Purdue-UAB Botanicals Center for Age-Related Disease

MALDI-TOF Mass Spectrometry Fingerprinting Technique

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MALDI-TOF mass spectrometry is an advanced technique for rapid protein identification that is based on mass spectra of peptide fragments. In this segment of the workshop, we will look at a sample of an unknown protein digest. This guide will offer a step-by-step protocol to follow in order to get to the desired conclusion. Preparation of the sample for *mass spec* analysis was performed prior to class due to time constraints. However, the procedure that the sample was taken through is as documented below:

In-Gel Digestion (based on a procedure developed at UCSF):

1. Prepare the following solutions:
   25 mM NH_4HCO_3 (100 mg/50 ml)
   25 mM NH_4HCO_3 in 50% CAN
   50% ACN/5% formic acid (may substitute TFA or acetic acid)
   12.5 ng/µL trypsin in 25mM NH_4HCO_3 (stored in -80°C in a freezer)
2. Dice each gel slice into small pieces (1 mm, or crush with razor blade) and place into 0.65 mL tubes.
3. Add ~50 µL (or enough to cover) of 25 mM NH_4HCO_3/50% ACN, vortex and spin for 30 min.
4. Discard the supernatant.
5. Repeat steps 3 and 4 two times. For Coomassie blue stained gels, if gel pieces are still very blue after 1st washing, you can rehydrate the gel pieces with ammonium bicarbonate before repeating the washes.
6. Speed Vac the gel pieces to complete dryness (~ 20 min).
7. Add 20 µL of 12.5ng/uL trypsin solution. This volume will vary from sample to sample depending on the size of the gel pieces.
8. Incubate at room temp. for 30 min. Add 10-20 µl 25 mM NH_4HCO_3 if needed to completely cover the gel pieces.
9. Spin briefly, and incubate at 37°C overnight (16-20 hrs).

NOTE: proteins in gel samples may be alkylated either prior to SDS-PAGE analysis or after th selection of the spot but prior to trypsin digestion.

Extraction of Peptides

:

1. Briefly vortex and spin the digest in the tubes. Add 50 µL (enough to cover) of 50% ACN/5% formic acid, vortex and spin gently for 30 min. Use 1 µL of the peptide mixture plus 1 mL of α-cyano-4-hydroxycinammic acid CHCA for preliminary analysis. Continue extraction with remaining sample.
2. Transfer the supernatant to a clean tube. Add 50 µL (enough to cover) of 50% ACN/5% formic acid to gel pieces, vortex and mix on the Nutator for 30 min. Pool extracted peptides together in one tube. Repeat one more time.
3. Vortex the extracted digests, spin and Speed Vac to reduce volume to dryness.
4. Add 5-10 µL 50% ACN/5% formic acid.

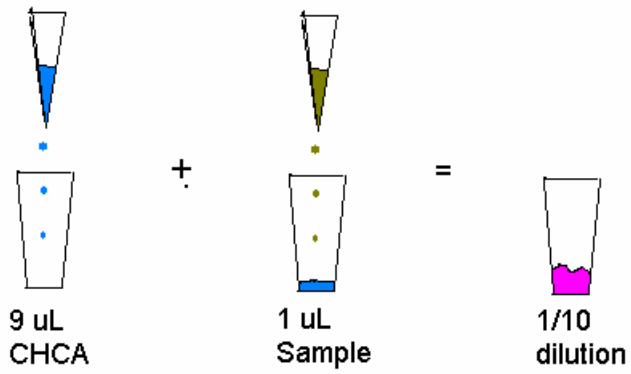
Spotting the MALDI Plate:

The sample in question is currently in a 0.65mL Eppendorf tube dissolved in a solution of 50/50 Acetonitrile, 1% Formic acid (0.1% TFA may be used as a substitute). As stated in the overview class, the primary matrix that is used to adhere the peptides to the MALDI plate is CHCA or α-cyano-4-hydroxycinammic acid. Other matrices that are used in for spot adhesion are as follows:

