Agilent Nanospray and HPLC-Chip/MS Protein Identification Solutions Quick Start Guide

An overview and quick reference for the Protein ID Solutions

Use this document for your first steps with the Protein ID Solutions, and as a quick reference after you have become familiar with the protocols.

What are the Nanospray and HPLC-Chip/MS Protein Identification Solutions?

The Nanospray and HPLC-Chip/MS Protein Identification Solutions are complete solutions for nanospray LC/MS/MS analysis of proteins from complex samples. They include standard and optional components you need to fractionate, digest, clean up, analyze, and identify the proteins from biological samples. The next page shows a diagram of these solutions.

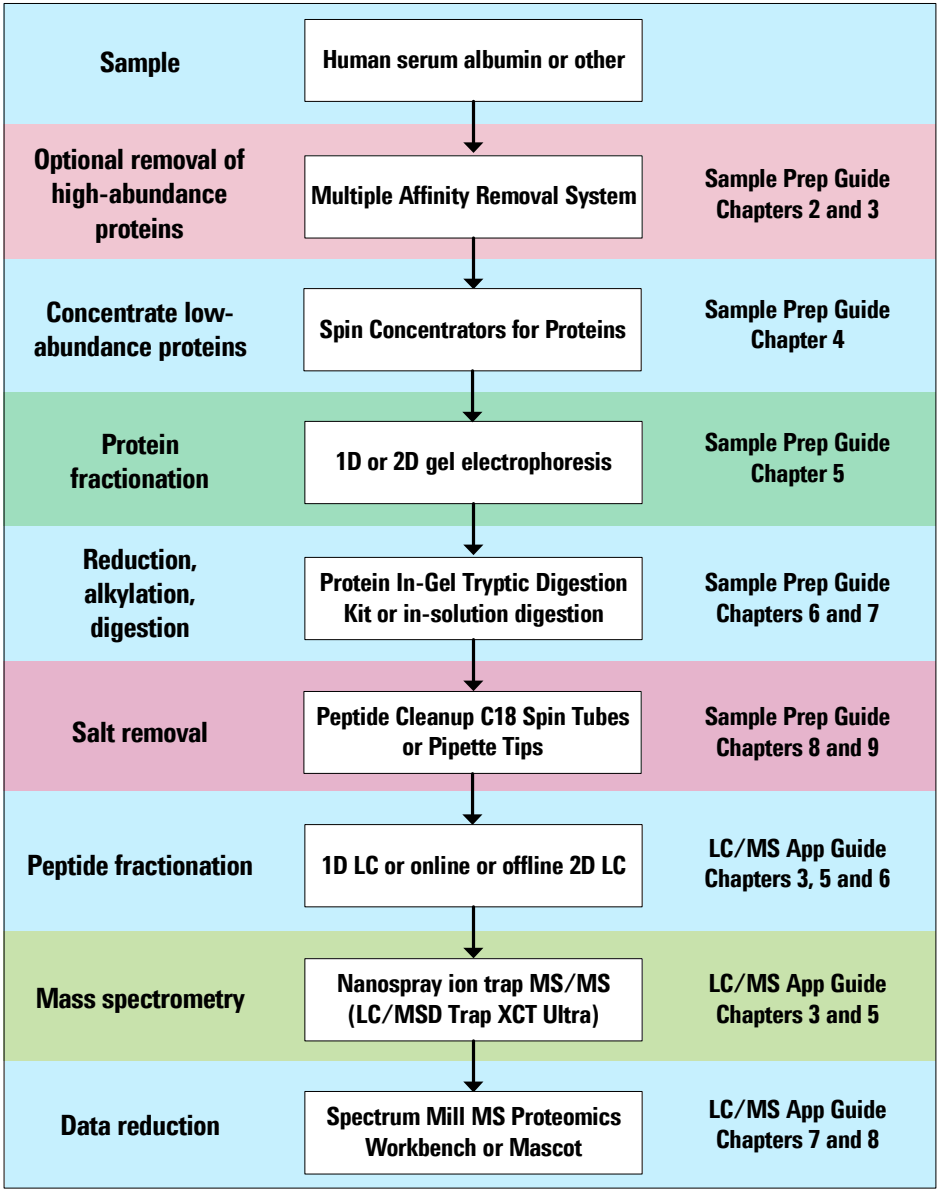
The fundamental difference between the Nanospray Protein Identification Solution and the HPLC-Chip/MS Protein Identification Solution is that the former uses a conventional nanoflow HPLC system, while the latter uses a microfluidic HPLC-Chip.

What documentation is available?

This *Quick Start Guide* includes summaries of the protocols, a list of part numbers for consumables, a list of common laboratory equipment and supplies you should have on hand, and a safety overview.

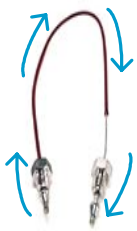
In addition to this *Quick Start Guide*, the Protein ID Solutions include a *Sample Preparation Guide* that provides details for all the protocols to prepare your samples, as well as an *LC/MS Application Guide* that covers peptide fractionation, MS/MS analysis, and data analysis. These guides include a familiarization

exercise that takes you through the entire sample preparation and protein identification process. Agilent also provides manuals with each of the instrument modules you use for these solutions.



