass spectrometer as directed in the *Orthogonal Nanospray Ion Source User's Guide*.

- Use the ESI tuning mix (G2431A).
- Use the nanospray source.
- Be sure to check the ion trap mass axis calibrations (scan, isolation and fragmentation).
- Calibrate the detector gain. In **MSD Trap Control**, click the **Calibration** tab, then the **Detector** button.

CAUTION The detector ages faster and must be calibrated more frequently when it is new.

You should calibrate the MS if:

- This is the first time you are using it.
- You observe mass shifts or other problems indicating the need to calibrate.
- Your background seems unusually low, which indicates aging of the detector.

3
Peptide Separation and Detection with Conventional Nanoflow LC/MS/MS

This chapter presents protocols for conventional 1D and 2D nanoflow LC/MS analyses. Choice of protocol depends on sample complexity, sample cleanliness (presence or absence of salts), peptide concentration, and how important it is to identify as many proteins as possible for your study. In general, the 2D methods provide more identifications, but at the cost of additional analysis time. For guidelines on protocol selection, see "Choosing the LC method" on page 8. Before you run any of the protocols, prepare the system as described in "To prepare the LC and MS just before an analysis" on page 15.

If you plan to use the HPLC-Chip/MS Ion Trap system, skip to Chapter 5.

Safety

WARNING **Always take proper precautions for handling and disposing of solvents and other chemicals. Consult the material data safety sheets supplied by the vendors.**

For additional safety precautions, see the manuals you received with the individual system components.

Procedure summary

The following summarizes the various modes of nanoflow LC/MS/MS analysis. If you have not yet chosen your operating mode, see "Choosing the LC method" on page 8.

1D LC - direct injection mode

A schematic 1D LC method with direct injection is shown in Figure 8, and a more detailed flow diagram is given in "Direct injection mode (1D LC)" on page 98. For amenable samples, this mode gives the best chromatography, the best sequence coverage, and the best sensitivity.

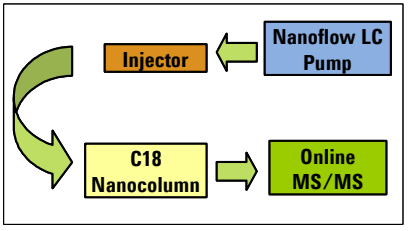


Figure 8 ID LC with direct injection

1. Inject no more than 1 µL sample.
2. Separate peptides on the nanoflow column.
3. Perform MS and MS/MS analysis of separated peptides to obtain molecular weight and sequence information.

1D LC - enrichment column mode

This 1D LC method uses an enrichment pre-column to remove salts and other water-soluble contaminants from samples, and to permit large-volume injections (several µL or greater) that would take a long time to load using nanoflow rates. A schematic is shown in Figure 9. For more detailed flow diagrams, see "Enrichment column mode (1D LC)" on page 99.

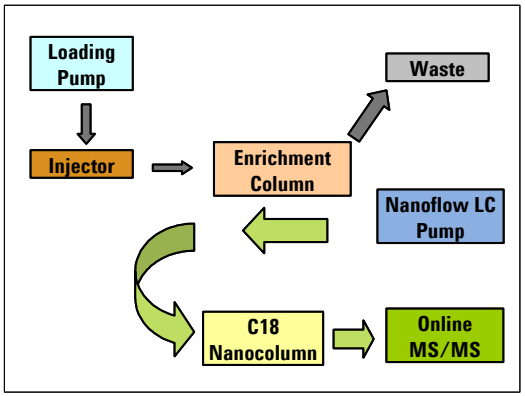


Figure 9 1D LC with enrichment column

1. Using a higher flow rate, load sample onto an enrichment column, where the peptides are trapped and the samples are desalted.
2. Switch enrichment column into the solvent path of the nanoflow pump and separate sample on the nanoflow column.
3. Perform MS and MS/MS analysis of separated peptides to obtain molecular weight and sequence information.

2D LC with salt steps

The 2D LC method, shown in Figure 10, uses both SCX and reversed phase separations. You load the sample onto an SCX column and then elute it in fractions by injecting sequential salt steps. Each injected salt solution elutes a fraction of the peptides from the SCX column for analysis by nanoflow LC/MS/MS. The result is an individual data file for each step of the salt gradient. For more detailed flow diagrams, see "Two-dimensional liquid chromatography (2D LC)" on page 101.

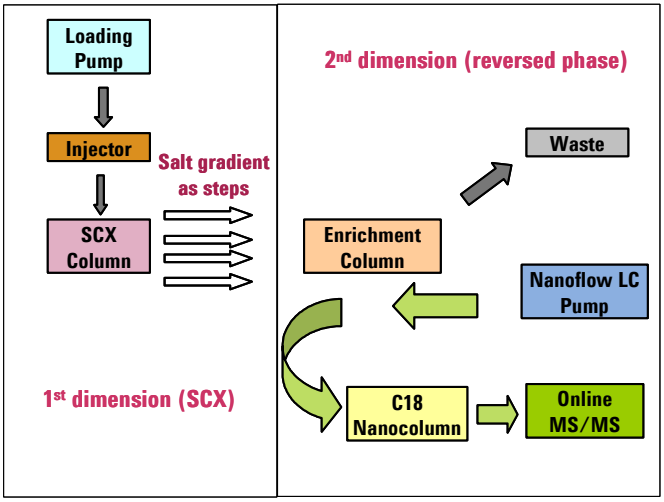


Figure 10 Online 2D LC with salt steps

1. Load sample onto a strong cation exchange column. Non-bound peptides flow through to the enrichment column, where the peptides are trapped and the samples are desalted.
2. Switch enrichment column into the solvent path of the nanoflow pump and separate sample on the nanoflow column.
3. Perform MS and MS/MS analysis of separated peptides to obtain molecular weight and sequence information.
4. After the first analysis is complete, switch enrichment column back into the path of the cation exchange column.
5. Inject salt solution to elute peptides onto enrichment column. In this step, you inject the lowest concentration first and increase salt concentration each time you perform the step.
6. Repeat steps 2 to 5 until you finish the highest salt concentration and all peptides have been analyzed.

2D LC with semi-continuous salt gradient

This 2D LC method, shown in Figure 11, is similar to 2D LC with salt steps, but provides better peptide separation via a salt gradient and use of two enrichment columns for alternate trapping and sample elution. For more details, refer to the Agilent application note, "Improved 2D Nano LC/MS for Proteomics Applications – a Comparison on Yeast," Agilent publication number 5989-0212EN.

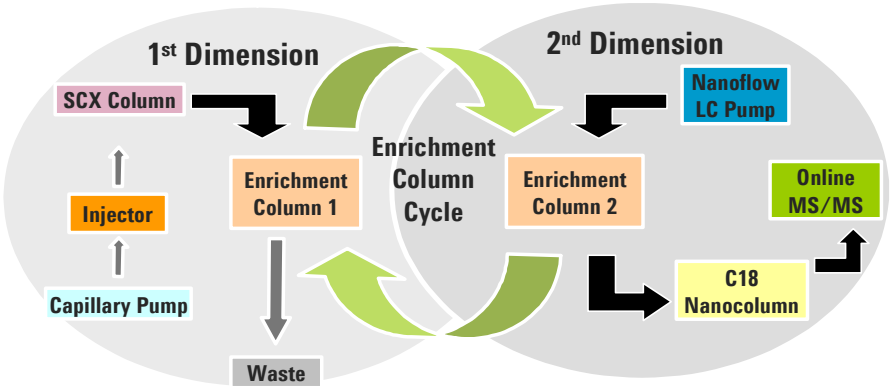


Figure 11 Online 2D LC with semi-continuous salt gradient

Additional materials required

In addition to the nanoflow LC/MS/MS system, you need the following solvents:

- Water, HPLC grade (18 megohm) - Agilent p/n 8500-2236 or equivalent
- Acetonitrile, HPLC grade - Agilent p/n G2453-85050 or equivalent
- Formic acid, analytical grade - Agilent p/n G2453-85060 or equivalent
- Ammonium formate, 5 M - Agilent p/n G1946-85021 (provided with the Nanospray Protein Identification Solution)

Protocol for 1D direct injection LC method

Column

ZORBAX 300SB-C18, 75 µm x 150 mm, 3.5 µm particle size or
ZORBAX 300SB-C18, 75 µm x 50 mm, 3.5 µm particle size
(The longer column gives better separations when there are more peaks.)

Maximum injection volume

1 µL (maximum volume with this method)

Nanoflow pump

A = 0.1% formic acid in water
B = 90% acetonitrile + 0.1% formic acid in water
Flow rate = 300 nL/min

Table 1 Nanoflow pump solvent gradient

Time (min)	% B
0	3
10	3
30	45
33	65
33.01	3

Stop time

40 min

Post time

5 min

Injector program

Table 2 Injector program

Step	Action
1	Draw default amount from sample, 10 µL/min speed, default offset
2	NEEDLE wash in flush port, 5.0 sec
3	INJECT
4	WAIT 10.00 min
5	VALVE bypass

Needle flush solvent 20% methanol + 0.1% formic acid in water

Bottom-sensing On, 0.9 mm offset for plastic conical micro-vials (Agilent p/n 9301-0978), or 0.2 mm for 96-well Eppendorf well plates (Agilent p/n 5042-8502).

Pre- and post-run macros For direct injection mode when the 40-µL injection loop is installed, use pre-run and post-run macros to reduce the pressure/flow fluctuation. The setup is shown in Figure 12.

The macros are available at www.agilent.com/chem/techsupport. Look under **Status Bulletins and Patches**. Copy the macros to **hpchem\core**.

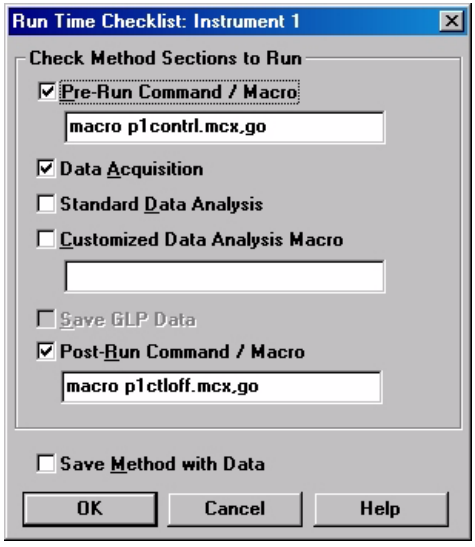


Figure 12 Pre-run and post-run macros for direct injection with 40-µL loop

Protocol for 1D enrichment LC - short method for less complex samples

Columns

- Enrichment column: ZORBAX 300SB-C18, 0.3 x 5 mm, 5 µm particle size
- Reversed-phase column: ZORBAX 300SB-C18, 75 µm x 150 mm, 3.5 µm or ZORBAX 300SB-C18, 75 µm x 50 mm, 3.5 µm particle size
  (The longer column gives better separations when there are more peaks.)

Maximum injection volume

8 µL or 40 µL, depending on loop installed

Enrichment column switch

Position 1 at 0 min, position 2 at 5 min, then return to position 1 at 61 min

Nanoflow pump

A = 0.1% formic acid in water
B = 90% acetonitrile + 0.1% formic acid in water
Flow rate = 300 nL/min
Primary flow = 200 to 500 µL/min (From the **Instrument** menu, choose **More pumps > Auxiliary**.)

Table 3 Nanoflow pump solvent gradient

Time (min)	% B
0	3
5	3
8	15
50	45
55	90
60	90
61	3

Stop time

75 min

Post time

5 min

Second pump

3% acetonitrile + 0.1% formic acid in water

Isocratic, quaternary or binary pumps use flow gradient given in Table 4 on page 27. Capillary pumps use no flow gradient; just set flow to 15 µL/min.

Table 4 Flow gradient for second (non-capillary) pump

Time (min)	Flow (mL/min)
initial	0.005
0.01	0.1
0.50	0.1
1.0	0.05
1.01	0.01
8.0	0.01
8.01	0.005
9.9	0.005
10.0	0
74	0
74.01	0.005

Injector program

Table 5 Injector program

Step	Action
1	Draw default amount from sample, 10 µL/min speed, default offset
2	NEEDLE wash in flush port, 5.0 sec
3	INJECT

Needle flush solvent

20% methanol + 0.1% formic acid in water

Bottom-sensing

On, 0.9 mm offset for plastic conical micro-vials (Agilent p/n 9301-0978), or 0.2 mm for 96-well Eppendorf well plates (Agilent p/n 5042-8502).

Protocol for 1D enrichment LC - long method for more complex samples

Columns

- Enrichment column: ZORBAX 300SB-C18, 0.3 x 5 mm, 5 µm particle size
- Reversed-phase column: ZORBAX 300SB-C18, 75 µm x 150 mm, 3.5 µm or ZORBAX 300SB-C18, 75 µm x 50 mm, 3.5 µm particle size
  (The longer column gives better separations when there are more peaks.)

Maximum injection volume

8 µL or 40 µL, depending on loop installed

Enrichment column switch

Position 1 at 0 min, position 2 at 5 min, then return to position 1 at 146 min

Nanoflow pump

A = 0.1% formic acid in water
B = 90% acetonitrile + 0.1% formic acid in water
Primary flow = 200 to 500 µL/min (From the **Instrument** menu, choose **More pumps > Auxiliary**.)

Table 6 Nanoflow pump solvent and flow gradient

Time (min)	% B	Flow (µL/min)
0	3	0.3
5	3	0.3
11	15	0.3
131	45	0.3
138	90	0.4
143	90	0.6
146	3	0.4
160	3	0.3

Stop time

160 min

Post time

5 min

Second pump

3% acetonitrile + 0.1% formic acid in water

Isocratic, quaternary or binary pumps use flow gradient given in Table 7 on page 29. Capillary pumps use no flow gradient; just set flow to 15 µL/min.

Table 7 Flow gradient for second (non-capillary) pump

Time (min)	Flow (mL/min)
initial	0.005
0.01	0.1
0.50	0.1
1.0	0.05
1.01	0.01
8.0	0.01
8.01	0.005
9.9	0.005
10.0	0
159	0
159.01	0.005

Injector program

Table 8 Injector program

Step	Action
1	Draw default amount from sample, 10 µL/min speed, default offset
2	NEEDLE wash in flush port, 5.0 sec
3	INJECT

Needle flush solvent

20% methanol + 0.1% formic acid in water

Bottom-sensing

On, 0.9 mm offset for plastic conical micro-vials (Agilent p/n 9301-0978) or 0.2 mm for 96-well Eppendorf well plates (Agilent p/n 5042-8502).

Protocol for online 2D LC method with salt steps

LC details

Columns

- SCX column: ZORBAX BioSCX Series II, 0.30 x 35 mm, 3.5 µm particle size.
- Enrichment column: ZORBAX 300SB-C18, 0.3 x 5 mm, 5 µm particle size
- Reversed-phase column: ZORBAX 300SB-C18, 75 µm x 150 mm, 3.5 µm or ZORBAX 300SB-C18, 75 µm x 50 mm, 3.5 µm particle size
  (The longer column gives better separations when there are more peaks.)

Injection volume

20 µL for salt steps (must have 40 µL loop installed)

Recommended salt steps

0, 15, 30, 45, 60, 75, 90, 120, 300, 500 mM ammonium formate (prepared in 3% acetonitrile + 0.1% formic acid in water). Choose the number of salt steps based on sample complexity.

Prepare salt solutions from 5 M ammonium formate stock (Agilent p/n G1946-85021). Prepare the solutions directly into the 2-mL glass autosampler vials supplied with the Nanospray Protein Identification Solution.

Table 9 Preparation of salt solutions for 2D LC with salt steps

Concentration (mM)	Volume of 5 M stock (µL)	Volume of water (µL)
15	3	997
30	6	994
45	9	991
60	12	988
75	15	985
90	18	982
120	24	976
300	60	940
500	100	900
1000	100	400

Enrichment column switch Position 1 at 0 min, position 2 at 5 min, then return to position 1 at 61 min if you use the short LC method or 146 min if you use the long LC method

Nanoflow pump Use the nanoflow pump settings from either the short or long enrichment methods. (See page 26 or page 28.) Choose the appropriate method based on the complexity of the sample.