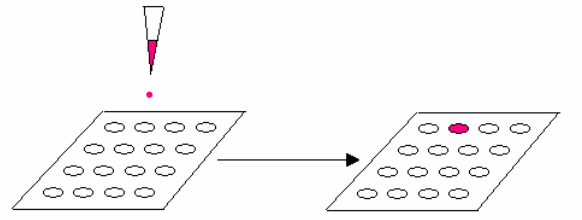
Matrix	*Uses*
2,5-Dihydroxybenzoic Acid (DHB)	Modified peptides, PEG
3,5-Dimethoxy-4-hydroxy-cinnamic Acid	Proteins, High Mass Lipids

When attempting to spot the plate, a series of dilutions such as 1/1, 1/5, or 1/10 are normally prepared in case of concentration issues that might arise. Thus, if the signal on the instrument is saturated (too strong), or weak to get an accurate reading, then a more diluted sample or a more concentrated one is required. The first step is to make the necessary dilutions. Since the protein in question shall be prepared in a 2 µg/µL concentration level, a dilution of 1/10 and 1/100 is more ideal for the instrument:

1) Obtain the CHCA matrix.
2) Aliquot 9 µL of CHCA into a separate Eppendorf tube. Then, obtain a 1 µL volume from the digested sample tube. Mix the two solutions together by using your pipette. This is the 1/10 dilution mixture.



3) Next, take 1 µL of the current mixture and carefully spot the plate (location is not that important on the plate).



4) To make the 1/100 dilution, simply add 1 µL of the 1/10 dilution to a 9 µL CHCA tube, and mix. Allow the spotted plate to air dry for a few moments.

Using the Voyager Software (Acquiring the Mass Spectrum):

Once the plate is dried after the application of the solution mixture, one can now begin the acquisition of data using the MALDI-TOF. The next few pages will describe some of the necessary controls needed to run the basics of the Elite MALDI-TOF and the Voyager Software it utilizes. The two main software programs for the Elite and DE-Pro MALDI-TOF mass spectrometers are *Voyager Control Panel* and *Data Explorer*. Some diagrams of the two respective setups are shown with some important tags to help guide you through the software.

(Voyager Control Panel)