Optional Multiple Affinity Removal Columns

1. If organic in LC, purge with water + inject water



1 mL/min, 15-20 min
Do not install column yet

2. Set up Buffer A and Buffer B

Do not install column yet

3. Purge lines with Buffer A and Buffer B

1 mL/min, 10 min

4. Set up LC timetable and run 2 method blanks

Time	%B
xxxx	xx
xxxx	xx
xxxx	xx
xxxx	xx

See Table 1 on next page

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5. Attach column and equilbrate in Buffer A

1 mL/min, 4 min

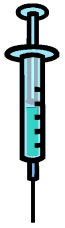
6. Dilute sample

See Table 1 on next page

7. Remove particulates with a 0.22 µm spin filter

1 min, 16,000 g

8. Inject the diluted serum into the LC



See Table 1 on next page

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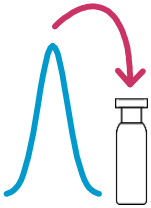
9. Collect flow-thru fraction and store at -20C



See Table 1 on next page

10. Elute bound proteins with Buffer B

See Table 1 on next page

11. Regenerate column with Buffer A

See Table 1 on next page

12. Repeat steps 6 - 11 for each sample

Table 1 Detailed steps for 50- and 100-mm affinity removal columns

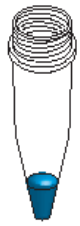
Step	50 mm column	100 mm column
4	Inject 100 µL Buffer A.	Inject 200 µL Buffer A.
6	p/n 5188-5332: Dilute human serum 4 times, e.g., 30 µL serum + 90 µL Buffer A	p/n 5188-5333: Dilute human serum 4 times, e.g., 80 µL serum + 240 µL Buffer A
	p/n 5185-5984: Dilute human serum 5 times, e.g., 15 µL serum + 60 µL Buffer A	p/n 5185-5985: Dilute human serum 5 times, e.g., 30 µL serum + 120 µL Buffer A
	p/n 5188-5217: Dilute mouse/rat serum 5 times, e.g., 40 µL serum + 160 µL Buffer A	p/n 5188-5218: Dilute mouse/rat serum 5 times, e.g., 90 µL serum + 360 µL Buffer A
8	p/n 5188-5332: Inject 120 to 160 µL p/n 5185-5984: Inject 75 to 100 µL p/n 5188-5217: Inject 200 to 225 µL	p/n 5188-5333: Inject 320 to 400 µL p/n 5185-5985: Inject 150 to 200 µL p/n 5188-5218: Inject 450 to 500 µL
9	Peak at 1.5 to 4.5 min for human column Peak at 3 to 6 min for mouse/rat column	Peak at 2.5 to 6.0 min for human column Peak at 3 to 7 min for mouse/rat column
10	Elute for 3.5 min.	Elute for 7.0 min.
11	Equilibrate for 7.4 min.	Equilibrate for 11 min.

Table 2 Detailed dilution step for affinity removal cartridges

Cartridge for human samples - high capacity (5188-5341)	Cartridge for human samples - standard capacity (5188-5230)	Cartridge for mouse/rat samples (5188-5289)
Dilute 14 to 16 µL serum to 200 µL with Buffer A. Example: 14 µL serum + 186 µL Buffer A	Dilute 7 to 10 µL serum to 200 µL with Buffer A. Example: 8 µL serum + 192 µL Buffer A	Dilute 25 to 30 µL serum to 200 µL with Buffer A. Example: 30 µL serum + 170 µL Buffer A

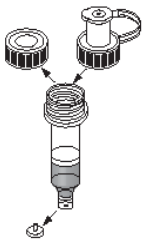
Optional Multiple Affinity Removal Spin Cartridges

1. Dilute and filter sample



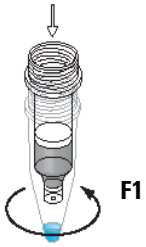
Dilute. (See Table 2 on previous page)
Filter.

2. Prepare spin cartridge



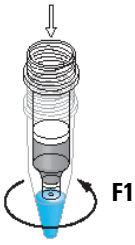
4 mL Buffer A

3. Apply sample



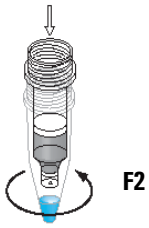
200 µL diluted serum
Centrifuge 1.5 min at 100 × g

4. Wash and collect flow-through F1



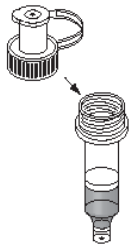
400 µL Buffer A
Centrifuge 2.5 min at 100 × g

