Agilent MALDI Protein Identification Solution

MS Application Guide

Agilent Technologies

Notices

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In This Guide...

The *MS Application Guide* presents the information you need to conduct the instrumental analyses for the Agilent MALDI Protein Identification Solution. In this guide you will learn:

- How to optionally fractionate peptides by liquid chromatography with MALDI plate spotting (LC MALDI)
- How to prepare and analyze samples by MALDI MS/MS
- How to analyze the resulting data with the Spectrum Mill MS Proteomics Workbench or the Mascot protein database search software

1 Overview of MALDI MS with the MALDI Protein Identification Solution

Get an overview of the entire process for optional peptide fractionation, MALDI MS analysis, and translation of spectra to protein identifications.

2 MALDI Sample Preparation and Manual Plate Spotting

Learn how to dilute peptide samples, mix them with MALDI matrix, and spot them onto a MALDI target plate.

3 Peptide Analysis with MALDI MS/MS

Follow the protocol to perform MALDI MS/MS analyses.

4 Optional LC MALDI

Learn how to fractionate peptides by reversed-phase liquid chromatography, and how to automatically spot the fractions onto a MALDI plate.

5 Data Analysis with Spectrum Mill MS Proteomics Workbench

Follow a general Spectrum Mill workbench protocol to process MALDI MS/MS data to generate tables of protein identifications.

Contents

1 Overview of MALDI MS with the MALDI Protein Identification Solution

Figure 1 gives an overview of the matrix-assisted laser desorption/ionization (MALDI) mass spectrometric (MS) analysis portion of the Agilent MALDI Protein Identification Solution. The applicable chapters from this *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*.

This guide describes how peptides are optionally fractionated by liquid chromatography Chapter 4), how they are mixed with matrix and spotted onto a MALDI plate (Chapter 2 and Chapter 4), and how they are analyzed by MALDI MS/MS (Chapter 3). This guide also describes how the MS/MS spectra are then searched against protein databases using either the Spectrum Mill MS Proteomics Workbench (Chapter 5) or Mascot (Chapter 6).

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

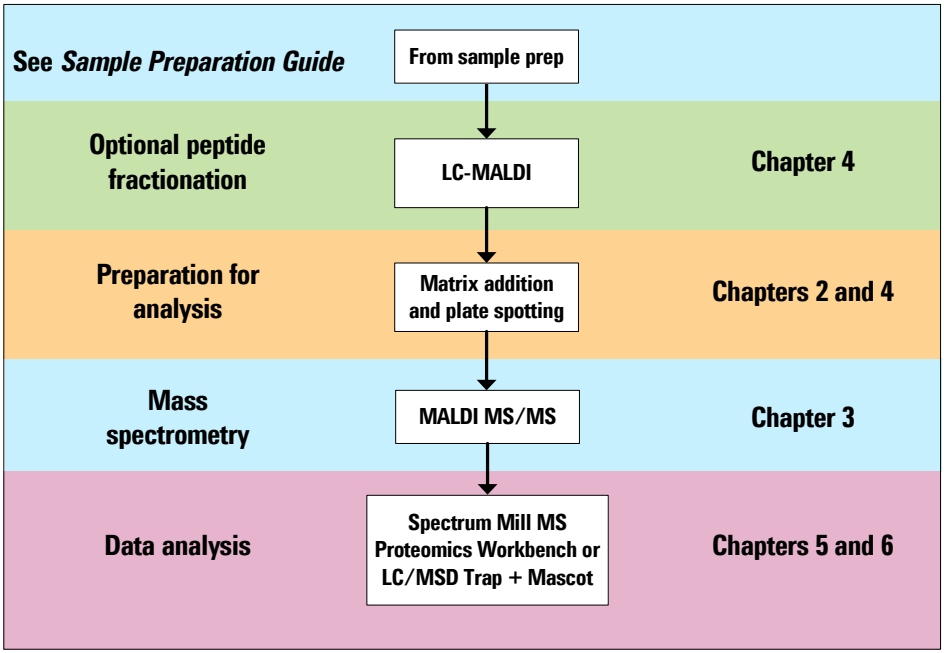

Figure 1 Workflow for MALDI MS steps of MALDI Protein Identification Solution

Optional LC fractionation and automatic plate spotting (LC MALDI)

When MALDI MS/MS analyses are performed to identify peptides, more proteins can be identified when there are fewer peptides per spot. Liquid chromatography (LC) is commonly used to fractionate peptides. This application guide describes an optional reversed-phase (RP) LC separation with matrix addition and real-time deposition of the LC run onto a MALDI plate. This technique is known as LC MALDI.

Preparation for analysis (matrix addition and plate spotting)

If you perform LC MALDI, the matrix addition and plate spotting are automated along with the LC separation, so you skip this step. Otherwise, you perform the matrix addition and plate spotting manually, as described here.

To do this step manually, you first adjust the peptide samples to the proper concentration. Then you mix each one with a MALDI matrix solution and spot the mixture onto a MALDI plate. The MALDI matrix contains a chromophore that absorbs the laser energy and transfers it to the sample, aiding in ionization.

Mass spectrometry

The next step in the workflow is MALDI MS analysis. Following ionization in the PDF-MALDI source, the peptides enter the ion trap mass spectrometer (MS). 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 MALDI Protein Identification Solution, 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 sample fractions (e.g., gel spots) to be automatically consolidated, and permits ready sample comparison.

1 Overview of MALDI MS with the MALDI Protein Identification Solution

2 MALDI Sample Preparation and Manual Plate Spotting

The MALDI Protein Identification Solution includes the matrix solution, dilution solvent, and target plate you need for successful analyses. This chapter describes how to adjust the concentration of your sample, combine the sample with matrix, and spot the target plate.

If you wish to fractionate your peptides prior to spotting them onto the MALDI plate, see Chapter 4, “Optional LC MALDI,” starting on page 29. In this case, you can skip the steps in this chapter since the matrix addition and plate spotting are integral parts of the LC MALDI procedure.

Agilent reagents and equipment

To prepare samples for MALDI analysis, you need the following Agilent supplies:

Table 1 MALDI sample preparation – available products

Part number	Product name	Product description
G2037A	α-Cyano-4-hydroxycinnamic acid, 3 x 3 mL	MALDI matrix, 6 mg/mL
G1974-85000	MALDI Matrix Dilution Solvent, 20 mL	Solution of 75% (by volume) water, 24.9% isopropyl alcohol, and 0.1% trifluoroacetic acid (TFA)
G1972-60025	Target plate	Plate for MALDI sample spotting and analysis

Storage

Store the MALDI matrix (p/n G2037A) and MALDI Matrix Dilution Solvent (p/n G1974-85000) at 4 °C.

Prior to using the MALDI matrix (p/n G2037A), warm it to room temperature and mix well to ensure that all the matrix is in solution.

Safety

WARNING

When preparing samples for MALDI-MS/MS analysis, follow safety guidelines for handling biological and chemical materials and wear protective eyewear and gloves.

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

Procedure summary

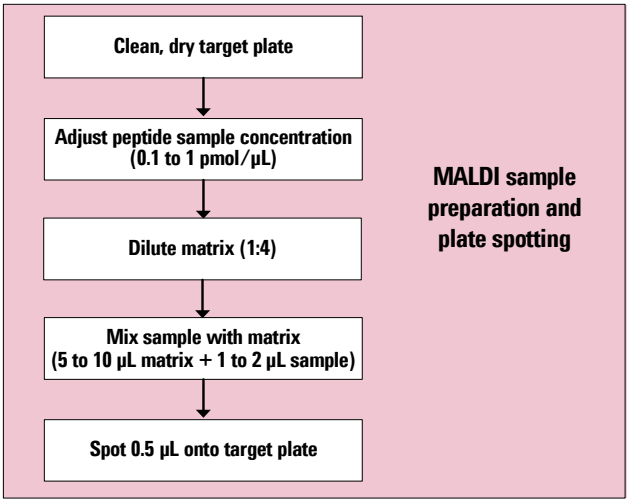

Figure 2 Procedure for MALDI sample preparation

Additional materials required

The following is a list of materials for sample preparation and plate spotting:

- Adjustable micropipettors (0.5 to 1000 µL)
- Sample vials or tubes
- Vortex
- Centrifuge

The following are needed to clean MALDI plates per the Agilent procedure (page 11):

- Acetone
- Methanol
- Deionized water
- Ammonium formate

Protocol to combine samples with matrix

Step 1. Adjust peptide sample concentration

- Adjust sample concentration for optimal performance. Ideally, the peptide concentration should be about 0.1 to 1 pmol/µL before you dilute it in the matrix solution.