Second pump 3% acetonitrile + 0.1% formic acid in water

Isocratic, quaternary or binary pumps use flow gradient given in Table 10.

Capillary pumps use no flow gradient; just set flow to 15 µL/min.

Table 10 Flow gradient for second (non-capillary) pump

Time (min)	Flow (mL/min)
initial	0.005
0.01	0.1
0.50	0.1
1.0	0.05
1.01	0.01
10	0.01
10.01	0.005
15	0.005
15.1	0
74 (short method) or 159 (long method)	0
74.01 (short method) or 159.01 (long method)	0.005

Injector program

Table 11 Injector program

Step	Action
1	Draw default amount from sample, 10 µL/min speed, default offset
2	NEEDLE wash in flush port, 5.0 sec
3	INJECT

Sequence table

A typical sequence table for salt steps is given in Table 12.

Table 12 Sequence table to inject salt steps

Line	Location	Sample Name	Method Name	Inj/ Location	Sample Type	DataFile	Inj Volume
1	P1-A-01	Sample	2DLCX.M	1	Sample	0mM	5
2	Vial 1	15mM salt	2DLCX.M	1	Sample	15mM	20
3	Vial 2	30mM salt	2DLCX.M	1	Sample	30mM	20
4	Vial 3	45mM salt	2DLCX.M	1	Sample	45mM	20
5	Vial 4	60mM salt	2DLCX.M	1	Sample	60mM	20
6	Vial 5	75mM salt	2DLCX.M	1	Sample	75mM	20
7	Vial 6	90mM salt	2DLCX.M	1	Sample	90mM	20
8	Vial 7	120mM salt	2DLCX.M	1	Sample	120mM	20
9	Vial 8	300mM salt	2DLCX.M	1	Sample	300mM	20
10	Vial 9	500mM salt	2DLCX.M	1	Sample	500mM	20
11	Vial 10	1000mM salt	2DLCX.M	1	Sample	1000mM	20

Protocol for 2D LC method with semi-continuous salt gradient

For this method, refer to the Agilent application note, “Improved 2D Nano LC/MS for Proteomics Applications – a Comparison on Yeast,” Agilent publication number 5989-0212EN.

MS/MS method for LC/MSD Trap XCT Plus or XCT Ultra

Source and ion optics conditions

Ionization mode	Positive nanospray with Agilent orthogonal source (G1982B)
Drying gas flow	5 L/min
Dry gas temperature	300 °C
Vcap	Typically 1600-2000 V (Check for stable current of 80-100 nA at starting solvent composition and flow.)
Skim1	30 V
Capillary exit	100 V

Ion trap conditions - MS scan

Trap drive	85
Averages	1
ICC	On
Maximum accumulation time	150 ms
Smart target	500,000
MS scan range	300-2200

Ion trap conditions - automatic MS/MS

Scan mode	Peptide
Number of precursor ions	3 or 4 for XCT Plus; 5 or 6 for XCT Ultra
Averages	1
Fragmentation amplitude	1.3 V
SmartFrag	On, 30-200%

Active exclusion	On, 2 spectra, 1 min
Prefer +2	On
MS/MS scan range	100-2000
Ultra scan	On
ICC target	500,000

To shut down the nanoflow LC/MS/MS system

To shut down the system, do the following at the end of your sample sequence:

1. Inject two solvent blanks to remove residual peptides from the injector. Acquire data for these injections.
   - For simple samples, inject the solvent you used to dissolve your peptides.
   - For complex samples, inject 50% TFE in 0.1% formic acid in water to remove hydrophobic peptides.
2. In the **Sequence Parameters**, set the post-sequence command/macro to **macro "SHUTDOWN.MAC", go**.

NOTE Note that the **Standby** command stops the LC pumps, but does *not* put the ion trap into standby mode. The **Shutdown** macro stops the LC pumps and puts the ion trap into standby mode.

3. Before you run your next sequence, open the data files for the blanks and check for peptide peaks. If there were substantial peaks in the last injection, inject more blanks.

CAUTION If you do not plan to use the system for several days, remove the nanospray needle/column holder assembly and store in protective plastic sleeve. This will extend the life of the needle.

Troubleshooting

If you have flow path blockages

Cause	Solution
1 Fused silica particles in system	• Solvent-rinse capillaries and fittings prior to installation. • Avoid overtightening fittings. • Avoid excessive bending or coiling of the capillaries. If you coil to a radius of less than 40 mm, you will damage the capillaries. • Avoid kinking, bending or crushing capillaries with LC doors or cover panels. • Backflush capillaries if they are already blocked.
2 Particles from solvent or sample	• Always filter solvents through 0.4 µm filters. • Clean up samples per *Sample Preparation Guide*.
3 Nanospray needle blocked due to heat without flow	• If you do not plan to use the system for several days, remove the nanospray needle/column holder assembly and store in protective plastic sleeve. • If the needle is blocked, flush with 100% B1 for 30 min. This sometimes removes the blockage.

If you have unstable flow

Cause	Solution
1 Flow blockages (can be especially troublesome in direct injection mode with sampler in mainpass)	• See "If you have flow path blockages" (above).
2 System pressure too low	• Keep system pressure higher than 20 bar at the pump outlet.
3 EMPV needs maintenance	• Run the EMPV cleaning procedure. See the nanoflow pump manual.
4 Air bubbles in liquid flow path	**a** Purge pumps if you have not already done so. **b** If bubbles persist, pump 500 nL/min of 50% B for 20 min. **c** If bubbles still persist, disconnect first capillary at pump end of system and purge at higher flow rate. Reconnect first capillary and disconnect next one. Purge again. Continue disconnecting capillaries sequentially and purging until bubbles are gone.

If you have unstable spray

Cause	Solution

1 Capillary voltage not set correctly	• Adjust the capillary voltage.
	  ◦ Using the 8 µm needle tips and liquid flow between 175 and 300 nL/min., the capillary voltage should be between 1600 and 2000 volts.
	  ◦ New needles usually require less voltage, but need slightly more as the needle ages and the tip becomes eroded or enlarged.

2 Nanospray needle not positioned correctly	• Adjust needle position as described in the *Agilent G1982B Orthogonal Nanospray Ion Source User's Guide*.
3 Flow path blockages	• See "If you have flow path blockages" on page 35 of this chapter.
4 Nanospray needle tip is damaged or partly blocked. (You observe sputtering or split spray)	• Replace the needle, reinstall the needle/column holder assembly in the source, and adjust needle position.
5 Ferrule not making good seal with needle	• Replace the ferrule, reinstall the needle/column holder assembly in the source, and adjust needle position.
6 Flow too great or needle tip enlarged (You observe steady bowed stream of liquid.)	• Reduce the LC flow. • Replace the needle as above.

If you have poor chromatography

Cause	Solution
1 Gaps at LC connections	• When you connect a capillary to a fitting or the column, push the capillary into the fitting firmly and smoothly to avoid gaps. • When connections are leaking, set column flow to zero, loosen the fitting, reinsert the fused silica and retighten the fitting. If you tighten the fitting without re-seating the fused silica tube, you may allow a gap to remain between the fused silica and the fitting. This will result in peak dispersion.

2 Note: Chromatography in enrichment column mode is generally not as good as in direct injection mode.	• Switch to direct injection mode if that is an option. Consider:
	  ◦ Your injection volume
	  ◦ Level of salts and other water-soluble contaminants.

If you have sample carryover

Cause	Solution
1 No needle wash	• Set up needle wash for well-plate sampler.
2 Inappropriate needle wash solvent	• Switch to a solvent combination in which your sample is completely soluble.

3 Residual hydrophobic peptides in the injection system	• Inject the following to remove hydrophobic peptides:
	  ◦ First inject 50% TFE in water.
	  ◦ Then inject several solvent blanks with a high percentage of organic.

If you have poor sensitivity

Cause	Solution
1 Detector gain adjustment needs to be redone	• See instructions for verifying detector setting in your LC/MSD Trap documentation.
2 Capillary voltage too high, causing corona which can destroy peptides	• Reduce the capillary voltage.
3 Sample degradation from sitting at room temperature	• Prepare fresh samples. • If you have the optional thermostat on the micro well-plate sampler, make sure it is turned on and set to 4 °C.
4 Peptides adsorbed on vial surface	• Switch to a different vial material (glass or plastic).
5 Bad injection due to air bubble at bottom of vial	• Tap vial gently to dislodge air bubble.
6 Unstable spray	• See "If you have unstable spray" on page 36 of this chapter.
7 Other nanospray problems	• See the troubleshooting chapter in *Agilent G1982B Orthogonal Nanospray Ion Source User's Guide*.

8 **Find Compounds** settings not optimized for low-level samples, giving the appearance of poor sensitivity
Note: Because the **Find Compounds** function is so efficient, you can often obtain a good Mascot search even when peaks are not evident in the base peak chromatogram.
	• For low-level samples, reduce these thresholds in the data analysis method parameters:
	  ◦ Find-AutoMS(n) compound detection threshold
	  ◦ Mass List-Apex absolute intensity threshold

If you have low database search scores or poor sequence coverage

Cause	Solution

1 Sample preparation or data acquisition problem	• Open the data file and see how many peaks you have in the MS BPC.
	  ◦ If you have very few peaks, you have a problem with the sample preparation or with the data acquisition settings. See causes 2 through 5 below, as well as the troubleshooting in the *Sample Preparation Guide*.
	  ◦ If you have a reasonable number of peaks, your problem resides in the data processing. See causes 6 through 10 below.

2 Number of precursors for data-dependent MS^n set too low	• Go to the **MS(n)** tab on the Trap control screen and set **No. of Precursor Ions** to 3 or 4 for the XCT Plus or 5 to 6 for the XCT Ultra.
3 Threshold for triggering MS^n set too high	• Examine your data and set a lower threshold if it appears inadequate MS^n experiments were generated. • To set a lower threshold, go to the **MS(n)** tab on the Trap control screen and set **Threshold Abs** and/or **Threshold Rel** to a lower number.
4 SmartFrag turned off, resulting in inadequate fragmentation	• Click the **Fragmentation** button on the **MS(n)** page of the Trap control screen. • Make sure **SmartFrag** is checked and set to 30-200%.
5 Poor sensitivity	• See "If you have poor sensitivity" on page 37 of this chapter.
6 Default settings for LC/MSD Trap data analysis software not appropriate for sample (applies only if you use Mascot search)	• See "Troubleshooting" on page 95 of Chapter 8, "Data Analysis with Mascot Protein Database Search".
7 Mascot parameters not optimized (applies only if you use Mascot search)	• See "To use Mascot protein database search" on page 90 of Chapter 8, "Data Analysis with Mascot Protein Database Search".
8 Spectrum Mill Data Extractor parameters not set properly (applies only if you use Spectrum Mill workbench)	• See the documentation you received with the Spectrum Mill MS Proteomics Workbench.
9 Spectrum Mill Search parameters not set properly (applies only if you use Spectrum Mill workbench)	• See the documentation you received with the Spectrum Mill MS Proteomics Workbench.
10 Chosen database did not give good results.	• Try searching another database.

Tips

See the tips in the *Agilent Nanoflow LC System for Mass Spectrometry (MS) G2229 Quick Start Guide* and the *Agilent Nanoflow Proteomics Solution Quick Reference Guide*.

4 Setup of HPLC-Chip/MS Ion Trap System

The HPLC-Chip/MS Ion Trap system used for the HPLC-Chip/MS Protein Identification Solution consists of Agilent nanoflow LC modules, the HPLC-Chip/MS interface, the Protein ID Chip, and the LC/MSD Trap XCT Ultra. The LC/MSD Trap software is used for complete system control and automation.

The Agilent HPLC-Chip is a polyimide-based chip with microfluidic channels. The Protein ID Chip you use for the HPLC-Chip/MS Protein Identification Solution is fabricated in a one-dimensional LC configuration. It incorporates an enrichment column to remove salts and concentrate the sample, an analytical column, a nanoelectrospray tip, and the microfluidic channels that connect them. The chip design significantly reduces the number of capillaries and fittings you need for nanoflow HPLC/MS. It is reusable and is easier to set up and use.

The Protein ID Chip inserts into the Agilent HPLC-Chip/MS interface, which couples the LC system and the LC/MSD Trap. The interface includes the valve to switch flows between the columns. This chapter describes how to set up this system and how to prepare it for an analysis.

System components

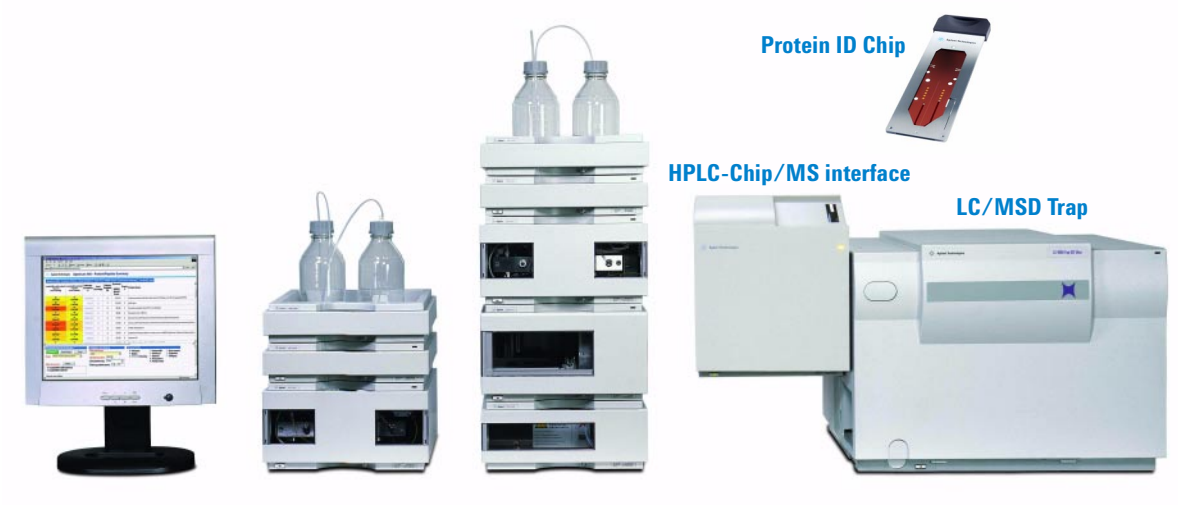


Figure 13 HPLC-Chip/MS Ion Trap system

The HPLC-Chip/MS system (Figure 13) consists of:

- Agilent 1100 Series nanoflow pump with micro vacuum degasser
- Agilent 1100 Series micro well-plate autosampler with optional thermostat
- Second pump for sample loading: Agilent 1100 Series capillary pump
- Agilent Protein ID Chip
- Agilent HPLC-Chip/MS interface, which includes:
  - HPLC-Chip loading and ejection mechanism
  - Microvalve for flow switching
  - LC connections to the nanoflow LC pump and to the micro well-plate autosampler with loading pump.
  - Orthogonal nanospray ion source with optics for spray visualization
- Agilent ChemStation B.01.03 and LC/MSD Trap software 6.0
- Agilent 1100 Series LC/MSD Trap XCT Ultra

Safety

WARNING **Always take proper precautions for handling and disposing of solvents and other chemicals. Consult the material data safety sheets supplied by the vendors.**

For additional safety precautions, see the manuals you received with the individual system components.

Additional materials required

In addition to the HPLC-Chip/MS Ion Trap system, you need the following solvents:

- Water, HPLC grade (18 megohm) - Agilent p/n 8500-2236 or equivalent
- Acetonitrile, HPLC grade - Agilent p/n G2453-85050 or equivalent
- Formic acid, analytical grade - Agilent p/n G2453-85060 or equivalent

To prepare solvents

1 To avoid bacterial growth, use fresh, clean solvent bottles. If you plan to analyze a lot of samples, use a 2-L bottle (Agilent p/n 9301-6342) for the aqueous solvent.

NOTE The solvent channels used for the nanoflow pump are A1 and B1.

2 Prepare 0.1% formic acid in water for solvent A1.

3 Prepare 90% acetonitrile + 0.1% formic acid in water for solvent B1.

CAUTION Replace the solvent for the B1 channel at least weekly. The A1 solvent is stable longer.