5. Wash and collect flow-through F2

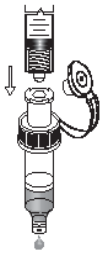


400 µL Buffer A
Centrifuge 2.5 min at 100 × g

6. Prepare for elution

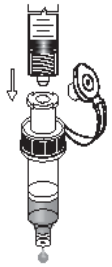


7. Elute bound fraction



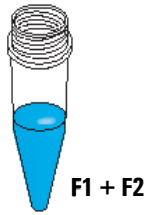
2 mL Buffer B
Save or discard

8. Re-equilibration



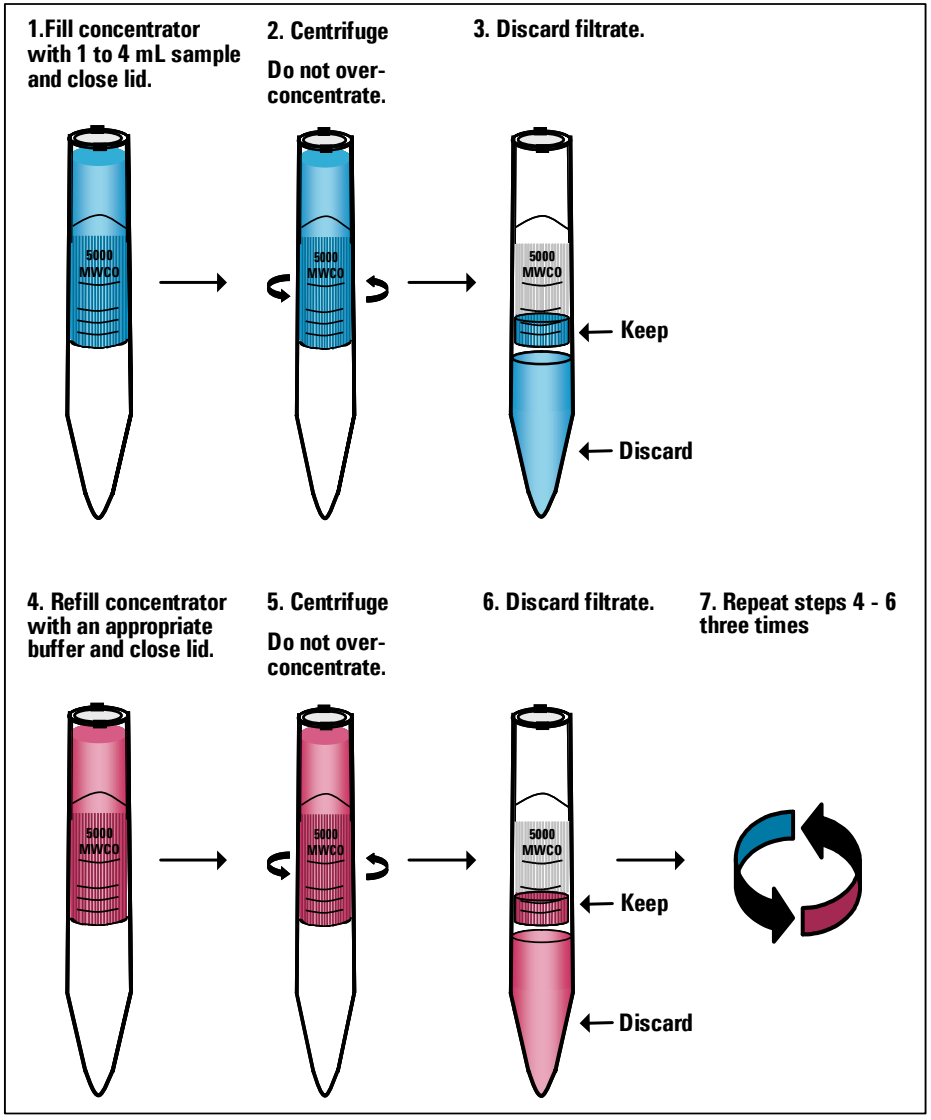
4 mL Buffer A

9. Analyze F1 and F2

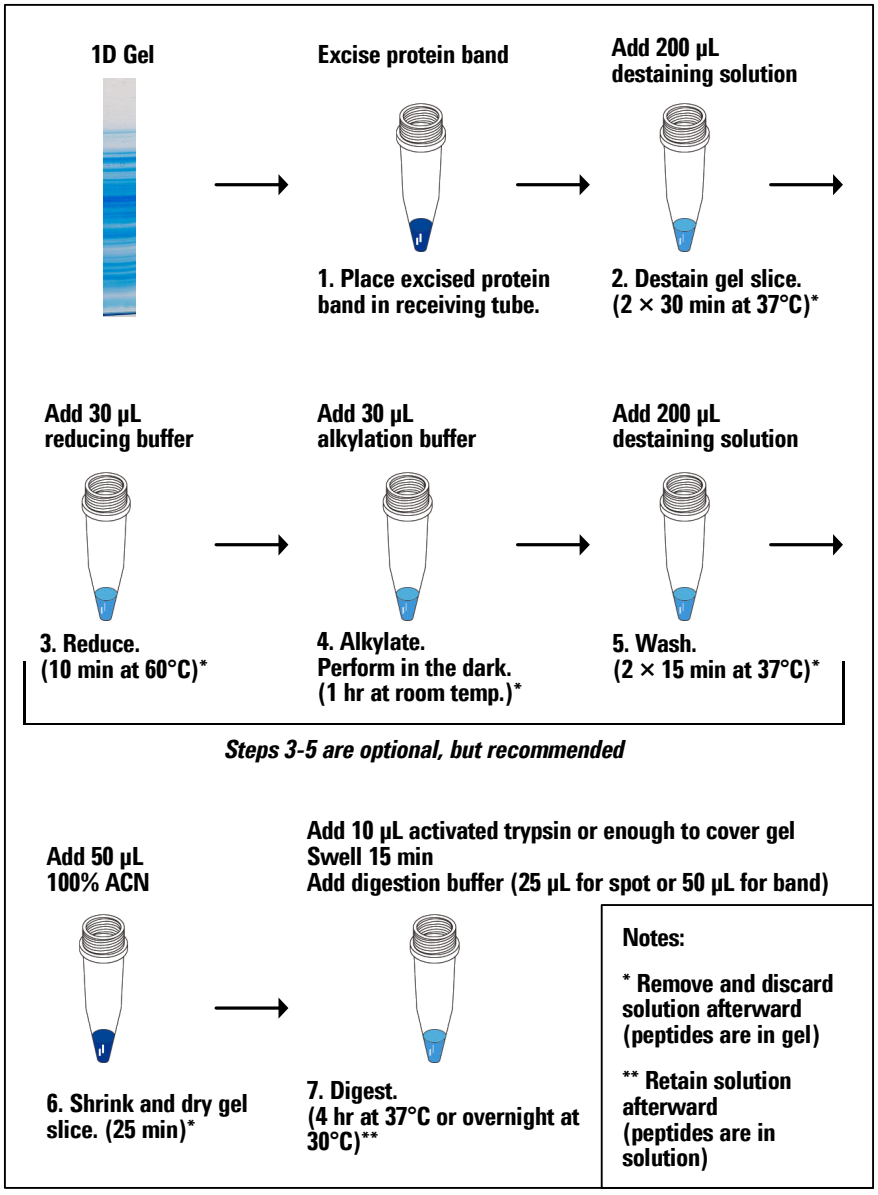


Individually or combined
Concentrate first

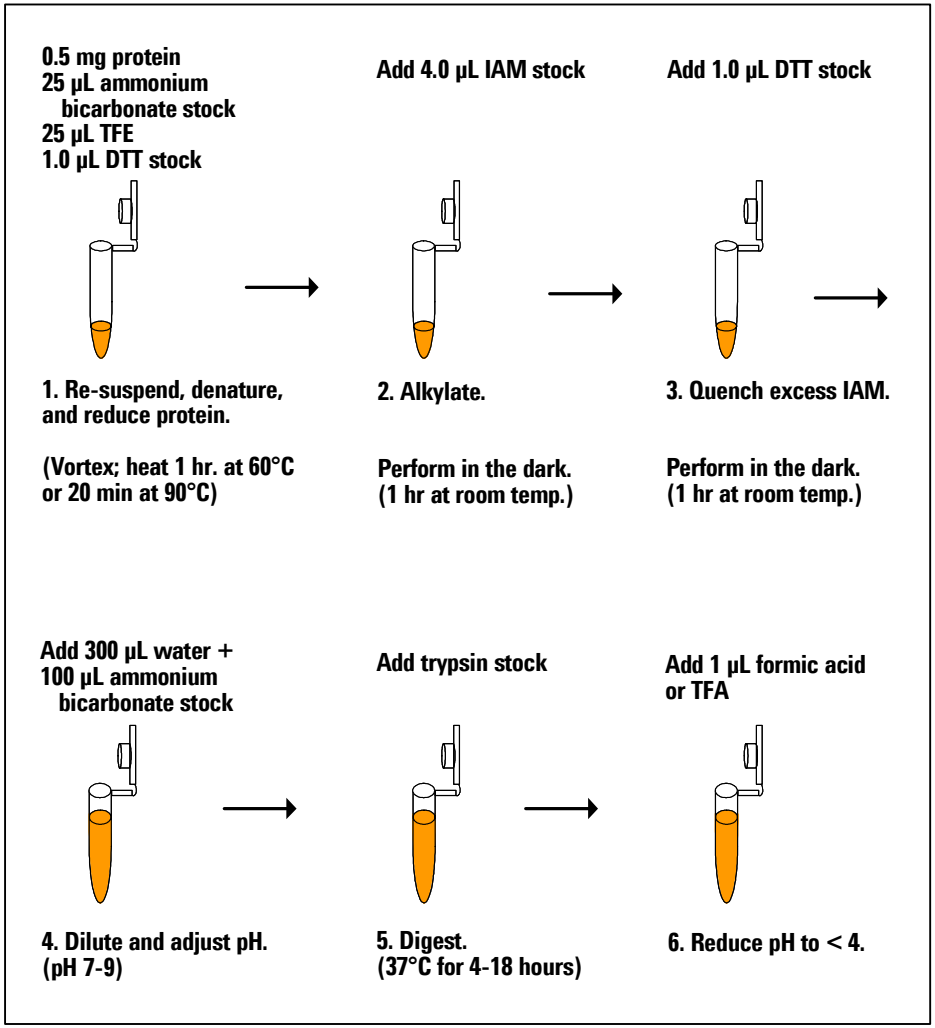
Agilent Spin Concentrators for Proteins



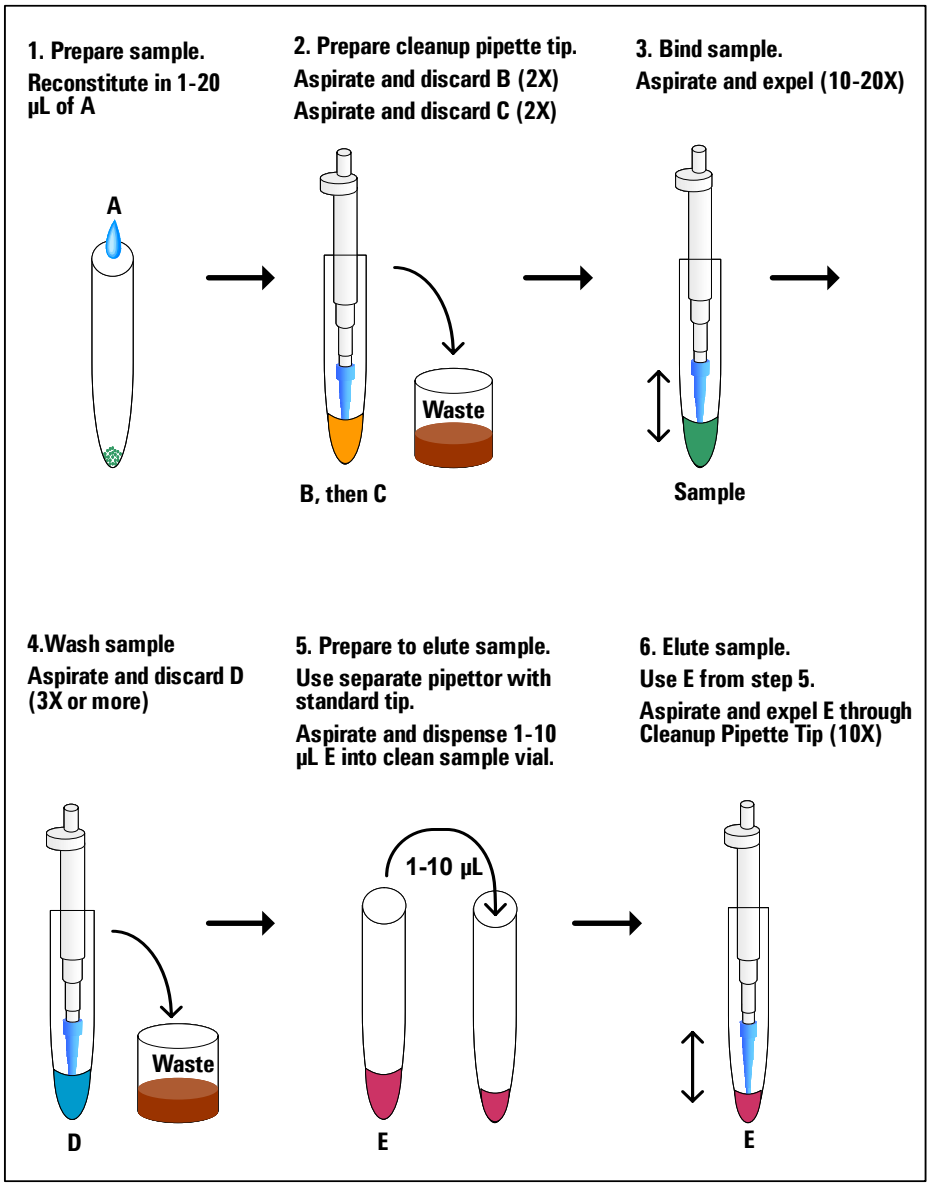
Agilent Protein In-Gel Tryptic Digestion Kit