Step 2. Dilute matrix

1 Obtain the following from the MALDI Protein Identification Solution kit:
   - α-Cyano-4-hydroxycinnamic acid (p/n G2037A, 6 mg/mL)
   - MALDI Matrix Dilution Solvent (p/n G1974-85000), which consists of 75% (by volume) water, 24.9% isopropyl alcohol, and 0.1% trifluoroacetic acid (TFA)

NOTE Prior to using the MALDI matrix (p/n G2037A), warm it to room temperature and mix well to ensure that all the matrix is in solution.

2 Dilute α-cyano-4-hydroxycinnamic acid matrix 1:4 with MALDI Matrix Dilution Solvent.
   - For example, combine 80 µL matrix solution + 240 µL MALDI Matrix Dilution Solvent.
   - This gives a 1.5 mg/mL matrix solution, which translates to 1.25 mg/mL after you mix with sample.

NOTE

For most cases, a final matrix concentration of 1.25 mg/mL will provide good results. If you know your sample concentration or want to optimize MS response, adjust the matrix concentration as follows:

- For sample loading higher than 50 fmol, the final matrix concentration should be about 2 mg/mL.
- For sample loading from 1 to 50 fmol, the final matrix concentration should be about 1.25 mg/mL.
- For sample loading below 1 fmol, the final matrix concentration should be approximately 0.7 mg/mL.

Final matrix concentration refers to concentration in the mixture that is actually spotted onto the MALDI plate.

Step 3. Mix samples with matrix

3 Combine a 5- to 10-µL aliquot of matrix and a 1- to 2-µL aliquot of peptide sample.

4 Mix thoroughly by vortexing for at least 30 seconds.

5 Centrifuge.

6 Deposit up to 1 µL of the supernatant onto the target plate, as described in "Protocol to spot samples onto target plate".

NOTE

If you store pre-mixed samples between -20 °C and 4 °C, warm the tubes to room temperature and thoroughly re-mix prior to use. The best way to store samples with matrix is crystallized on a target plate.

Protocol to spot samples onto target plate

To spot premixed samples

1 Be sure that the target plate is clean and dry. For cleaning instructions, see "To clean target plates" on page 11.

CAUTION Even if you have a cleaning procedure for another manufacturers' MALDI plates, it is imperative that you use the Agilent cleaning procedure described on page 11.

2 Check that the target plate is placed on a *flat* surface. If the plate is not level, the spot could spread, reducing the effective sample concentration.

3 Use a micropipettor to draw 0.5 µL of sample + matrix into a disposable pipette tip. Carefully position the tip to 0.5 to 1 mm from the plate surface. Brace the pipettor (not your arm) with your other hand and angle the tip at about 60 to 90 degrees with respect to the plate. See Figure 3.

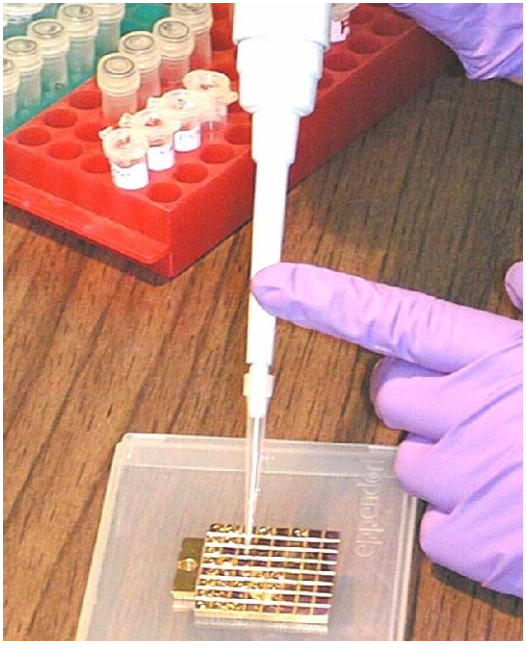

Figure 3 Spotting samples: best arm position and distance from plate

4 Gently expel the liquid. Check that only the droplet, not the tip, touches the plate. If the tip is placed too high the droplet will spread. If the tip touches the plate a thick cluster of crystals will form in the middle of the spot, which destroys its homogeneity.

5 Allow the spots to air dry at room temperature.

6 Store the covered plate at room temperature.

To spot peptides that have not been premixed

1 Be sure that the target plate is clean and dry. For cleaning instructions, see "To clean target plates".

2 Check that the target plate is placed on a *flat* surface. If the plate is not level, the spot could spread, reducing the effective sample concentration.

3 Use a micropipettor to deposit 0.3 µL of sample onto the target plate.

4 Inject 0.3 µL of matrix into the sample droplet. Be careful not to create an air bubble.

5 Allow the spot to air dry at room temperature.

To clean target plates

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

To avoid sample carryover, clean reusable target plates before you spot samples. This is especially important when you work with low-level samples.

To clean target plates:

1 Submerge plates in acetone and sonicate for about three minutes.

2 Submerge plates in a solution of 1:1 methanol:deionized water with 50 mM ammonium formate (3 to 4 g/L). Sonicate for about three minutes.

NOTE It is convenient to prepare extra 1:1 methanol:deionized water with 50 mM ammonium formate. Prepare 500 mL and store it in a capped bottle in the fume hood. You do not need to freshly prepare this solution each time.

3 Submerge plates in deionized water and sonicate for about 3 minutes.

4 Submerge plates in methanol and sonicate for about three minutes.

5 Heat the plates in the PDF-MALDI source for 20 minutes each or with a heat gun to completely dry.

6 Let the plates cool completely before spotting samples.

Troubleshooting

For troubleshooting information, see the troubleshooting chapter in the manual you received with the PDF-MALDI source.

Tips

Spot uniformity For best results, MALDI requires uniform co-crystallization of a layer of sample and matrix on a target plate. Sample exposure to strong ionic detergents, formic acid and contaminants may inhibit good crystallization.

Vortexing and centrifuging You obtain the best sensitivity when samples are pre-mixed with matrix. Since samples are usually in an aqueous buffer and matrix is dissolved in organics, matrix and sample must be *thoroughly* vortexed and centrifuged prior to spotting. Matrix and sample mix quite slowly, so vortex for at least 30 seconds. Centrifuging eliminates air bubbles and pulls the sample to the bottom of the vial, increasing homogeneity.

Peptide concentration Avoid using higher sample concentrations than recommended; the final peptide concentration should be 0.1 to 1 pmol/µL before dilution in the matrix solution. Reducing the peptide concentration (not increasing it) frequently increases the signal intensity.

Matrix dilution Use freshly diluted matrix solution whenever possible. Mix small volumes of solution as needed, or use refrigerated or frozen aliquots. Bring them to room temperature before mixing.

More information For more information, see the documentation you received with the PDF-MALDI source.

3
Peptide Analysis with MALDI MS/MS

This chapter presents the protocol for the MALDI MS/MS analysis. You can acquire the data either manually or automatically. For manual operation, you analyze one spot at a time and you must be present to start the next sample. For automatic operation, you establish settings to run a series of spots without further intervention. For more details on operating the MALDI MS/MS system, see the documentation you received with the PDF-MALDI source and the LC/MSD Trap.

System components

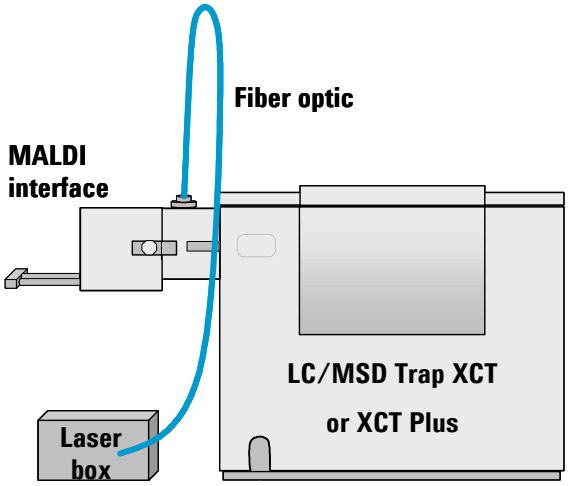

Figure 4 MALDI MS/MS system

The MALDI MS/MS system (Figure 4) for the MALDI Protein Identification Solution consists of:

- Agilent G1974A Pulsed Dynamic Focusing (PDF) MALDI Source
- Agilent 1100 Series LC/MSD Trap XCT or XCT Plus
- Target software for control of the PDF-MALDI source
- LC/MSD Trap software 5.2 SR1

Laser compliance

The Agilent G1974A PDF MALDI Source is a Class 1 Laser Product and complies with 21 CFR 1040.10.