Remember to check your waste bottle frequently. The bottle fills more quickly because you split your flow.

To plumb the system

1. Decide which LC configuration is most appropriate for your samples. If you are unsure, read "Choosing the LC method" below.
2. Read and follow all instructions in the *Agilent Nanoflow LC System for Mass Spectrometry (MS) G2229 Quick Start Guide*. (Skip this step if you did it when the system was installed.)
3. Arrange your LC modules as described in the *Agilent 1100 Series G4240A HPLC-Chip/MS Cube User's Guide*.
4. Read step 1 of "Plumbing overview" on page 11. Also read step 2 of that section if you plan to do online 2D LC because you will need to install an SCX column. The enrichment and analytical columns are incorporated into the Protein ID Chip.
5. Plumb the system. For the capillaries to use, refer to the *Agilent 1100 Series G4240A HPLC-Chip/MS Cube User's Guide*.

Choosing the LC method

Before you can plumb the system per instructions in this chapter, you need to decide which LC configuration is most appropriate for your samples:

- For very complex samples (e.g., whole cell lysates without prior protein or peptide separation), choose 2D LC. Within this category, select one of the following:
  - If you want the maximum number of proteins identified (and are willing to devote the longest analysis time), choose offline SCX. See "Optional Peptide Fractionation with Offline SCX" on page 65.
  - For a sample of only moderate complexity (such as a gel band with less than 20-30 proteins), choose online SCX with salt steps. You will need to plumb the SCX column.
- For less complex samples, choose 1D LC.

For more details on 1D and 2D LC configurations, see Chapter 9, "Reference: Sample Analysis Strategy," starting on page 97.

To configure the Chip Cube

From the **Instrument** menu, choose **More Chip Cube > Configuration**. Refer to Figure 14 and configure the Chip Cube as follows:

Injection Flush Volume 8 µL (for 1D LC) or 16 µL (for 2D LC) with 75-µm needle seat capillary

Loading Pump G1376A

Analytical Pump G2226A

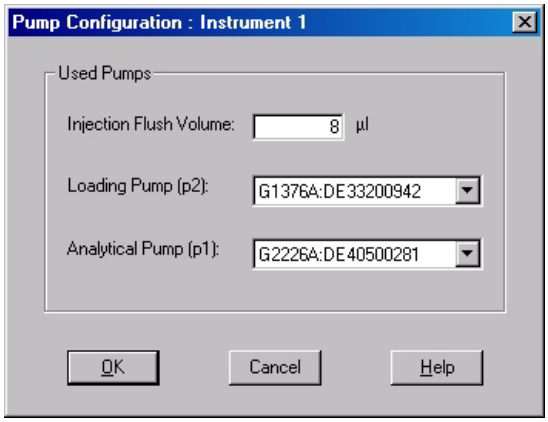


Figure 14 Chip Cube configuration

Explanation

To allow maximum reuse of methods, some configuration parameters are specific to the system and are not stored with the method. Examples include the injector loop configuration and the plate configuration for the micro-well plate sampler.

When you establish the above configuration settings for the Chip Cube, the system automatically calculates (based on injection flush volume and sample injection volume) when to switch the valve between sample loading and sample analysis. Also, when you wish to eject the Protein ID Chip, the system calculates how long to wait to release LC pump pressure before it actually ejects the HPLC-Chip. So it is critical that you properly configure the Chip Cube.

To insert the HPLC-Chip and start the spray

Inserting the HPLC-Chip

1 Insert the Protein ID Chip (p/n G4240-62001) into the slot on the HPLC-Chip/MS interface.

2 In the ChemStation software, right-click the HPLC-Chip icon and select **Operate**.

The automatic loading mechanism loads the HPLC-Chip into the HPLC-Chip/MS interface and positions the needle tip.

3 Check the video monitor to be sure the Protein ID Chip is in the correct position.

Starting the nanoflow pump

1 Set the flow rate to 300 nL/min.

2 Set the solvent composition to 97:3 A1:B1 (the starting composition for the methods).

3 Turn the pump on.

Starting spray

1 Make sure the capillary voltage is set to 1800 V.

2 Confirm that you have a stable current (typically 40 to 50 nA).

The maximum voltage for the HPLC-Chip/MS orthogonal nanospray source is 2200 V, and a typical voltage with a new chip is 1800 V.

3 Save the method to store the new **Vcap** setting.

NOTE

Increase the **Vcap** to the point where stable spray (without spiking) is achieved. Splitting of the stream usually indicates that the voltage is too high. Look for both a stable and reasonable MS spectrum as well as several minutes of stable chromatographic signal (no spiking).

The capillary current is typically 40 to 50 nA at the starting composition of 97:3 A1/B1, and will drop as the percentage of B1 increases.

To prepare the LC and MS just before an analysis

After you have plumbed your HPLC-Chip/MS system, you prepare it for stable analyses. You purge the LC, calibrate the MS (if not already done), and connect the two. Repeat the LC purge any time the system has been shut down for more than a day.

To prepare the nanoflow pump

Follow directions in "To prepare the nanoflow pump" on page 15.

To prepare the capillary pump

1. Purge the capillary pump. Purge the solvent channel(s) you will use at 2.5 mL/min for four minutes each.
2. Switch to your composition and flow rate for sample loading.

To prepare the well-plate sampler

If the well-plate sampler has not been used for two days, prime the needle wash for 120 seconds. Go to **Instrument > More Injector > Prime/Flush Pump**.

To prepare the Protein ID Chip

The first time you use a new Protein ID Chip, condition it as follows:

1. Make two to three injections of a high-level standard protein digest sample (e.g., 100 fmol BSA digest) using the short method on page 51.
2. Inject a solvent blank.

To calibrate the MS

1. Just before use, prepare a 1:5 dilution of the electrospray calibrant (p/n G2421A) in acetonitrile.
2. Install the MS Calibration and Diagnosis Chip (p/n G4240-61001).
3. Connect the dedicated calibrant line from the HPLC-Chip/MS interface to the syringe pump.
4. Load the syringe pump with diluted calibrant and start the flow at 18 µL/hr (about 300 nL/min).
5. Calibrate the ion trap mass spectrometer as directed in the *Agilent Chip Cube Interface Assembly for LC/MSD Trap User's Guide*.

- Be sure to check the ion trap mass axis calibrations (scan, isolation and fragmentation).
- Calibrate the detector gain. In **MSD Trap Control**, click the **Calibration** tab, then the **Detector** button.

CAUTION

The detector ages faster and must be calibrated more frequently when it is new.

It is a good idea to check the detector gain more frequently if the system is in constant use.

You should calibrate the MS if:

- This is the first time you are using it.
- You observe mass shifts or other problems indicating the need to calibrate.
- Your background seems unusually low, which indicates aging of the detector.

5
Peptide Separation and Detection with HPLC-Chip/MS

This chapter presents protocols for 1D enrichment mode and 2D nanoflow HPLC-Chip/MS analyses. Choice of the 1D or 2D protocol depends on sample complexity, peptide concentration, and how important it is to identify as many proteins as possible for your study. In general, the 2D methods provide more identifications, but at the cost of additional analysis time. For guidelines on protocol selection, see "Choosing the LC method" on page 44. Before you run any of the protocols, prepare the system as described in "To prepare the LC and MS just before an analysis" on page 47.

Safety

WARNING **Always take proper precautions for handling and disposing of solvents and other chemicals. Consult the material data safety sheets supplied by the vendors.**

For additional safety precautions, see the manuals you received with the individual system components.

Procedure summary

For a summary of the 1D LC procedure for the HPLC-Chip/MS, see "1D LC - enrichment column mode" on page 21. For more detailed flow diagrams, see "Enrichment column mode (1D LC)" on page 99.

For a summary of the 2D LC procedure for the HPLC-Chip/MS, see "2D LC with salt steps" on page 21. For more detailed flow diagrams, see "Two-dimensional liquid chromatography (2D LC)" on page 101.

NOTE While some of the figures in the referenced sections depict a conventional nanoflow system, the same principles apply for the HPLC-Chip. Note that the Protein ID Chip contains both the enrichment column and the analytical column (C18 nanocolumn). The Protein ID Chip does not contain an SCX column.

Additional materials required

In addition to the HPLC-Chip/MS system, you need the following solvents:

- Water, HPLC grade (18 megohm) - Agilent p/n 8500-2236 or equivalent
- Acetonitrile, HPLC grade - Agilent p/n G2453-85050 or equivalent
- Formic acid, analytical grade - Agilent p/n G2453-85060 or equivalent
- Ammonium formate, 5 M - Agilent p/n G1946-85021 (provided with the HPLC-Chip/MS Protein Identification Solution)

Protocol for 1D enrichment LC with Protein ID Chip - short method for less complex samples

Configuration

See "To configure the Chip Cube" on page 45.

HPLC-Chip

Use the Protein ID Chip, p/n G4240-62001. The chip integrates the following columns:

- Enrichment column: ZORBAX 300SB-C18, 40 nL, 5 µm particle size
- Reversed-phase column: ZORBAX 300SB-C18, 75 µm x 43 mm, 5 µm particle size

Maximum injection volume

8 µL or 40 µL, depending on loop installed

Chip Cube

- Inner valve: Enrichment
- Valve timetable: Enrichment at 7 min

Nanoflow pump

A = 0.1% formic acid in water
B = 90% acetonitrile + 0.1% formic acid in water

Flow rate = 300 nL/min

Primary flow = 500 to 800 µL/min (From the **Instrument** menu, choose **More pumps > Auxiliary**.)

Table 13 Nanoflow pump solvent gradient

Time (min)	% B
0	3
7	80
7.01	30

Stop time

9 min

Post time

none

Capillary pump

3% acetonitrile + 0.1% formic acid in water
Capillary pumps use no flow gradient; just set flow to 4 µL/min.

Needle flush solvent

20% methanol + 0.1% formic acid in water

Bottom-sensing On, 0.9 mm offset for plastic conical micro-vials (Agilent p/n 9301-0978), or 0.2 mm for 96-well Eppendorf well plates (Agilent p/n 5042-8502).

Protocol for 1D enrichment LC with Protein ID Chip - long method for more complex samples

Configuration See "To configure the Chip Cube" on page 45.

HPLC-Chip Use the Protein ID Chip, p/n G4240-62001. The chip integrates the following columns:

- Enrichment column: ZORBAX 300SB-C18, 40 nL, 5 µm particle size
- Reversed-phase column: ZORBAX 300SB-C18, 75 µm x 43 mm, 5 µm particle size

Maximum injection volume 8 µL or 40 µL, depending on loop installed

Chip Cube

- Inner valve: Enrichment
- Valve timetable: Enrichment at 37 min

Nanoflow pump A = 0.1% formic acid in water
B = 90% acetonitrile + 0.1% formic acid in water

Primary flow = 200 to 500 µL/min (From the **Instrument** menu, choose **More pumps > Auxiliary**.)

Table 14 Nanoflow pump solvent and flow gradient

Time (min)	% B	Flow (µL/min)
0	3	0.30
30	45	0.30
35	80	0.30
37	80	0.30
37.01	30	0.45

Stop time 45 min

Post time	none
Capillary pump	3% acetonitrile + 0.1% formic acid in water Capillary pumps use no flow gradient; just set flow to 4 µL/min.
Needle flush solvent	20% methanol + 0.1% formic acid in water
Bottom-sensing	On, 0.9 mm offset for plastic conical micro-vials (Agilent p/n 9301-0978), or 0.2 mm for 96-well Eppendorf well plates (Agilent p/n 5042-8502).

Protocol for online 2D LC method with Protein ID Chip

LC details

SCX column ZORBAX BioSCX Series II, 0.30 x 35 mm, 3.5 µm particle size.

HPLC-Chip Use Protein ID Chip, p/n G4240-62001. The chip integrates the following columns:

- Enrichment column: ZORBAX 300SB-C18, 40 nL, 5 µm particle size
- Reversed-phase column: ZORBAX 300SB-C18, 75 µm x 43 mm, 5 µm particle size

Injection volume 20 µL for salt steps (must have 40 µL loop installed)

Recommended salt steps 0, 15, 30, 45, 60, 75, 90, 120, 300, 500 mM ammonium formate (prepared in 3% acetonitrile + 0.1% formic acid in water). Choose the number of salt steps based on sample complexity.

Prepare salt solutions from 5 M ammonium formate stock (Agilent p/n G1946-85021). Prepare the solutions directly into the 2-mL glass autosampler vials supplied with the HPLC-Chip/MS Protein Identification Solution.

Table 15 Preparation of salt solutions for 2D LC with salt steps

Concentration (mM)	Volume of 5 M stock (µL)	Volume of water (µL)
15	3	997
30	6	994
45	9	991
60	12	988
75	15	985
90	18	982
120	24	976
300	60	940
500	100	900
1000	100	400

Nanoflow pump Use the nanoflow pump settings from either the short or long enrichment methods for the Protein ID Chip. (See page 51 or page 52.) Choose the appropriate method based on the complexity of the sample.

Capillary pump 3% acetonitrile + 0.1% formic acid in water

Capillary pumps use no flow gradient; just set flow to 4 µL/min.

Sequence table A typical sequence table for salt steps is given in Table 16.

Table 16 Sequence table to inject salt steps

Line	Location	Sample Name	Method Name	Inj/ Location	Sample Type	DataFile	Inj Volume
1	P1-A-01	Sample	2DLCX.M	1	Sample	0mM	5
2	Vial 1	15mM salt	2DLCX.M	1	Sample	15mM	20
3	Vial 2	30mM salt	2DLCX.M	1	Sample	30mM	20
4	Vial 3	45mM salt	2DLCX.M	1	Sample	45mM	20
5	Vial 4	60mM salt	2DLCX.M	1	Sample	60mM	20
6	Vial 5	75mM salt	2DLCX.M	1	Sample	75mM	20
7	Vial 6	90mM salt	2DLCX.M	1	Sample	90mM	20
8	Vial 7	120mM salt	2DLCX.M	1	Sample	120mM	20
9	Vial 8	300mM salt	2DLCX.M	1	Sample	300mM	20
10	Vial 9	500mM salt	2DLCX.M	1	Sample	500mM	20
11	Vial 10	1000mM salt	2DLCX.M	1	Sample	1000mM	20

MS/MS method for LC/MSD Trap XCT Ultra

Source and ion optics conditions

Ionization mode	Positive nanospray with Agilent orthogonal source included within HPLC-Chip/MS interface (G4240A)
Drying gas flow	4 L/min
Dry gas temperature	300 °C
Vcap	Typically 1800 V (Check for stable current of about 40-50 nA at starting solvent composition and flow.)
Skim1	30 V
Capillary exit	100 V

Ion trap conditions - MS scan

Trap drive	85
Averages	1
ICC	On
Maximum accumulation time	150 ms
Smart target	500,000
MS scan range	300-2200

Ion trap conditions - automatic MS/MS

Scan mode	Peptide
Number of precursor ions	5 or 6
Averages	1
Fragmentation amplitude	1.3 V

SmartFrag	On, 30-200%
Active exclusion	On, 2 spectra, 1 min
Prefer +2	On
MS/MS scan range	100-2000
Ultra scan	On
ICC target	500,000

To shut down the HPLC-Chip/MS system

To shut down the system, do the following at the end of your sample sequence:

1 Inject two solvent blanks to remove residual peptides from the injector. Acquire data for these injections.

- For simple samples, inject the solvent you used to dissolve your peptides.
- For complex samples, inject 60% acetonitrile + 0.1% formic acid in water to remove hydrophobic peptides.

2 In the **Sequence Parameters**, set the post-sequence command/macro to **macro "SHUTDOWN.MAC", go**.

NOTE Note that the **Standby** command stops the LC pumps and ejects the Protein ID Chip, but does *not* put the ion trap into standby mode. The **Shutdown** macro stops the LC pumps and puts the Protein ID Chip and the ion trap into standby mode.

3 Before you run your next sequence, open the data files for the blanks and check for peptide peaks. If there were substantial peaks in the last injection, inject more blanks.