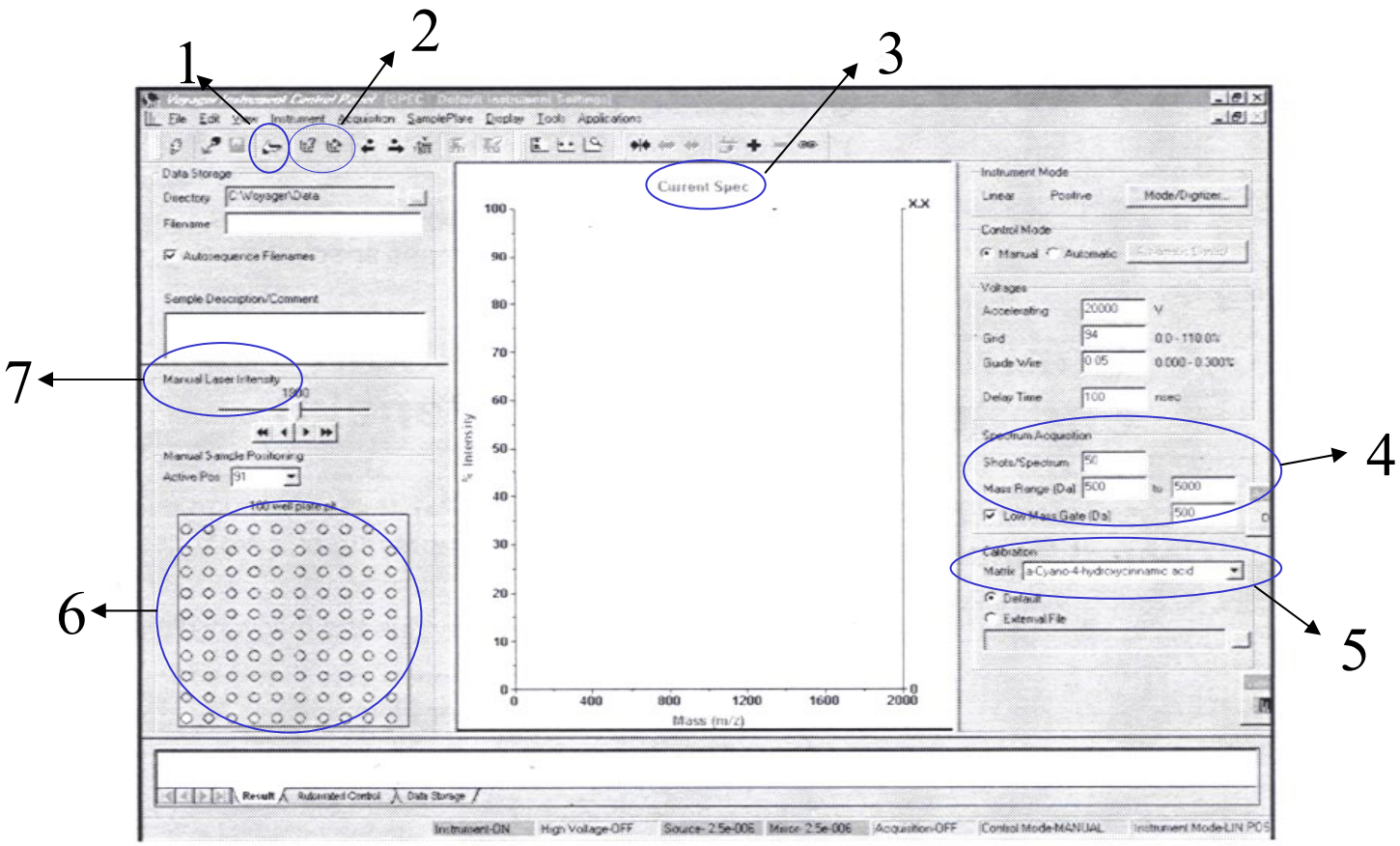


Figure 1-23 Voyager Instrument Control Panel

1) Eject/Load MALDI Plate
2) Sum/Subtract Spectra
3) Acquired Spectra
4) Mass Range/Data Collection Potential
5) Matrix Used in Sample
6) Well Plate Used for Analysis
7) Laser Intensity

(Data Explorer Software)