Safety

The PDF-MALDI source is safe when operated properly. *Always* observe the following safety rules:

WARNING ***Never*** **switch the power ON at the rear panel of the AP/MALDI PDF control unit before the source is *completely installed*, and the optical fiber is properly connected at *both ends*.**

WARNING **Whenever you detach or connect the optical fiber to the control unit or the source housing, *make sure* the power switch on the control unit is OFF.**

WARNING **The target plate will be hot when you remove it from the source. Be extremely careful not to burn yourself when you remove the plate.**

WARNING **Never defeat or bypass interlocks.**

Never open the cover of the source control unit.

WARNING **Use of controls or adjustments or performance procedures other than those specified herein may result in hazardous radiation exposure.**

WARNING **See the additional safety information in the documentation you received with the PDF-MALDI source and the LC/MSD Trap.**

Procedure summary

The following summarizes the protocol for MALDI MS/MS.

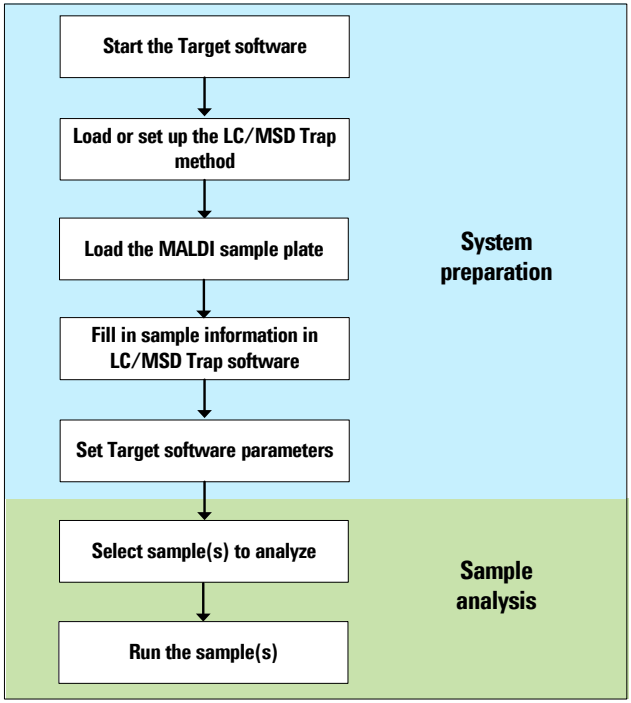

Figure 5 MALDI MS/MS procedure

PDF-MALDI ion source operation

Step 1. Start the Target software

1 Review the safety rules for the PDF-MALDI ion source. See the documentation you received with the source.

2 Close the PDF-MALDI source.

3 Turn on the PDF-MALDI source control unit.

4 Start the Target software.

5 Wait until initialization is completed and the Target software status field indicates **Ready**.

Figure 6 shows the main screen for the Target software.

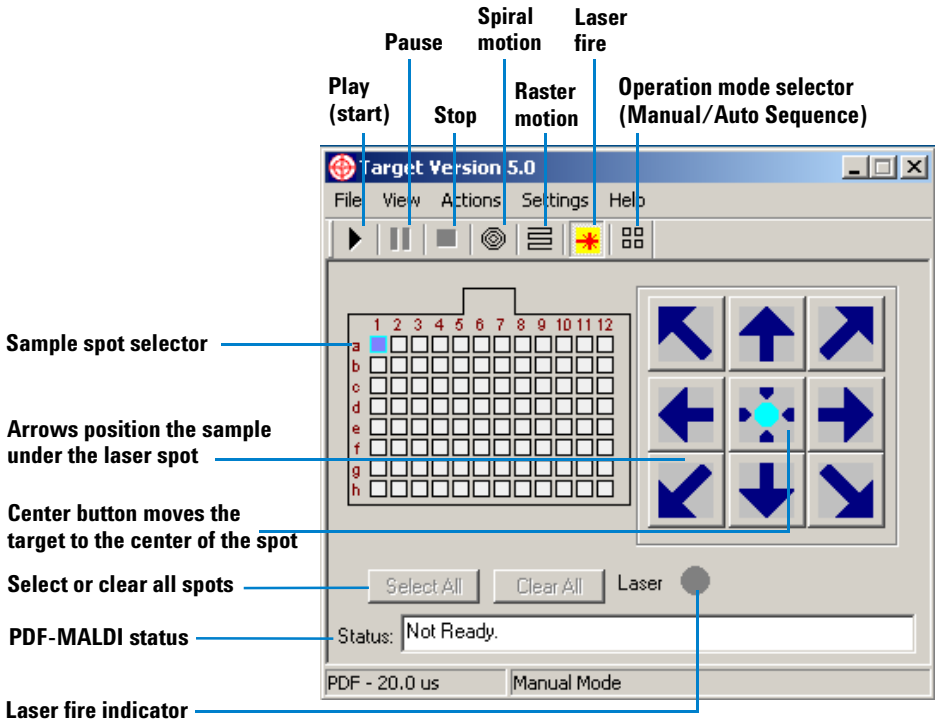

Figure 6 Main Target program screen

Step 2. Set the LC/MSD Trap parameters

1 Start the LC/MSD Trap control software.

2 Load the appropriate method. To create a method, see settings under "Settings for MS/MS analysis with LC/MSD Trap XCT or XCT Plus" on page 24.

Step 3. Load the target plate

1 Open the PDF-MALDI source.

2 Insert the target plate.

3 Close and lock the source. Verify that you hear the two halves of the source click together.

Step 4. Run the samples

- If you will run samples manually one spot at a time, go to "To acquire data manually" below.
- If you will run samples in an automated sequence, go to "To acquire data automatically" on page 20.

To acquire data manually

Do this step only if you want to acquire your data in manual mode.

1 Select the **Sample Info** tab in the LC/MSD Trap Control window and fill in sample information.

2 In the Target software, if the **Auto Sequence** button is activated, click to deactivate it. 

3 Set the Target software parameters:

   a From the Target main screen, choose **Settings > Set Parameters**.

   b Establish settings as described in "Target program settings" on page 23.

4 On the Target software main screen, click buttons to activate the following:

   - Laser fire 
   - Spiral motion 

5 Select sample to analyze.

a Click the sample position on the Target main screen sample map.

b Verify via the Video Capture imaging system that the target plate moves to this sample position and stops near its center.

c Double-click the spot center to position the laser on the spot.

6 Start the LC/MSD data acquisition by clicking the green arrow at the top of the LC/MSD Trap Control software window.

7 Click the **Play** (start) button in the Target window to start the laser firing.

NOTE

After you click **Play**, you can still adjust the position of the sample plate. For fast adjustment, view the Video Capture window and double-click the spot center. For slower adjustment, click the arrows on the Target main screen.

You can also turn the laser and spiral motion on and off by clicking the appropriate buttons on the Target main screen.

8 After data acquisition is complete, click the **Stop** button in the Target software to stop the laser fire.

The LC/MSD Trap will stop automatically when the method runtime is reached. If you want to manually stop the analysis, click the **Stop** button in the Trap control software.

9 Repeat step 5 through step 8 for each sample to analyze.

10 After all samples have been acquired, click **Standby** in the instrument control window.

11 In the Target software, click the **Stop** button to stop the PDF-MALDI operation.

12 Open the target flange and wait a few minutes for the plate to cool, then with gloves on, carefully remove the target plate.

WARNING

The target plate will be at 80 °C. Be extremely careful not to burn yourself when you remove the plate.

To acquire data automatically

Do this step only if you analyze your samples in automated mode.

1 Make sure that the cable has been installed between the **External Control** connector on the rear panel of the PDF-MALDI source control unit and the **Remote** and **Aux Port** connectors on the LC/MSD Trap.

2 In the LC/MSD Trap Control software, enable remote control:

   a Select the **Mode** tab.

   b Click the **Configure...** button.

   c Set up the remote control as shown in Figure 7.