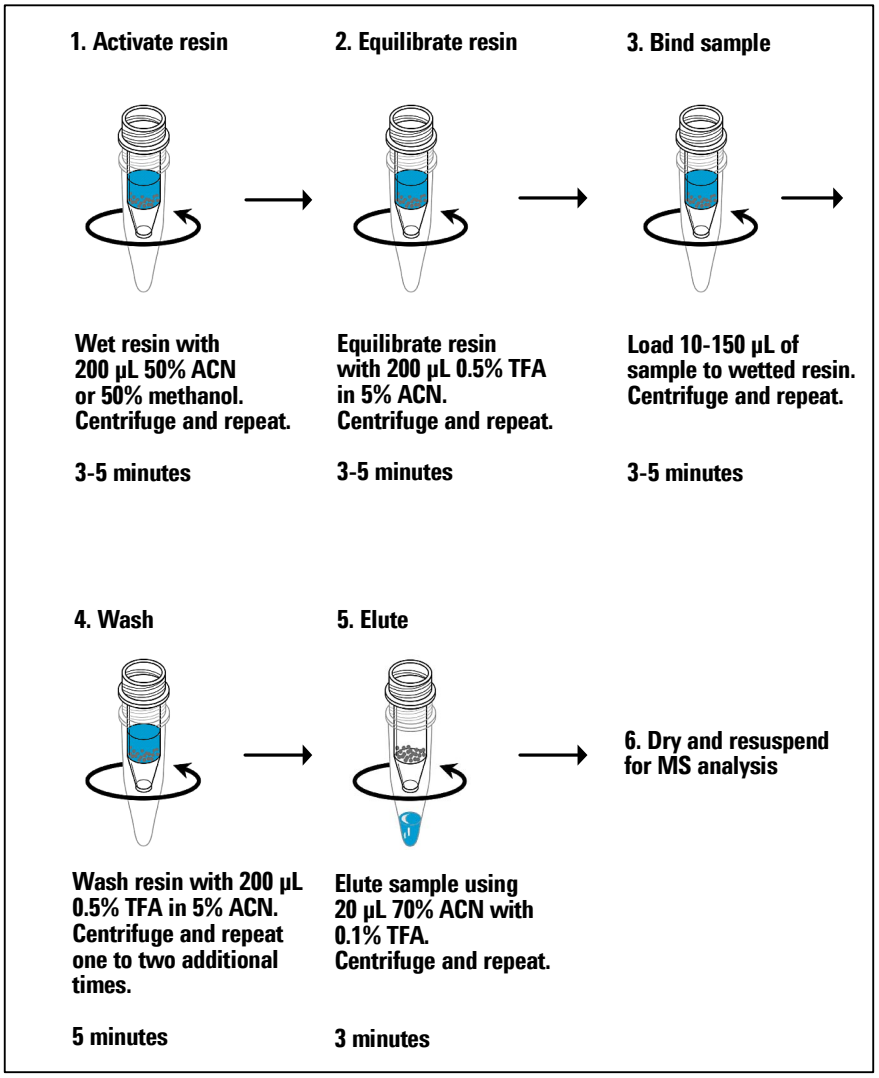
In-Solution Tryptic Digestion



Agilent Peptide Cleanup C18 Pipette Tips

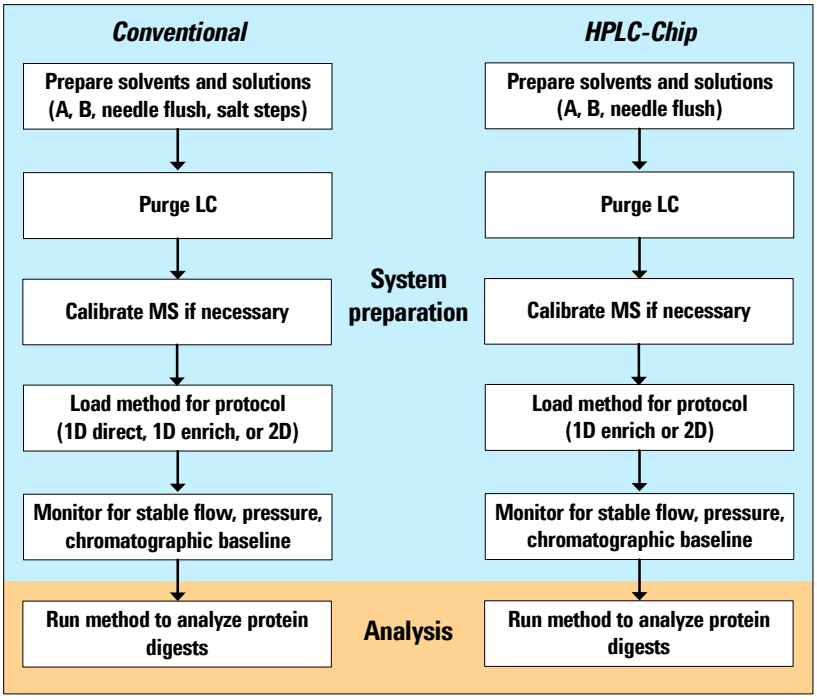


Agilent Peptide Cleanup C18 Spin Tubes



Online 1D and 2D LC/MS/MS

Summary of steps



Summary of installation of nanospray needle for conventional system

Insertion of C18 particles to prevent needle blockage

1. Prepare suspension of C18 particles.
2. Dip the blunt end of a nanospray needle into the suspension of C18 particles for only 1 second.

Needle installation

1. Insert the blunt end of the needle through the nanospray needle nut and conductive ferrule.
2. Connect the fitting directly to the outlet of the LC column, as shown in Figure 1.
3. Tighten the needle *very gently*.
4. Verify that the LC solvent continues to flow out of the needle.