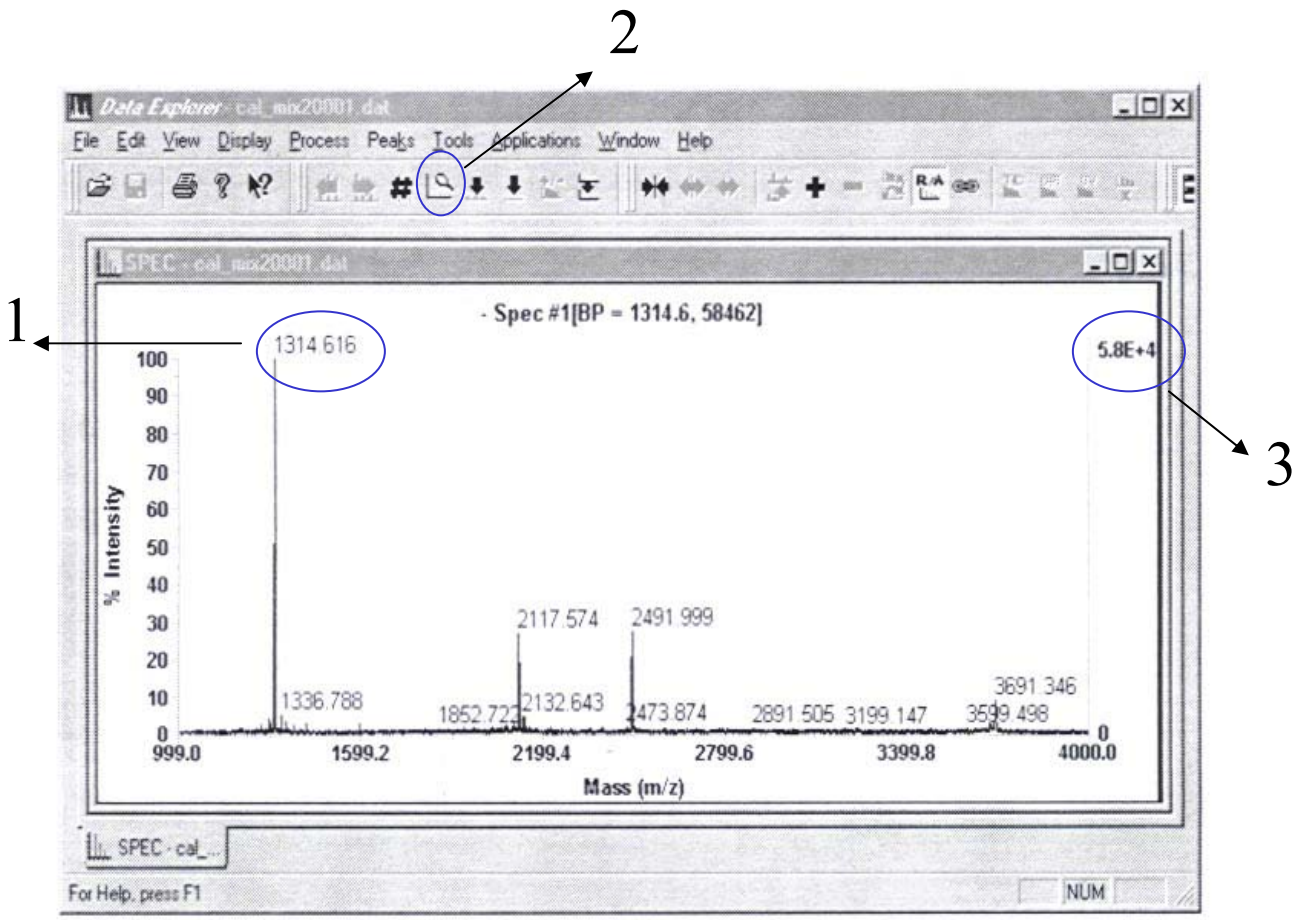


Figure 1-25 Data Explorer Window

1) Peptide Fragment m/z value
2) Intensity Filter
3) Intensity of Spectrum

Before starting the analysis process, get acquainted with the parts of the MALDI. First, notice the video monitor and the Oscilloscope. The plate can be monitored during acquisition of data by looking at the video screen. The peaks are determined by adjusting the scope. Do not concern yourself with adjusting the scope. Now we will start the actual process acquiring data. Eject the MALDI plate from the machine by pressing the hand-shaped button on the Control Panel (labeled 1 on diagram). A screen will appear asking you to eject the plate. Hit the OK Button now. This will allow the loading stage to be released from the source chamber. You will see the loading stage eject from the machine quite clearly near the front of the Elite. When putting in a new plate to the stage, have the left side of the plate (one with the solid surface versus the opening) facing towards you. The MALDI will not load the plate if the grooves on the bottom do not match; thus, do not worry about damaging the instrument. Once you have loaded the new plate, use the Eject/Load button again to load the plate. A screen will appear asking

you what parameters are desired in loading. Choose "Plate Type Plate 1," and then hit Load Plate. The loading stage will then reenter the instrument. It will take a few minutes to properly load the plate. Once this is complete, we can begin acquiring data.

You have spotted a location on the plate; choose the corresponding number in the Control Panel Window (#6). Once the plate number has been picked, you will see the plate mobile in the video screen. Once the stage has stopped moving, we can begin shooting the laser. A joystick should be located on the Elite. Press the button furthest to the left. This will tell the Voyager software to begin acquiring data. After a few moments, you will notice a pulsing light on the video screen. This is the laser reflecting off the plate ionizing the peptides that are to be examined. To determine if peptides are ionizing, we need to look at the oscilloscope to see that peptide peaks are registering (should look roughly like stalagmites). Some adjustments may be needed on the scope to determine if ionization is occurring. If no peaks are being detected, then moving the MALDI plate slightly might help. One must locate a concentration, or *sweet spot*, of peptides for the peaks to show up. Once you have found a suitable location, allow the laser acquisition to continue until the spectrum appears on the Voyager Control Panel. Once this point is reach, we need to determine if the spectrum is intense or resolved for analysis purposes. Keys at identifying decent spectra are low levels of background noise (few peaks), but the peaks that do show are intense and resolved. Check to see if strong peaks are present in your spectrum. Once we have a pertinent spectrum, we can save the data and move on to the Data Explorer Software.