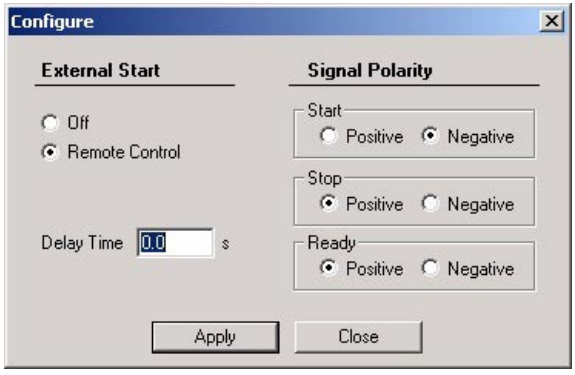

Figure 7 Settings for remote control

3 Select the **Sample Info** tab in the LC/MSD Trap Control window and fill in sample information, similar to what is shown in Figure 8.

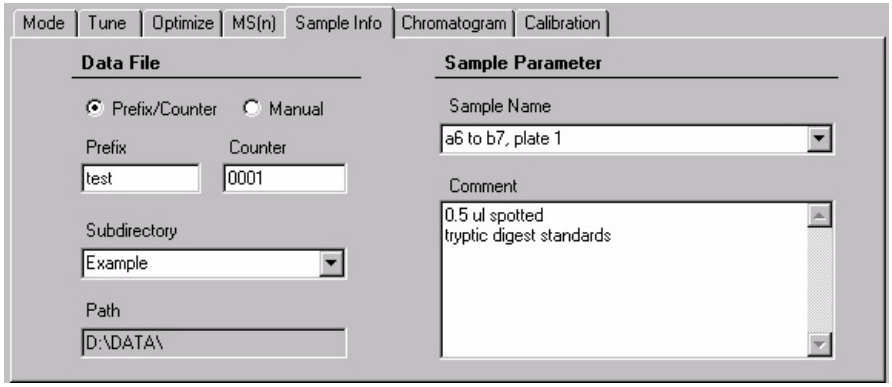

Figure 8 Sample information example

4 In the Target software, click to activate the **Auto Sequence** button.

5 Set the Target software parameters:

   a From the Target main screen, choose **Settings > Set Parameters**.

   b Establish settings as described in "Target program settings" on page 23.

6 On the Target software main screen, click buttons to activate the following:

   - Laser fire
   - Spiral motion

7 Select samples to analyze.

   a In the Target software main screen, click **Clear All** or **Select All**.

   b Click one of the sample positions you wish to run.

   c Select the remaining samples.

   - Use the **Shift** key to select a contiguous group of samples.
   - Use the **CTRL** key to select (or clear) a non-contiguous group of samples.

NOTE Regardless of the order that samples are selected, samples are analyzed from left to right, starting from the top to the bottom row.

8 In the Target software, click the **Play** button to start the data acquisition.

9 If you selected the **Manual** centering method, you will see the message "Waiting for user's input." This mode requires user verification of spot centers.

   a Observe the Video Capture image and double-click the spot center to re-center the spot. (Although you will not see confirmation, the software remembers the spot center.)

   b Verify that the plate moves to the next spot.

   c Repeat steps a and b.

NOTE

When the data acquisition is complete for the first sample, the laser will stop firing and the target will move to the next sample spot. This process will repeat until all samples are analyzed, or until you click the **Stop** button. 

On the sample map in the Target software main screen, a solid color indicates a sample that has already been analyzed, while a blinking color indicates a sample that is currently being analyzed.

10 After all samples have been acquired, click **Standby** in the instrument control window.

11 In the Target software, click the **Stop** button to stop the PDF-MALDI operation. 

12 Open the target flange and wait a few minutes for the plate to cool, then with gloves on, carefully remove the target plate.

WARNING

The target plate will be at 80 °C. Be extremely careful not to burn yourself when you remove the plate.

Target program settings

General tab

Use the settings that were determined at installation. These are loaded automatically each time you start the software.

Sample Plate tab

Target Position Offset If the typical center position is not physically at the center of a target spot, change the X and Y values. Click **Apply**. Then check the four corner positions of the target plate and adjust the offsets if necessary. You may need to compromise slightly to align all the corners equally well.

Plate Format Standard 96

Spiral Motion tab

Use defaults if your spots contain 0.5 to 1.0 µL of combined sample and matrix. If you performed LC MALDI or manually deposited larger or smaller volumes, adjust for the spot size you deposited.

Raster Motion tab

Use defaults

PDF tab

PDF On Check box marked

PDF Delay The optimum PDF delay is established at installation and subsequently remains unchanged.

Auto Sequence tab

External Timing Selected

Synchro pulse duration 500 ms

Centering Method.

- Select **Geometry** if you use an automated, precisely controlled spotter (such as LC MALDI) and all your spots are in the same position in each sample cell.
- Select **Manual** if you wish to manually determine the center of each spot.

Settings for MS/MS analysis with LC/MSD Trap XCT or XCT Plus

CAUTION If you have not calibrated the ion trap recently, see "To calibrate the MS" on page 27.

	Mode
Scan mode	Ultra scan
Segments	Segment 1 end time 0.25 min; segment 2 end time 7 min
	Tune
Source	PDF-MALDI (positive)
Vcap	3000 V
Drying gas flow	5.0 L/min
Dry gas temperature	325 °C
Skim1	40 V
Capillary exit	200 V
Oct 1 DC	12.0 V
Oct 2 DC	3.2 V
Trap drive	110 V
Oct RF	200 V
Lens 1	-5 V
Lens 2	-60 V

Block voltages	Mark the check box for **Follow values on Page Tune/Expert**.

ICC values

Target	200,000 for XCT; 500,000 for XCT Plus
Maximum accumulation time	535 ms (corresponds to 6 laser shots)
MS scan range	400 to 2200 *m/z*
Averages	Segment 1 uses 4 averages; segment 2 uses 8 averages

MS/MS fragmentation conditions

Auto MS(n)	2, enabled for segment 2 only
Fragmentation amplitude	1.50 V
Precursor selection	Include 600 to 2200 *m/z*
Number of precursor ions	3 (Set to a smaller number if your spots are less than 0.5 µL in total volume.)
SPS	Enabled
Threshold absolute	200 counts
Threshold relative	2%
SmartFrag	On, 30-200%
Active exclusion	On, exclude after 1 spectrum, release after 20 min

Advanced Auto MS(n) parameters

MS(n) averages	16
Isolation width	4.0
Use values from MS scan	Yes
Use ultra scan	Yes

Troubleshooting

For troubleshooting information, see the troubleshooting chapter in the manual you received with the PDF-MALDI source.

Tips

MS stop time You can adjust the MS stop time for your sample complexity. The protocol specifies 7 minutes, but a less complex sample may require only 3 to 4 minutes. A more complex sample may require 15 to 20 minutes and a larger volume spotted.

To calibrate the MS

Calibrate the ion trap mass spectrometer as directed in the *Orthogonal Nanospray Ion Source User's Guide* or the *LC/MSD Trap Installation Guide*.

- Use the ESI tuning mix (G2431A).
- Use the electrospray source or the orthogonal 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 have not calibrated for 30 days.
- You observe mass shifts or other problems indicating the need to calibrate.
- Your background seems unusually low, which indicates aging of the detector.

NOTE Use a MALDI analysis of a standard protein digest to track performance of the MS. If you observe mass shifts or other performance inconsistencies, recalibrate the MS.

To shut down the MALDI MS/MS system

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

1. After all samples have been acquired, click **Standby** in the LC/MSD Trap Control window.
2. In the Target software, click the **Stop** button to stop the PDF-MALDI operation. 
3. Open the target flange and wait a few minutes for the plate to cool, then with gloves on, use needle nose pliers to carefully remove the target plate.

WARNING **The target plate will be at 80 °C. Be extremely careful not to burn yourself when you remove the target plate.**

4
Optional LC MALDI

This chapter describes LC MALDI, which is peptide fractionation by reversed-phase liquid chromatography, followed by real-time spotting onto a MALDI plate. For MALDI MS/MS analysis, you get best results if you have no more than two to three proteins per spot. This becomes even more critical if your proteins vary widely in concentration. If you have 1D gel bands from a fairly complex sample, you need to further fractionate the sample to reduce the sample complexity. LC fractionation is useful for this purpose. This chapter outlines an optimized method that includes online matrix addition and MALDI plate spotting.