CAUTION If you do not plan to use the system for the next eight hours, turn off the LC pumps and put the ion trap in standby mode. This extends the life of the electron multiplier.

Troubleshooting

If you have flow path blockages

Cause	Solution
1 Particles from solvent or sample	• Always filter solvents through 0.4 µm filters. • Clean up samples per *Sample Preparation Guide*.

If you have unstable flow

Cause	Solution
1 Flow blockages	• See "If you have flow path blockages" (above).
2 System pressure too low	• Keep system pressure higher than 20 bar at the pump outlet.
3 EMPV needs maintenance	• Run the EMPV cleaning procedure. See the nanoflow pump manual.
4 Air bubbles in liquid flow path	**a** Purge pumps if you have not already done so. **b** If bubbles persist, pump 500 nL/min of 50% B for 20 min. **c** If bubbles still persist, disconnect first capillary at pump end of system and purge at higher flow rate. Reconnect first capillary and disconnect next one. Purge again. Continue disconnecting capillaries sequentially and purging until bubbles are gone.

If you have unstable spray

Cause	Solution
1 Unstable flow	• See "If you have unstable flow" (above).

2 Capillary voltage not set correctly (You observe sputtering or split spray.)	• Adjust the capillary voltage.
	  ◦ With a flow of 300 nL/min, the capillary voltage should be 1800 volts.
	  ◦ If you observe a split stream, this typically means Vcap is too high.

3 Flow path blockages	• See "If you have flow path blockages" (above).

If you have poor chromatography

Cause	Solution
1 New chip not conditioned	• Before you use a new Protein ID Chip for analyses, condition it as described in "To prepare the Protein ID Chip" on page 47.
2 Sample overloaded the chip	• Inject 1/10 the sample volume or dilute the sample 1:10 and inject again. Observe whether the chromatography improves.

If you have sample carryover

Cause	Solution
1 No needle wash	• Set up needle wash for well-plate sampler.
2 Inappropriate needle wash solvent	• Switch to a solvent combination in which your sample is completely soluble.
3 Residual hydrophobic peptides in the injection system	• Analyze blanks with high organic until the peaks are gone.

If you have poor sensitivity

Cause	Solution
1 Detector gain adjustment needs to be redone	• See instructions for verifying detector setting in your LC/MSD Trap documentation.
2 Capillary voltage too high, causing corona which can destroy peptides	• Reduce the capillary voltage.
3 Sample degradation from sitting at room temperature	• Prepare fresh samples. • If you have the optional thermostat on the micro well-plate sampler, make sure it is turned on and set to 4 °C.
4 Peptides adsorbed on vial surface	• Switch to a different vial material (glass or plastic).
5 Bad injection due to air bubble at bottom of vial	• Tap vial gently to dislodge air bubble.
6 Unstable spray	• See "If you have unstable spray" on page 59 of this chapter.
7 **Find Compounds** settings not optimized for low-level samples, giving the appearance of poor sensitivity (applies only if you use Mascot search) Note: Because the **Find Compounds** function is so efficient, you can often obtain a good Mascot search even when peaks are not evident in the base peak chromatogram.	• For low-level samples, reduce these thresholds in the data analysis method parameters: ◦ Find-AutoMS(n) compound detection threshold ◦ Mass List-Apex absolute intensity threshold

If you have low database search scores or poor sequence coverage

Cause	Solution

1 Sample preparation or data acquisition problem	• Open the data file and see how many peaks you have in the MS BPC.
	  ◦ If you have very few peaks, you have a problem with the sample preparation or with the data acquisition settings. See causes 2 through 5 below, as well as the troubleshooting in the *Sample Preparation Guide*.
	  ◦ If you have a reasonable number of peaks, your problem resides in the data processing. See causes 6 through 10 below.

2 Number of precursors for data-dependent MS^n set too low	• Go to the **MS(n)** tab on the Trap control screen and set **No. of Precursor Ions** to 5 or 6.
3 Threshold for triggering MS^n set too high	• Examine your data and set a lower threshold if it appears inadequate MS^n experiments were generated. • To set a lower threshold, go to the **MS(n)** tab on the Trap control screen and set **Threshold Abs** and/or **Threshold Rel** to a lower number.
4 SmartFrag turned off, resulting in inadequate fragmentation	• Click the **Fragmentation** button on the **MS(n)** page of the Trap control screen. • Make sure **SmartFrag** is checked and set to 30-200%.
5 Poor sensitivity	• See "If you have poor sensitivity" on page 61 of this chapter.
6 Default settings for LC/MSD Trap data analysis software not appropriate for sample (applies only if you use Mascot search)	• See "Troubleshooting" on page 95 of Chapter 8, "Data Analysis with Mascot Protein Database Search".
7 Mascot parameters not optimized (applies only if you use Mascot search)	• See "To use Mascot protein database search" on page 90 of Chapter 8, "Data Analysis with Mascot Protein Database Search".
8 Spectrum Mill Data Extractor parameters not set properly (applies only if you use Spectrum Mill workbench)	• See the documentation you received with the Spectrum Mill MS Proteomics Workbench.
9 Spectrum Mill Search parameters not set properly (applies only if you use Spectrum Mill workbench)	• See the documentation you received with the Spectrum Mill MS Proteomics Workbench.
10 Chosen database did not give good results.	• Try searching another database.

Tips

See the tips in the *Agilent Nanoflow LC System for Mass Spectrometry (MS) G2229 Quick Start Guide*. See also the operation and maintenance chapters in the *Agilent 1100 Series G4240A HPLC-Chip/MS Cube User's Guide*.

6 Optional Peptide Fractionation with Offline SCX

This chapter describes an optional peptide fractionation by offline strong cation exchange (SCX) chromatography. This technique can be used to fractionate complex samples such as whole cell lysates and biological fluids. The collected fractions are later analyzed by LC/MS/MS. When samples are less complex or there is a need for greater automation, SCX can be accomplished online as the first step in online two-dimensional (2D) LC/MS/MS. With the online technique, described in Chapter 3, peptides are eluted in fractions from the SCX column by injection of salt solutions of increasing ionic strength, and then are analyzed by reversed-phase LC/MS/MS. With the offline technique, described in this chapter, peptides are eluted continuously from the SCX column by use of a salt gradient, are collected in fractions, and then are analyzed by reversed-phase LC/MS/MS. The advantage of doing the SCX offline is increased resolution and fewer peptides per fraction, which enables identification of more of the low-abundance proteins. The disadvantage is that there are more fractions, and this requires more LC/MS/MS and data reduction time.

System components

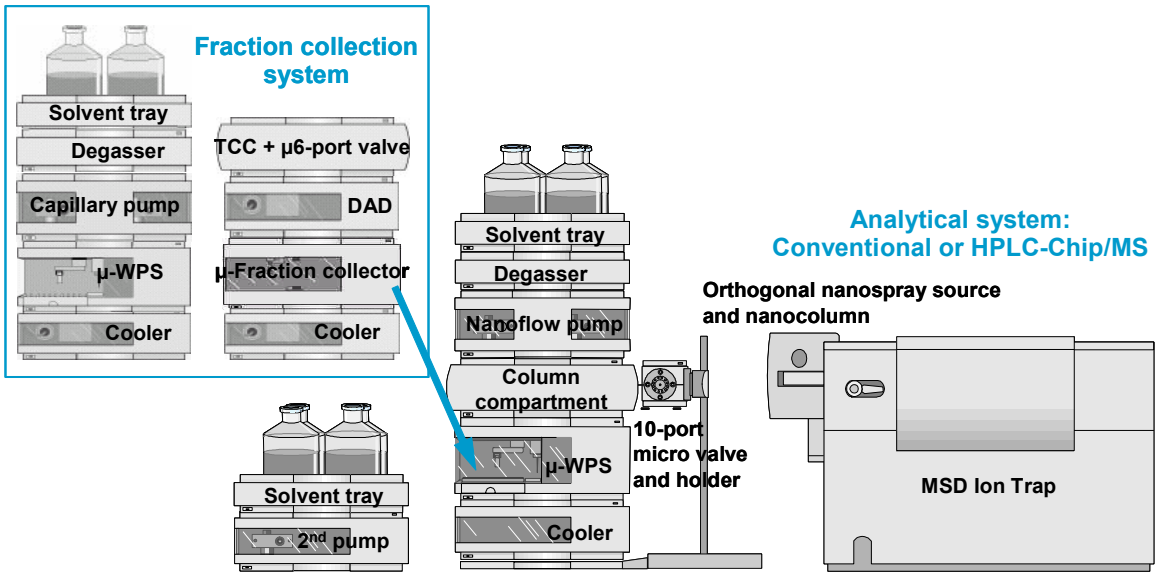


Figure 15 Offline SCX in combination with nanoflow LC/MS/MS

The fraction collection system consists of:

- Agilent 1100 Series capillary pump with micro vacuum degasser
- Agilent 1100 Series thermostatted micro well-plate autosampler
- Agilent 1100 Series thermostatted column compartment with micro 2-position/6-port valve
- Agilent 1100 Series diode array detector (DAD) with 500-nL flow cell
- Agilent 1100 Series thermostatted micro collector/spotter
- Agilent ChemStation B.01.03 software

Safety

WARNING **Always take proper precautions for handling and disposing of solvents and other chemicals. Consult the material data safety sheets supplied by the vendors.**

See the manuals that you received with the 1100 Series modules for additional safety precautions.

Procedure summary

Schematic overview

Figure 16 shows a schematic diagram of offline 2D LC/MS. You separate peptides in the first dimension by offline SCX with fraction collection (the topic of this chapter). You then separate the peptides in the second dimension with nanoflow LC/MS/MS, as described in Chapter 3 or Chapter 5.

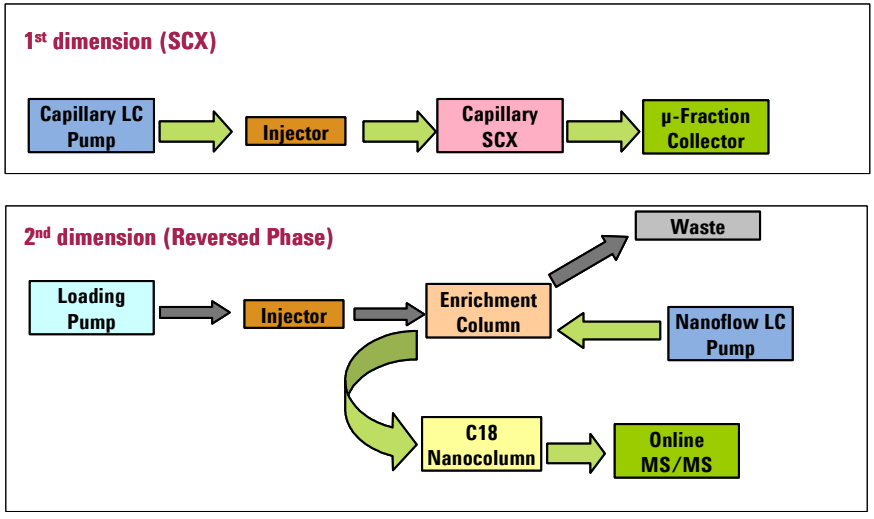


Figure 16 Overall schematic for offline 2D LC/MS

Summary of steps

1 Load tryptic peptides onto the SCX column.

2 Elute peptides with a linear salt gradient. Some general guidelines are given in Table 17. More details for two of the gradients are given in Table 18 and Table 19.

Table 17 Salt gradients for peptide mixtures of increasing complexity

Sample complexity	Goal	SCX gradient (min) 1st part (0-100 mM ammonium formate)	SCX gradient (min) 2nd part (100-500 mM ammonium formate)	Approximate number of SCX fractions*
Simple mixture, e.g., highly resolved 2D gel spot	Maximum number of proteins identified	15	3	7
Subcellular fraction	Reasonable number of proteins identified with shorter analysis time	30	6	14
Whole cell lysate	Reasonable number of proteins identified with shorter analysis time	60	12	28
Whole cell lysate	Maximum number of proteins identified	120	24	45

* Assuming fractions collected as described on page 71

3 Collect fractions of about 15 µL each with the 1100 Series micro-fraction collection system. You can collect smaller fractions, but this will increase LC/MS analysis time.

4 Transfer the well-plates containing all collected fractions to the nanoflow LC/MS/MS system.

5 Analyze the fractions using one of the following protocols:

- "Protocol for 1D enrichment LC - short method for less complex samples" on page 26

- “Protocol for 1D enrichment LC - long method for more complex samples” on page 28
- “Protocol for 1D enrichment LC with Protein ID Chip - short method for less complex samples” on page 51
- “Protocol for 1D enrichment LC with Protein ID Chip - long method for more complex samples” on page 52.

More details

For more details, see the application note entitled “Tools and considerations to increase resolution of complex proteome samples by two-dimensional offline LC/MS,” Agilent publication number 5989-0213EN.

Additional materials required

In addition to the 1100 Series modules, you need the following:

- Water, HPLC grade (18 megohm) - Agilent p/n 8500-2236 or equivalent
- Acetonitrile, HPLC grade - Agilent p/n G2453-85050 or equivalent
- Formic acid, analytical grade - Agilent p/n G2453-85060 or equivalent
- Ammonium formate, reagent grade

Protocol for offline SCX

Column Choose one of the following:

- ZORBAX BioSCX Series II, 0.30 x 35 mm, 3.5 µm particle size, p/n 5065-9912
- ZORBAX BioSCX Series II, 0.80 mm x 50 mm, 3.5 µm particle size, p/n 5065-9942

Capillary pump A = 5% acetonitrile + 0.03% formic acid; B = 5% acetonitrile, 500 mM ammonium formate + 0.03% formic acid

Flow 5 µL/min for the 0.3 mm column, or 15 µL/min for the 0.8 mm column

Gradient See Table 18 for a protein mixture of moderate complexity (e.g., a subcellular fraction) or Table 19 for a highly complex protein mixture (e.g., a whole cell lysate). In both tables, the gradient balances the need to separate proteins with the need to optimize analysis time.

Table 18 SCX gradient for fraction collection for moderately complex peptide mixture

Time (min)	% B
0	0
30	20
36	100
44	100
44.01	0

Table 19 SCX gradient for fraction collection for highly complex peptide mixture

Time (min)	% B
0	0
60	20
72	100
86	100
86.01	0

Injection volume Up to 8 µL or 40 µL, depending on loop installed. Typically, you set your injection volume to inject no more than 100 to 150 µg total protein per run.

Fraction collection Time-based, first fraction 0 - 5 min, remaining fractions each 3 min, liquid contact control mode with distance of 0 mm, fraction size 15 µL, cooling 4 °C

Troubleshooting

Problem	Cause	Solution
SCX column plugs frequently.	Particles from biological samples	Filter or use Agilent Spin Filters (p/n 5185-5990) to remove particulates.
		If your column is plugged, you can sometimes recover by backflushing the column or replacing the screen at the head of the column. Check your column information sheet for the part number for the screen.
All peptides elute in the first few fractions.	Salts from biological samples	Remove salts with Agilent Cleanup C18 Spin Tubes or Cleanup C18 Pipette Tips.
	Urea or guanidine HCl was used in the tryptic digestion	Remove salts with Agilent Cleanup C18 Spin Tubes or Cleanup C18 Pipette Tips.
	Excess ammonium bicarbonate from digestion.	Dry sample. Add 100 to 200 µL water. Repeat drying and water addition once or twice, ending with a dry sample. Dissolve sample in 0.1% formic acid or other appropriate buffer.
Some peptides do not elute from the SCX column.	Highly-charged peptides	Run a pH gradient in combination with a salt gradient. • The SCX method is optimized for tryptic peptides, which typically carry no more than 3 or 4 charges. • For more highly-charged peptides, you may need to run a combination salt/pH gradient. For details, see the note on page 104.

For additional troubleshooting tips, see the manuals you received with the modules in your fraction collection system.

Tips

Gradient for fraction collection

Optimize the gradient for fraction collection for the complexity of your samples. For moderately complex samples, use a steeper gradient, as outlined in Table 18. For more complex samples, use a shallower gradient, as outlined in Table 19. For all gradients described here, you collect a fraction every three minutes.