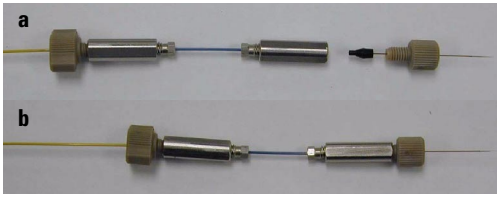


Figure 1 Nanospray needle installation

Installation of assembly into nanospray source

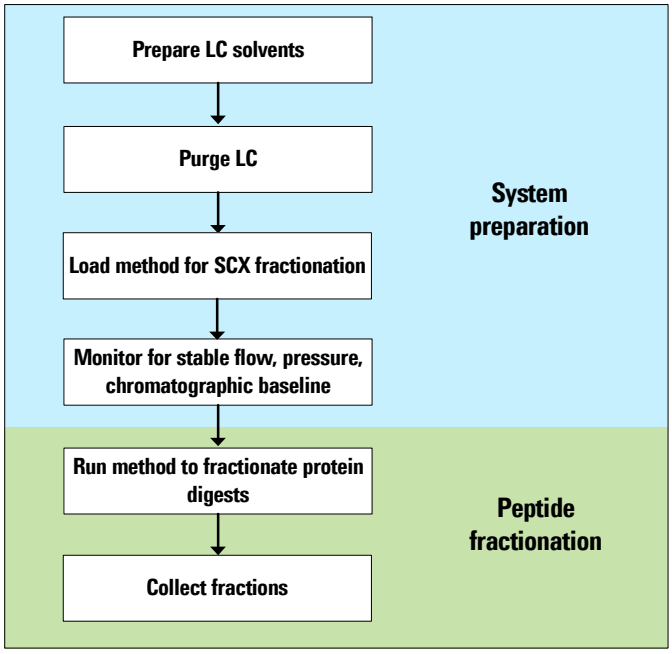
1. Make sure the capillary voltage is set to 1400 V.
2. Insert the needle / column assembly into the needle holder assembly mount on the nanospray source. See Figure 2.

Figure 2 Needle holder in needle holder assembly

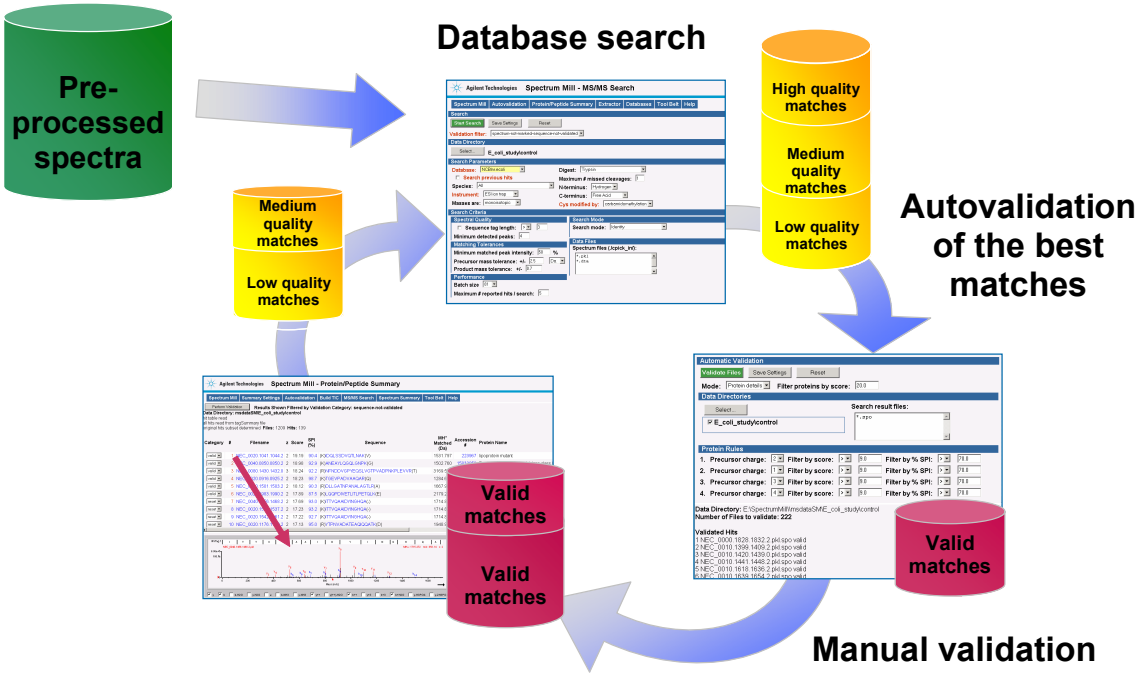
Needle positioning and starting of spray

1. With the needle assembly installed, use the microscope to position the needle within 3 mm of the flat electrode (modified capillary cap).
2. Start the spray with caution:
   - **a** Verify that the flow rate is 300 nL/min, that the solvent composition is 97:3 A1:B1, and that the dry gas is set at 300 °C.
   - **b** Be sure that a droplet has formed at the needle tip or that a stream has started.
   - **c** 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.
   - **d** Adjust the capillary voltage until a stable current is reached (typically 80 to 100 nA). Do not exceed 2200 V. A typical voltage with a new needle is 1600 to 1700 V.
3. Save the method to store the new **Vcap** setting.