System components

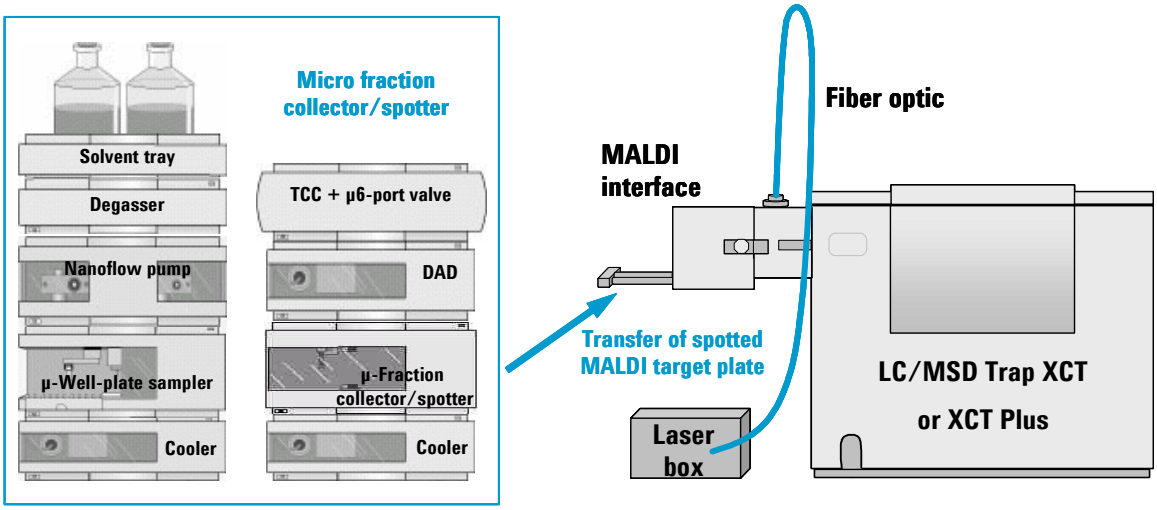

Figure 9 Micro fraction collector/spotter and interface to MALDI MS/MS system

Fractionation/spotting system

- Agilent 1100 Series nanoflow 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 (optional; used if you want to control the temperature of the column or use more than one column)
- Agilent 1100 Series diode array detector (DAD) with 80-nL flow cell
- Agilent 1100 Series thermostatted micro fraction collector/spotter

MALDI MS/MS system

- Agilent 1100 Series LC/MSD XCT or XCT Plus
- Agilent PDF-MALDI source
- Agilent ChemStation A10.02 software
- Agilent LC/MSD Trap software version 5.2
- Agilent software for the control of the PDF-MALDI source

Accessories for online matrix addition

To perform the online matrix addition, you need

- Syringe pump, KDS 200 or equivalent

  Note that the syringe pump is not supplied by Agilent. The KDS 200 pump from KD Scientific (www.kdscientific.com/Products/KDS200/kds200.html) is recommended because you can control it via external contacts. External control allows you to turn the flow of matrix solution off and on depending on whether you are actually spotting.

- Agilent Online Matrix Kit for MALDI Spotting (G1364-68706)

  The contents of the kit are listed in Table 2.

  Note that the kit contains an External Contact BCD Board to control the syringe pump, as well as a cable to connect the KDS 200 syringe pump.

  For more information on setting up online matrix addition, see the documentation you received with the micro fraction collector/spotter.

Table 2 Online Matrix Kit for MALDI Spotting (G1364-68706)

Item	Part number
External contact BCD board kit	G1351-68701
Cable, BCD board to KDS syringe pump	5181-1536
PEEK coated fused silica capillary, 550 mm x 125 µm	G1375-87318
Needle, LL, 22/51/3, 2/pk	5183-4614
Syringe, ¼-28, 1 mL	5181-1541
Micro-connector, T-type	5042-8519
Union, ¼-28 to Luer	5042-8518
Adapter body, 10-32 to ¼-28	5042-8517

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 10 shows a plumbing diagram for micro fractionation/spotting with online matrix addition.

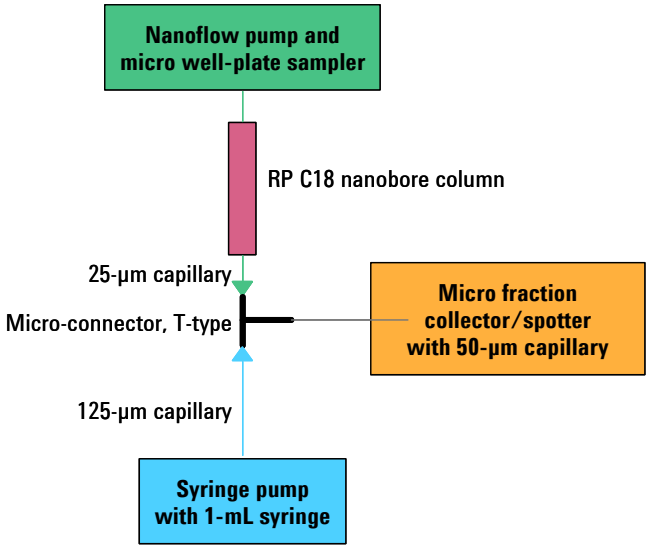

Figure 10 Schematic for micro fractionation/spotting with online matrix addition

Summary of steps

1 Separate tryptic peptides with a C18 nanobore column.

2 Add matrix solution to column effluent.

3 Spot column effluent plus matrix solution onto MALDI plate. Adjust the number of fractions (spots/min) to meet your separation needs. Use Table 3 as a guide.

Table 3 Spotting rate for peptide mixtures of increasing complexity*

Sample complexity	Goal	Spots/min	nL/spot†	Number of spots
Subcellular fraction or 1D gel band	Reasonable number of proteins identified with shorter analysis time	2	400	40
Subcellular fraction or 1D gel band	Maximum number of proteins identified	4	200	80
Whole cell lysate, sera, or fractions from these	Reasonable number of proteins identified with shorter analysis time	8	100	160

* Assumes LC conditions from "Protocol for micro fractionation/spotting" on page 35

† LC effluent only; same amount of matrix solution is added online

4 Transfer the MALDI plate to the MALDI MS/MS system.

5 Analyze the fractions using the protocol described in Chapter 3, "Peptide Analysis with MALDI MS/MS," starting on page 13.

More details

For more details, see the application note entitled "Coupling of nano-LC to MALDI mass spectrometry – Real-time LC run mapping on a MALDI plate," Agilent publication number 5989-1479EN.

Additional materials required

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

- Accessories for online matrix addition (see page 31)
- Water, HPLC grade (18 megohm) - Agilent p/n 8500-2236 or equivalent
- Acetonitrile, HPLC grade - Agilent p/n G2453-85050 or equivalent
- Trifluoroacetic acid (TFA), sequencing grade
- α-Cyano-4-hydroxycinnamic acid (α-CHCA) - Agilent p/n G2037A or equivalent
- Isopropyl alcohol, HPLC grade
- Acetic acid

Protocol for micro fractionation/spotting

Column Agilent ZORBAX 300SB-C18, 0.10 mm x 150 mm, 3.5 µm particle size, p/n 5065-9910

Nanoflow pump A = water + 0.05% TFA; B = 90% acetonitrile + 0.05% TFA in water

Column flow 800 nL/min

Primary flow 500 to 800 µL/min

Gradient See Table 4.

Table 4 Gradient for micro fractionation/spotting

Time (min)	% B
0	5
10	5
12	15
52	65
55	85
60	85

Stop time 70 min

Post time 15 min

Injection volume Up to 6 µL (For a larger injection, increase the hold time at the beginning of the gradient and increase all times in the gradient table accordingly.)