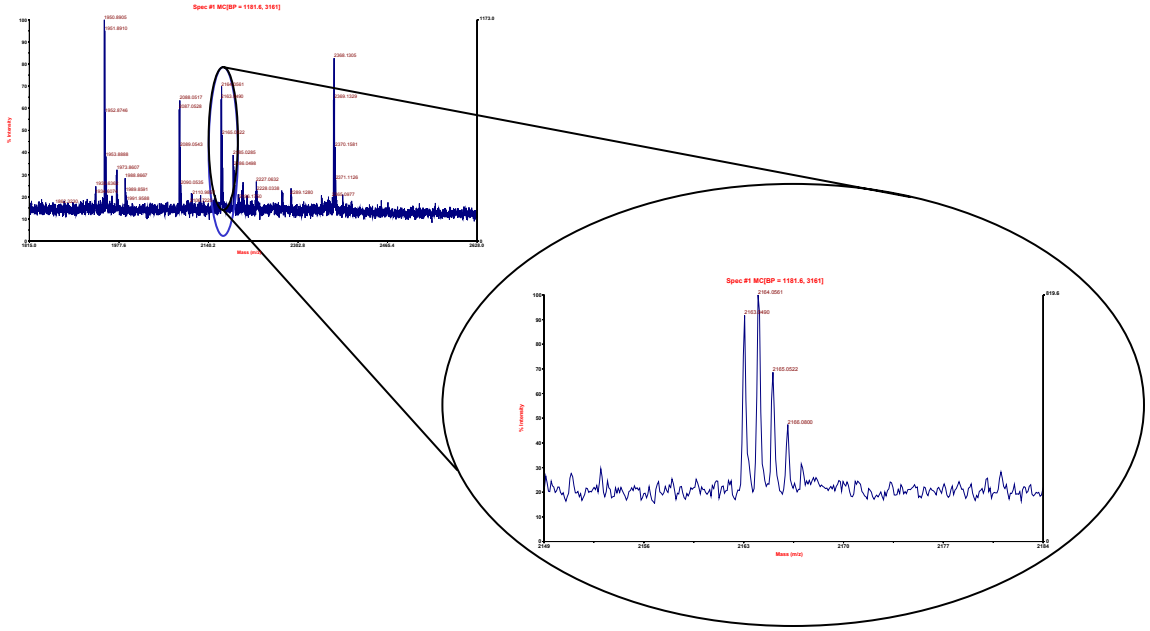
Using the Voyager Software (Data Analysis):

Once the spectrum is in the data explorer, we can begin to manipulate minor aspects of the spectrum to better resolve and clarify certain peaks. The first step, if necessary, is to filter some of the noise in the background. The diagram for the Data Explorer, unfortunately, does not have a programmed setup that we utilize in lab, but the process will be described as best as possible. In the setup, there is a tool bar located at the top of the window. You will see a series of icons the represent certain functions. If you place your cursor over the button in question, a small description will appear about its function. We need to find the Noise Filter Function that is identified as a black arrow pointing down, with a thick black line under the arrow. Click this button and then OK. This will help reduce some of the residual background noise. The next step is the calibration step. With these peptide digests, we used trypsin as the enzyme for digestion. Because of trypsin readily digests itself, we can use the characteristic autolysis peptide peaks of trypsin as a means to calibrate the instrument. First, here is a list of some of the major trypsin peaks that readily appear in tryptic digest spectra:

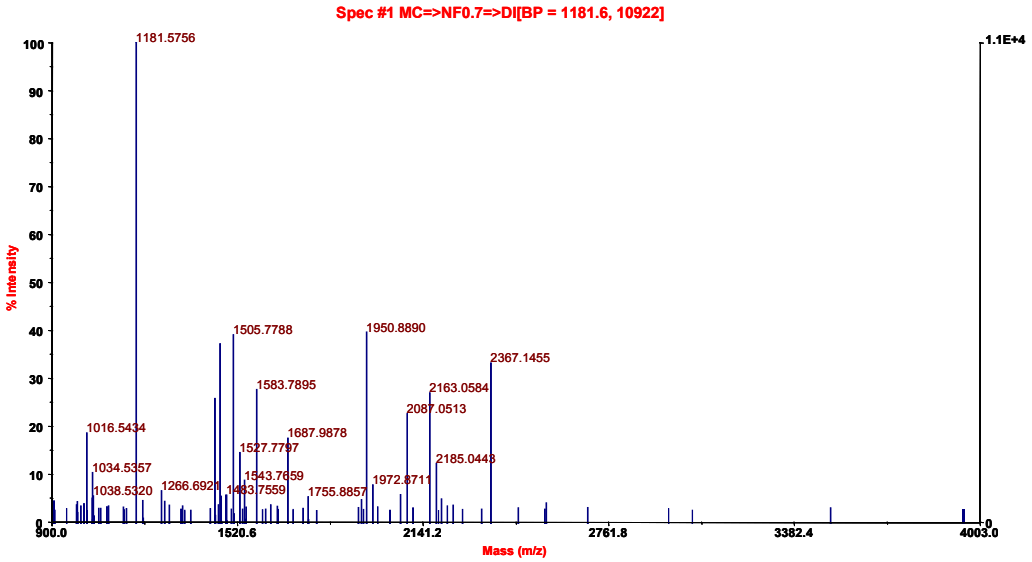
m/z values Calculated:

1) 905.497 2) 1019.496 3) 1152.566 4) 1636.684
5) 1432.713 6) 2162.049 7) 2192.987 8) 2272.152

The most common and usually strongest trypsin peak to calibrate with would be 2162.049. Since the MALDI is an ionizing instrument, analysis is carried out in the "positive mode." This adds a +1 *m/z* value to each peak seen on the spectrum. So, a peak ~ 2163 *m/z* will be found. In order to clearly get the correct calibration, we must zoom in on the area of the trypsin autolysis peptide peak to get the right isotopic form. Since peptides have many carbon atoms, each contributes to an increasing possibility of the peptide having one or more carbon-13 atoms:



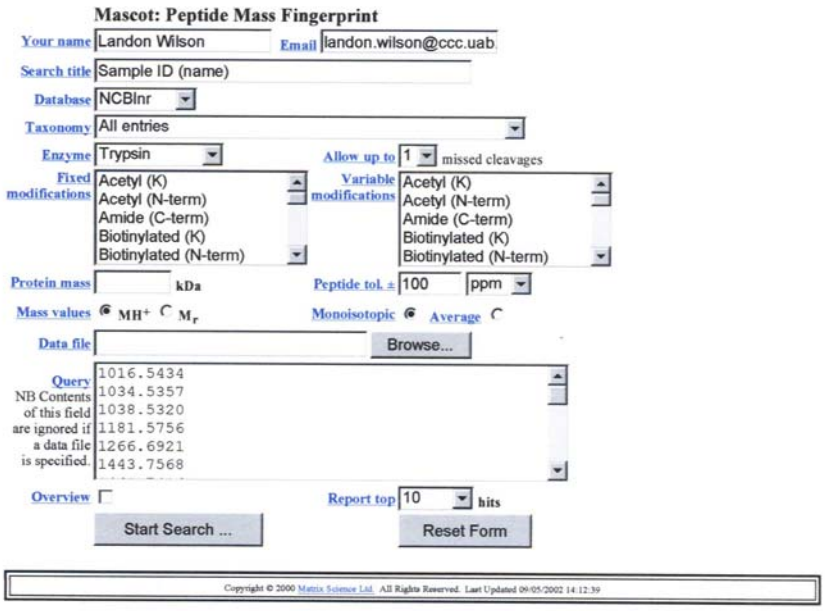
The left most peak in the array is the isotope with all C^{12} atoms that we will choose to calibrate with in the spectrum. To do this, choose the icon on the tool bar that looks like a balance scale. This is the calibration button. Now focus in on the trypsin peak you desire, and right click on the first peak. This will bring up a menu of multiple peaks to calibrate. Trypsin resolved peak 2163.049 should already be highlighted. Now hit OK. Then select the Solve and Plot Button, then Apply Calibration Button. This should adjust the spectrum accordingly. Once this is done, we must choose to de-isotope the Spectrum. To do this, choose the tool bar option labeled as Macro 2. This is located on the right side of the tool bar. Once this is done, the peaks in the spectrum should all convert to the mono-isotopic form.