To identify more proteins, first try extending the gradient, then try collecting fractions more frequently. Note that each additional fraction adds to the LC/MS analysis time.

Preventing degradation of fractions

Be sure the thermostat on the fraction collector is turned on and set to 4 °C. Since you do not want to hold samples too long at 4 °C, do not collect more fractions than you can analyze in a couple of days.

Plumbing of capillaries

The capillary you plumb between the diode array detector and the well-plate sampler depends on the flow rate you use for fraction collection. For the 5 µL/min flow rate you use with this application, it is best to use the green 50-µm ID capillary (p/n G1364-87305).

7
Data Analysis with Spectrum Mill MS Proteomics Workbench

This chapter provides a general protocol and tips for processing your data with the Spectrum Mill MS Proteomics Workbench. If you do not have the Spectrum Mill workbench, proceed to Chapter 8.

The Spectrum Mill workbench is a comprehensive software package that starts with raw mass spectral data files and provides tools to rapidly convert these to tables of protein identities. This software includes modules for extraction of high-quality spectra from raw data files, rapid protein database search of MS or MS/MS spectra, data review and validation, and results summary for single samples or groups of samples that span complex studies. It also includes capabilities for identification of post-translational modifications, for quantitative analysis (including but not limited to ICAT quantitation), and for *de novo* sequencing. The software accepts data from multiple vendors' instrument types in multiple file formats. Both public and proprietary databases are supported.

The Spectrum Mill workbench provides a means to segregate search results that contain a valid interpretation of an MS/MS spectrum from those that do not. Results that *are* validated can be summarized in a results table. Results that are *not* validated can then be subjected to subsequent rounds of searches (against other databases or in homology mode, for example). This iterative approach allows for efficient processing that can be customized to the needs of the study.

Procedure summary

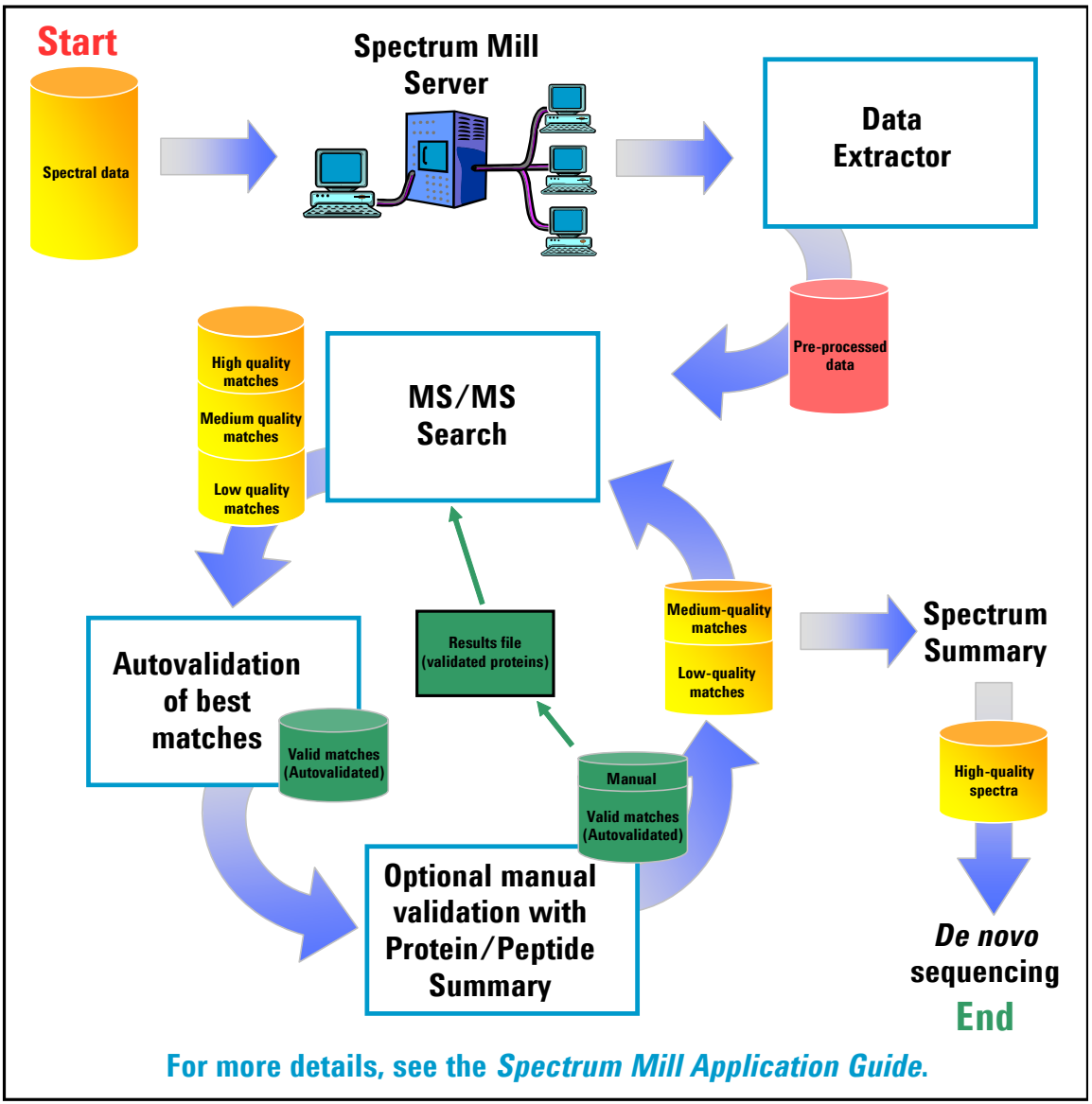


Figure 17 Summary of iterative processing with the Spectrum Mill workbench

Figure 17 shows the iterative MS/MS search strategy with the Spectrum Mill workbench. Assuming the goal is to identify as many proteins as possible, the following summarizes the process:

1. Copy or move data to the Spectrum Mill server.
2. Preprocess the raw data files with the Data Extractor.
3. Search a database in identity mode, preferably a species subset database for the first search.
4. Autovalidate the results with the highest scores. (Validation means that the proposed database match is accepted as the correct match for the MS/MS spectrum.)
5. Optional: Manually review the medium-quality spectra and validate additional results.
6. Use Tool Belt to create a saved results file of validated protein hits (***.res** file).
7. Search the spectra that have not been validated:
   - **a** Search against validated protein hits for the combination of oxidized methionine and pyroglutamic acid.
   - **b** If you suspect phosphorylation, search against validated protein hits for the combination of phosphorylated S, phosphorylated T, and phosphorylated Y.
   - **c** If you suspect other modifications, search for them.
   - **d** Search in no enzyme mode against validated protein hits.
   - **e** Search in identity mode against a larger database.
8. Continue to perform iterative cycles of database search and validation to identify as many proteins as desired.
9. Use Spectrum Summary to examine the remaining unmatched spectra to determine if there are high-quality spectra.
10. Perform *de novo* sequencing on high-quality spectra.

Protocol for data processing with Spectrum Mill workbench

The following describes in detail the iterative search strategy to process LC/MS/MS data with the Spectrum Mill workbench. The strategy assumes that the goal is to identify as many proteins as possible. If your study does not require you to identify so many proteins, you may omit step 5 through step 13. For details on use of the Spectrum Mill workbench, see the manuals and online help you received with the product.

1 Copy or move the raw LC/MS/MS data files to the Spectrum Mill server.
   - Be sure to set up a directory structure on the server that makes it easy to summarize and compare your results. See "Copying data to server" on page 83.

2 Extract the data.
   - Set the correct cysteine modification.
   - For **Merge scans with same precursor m/z**, if necessary change the time range to be compatible with your chromatographic data. A good starting point is the default of 15 seconds with a +/- 1.4 *m/z* window.

3 Do the first database search.
   - Use a database subset (e.g. mammals).
   - Search in identity mode.
   - For Spectrum Mill version A.03.02 or later, mark the check box to **Calculate reversed database scores**.

4 Autovalidate in **Protein Details** mode, then (optionally) in **Peptide** mode (using default settings).

5 Manually validate (down to score of 6, SPI 70 for simple sets).
   - Depending on the size of the data set, it may be easiest to do this in batches (i.e., first score > 8, then score > 6).
   - For guidelines, see "To manually validate results" on page 79.
   - If your goal is to maximize the number of identified proteins, do a thorough job at this step because your validated results will be the basis for further searches.
   - If you do not need to maximize the number of identified proteins, skip this step because it can be time-consuming and may not produce a large number of identifications.

6 Use Tool Belt to create a saved results file of validated protein hits (***.res** file).

- Be sure to indicate the database you searched since this maps to accession numbers.

7 Perform database searches for suspected modifications. Search against previously validated protein hits. Search as follows with autovalidation and manual validation between each set of conditions:

a Search for pyroglutamic acid and oxidized methionine:

For Spectrum Mill version A.03.02 or later, search in **Variable** mode for pyroglutamic acid and oxidized methionine.

For Spectrum Mill versions prior to A.03.02, search in **Homology Multi - mq** mode.

b If you suspect phosphorylation:

For Spectrum Mill version A.03.02 or later, search in **Variable** mode for the combination of phosphorylated S, phosphorylated T, and phosphorylated Y.

For Spectrum Mill versions prior to A.03.02, search in **Homology Multi - sty** mode.

c Search for any other modifications you suspect for your sample. (This will increase sequence coverage).

8 Search the previously validated results in no enzyme mode to find non-specific cleavages for proteins you have already identified. (Set **Digest:** to **No enzyme**.) Repeat autovalidation and manual validation.

9 When you think you are done, list sequence-not-validated spectra in Protein Details mode and look for proteins with multiple peptides. These may represent legitimate proteins at low levels. Re-examine the spectra to confirm.

10 Optional: Search again using a larger database (entire database or larger subset). This is most important when the original subspecies is not well-represented in the database. Autovalidate and manually validate.

11 Check statistics in Tool Belt. If there is a significant number of unmatched filtered spectra, continue searching.

NOTE

If the Tool Belt statistics show that only a small percentage of your collected MS/MS spectra were filtered, use the LC/MSD Trap DataAnalysis to check your data file to see if you collected a large number of MS/MS spectra on background. If so, your MS/MS data acquisition threshold may be set too low. To set a higher threshold, go to the **MS(n)** tab on the Trap control screen and set **Threshold Abs** and/or **Threshold Rel** to a larger number.

12 Use Spectrum Summary to check for sequence-not-validated spectra with sequence tags greater than 6 or 7. Review these and mark as **Good Spectrum** as appropriate.

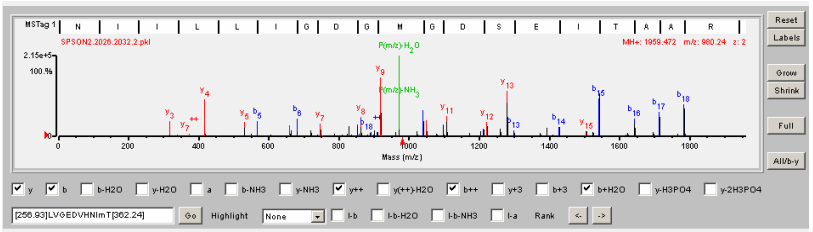
13 Subject the good spectra to *de novo* sequencing.

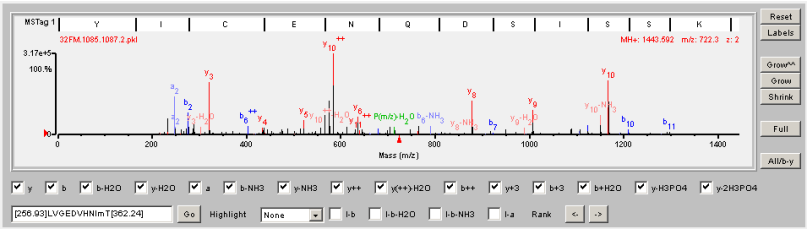
14 When you have gained enough information from your data, summarize the results.

To manually validate results

See guidelines in Table 20.

Table 20 Guidelines for manual results validation

Guideline	Example
The best spectra will generally be found by autovalidation, so do not expect to see spectra like the one to the right.	

• Loss of NH_3 occurs from R, K, Q and N residues.
• Loss of H_2O occurs from S, T, E and D.
• R, H, K and N are charge-bearing residues and increase the maximum charge state allowed for a peptide fragment.
• Enhanced fragmentation may be observed at the following bonds:
  ◦ His – Xaa
  ◦ Xaa – Gly
  ◦ Xaa – Ser
	

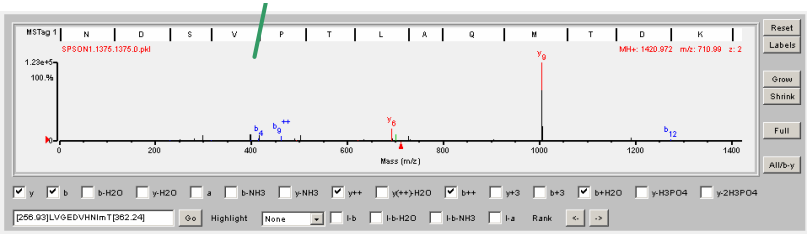
The peptide bond on the N-terminal side of Pro is particularly labile (high local proton affinity) and usually results in an MS/MS spectrum dominated by the y-ion ending in Pro.	

Table 20 Guidelines for manual results validation

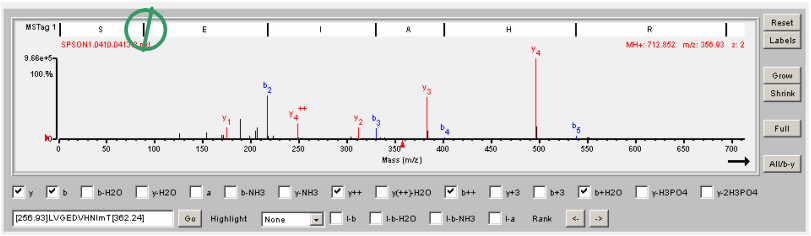
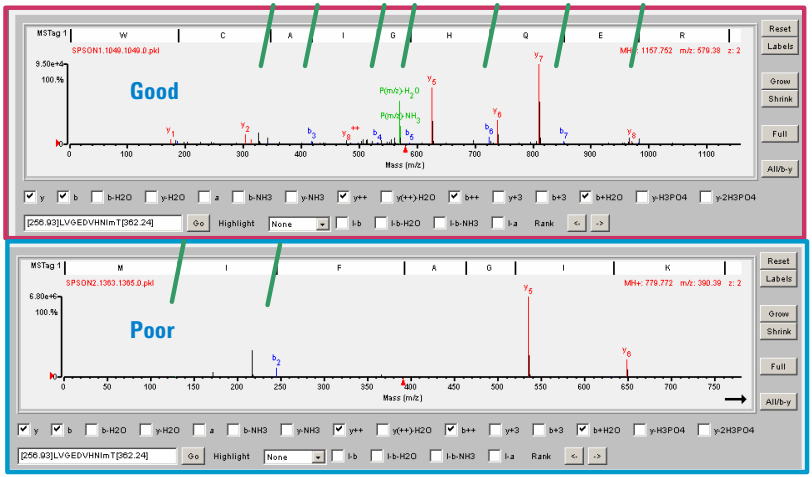
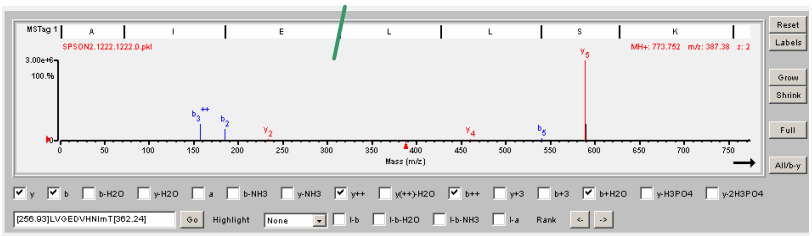
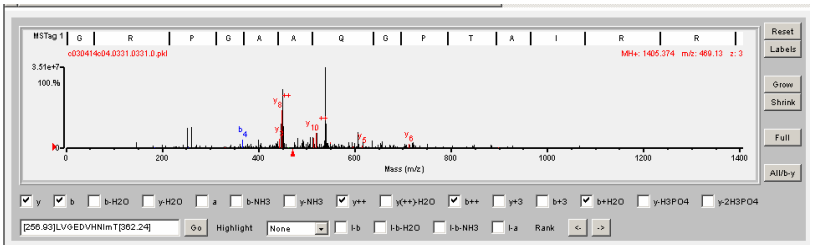
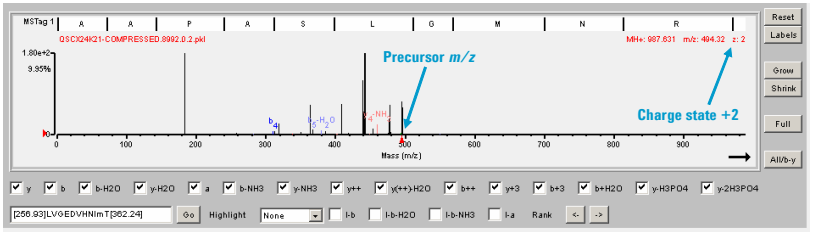
Guideline	Example
It is typical to lack fragmentation between the first two N-terminal amino acids (i.e., no b_1 or y_{x-1} ions).	
Look for coverage over a significant portion of the backbone.	
The peptide bond on the C-terminal side of acidic residues (D, E) may show enhanced cleavage.	