Optional Offline SCX

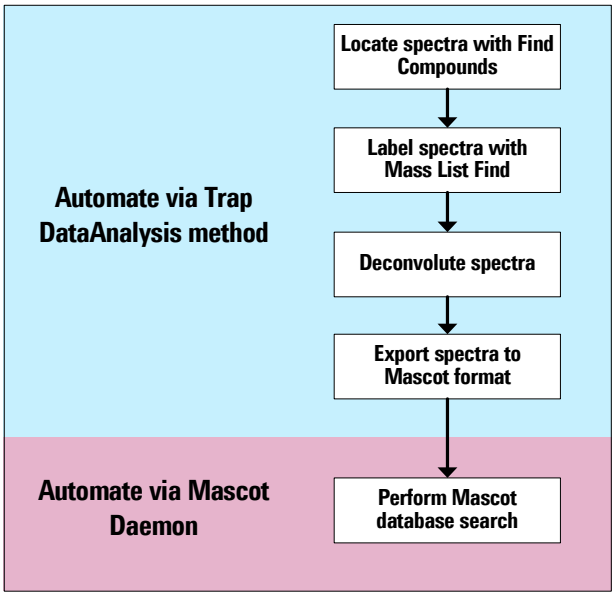


Spectrum Mill MS Proteomics Workbench



1. Copy or move data to the Spectrum Mill server.
2. Preprocess the raw data files with the Data Extractor.
3. Search a database, preferably a species subset database for the first search.
4. Autovalidate the results with the highest scores.
5. (Optional) Manually review and validate additional results.
6. Search the spectra that have not been validated:
   - In variable mode (homology mode for versions prior to 3.02) against previous protein hits
   - In no enzyme mode against previous protein hits
   - In identity mode against a larger database
7. Continue to perform iterative cycles of database search and validation to identify as many proteins as desired.
8. Use Spectrum Summary to examine the remaining unmatched spectra to determine if there are high-quality spectra.
9. Perform *de novo* sequencing on high-quality spectra.

Mascot Protein Database Search



1 Add DataAnalysis method **Auto_MIS_MgfExport.ms** to your method for 1D or 2D LC/MS/MS analysis. This adds a Visual Basic script that:

   a Uses **Find Compounds** to locate and hierarchically organize related MS and MS/MS spectra

   b Uses **Mass List Find** to label spectra with masses

   c Performs (optional) charge deconvolution

   d Exports spectra to Mascot format (*.mgf file).

2 Perform Mascot database search at www.matrixscience.com or via Mascot Daemon.

Part numbers for Agilent consumables

Part number	Product name	Product description
5188-5332	High Capacity Multiple Affinity Removal Column Hu-6HC, 4.6 × 50 mm, 30 to 40 µL human serum capacity per injection	Removes human albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin
5188-5333	High Capacity Multiple Affinity Removal Column Hu-6HC, 4.6 × 100 mm, 80 to 100 µL human serum capacity per injection	Removes human albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin
5185-5984	Multiple Affinity Removal Column Hu-6, 4.6 × 50 mm, 15 to 20 µL human serum capacity per injection	Removes human albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin
5185-5985	Multiple Affinity Removal Column Hu-6, 4.6 × 100 mm, 30 to 40 µL human serum capacity per injection	Removes human albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin
5188-5217	Multiple Affinity Removal Column Ms-3, 4.6 × 50 mm, 37 to 50 µL mouse serum capacity per injection	Removes mouse/rat albumin, IgG, and transferrin
5188-5218	Multiple Affinity Removal Column MS-3, 4.6 × 100 mm, 75 to 100 µL mouse serum capacity per injection	Removes mouse/rat albumin, IgG, and transferrin
5185-5987	Buffer A, 1 liter	Ready-to-use, optimized buffer for loading, washing and equilibrating affinity columns and spin cartridges
5185-5988	Buffer B, 1 liter	Ready-to-use, optimized buffer for elution of bound proteins from affinity columns and spin cartridges
5185-5990	Spin filters, 0.22 µm, 1 pack of 25	For sample cleanup before loading affinity column or spin cartridge
5185-5991	Concentrators, 4 mL, 5 kDa MWCO, 1 pack of 25	For concentrating flow-through fractions from affinity column or spin cartridge
5188-2798	Concentrators, 15 mL, 5 kDa MWCO, 1 pack of 10	For concentrating flow-through fractions from affinity column

Part number	Product name	Product description
5185-5989	Human serum albumin (HSA) standard, 20 mg/mL, 1 mL (for Human Multiple Affinity Removal Columns) in Buffer A	Dilute standard for checking column capacity and for familiarization exercise
5185-5995	Replacement end fitting with frit for column (1 each)	Replaced when column frits are clogged. Replace inlet and outlet fittings at the same time.
5185-5986	Starter Reagent Kit for Affinity Columns Buffer A: 2 ×1 liter Buffer B: 1 liter Spin filters 0.22 µm: 2 packs of 25 Protein concentrators: 1 pack of 25	Reagent kit that should last for approximately 200 injections for 4.6 x 50 mm columns, and 100 injections for 4.6 x 100 mm columns
5188-5341	High Capacity Multiple Affinity Removal Spin Cartridge Hu-6HC, 0.45 mL, 1 each, for human fluids, 14 to 16 µL serum capacity per use	Removes human albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin
5188-5230	Multiple Affinity Removal Spin Cartridge Hu-6, 0.45 mL, 1 each, for human fluids, 7 to 10 µL serum capacity per use	Removes human albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin
5188-5289	Multiple Affinity Removal Spin Cartridge Ms-3, 0.45 mL, 1 each, for mouse/rat fluids, 25 to 30 µL serum capacity per use	Removes mouse/rat albumin, IgG, and transferrin
5188-5249	Luer-Lock adapters, pack of 2	Allows attachment of Luer-Lock syringes to spin cartridge
5188-5250	5-mL plastic Luer-Lock syringes, 1 pack of 2	For dispensing the washing, eluting, and re-equilibrating buffers through spin cartridge
5188-5251	1.5-mL screw-top microtubes, 1 pack of 100	Eppendorf-style tubes used to collect fractions from spin cartridge
5188-5252	Spin cartridge screw caps and plugs, 1 pack of 6	Extra caps and plugs to seal the top and bottom of affinity spin cartridges
5188-5253	Teflon Luer-Lock needles, 1 pack of 10	For transferring solutions with Luer-Lock syringes