Injector bypass After 10 min

Diode-array detector (DAD) 210 nm +/- 8 nm, reference 360 nm

DAD flow cell 80 nL

Matrix 2 mg/mL α-CHCA in 50% isopropyl alcohol / 49% water / 1% acetic acid

Matrix flow (syringe pump) 800 nL/min

Micro fraction collector/spotter	Time-based spotting onto Agilent MALDI plates between 14 and 34 minutes during the LC run with spotting rate chosen from Table 3 on page 33
Liquid contact adjustment	2
Contact control distance	0.5 mm (You may need to adjust depending on flow rate and how you did the plate calibration.)
Fraction collector thermostat	20 °C
External contact control	Open at 0 min; close at 12 min; open at 34 min. (Enter these settings in the dialog box for the 1100 module in which you installed the external contact control board. These settings close the contact and start the matrix flow two minutes before the start of plate spotting.)
Bottom-sensing	On, 0.9 mm offset for plastic conical micro-vials (Agilent p/n 9301-0978)

Troubleshooting

Problem	Cause	Solution
LC 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.
MALDI spots are not reproducible in size and shape.	Liquid contact adjustment not set properly	Set the liquid contact adjustment on the micro fraction collector/spotter to 2. The liquid contact adjustment is the ratio of LC pump flow + matrix flow to LC pump flow.
	Capillary tip not properly positioned relative to MALDI plate	Perform a position accuracy calibration for MALDI targets. See the manual you received with the micro fraction collector/spotter.
	Contact control distance not set properly (see Figure 11)	Adjust the contact control distance. You will find this setting in the configuration dialog box.

Problem	Cause	Solution
Spots are skipped on the MALDI target.	Liquid contact adjustment not set properly	Set the liquid contact adjustment on the micro fraction collector/spotter to 2.
	Capillary tip not properly positioned relative to MALDI plate	Perform a position accuracy calibration for MALDI targets. See the manual you received with the micro fraction collector/spotter.
	Contact control distance not set properly	Adjust the contact control distance. You will find this setting in the configuration dialog box.
MALDI crystals are not homogeneous.	Matrix dissolved in wrong solvent	Be sure to use 50% isopropyl alcohol / 49% water / 1% acetic acid.
	MALDI plate not at the correct temperature	Set the temperature control on the micro fraction collector/spotter to 20 °C.
The first few MALDI spots do not crystallize well.	Salts from biological samples	Use Agilent Cleanup Pipette Tips (p/n 5188-5239) to remove salts, or start spotting after the column void volume.

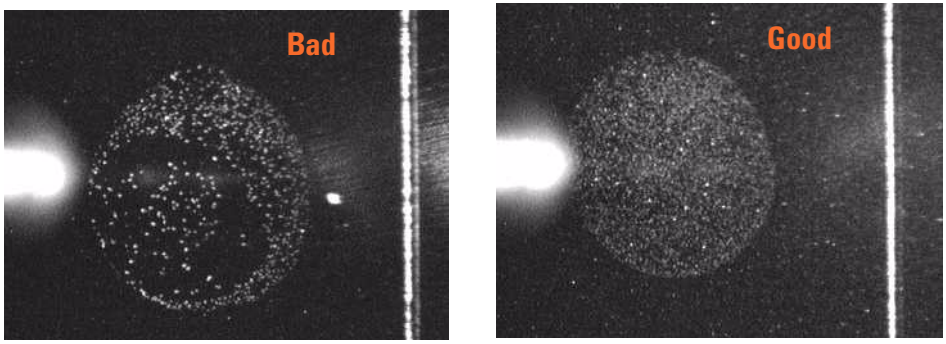

Figure 11 A bad LC MALDI spot formed when the contact control distance was set too low, and a good spot shown after increasing this distance

For additional troubleshooting tips, see the manuals you received with the modules in your micro fractionation/spotting system.

Tips

Plate spotting rate

Optimize the plate spotting rate for the complexity of your samples. For less complex samples, collect fewer spots/minute. For more complex samples, collect more spots/minute or run a shallower gradient for better separation. See guidelines in Table 3 on page 33. More spots require additional analysis time, but translate to additional peptides and proteins identified.

Sample/matrix crystallization on MALDI plate

For optimum MALDI analysis, be sure your crystals are uniform in number and size over the entire MALDI spot. Two factors are important to achieve this goal.

- Solvent used for the matrix solution: The recommended solvent, 50% isopropyl alcohol / 49% water / 1% acetic acid, was chosen to minimize the effects of the changing solvent composition produced by the LC gradient. Other solvents may give poor results.
- Rate of droplet drying: This is regulated by the temperature of the micro fraction collector/spotter. For best results, set this to 20 °C.

Diode-array detector

Use the diode-array detector to monitor and optimize the peptide separation.

Adjusting analysis for spot size

- For spots that are smaller than 0.5 µL or larger than 1.0 µL, adjust the **Spiral Motion** settings in the Target software.
- For spots that are smaller than 0.5 µL, use the Trap Control software to reduce the analysis time for the MS/MS segment.

5 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 6.

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. 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.

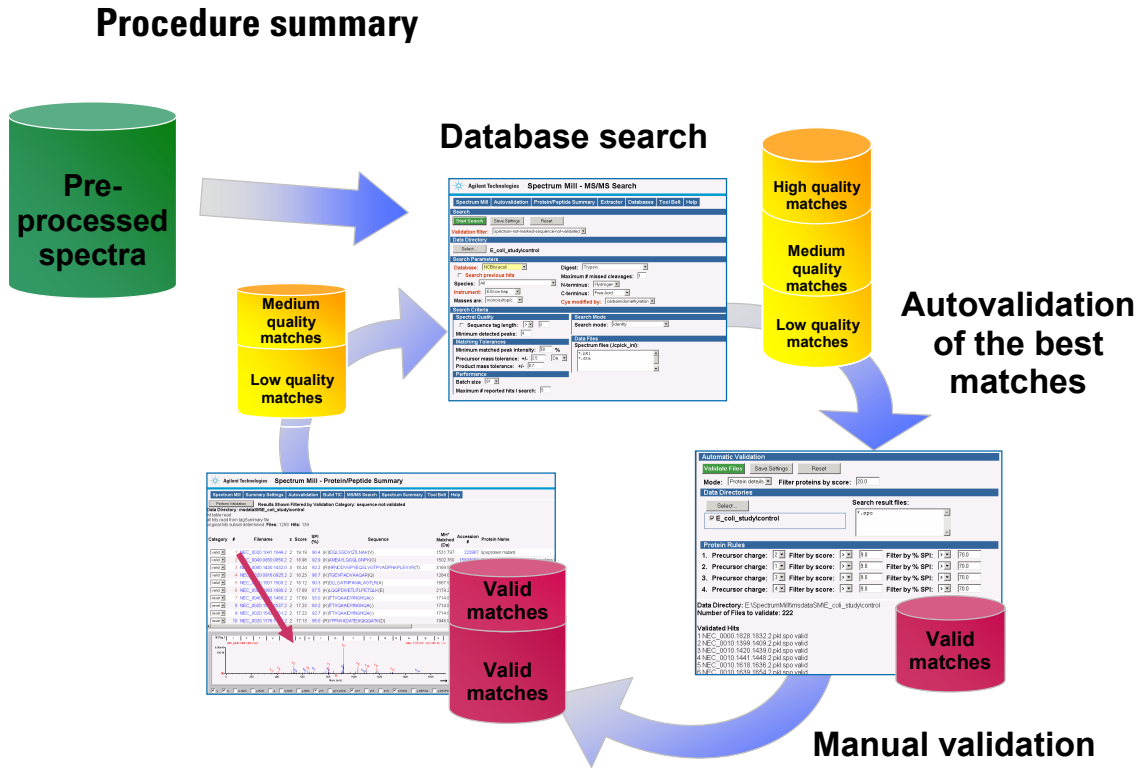

Figure 12 Summary of iterative processing with Spectrum Mill workbench

Figure 12 shows the general procedure for data analysis with the Spectrum Mill workbench. 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, 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. Manually review and validate additional results.
6. Search the spectra that have not been validated:
   - In homology mode or no enzyme mode against previous protein hits

- In identity or homology mode against a different database

7 Continue to perform iterative cycles of database search and validation to identify as many proteins as desired.

Protocol for data processing with Spectrum Mill workbench

The following protocol describes how to efficiently process MALDI MS/MS data with the Spectrum Mill workbench. The protocol assumes that the goal is to identify as many proteins as possible. If your study does not require this, you may omit some steps (those between step 5 and step 10). 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 MALDI MS/MS data files to the Spectrum Mill server.

- Be sure to set up a directory structure on the server that will make it easy to summarize and compare your results. See "Tips" on page 44.

2 Extract the data.

- Be sure to set the correct cysteine modification.
- Set **Sequence tag length** to -1. (This turns off the sequence tag filter.)
- Set **Merge scans with same precursor m/z** to 600 sec, or long enough to cover the entire analysis time for a spot.

3 Do the first database search.

- Use a database subset (e.g. mammals).
- Search in identity mode.