Table 20 Guidelines for manual results validation

Guideline	Example
Be suspicious of a clumped pattern that occurs frequently throughout the LC run (usually a background ion).	
Be suspicious if no ions appear above the precursor ion (when precursor charge state is >1).	
Remember - When in doubt, *don't* validate. • The goal is *no* false positives. • Validating a questionable hit removes it from subsequent searches.	

To summarize data

When you summarize data with the Protein/Peptide Summary page, remember that you can:

Validate results

For **Mode**, select **Peptide**, **Protein Details**, **Protein - Single Peptide ID**, or **Protein - Sample Centric Rows Details.**

Compare results across samples

For **Data Directory**, select more than one sample folder.

Evaluate fractionation

For **Mode**, select **Protein-Peptide Distribution Columns**.

Do light/heavy calculations

Under **Review Fields**, mark the **L/H** check box and select the site of the light/heavy modification.

Print results

1. Enable **Print background colors and images** in Internet Explorer. Select **Tools > Internet Options...**. Click the **Advanced** tab and mark the check box for **Print background colors and images**.
2. Select **File > Page setup...** to set the page to landscape mode.
3. Click in the frame you wish to print.
4. Select **File > Print Preview...**.
5. At the top of the **Print Preview** window, select **Only the selected frame**.
6. Click the **Print...** button.

Import data into Excel, Synapsia Informatics Workbench, or LIMS

1. Mark the **Excel Export** check box.
2. Click the **Summarize** button.
3. Check that you see a display with two buttons, one to upload to LIMS and another to display the file.
4. Do one of the following:
   - To import the data into Excel, import as semicolon-delimited data.
   - To import the data into Synapsia, see the Synapsia online help. Be sure to copy and paste into the Synapsia **Import Data** screen only the bold portion of the **Upload Path** from the Spectrum Mill screen.
   - To upload to LIMS, see the Server Administration online help.

Tips

Copying data to server

To make it easy to compare data sets, set up the appropriate directory structure on the Spectrum Mill server. Whenever you want to compare samples in a set, you need to set up a subdirectory for each sample. This sample directory may contain data files from multiple sample fractions or gel slices. Here are some examples:

- If you perform a two-dimensional LC/MS/MS analysis of a single sample, transfer all the files to a single directory on the server.
- If you run the same two-dimensional LC/MS/MS analysis on a second sample, or if you repeat the run on the first sample, transfer all these files to a second directory.
- If you conduct a differential expression study, transfer samples from one cell state into one directory and the second cell state into a second directory.

Extracting spectra

When you extract your data, be sure to remember the following:

- You extract only one directory at a time.
- For **Merge scans with same precursor m/z**, if necessary change the time range to be compatible with your chromatographic data.
  - This option sets the time range for merging scans from the same peak.
  - A good starting point is 15 seconds with a +/- 0.5 *m/z* window.
  - If the time range or *m/z* windows are too wide, you may merge scans for more than one peptide.
  - If they are too narrow, searches will take longer and you will observe redundant hits.

Searching spectra

Before you search a subset database, review the Spectrum Mill species definitions and modify if necessary. For the default species definitions, see the online help for the MS/MS Search page.

Validating results

For efficient validation of MS/MS search results:

- Use the Autovalidation page to validate the highest-scoring results — those that do not require manual review.
- Use the Protein/Peptide Summary page for manual review and validation of medium-scoring results.

7 Data Analysis with Spectrum Mill MS Proteomics Workbench

8 Data Analysis with Mascot Protein Database Search

In this chapter you learn how to perform data analysis using the LC/MSD Trap DataAnalysis software and Mascot database search. Mascot from Matrix Science Limited is a search engine that uses mass spectra to identify proteins from sequence databases. Mascot searches can be performed free of charge at www.matrixscience.com. Optional Mascot Daemon software automates the searches, but can only be used with an in-house Mascot Server license.

If you have the Spectrum Mill MS Proteomics Workbench, disregard this chapter and read Chapter 7 instead.

Procedure summary

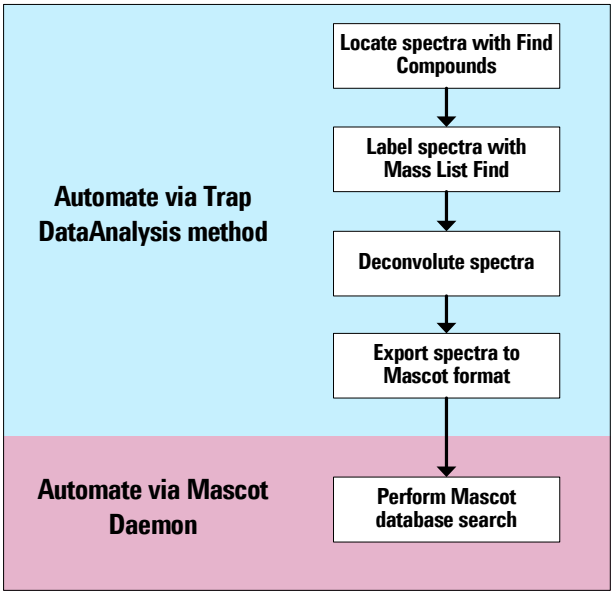


Figure 18 Data analysis with the LC/MSD Trap software and Mascot search

When you use Mascot, data analysis for protein digest samples consists of five steps:

1. Use **Find Compounds** to locate and hierarchically organize related MS and MS/MS spectra.
2. Use **Mass List Find** to label spectra with masses.
3. Perform (optional) charge deconvolution.
4. Export spectra to Mascot format.
5. Perform Mascot database search.

The first four steps are automated via a Visual Basic script that you add to your LC/MSD Trap methods. Once you have generated a Mascot generic format (*.mgf) file from your mass spectral data, you conduct an online search on a single file at www.matrixscience.com, or an automated search using the optional Mascot Daemon software on an in-house server.

To use LC/MSD Trap Visual Basic script to export data to Mascot

To activate the Visual Basic script

1 In **MSD Trap Control**, open the LC/MSD Trap method you created to do the 1D or 2D LC/MS/MS analysis of peptides.

2 Select **Method > Add DataAnalysis Part**.

3 Select the DataAnalysis method **Example DA Methods with Scripts\ Auto_MIS_MgfExport.ms.**

4 Select **Method > Save entire Method As** to save the LC/MS/MS method with the DataAnalysis part attached.

To change processing method parameters

The method **Auto_MIS_MgfExport.ms** includes:

- The Visual Basic script
- Starting parameters for **Find Compounds AutoMS(n)**, **Mass List Find**, **Deconvolution**, and **Mascot Export Options**

The script does not change or optimize the starting method parameters. If you change method parameters (i.e., based on recommendations from "Troubleshooting" on page 95), test and save your changes as follows:

1 Select **Method > Run** to execute the script again on your test data file.

2 Select **Method > Save As** to save a new copy of the processing method.

To change the Visual Basic script

The Visual Basic script is shown in Figure 19. To change the script:

1 Select **Method > Script**.

2 Make the desired change.

3 Save a new copy of the processing method by selecting **Method > Save As**.

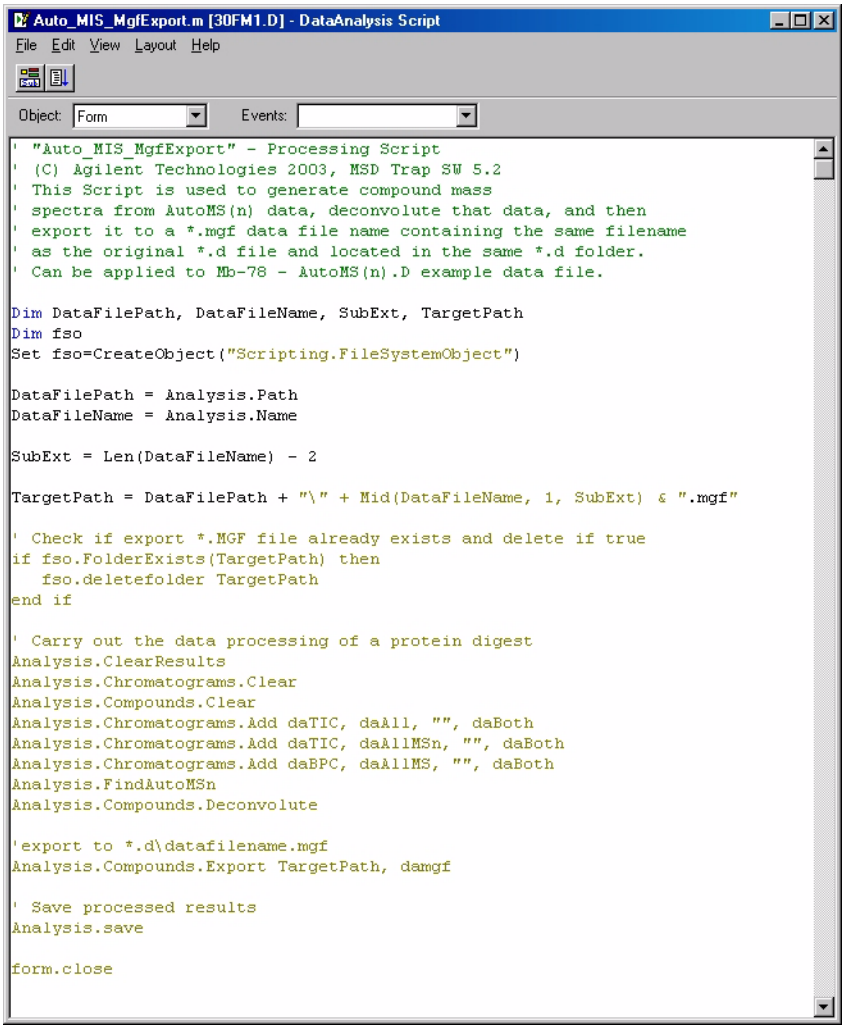


Figure 19 Visual Basic script to automate data analysis

For more information on modifying this script or writing new ones, see the following help:

- DataAnalysis Online Help (**Help > Help Topics > Using processing methods and scripting**)
- Visual Basic Script Language Reference (**Help > VBScript Language Reference**)

To deactivate the script

If you would prefer *not* to run this script automatically as part of your sequence, do one of the following:

To deactivate in ChemStation:

1 Go to the **Method and Run Control** view of the ChemStation software.

2 Select **Method > Do MS Post-Run Processing** to remove the check mark.

3 Select **Method** again to verify that the check mark is removed from **Do MS Post-Run Processing**.

To deactivate in LC/MSD Trap software:

1 Go to the **MSD Trap Control** screen.

2 Select **Method**.

3 Clear the **Run Processing Script** check box.

To use the MASCAT concatenator for multiple sample fractions

MASCAT concatenates multiple *.mgf files for Mascot data-base search, enabling consolidated search of spectra from two-dimensional LC/MS/MS analyses. To use MASCAT:

1 Click the MASCAT icon.

2 Select data files you wish to combine. See Figure 20 as an example.

3 Enter an output file path and name.

4 Click **Concatenate**.

5 Click **Calculate Number of Queries** and decide if the number fits within your time and computer memory constraints.

- If so, proceed to do the Mascot search (page 90).
- If not, go to step 6.

6 Regenerate the individual Mascot generic files using a higher threshold for compound finding and/or more stringent requirements for input spectral quality.

7 Repeat step 1 to step 5.

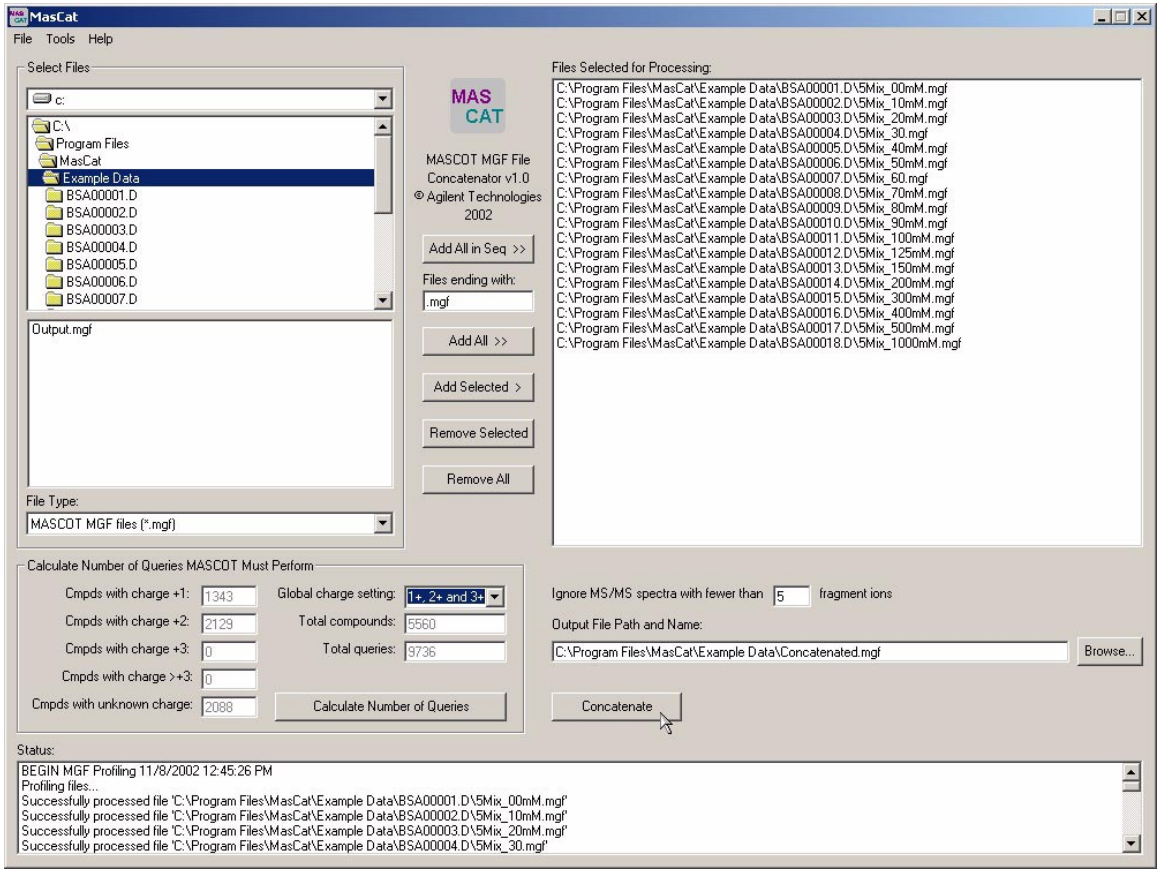


Figure 20 MASCAT concatenator to combine *.mgf files from multiple sample fractions

To use Mascot protein database search

1 Decide how you will do the search. The options are:

- Conduct an online search at www.matrixscience.com.
- Conduct an automated search using the optional Mascot Daemon software on an in-house server.