Now that we have the mono-isotopic peaks, we can determine the threshold of peaks we wish to use in our identification process. For most spectra, a threshold of 5-10% is adequate to remove any background or matrix peak contributions to the peak list. To set the threshold intensity, we simply choose the Peak Detection icon that looks like a magnifying class on the tool bar. Choose the option for Max Peak Area and input the desired percentage. Hit OK afterwards. Now, we are going to save the peak list by using Macro 1, which is located on the right side of the icon tool bar list, next to Macro 2. This will save a list of the peaks that are represented on the de-isotopic peak list. Now that we have the list of possible peaks, we can now move on to the MASCOT search engine procedure.

Mascot Database (Identification Process):

The database utilized in the identification is called Matrix Science: Mascot. http://www.matrixscience.com/. The database allows for multiple parameters to be adjusted to better consult and identify your unknown protein. Each protein has a characteristic peptide digest array, so the database has the peptide maps sorted to each protein. The peak list that was gathered from the Data Explorer Software can be inputted here. Parameters to adjust before moving to identification include:



Set Peptide Tolerance (100 ppm)
Report Top Ten Hits for Protein
Name Your Search

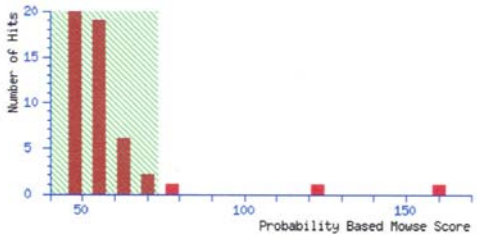
Once this is completed, the peak list may be entered into the query search. Input the numbers now by simply pasting them into the query box. Once this is done, hit the SEARCH button. The database will the search for best matching fits to the peptide fragments. In Mascot, a score of 73 or higher is considered significant. The report can also include the peptides that matched and their probable sequence.

Mascot Search Results

User : Landon Wilson
Email : landon.wilson@ccc.uab.edu
Search title : Sample ID (name)
Database : NCBInr 20020830 (1042297 sequences; 329709346 residues)
Timestamp : 5 Sep 2002 at 19:30:42 GMT
Top Score : 160 for gi|17136632, (NM_057465) porin-P1; Voltage-dependent anion-selective channel [D

Probability Based Mowse Score

Score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 73 are significant (p<0.05).



Protein Summary Report

Switch to Concise Protein Summary Report

To create a bookmark for this report, right click this link: Protein Summary Report (Sample ID (name))

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	gi|17136632	30531	160	(NM_057465) porin-P1; Voltage-dependent anion-selective channel
2.	gi|1526607	30587	123	(X92408) mitochondrial porin [Drosophila melanogaster]
3.	gi|21300816	49448	77	(AAAB01008966) agCP14446 [Anopheles gambiae str. PEST]
4.	gi|14520230	34784	70	(NC_000868) QUINOLINATE SYNTHETASE A [Pyrococcus abyssi]
5.	gi|15642770	36538	67	(NC_002505) O-sialoglycoprotein endopeptidase [Vibrio cholerae]
6.	gi|1364073	65674	66	genome polyprotein - border disease virus (isolate L83/84) (fr
7.	gi|1346637	222678	61	Myosin heavy chain, fast skeletal muscle, embryonic
8.	gi|86355	222834	61	myosin heavy chain, fast skeletal muscle, adult [validated] - c
9.	gi|13432175	223006	61	Myosin heavy chain, skeletal muscle, adult
10.	gi|1842051	223040	61	(U87231) myosin heavy chain [Gallus gallus]

If the score was not a good match, one can try different techniques such as removing trypsin signature peaks from the peak list, or look for patterns such as salt adducts (plus 22 *m/z*), and other possible modifications. Modifications to proteins can be inputted as a search parameter and this may improve your score as well.