Part number	Product name	Product description
5185-5254	Starter Reagent Kit for Affinity Spin Cartridges • Buffer A: 1 liter • Buffer B: 1 liter • Spin filters 0.22 µm: 2 packs of 25 • Protein concentrators: 1 pack of 25 • Luer-Lock adapters: 1 pack of 2 • 1.5-mL microtubes: 6 packs of 100 • Spin cartridge extra caps and plugs, 1 pack of 6 • Teflon Luer-Lock needles, 1 pack of 10	Reagent kit that should last for approximately 200 spin cartridge uses
5188-2749	Tryptic In-Gel Digestion Kit	For reduction, alkylation and in-gel digestion
5188-2750	Peptide Cleanup C18 Spin Tubes	For sample cleanup prior to MS analysis (for greater sample loads, e.g., in-solution digests)
5188-5239	Peptide Cleanup C18 Pipette Tips	For sample cleanup prior to MS analysis (for smaller sample loads, e.g., gel spots)
5065-9913	ZORBAX 300SB-C18, 0.3 x 5 mm, 5 µm particle size	Enrichment column for 1D and 2D LC/MS/MS analyses
5065-9911	ZORBAX 300SB-C18, 75 µm x 150 mm, 3.5 µm particle size	Reversed-phase column for 1D and 2D LC/MS/MS analyses
5065-9924	ZORBAX 300SB-C18, 75 µm x 50 mm, 3.5 µm particle size	Reversed-phase column for 1D and 2D LC/MS/MS analyses
5065-9912	ZORBAX BioSCX Series II, 0.30 x 35 mm, 3.5 µm particle size	Column for online SCX and offline SCX with fraction collection
5065-9942	ZORBAX BioSCX Series II, 0.80 x 50 mm, 3.5 µm particle size	Column for offline SCX with fraction collection, for higher sample loads
G1946-85021	Ammonium formate stock solution, 5 M, 6 ampoules, 2.2 mL each	For preparation of solutions for salt steps
9301-0978	Plastic conical micro-vials	For MS analysis
5182-0541	Caps for micro-vials	For MS analysis
5042-8502	Eppendorf well plates, 96-well, 150 µL, skirted	For fraction collection and MS analysis

Part number	Product name	Product description
9301-6378	Nanospray needles	Needles for orthogonal nanospray source
0100-2262	Nanospray ferrules	Ferrules for installation of nanospray needles
8500-2236	Water, 4 L	For HPLC and solution preparation
G2453-85050	Acetonitrile, 1 L	For HPLC and solution preparation
G2453-85060	Formic acid, 5 mL	For HPLC and solution preparation
G4240-62001	Protein ID Chip	Microfluidic chip that integrates enrichment column, analytical column, nanospray tip, and connections between them
G4240-61001	MS Calibration and Diagnosis Chip	Microfluidic chip for infusion of MS calibrant or samples that do not require chromatographic separation

Related Agilent products

See the following list for related Agilent products.

Product number	Description
5188-2747	**Lys Tag 4H Kit for MALDI-MS**, mass tagging reagent that improves sensitivity and mass spectral quality for lysine-containing peptides in MALDI-MS analysis
5188-2748	**Mass Tagging Accessories Kit**, kit containing products used in conjunction with mass tagging reagents for preparing samples for MS analysis (includes Peptide Cleanup C18 Pipette Tips, Tryptic Digestion Kit, MALDI matrix, and a peptide standard)
G2037A	**Alpha-Cyano-4-Hydroxycinnamic Acid**, MALDI Matrix, 3 x 3 mL

Necessary laboratory equipment and supplies

While the Nanospray and HPLC-Chip/MS Protein Identification Solutions include most of the instrumentation, reagents, columns, and supplies you need for protein identification, there are some common laboratory items that you must supply. These are listed below.

Item	Where needed
Centrifuge capable of 7500 x *g*, up to 15-mL tubes	Multiple Affinity Removal Columns, spin concentrators, spin tubes
HPLC, including binary pump, autosampler, and UV detector	Multiple Affinity Removal Columns
Microcentrifuge with adjustable centrifugal force (capable of spinning at 100 x g) and timer	Multiple Affinity Removal Spin Cartridges
50-mL vessels (for example, polypropylene tubes)	Multiple Affinity Removal Spin Cartridges
Transfer pipettes	Multiple Affinity Removal Spin Cartridges
600-µL microcentrifuge tubes (receiver tubes)	In-gel digestion
50-mL capped bottle or equivalent	In-gel digestion
10-mL storage bottle, tube or equivalent	In-gel digestion
HPLC grade water (18 megohm)	In-gel digestion, spin tubes, cleanup pipette tips, nanoflow LC/MS/MS, offline SCX
Micropipettors and tips: 1 to 1000 µL range	Multiple Affinity Removal Columns, Multiple Affinity Removal Spin Cartridges, in-gel digestion, in-solution digestion, spin tubes, cleanup pipette tips
Micropettor tips - thin gel loader type	Spin concentrators, in-gel digestion
Tube heater/shaker	In-gel digestion, in-solution digestion
Graduated cylinders	Spin tubes, in-gel digestion
Analytical balance	In-gel digestion, in-solution digestion, spin tubes
Vortex	In-solution digestion

Item	Where needed
Dithiothreitol (DTT), >99+%	In-solution digestion
Trifluoroethanol (TFE), 99+%	In-solution digestion
Eppendorf Safe-Lock microcentrifuge tubes, natural, not siliconized, 0.5 mL and 1.5 mL	In-solution digestion
pH indicator strips, pH ranges 2.5 to 4.5 and 7.0 to 9.0	In-solution digestion
Trifluoroacetic acid (TFA), sequencing grade	In-solution digestion, spin tubes, cleanup pipette tips
1.5-mL microcentrifuge tubes	Spin tubes, nanoflow LC/MS/MS
Methanol, HPLC grade (optional)	Spin tubes
Formic acid, analytical grade	In-solution digestion, spin tubes, nanoflow LC/MS/MS, offline SCX
Vacuum evaporator	Spin tubes
Sample vials or tubes	Cleanup pipette tips

Safety

WARNING **When preparing biological samples using the Nanospray and HPLC-Chip/MS Protein Identification Solutions, follow safety guidelines for handling biological and chemical materials and wear protective eyewear and gloves.**

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

NOTE For Material Safety Data Sheets (MSDSs) and Certificates of Analysis, visit www.agilent.com/chem/msds.

www.agilent.com

In this Book

The *Quick Start Guide* presents an overview of the protocols used with the Agilent Nanospray and HPLC-Chip/MS Protein Identification Solutions.

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Agilent Technologies