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

5 Manually validate (down to score of 6, SPI 50 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).

6 Go to Tool Belt and save the results as a *.res file.

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

7 Do the next database search. Search either a database subset (e.g. mammals) or previous results. Search in the following modes with autovalidation and manual validation between each set of conditions:

- Multi-homology **mq** or **mqsty** (if phosphorylation is expected)
- No enzyme mode (to find non-specific cleavages for proteins already identified)
- Using C- or N-terminal modifications if they are expected for the species
- If sample was exposed to urea, search for carbamylation as N-terminal modification and search in multi-homology **kmqsty** mode.

8 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.

9 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.

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

11 When you have gained enough information from your data, summarize the results. See "To summarize data" on page 43.

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 gel spots. Here are some examples:

- If you analyze multiple spots from a single sample, transfer all the files to a single directory on the server.
- If you prepare another sample in the same way, 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 that you extract only one directory at a time.

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.

6
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 5 instead.

Procedure summary

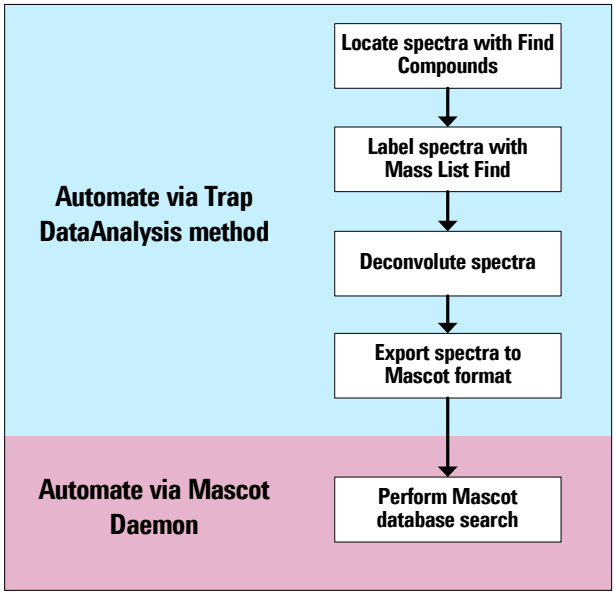

Figure 13 Data analysis with the LC/MSD Trap software and Mascot search

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 MALDI MS/MS analysis of peptides.

2 Select **Method > Add DataAnalysis Method 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 MALDI 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 55), 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 14. 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**.

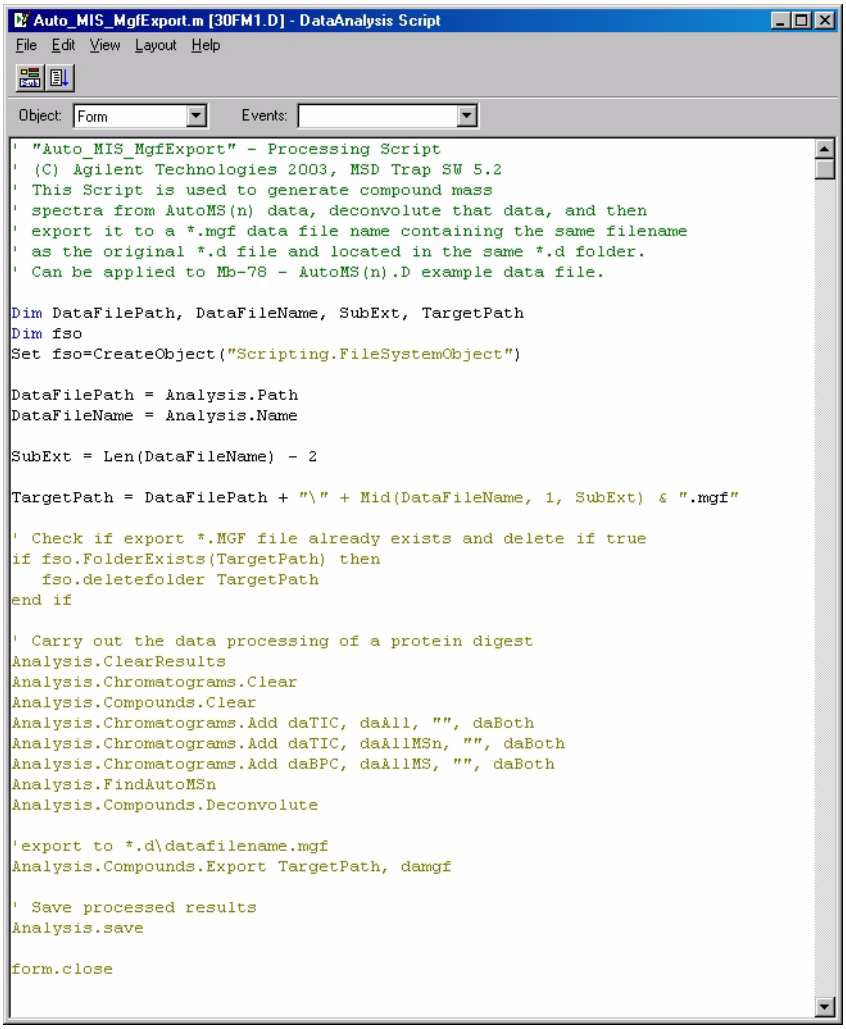

Figure 14 Visual Basic script to automate data analysis

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, deactivate it in the 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 sample fractions

MASCAT concatenates multiple *.mgf files for Mascot data-base search, enabling consolidated search of spectra from analysis of sample fractions (e.g., multiple gel spots). To use MASCAT:

1 Click the MASCAT icon.

2 Select data files you wish to combine. See Figure 15 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 51).
- 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.

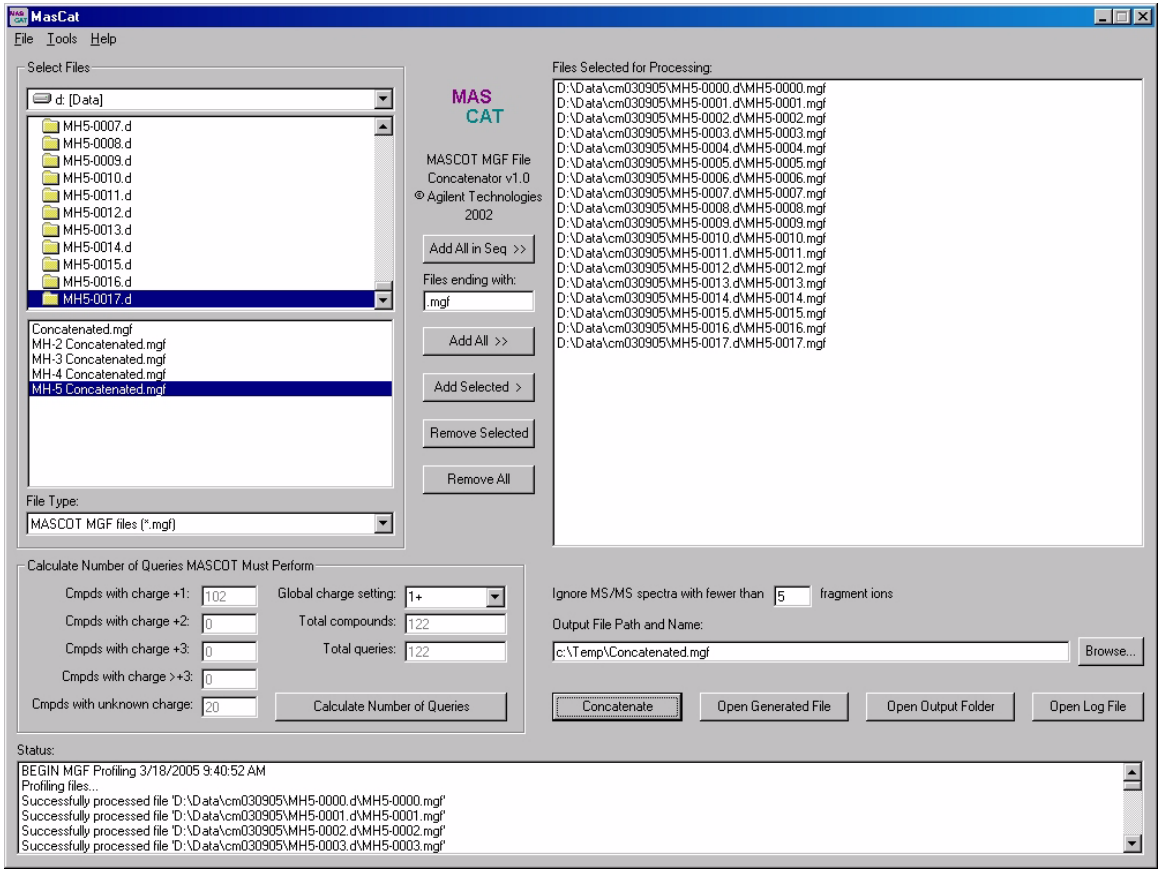

Figure 15 MASCAT concatenator to combine *.mgf files from multiple sample fractions (e.g., gel spots)

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 16 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.