2 Set parameters for either type of search.

- See Figure 21 and the explanations that follow.

- For more details, see the Mascot online help.

3 Click **Start Search...** to initiate the Mascot search.

4 View search results either immediately via your web browser, or later via email.

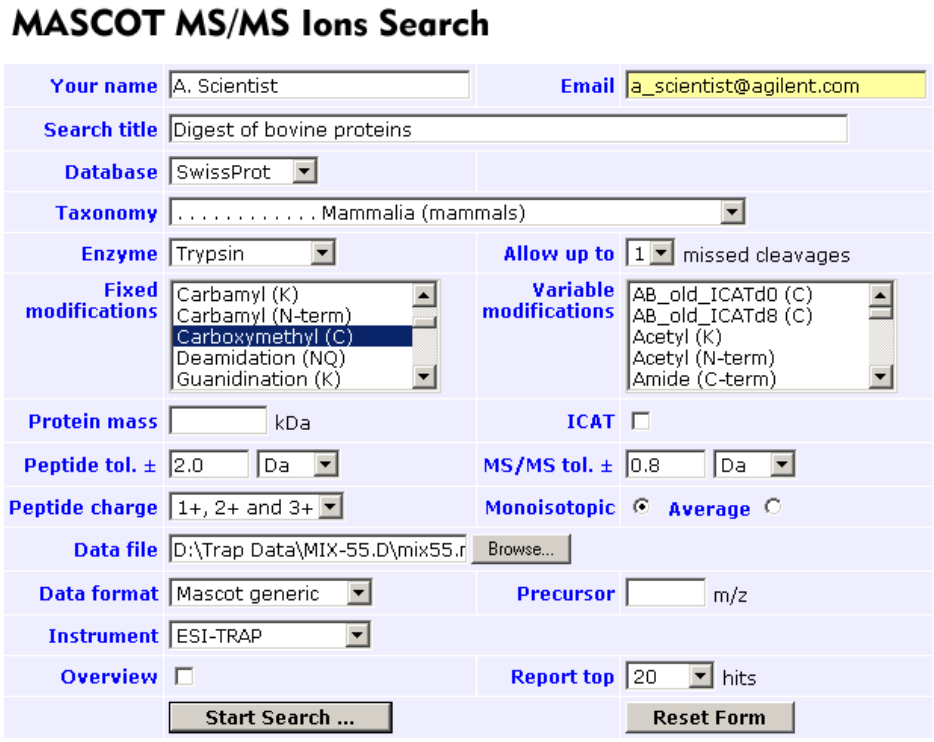


Figure 21 Parameters for Mascot MS/MS Ions Search

Database Search public databases at www.matrixscience.com, or search proprietary databases using an in-house server. Search a second database if your first choice fails to provide significant data.

Taxonomy For faster searches, select a species or group of species rather than **All entries**. Be aware that some databases do not rigorously classify taxonomy, so if you specify taxonomy you may obtain fewer legitimate hits. You may also miss homologous proteins from related species. If your search fails to give a result, try the **Species information unavailable** or **All entries** selections.

Enzyme Specify the enzyme used for protein digestion. If you are unsure, you can select **None,** but the search will take much longer because all cleavages must be considered.

Missed cleavages Unless your digestion is perfect, choose 1 or at most 2. You can choose larger numbers if you have confidence this is necessary, but larger numbers will increase search time and will increase the chance of false positives.

Fixed modifications These modifications are applied universally and add no additional search time.

Variable modifications Avoid specifying a large number without cause. These modifications are not applied universally and add significantly to the search time.

Peptide charge Select **1+, 2+, and 3+** unless you know you can more narrowly define the list of possible charge states. For example, if you have excluded singly-charged ions when setting up your data-dependent acquisition parameters, you can omit the 1+ possibility.

Data file Select your *.mgf file from the folder where you saved the exported file.

Instrument Select **ESI-Trap**.

To use Mascot Daemon

The optional Mascot Daemon software on an in-house server automates database searching. You set search parameters using the Mascot Daemon **Parameter Editor**, shown in Figure 22. Parameter entry is similar to that discussed in "To use Mascot protein database search" on page 90.

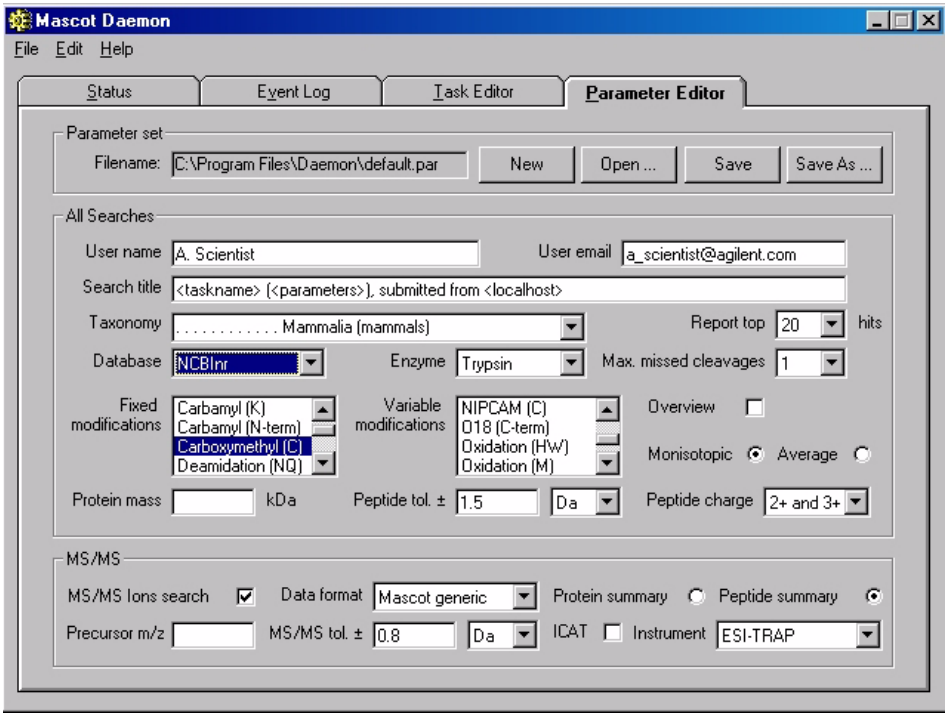


Figure 22 Mascot Daemon Parameter Editor

You set automation parameters using the Mascot Daemon Task Editor, shown in Figure 23. Some of these fields are discussed briefly below. Additional information is available via the Mascot Daemon online help.

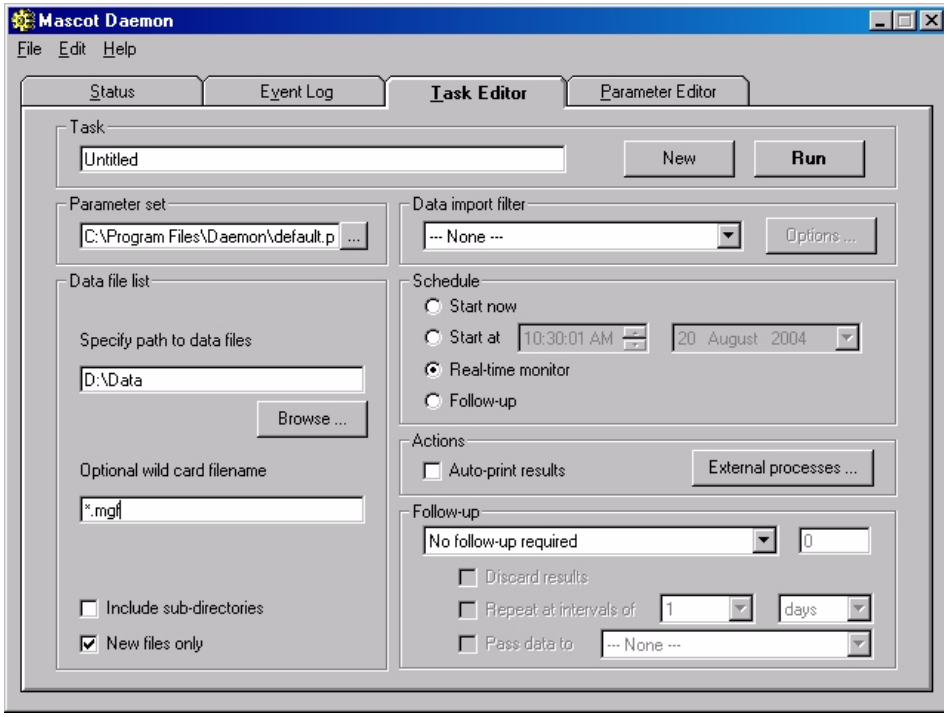


Figure 23 Mascot Daemon Task Editor

Schedule Schedule searches by time and date, or use the real-time monitor to run searches as Mascot generic files are written to a particular directory (e.g., by use of a Visual Basic script).

Follow-up Schedule score-dependent follow-up.

Optional wild card filename Search for Mascot generic format files (*.mgf) only.

Troubleshooting

Problem	Cause	Solution
Too few proteins identified	Default settings for LC/MSD Trap data analysis software not appropriate for sample	Check to see if the threshold for **Find Compounds** is set too high: **a** In the data analysis navigation tree, scroll to the bottom of the **Compound Mass Spectra** node. You should observe a reasonable number of compounds for your sample. **b** If this is not the case, select **Method > Parameters**. **c** Click the **Find** tab. **d** Click the **AutoMS(n)** subtab. **e** Enter a lower number for the **Compound detection, Intensity threshold**.
		Check to see if the retention time window for **Find Compounds** is set appropriately: **a** Select **Method > Parameters**. **b** Click the **Find** tab. **c** Click the **AutoMS(n)** subtab. **d** Set **Retention time window [min]** to base width of the chromatographic peaks.
		Check to see if the threshold for **Mass List Find** is set too high: **a** Select **Method > Parameters**. **b** Click the **Mass List** tab. **c** Make sure **Apex** is selected. **d** Click the **Apex** subtab. **e** Enter a smaller number for the **Absolute intensity threshold.**
	Incorrect settings for Mascot search	See "To use Mascot protein database search" on page 90.
	Chosen database did not give good results	Try searching a different database.
	Default settings for LC/MSD Trap data acquisition software not appropriate for sample	See the data acquisition troubleshooting tips under "If you have low database search scores or poor sequence coverage" on page 38 in Chapter 3.

Problem	Cause	Solution
	Problem with sample preparation	See the troubleshooting information in the *Sample Preparation Guide*.
Noisy spectra exported to Mascot		In Trap DataAnalysis, set a larger value for **Abundance cutoff [%]** for deconvolution. (The % refers to percent of the most intense peak in the mass spectrum.) **a** Select **Method > Parameters**. **b** Click the **Charge Deconvolution** tab. **c** Click the **Peptides/Small Molecules** subtab. **d** Set **Abundance cutoff [%]** to a higher value.

Tips

Threshold for Find Compounds

The intensity threshold for **Find Compounds** determines how many mass spectra are exported to Mascot. The appropriate threshold depends upon sample concentration and data acquisition parameters. To evaluate where to set the threshold, generate a total ion chromatogram containing all the MS^n data (**TIC, All MSn**). Set the threshold to a level that includes at least half the MS^n data points. The lower the threshold, the slower the data processing, but the better the chance for improved sequence coverage

Threshold for Mass List Find

Once you have located correlated MS and MS/MS spectra using **Find Compounds**, check to make sure the mass peaks are labeled. If mass peaks are not labeled, they will not be exported to Mascot. Check a few of your lower-intensity MS/MS spectra. Zoom in to see if the masses are labeled. You need to zoom in to check because the software prevents the labels from overlapping on your screen. If you observe good spectra (many fragments at intensities greater than the parent ion), but the mass peaks are not labeled (even when you zoom in), lower the threshold for **Mass List Find**.

9 Reference: Sample Analysis Strategy

This chapter provides an overview of three LC configurations for nanoflow LC/MS/MS and describes how they fit various sample analysis strategies. You learn when to use each configuration and how to plumb each one.

You can configure nanoflow LC/MS/MS for either one-dimensional liquid chromatography (1D LC) or two-dimensional liquid chromatography (2D LC). Use 2D LC for very complex protein digests or as a substitute for 2D gel electrophoresis. Otherwise, use 1D LC.

You can perform conventional 1D LC either with or without an enrichment column. (The Protein ID Chip is preconfigured for the enrichment column mode.) The choice of direct injection mode versus enrichment column mode depends upon sample characteristics, injection volume, chromatographic considerations, and peptide recovery needs.

Direct injection mode (1D LC)

The following discussion applies only for the Nanospray Protein Identification Solution. The direct injection mode is not applicable for the HPLC-Chip/MS Protein Identification Solution.

A diagram of the direct injection mode for the conventional nanoflow LC/MS/MS system is shown in Figure 24. The direct injection mode provides the best chromatography. There is no enrichment column to compromise peak resolution.

The direct injection mode also gives better sequence coverage and best sensitivity. You may lose peptides if you use the enrichment column. (The unrecovered peptides are generally those of lower chain length with less diagnostic value, but they may be important for your particular analysis.)

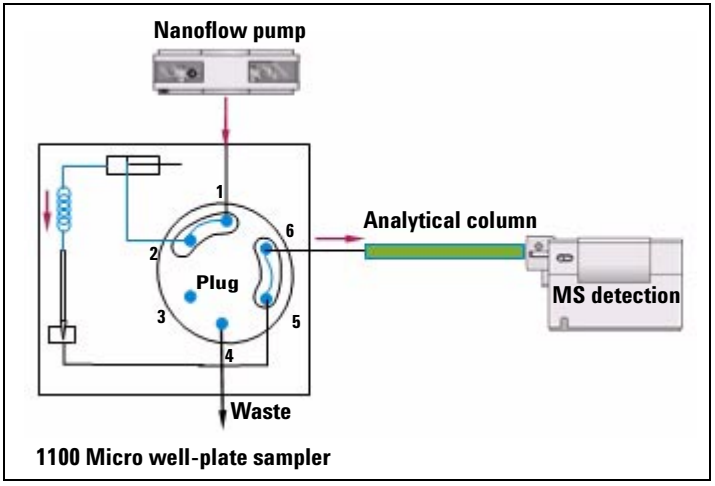


Figure 24 Direct injection mode

Enrichment column mode (1D LC)

The enrichment column is sometimes called a trapping column or a pre-column.

Flow diagrams for the conventional nanoflow LC/MS/MS system in enrichment column mode are shown in Figure 25 and Figure 26. Flow diagrams for the HPLC-Chip/MS system are similar, but some components are contained within the Protein ID Chip. For details on these microfluidic components, see the flow diagrams in the *Agilent 1100 Series G4240A HPLC-Chip/MS Cube User's Guide*.

You use the enrichment column mode to remove salts and other water-soluble contaminants from your sample. These are removed before the sample is backflushed from the enrichment column onto the analytical column. By removing these contaminants, the enrichment column helps to keep the system free of materials which can block narrow flow pathways and contribute to chemical background.

You also use the enrichment column mode for large-volume injections (several µL or greater) that would take a long time to load using nanoflow rates. The enrichment column has a larger internal diameter (id) than the analytical column and permits sample loading with a standard or capillary flow pump rather than a nanoflow pump.