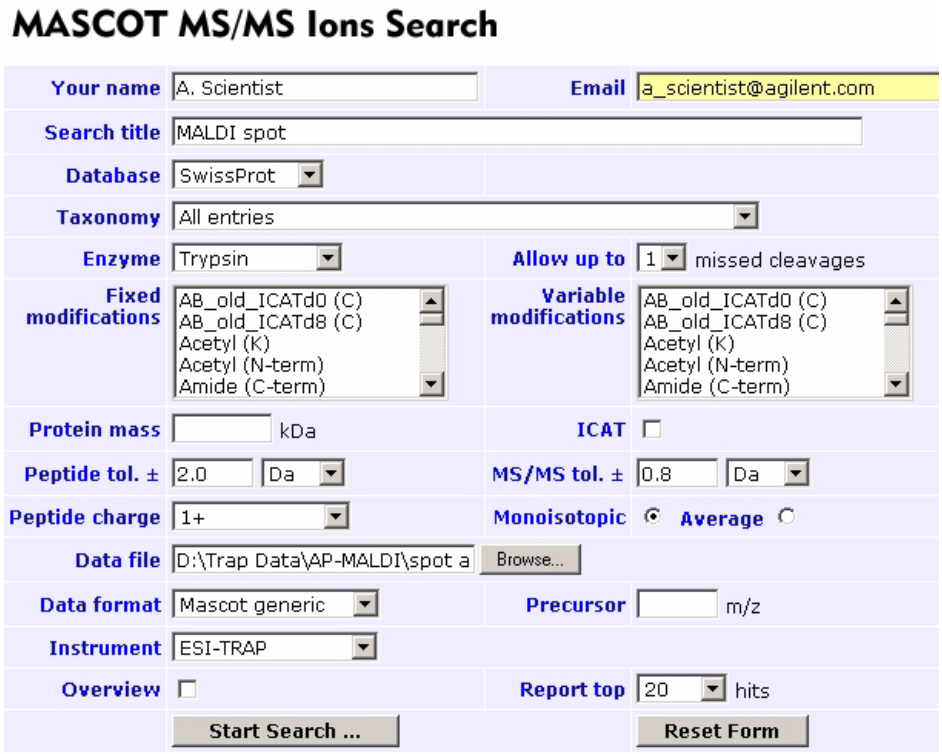

Figure 16 Parameters for Mascot MS/MS Ions Search

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+**, since MALDI ions are singly-charged.
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 17. Parameter entry is similar to that discussed in "To use Mascot protein database search" on page 51.

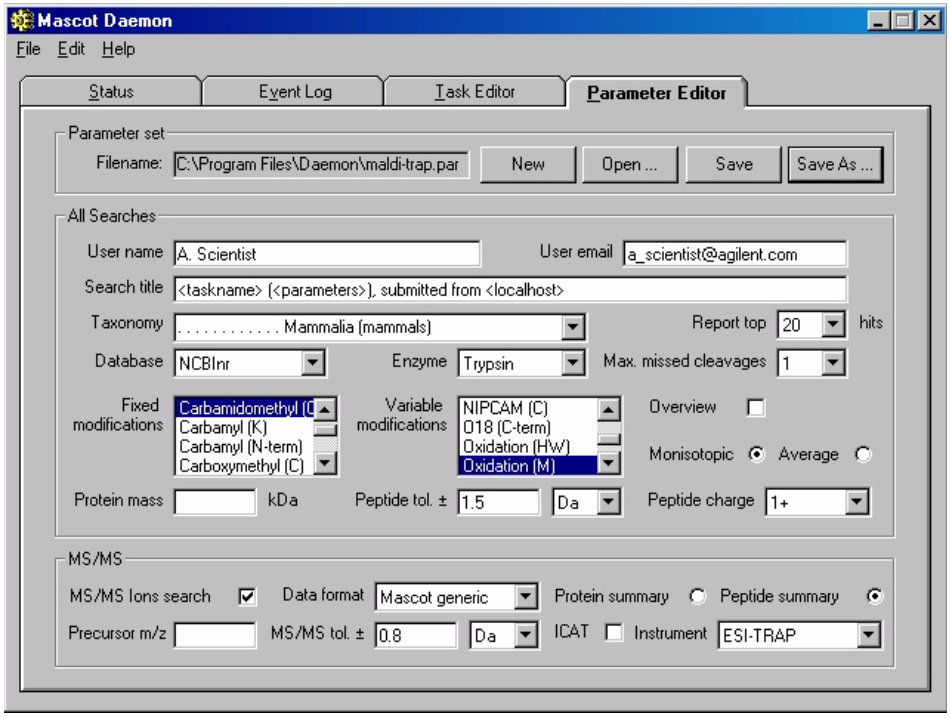

Figure 17 Mascot Daemon Parameter Editor

You set automation parameters using the Mascot Daemon Task Editor, shown in Figure 18. Some of these fields are discussed briefly below. Additional information is available via the Mascot Daemon online help.

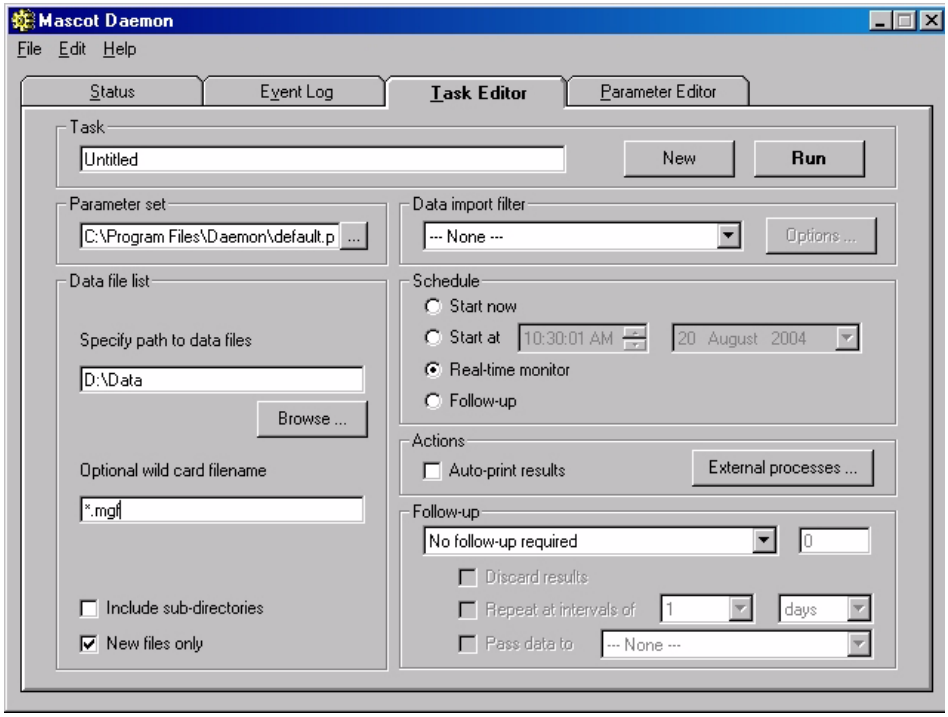

Figure 18 Mascot Daemon Task Editor

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 **10**. (Since there are no chromatographic peaks, you use the maximum setting.)
		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 51.
	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 troubleshooting information in the documentation you received with the PDF-MALDI source.

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 **Absolute intensity threshold** for **Mass List Find**.

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In this Book

The *MS Application Guide* presents the information you need to conduct the instrumental analyses for the MALDI Protein Identification Solution. In this guide you will learn:

- How to optionally fractionate peptides by liquid chromatography with MALDI plate spotting (LC MALDI)
- How to prepare and analyze samples by MALDI MS/MS
- How to analyze the resulting data with the Spectrum Mill MS Proteomics Workbench or the Mascot protein database search software.

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