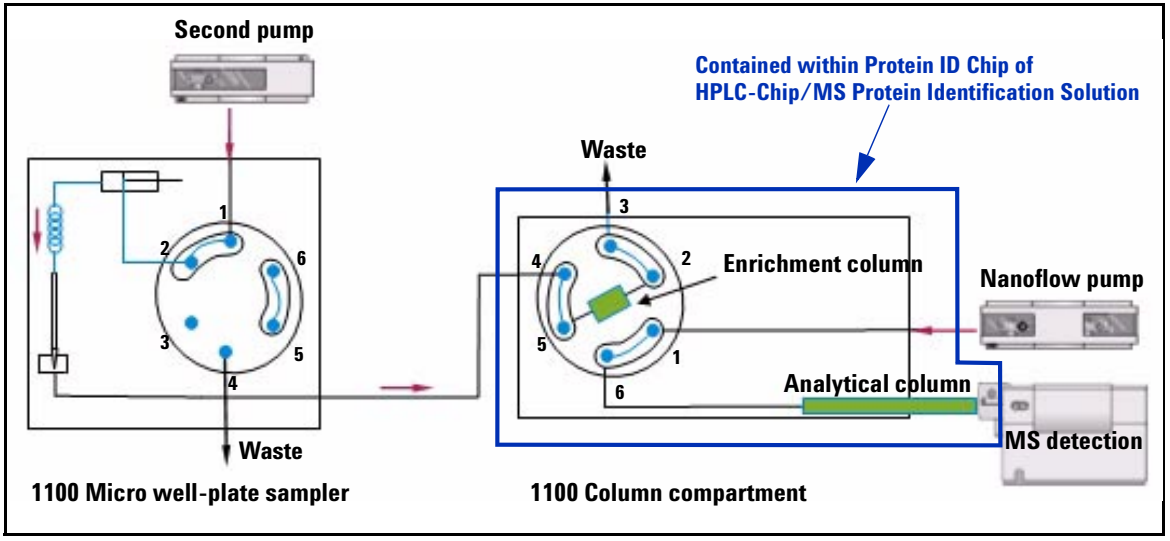


Figure 25 Enrichment column mode step 1: load sample on enrichment column

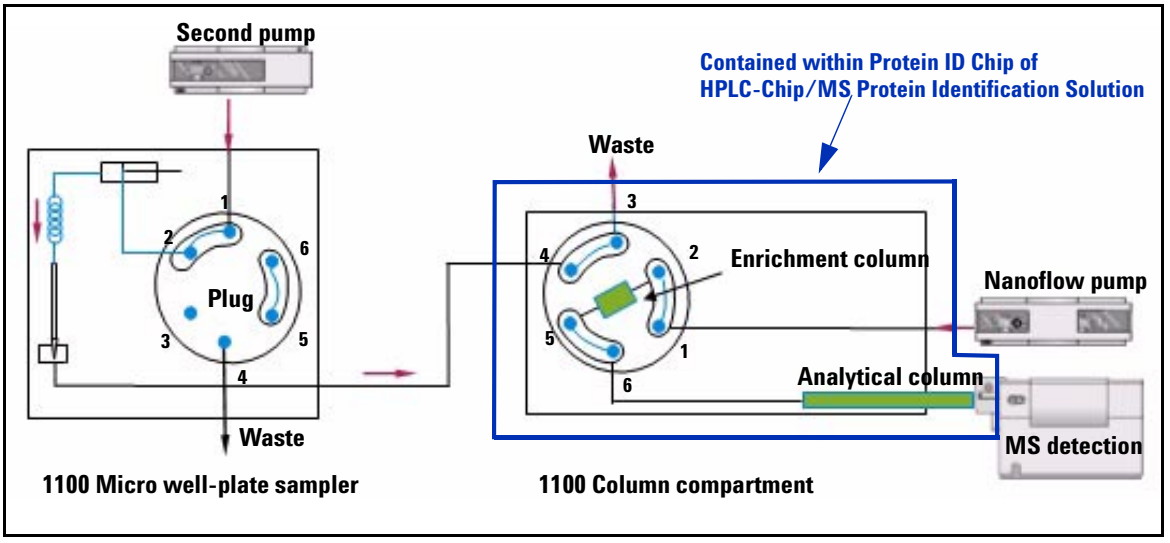


Figure 26 Enrichment column mode step 2: analyze sample

Two-dimensional liquid chromatography (2D LC)

You can configure nanoflow LC/MS/MS for two-dimensional liquid chromatography (2D LC) for instances where separation in a single dimension is insufficient. Two-dimensional LC adds a separation based on a different physical property. For example, if one of the separations is reversed-phase LC, then the second could be ion exchange, size exclusion, affinity, etc. Use two-dimensional LC for very complex protein digests or as a substitute for 2D gel electrophoresis.

A schematic of 2D LC is shown in Figure 27. In typical 2D LC for protein digests, you first separate the peptides on a strong cation exchange (SCX) column. You then further separate these fractions by reversed-phase LC. There are a number of configurations for the SCX separation:

- Offline SCX with fraction collection (described in Chapter 6)
- Online SCX with salt steps for sample elution (described in detail here)
- Online SCX with a semi-continuous salt gradient for sample elution (described in Agilent publication number 5989-0212EN)

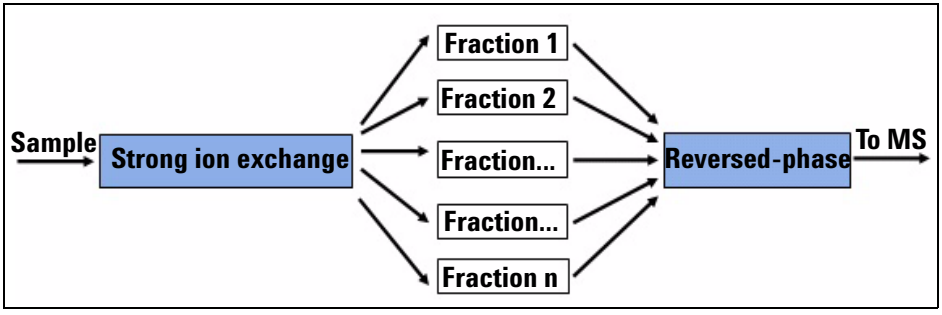


Figure 27 Two-dimensional LC concept

There are four steps for online two-dimensional LC with salt plugs. Flow diagrams for the conventional nanoflow LC/MS/MS system are shown in Figure 28 through Figure 31. Flow diagrams for the HPLC-Chip/MS system are similar, but some components are contained within the Protein ID Chip. For details on these microfluidic components, see the flow diagrams in the *Agilent 1100 Series G4240A HPLC-Chip/MS Cube User's Guide*.

In the first step of online 2D LC, you load the digest onto a strong cation exchange (SCX) column. (See Figure 28.) You use the Agilent 1100 well-plate sampler and a second (standard or capillary) LC pump for sample loading. The loading solvent is typically 96.9% water, 0.1% formic acid, and 3% acetonitrile. The enrichment column is in-line during sample loading to capture any peptides that are not retained on the SCX column.

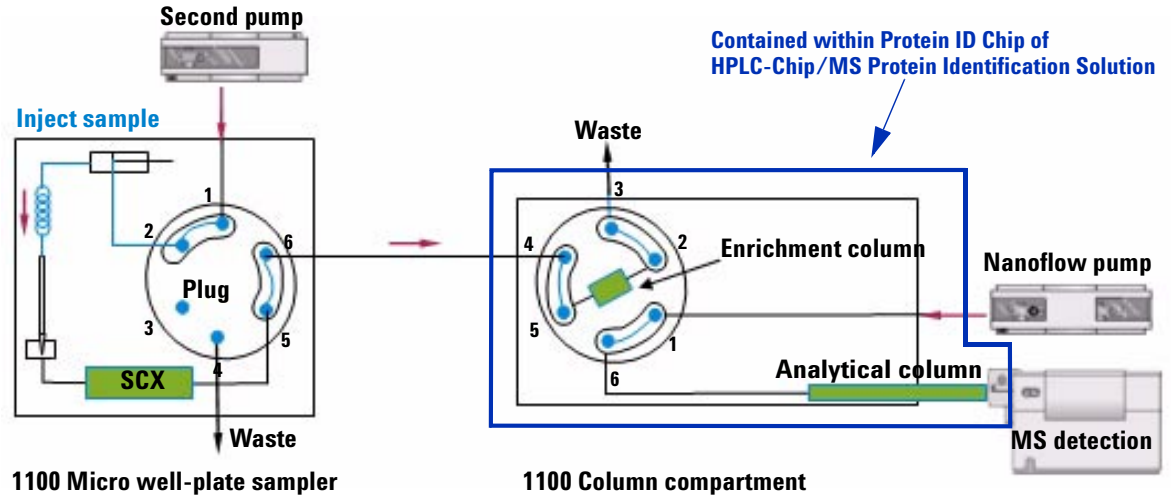


Figure 28 2D LC step 1: load sample on SCX column

In the second step (Figure 29), you elute the sample from the strong cation exchange column onto a C8/C18 enrichment column. You do this by injecting a sequential salt gradient with the autosampler. For LC/MS, the volatile salts (such as ammonium acetate and ammonium formate) are generally preferred. The salt step gradient typically consists of individual salt solutions ranging from 15 millimolar to 500 millimolar. These are injected in aliquots related to the size of the SCX column. For example, 20-µL aliquots are used for a 0.30 x 35 mm column. Each injected salt solution elutes a fraction of the peptides from the SCX column for analysis by nanoflow LC/MS/MS. The result is an individual data file for each step of the salt gradient.

You can optimize the spacing of the salt concentrations for your sample. Some samples fractionate more evenly with smaller concentration differences at the beginning of the stepwise gradient. For example, you might use 15, 30, 45, 60, 75, 90, 120, 300, and 500 millimolar concentrations, as opposed to concentrations spaced at 50 millimolar increments.

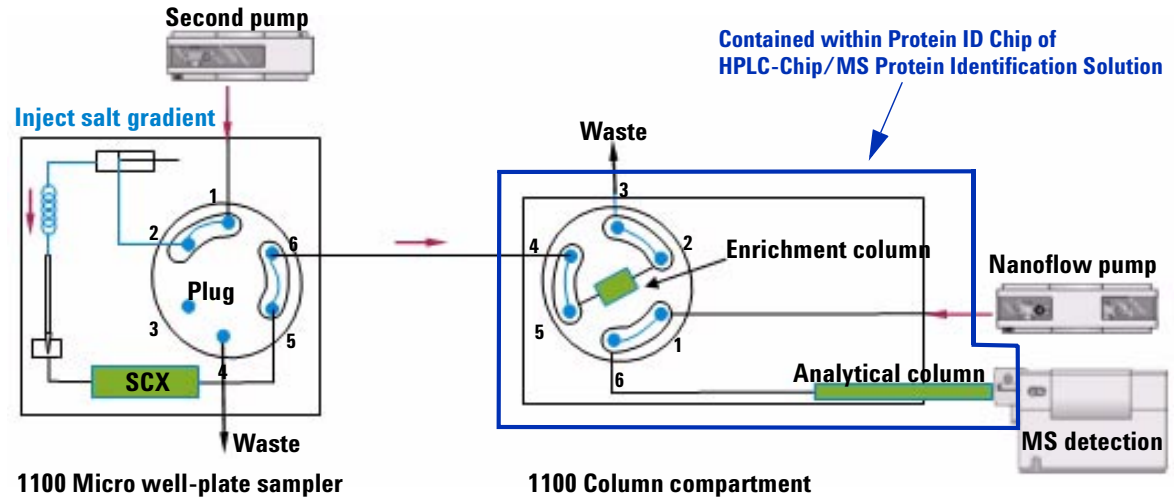


Figure 29 2D LC step 2: step-elute sample from SCX to enrichment column

In the third step, you wash and desalt the fraction on the enrichment column. To do this, you switch the six-port valve in the micro well-plate sampler and pump loading solvent or water with the second pump. (See Figure 30.)

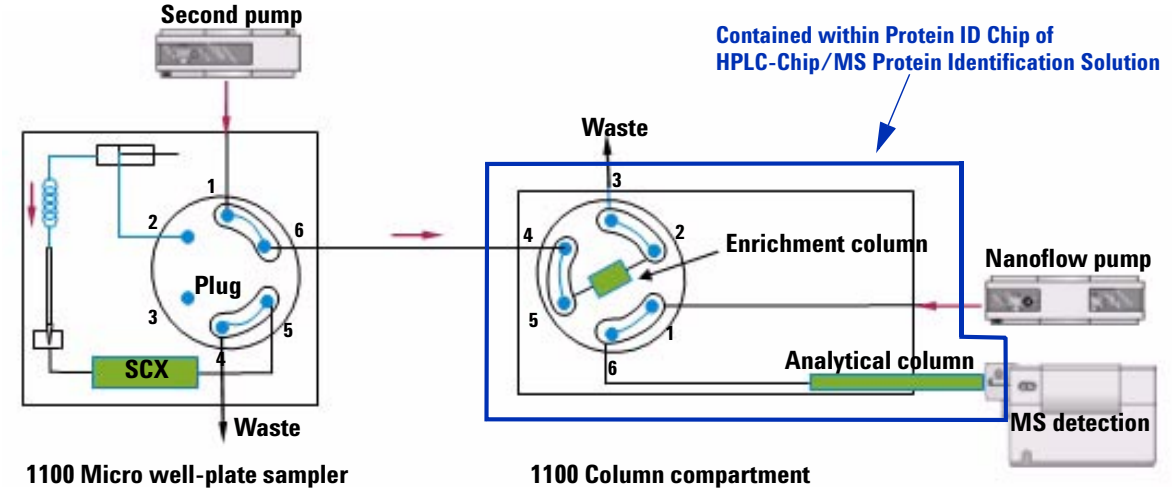


Figure 30 2D LC step 3: wash enrichment column

In the fourth step, you switch the nanoflow pump in-line, and backflush the peptide fraction off the enrichment column. You then analyze the fraction using nanoflow LC/MS/MS. (See Figure 31.)

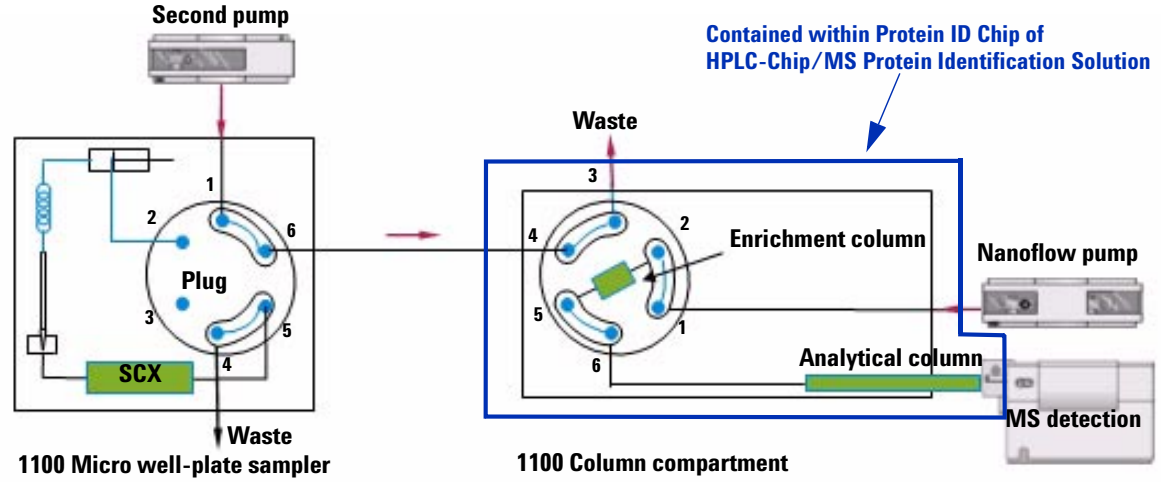


Figure 31 2D LC step 4: analyze sample

NOTE

The online and offline SCX protocols provided with the Nanospray and HPLC-Chip/MS Protein Identification Solutions are optimized for tryptic peptides. Because trypsin cleaves on the carboxy side of lysine and arginine, tryptic fragments most commonly have two charges, one for the terminal amino group and one for the basic lysine or arginine residue. With other enzymes, the number of charges will be different. Highly-charged peptides may not elute from the SCX column with salt steps alone. These may require that the pH be raised simultaneously to reduce the number of charges on these peptides. For highly-charged peptides, run a combination salt and pH gradient.

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In this Book

The *LC/MS Application Guide* presents the information you need to conduct the LC/MS steps for the Nanospray and HPLC-Chip/MS Protein Identification Solutions. In this guide you will learn:

- How to fractionate peptides by liquid chromatography
- How to perform MS/MS analyses of peptides
- How to analyze the resulting data with the Spectrum Mill MS Proteomics Workbench or the Mascot protein database search software.

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