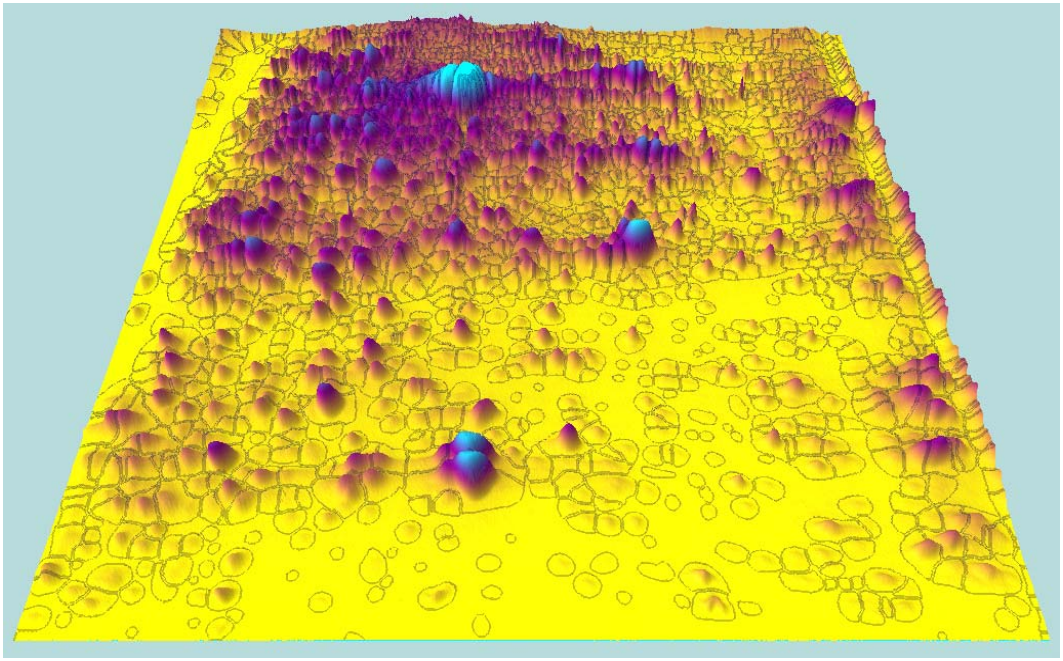
General Protocols of Proteomics and Mass Spectrometry



NSF Funded Proteomics Workshop

Contents

Soluble protein extraction from leaves

1. Weigh leaves (~ 0.2 g fresh weight) and put in labeled eppendorf tube.
2. Quick freeze in liquid nitrogen
3. Grind control into powder, dipping in liquid nitrogen every few seconds so the leaves do not defrost.
4. Re-suspend in 800ul extraction buffer. Continue to grind sample in buffer until solution is well homogenized. Place tube on ice and repeat procedure for the NaCl treated sample.
5. Vortex tubes briefly and sonicate 3 times for 15 seconds each time, putting on ice for 30 seconds and then vortexing in-between each sonication.
6. Spin tubes at highest speed for 10 min in a 4°C centrifuge
7. Pipette 720ul of each supernatant to a new tube and label them (using 1 ml pipette tip on top of a10 ul tip) . Avoid touching pellet!
   (If pellet comes loose or re-suspends, centrifuge again for 2 minutes)
8. Add 80ul 100% TCA to each tube.
9. Vortex 5 seconds.
10. Place samples on ice for 15 min.
11. Spin tubes at highest speed for 10 min in a 4°C centrifuge.
12. Pipette off supernatant and dispense in non-chlorinated liquid waste.
13. Add 1ml cold 100% acetone to pellet. Break up pellet with pipette as much as you can. Vortex to further break up pellet.
14. If pellet won't break up completely, sonicate for periods of 15 seconds until pellet is resuspended. Put on ice and vortex in-between each sonication.
15. Spin tubes at highest speed for 5 min in a 4°C centrifuge
16. Repeat steps 12-15 for a second 100% acetone wash.
17. Repeat steps 12-15 for a third time except with 1ml cold 80% acetone.
18. Fully pipette off supernatant.
19. Speedvac samples for a couple of minutes until dry
20. Solubilize pellet in 150ul IEF sample rehydration buffer

Extraction buffer:

40 mM Tris-HCl, pH 7.6, 1 mM DTT, 1x protease inhibitor cocktail

(Optional: 0.1 mg/ml DNase I, 0.025 mg/ml RNase and 5 mM $MgCl_2$)

Protein concentration assay using Bradford

For soluble proteins:

1. add MilliQ water to 0-10 µl protein sample or standard to make up 800 µl (800 µl water only is used as a blank)
2. add 200 µl of Bradford reagent, wait 5 min at 25 ^{o}C
3. measure the OD595 nm

For membrane proteins:

1. sample solution: add 3 ml 0.5% Triton X-100 into 200 ml MilliQ water
2. add the sample solution to 0-10 µl protein sample or standard to make up 800 µl, vortex an wait 10 min (800 µl sample solution only is used as a blank).
3. add 200 µl of Bradford reagent, wait 5 min at 25 ^{o}C
4. measure the OD595 nm

* the standard, .e.g BSA (1 µlg/µl) is used to calibrate and quantify the protein samples. We recommend including standards in your protein assay every time for accuracy.

Two-dimensional gel electrophoresis of proteins

Rehydration

21. Based on your calculations, dilute 300ug of each sample with IEF buffer for 185ul total volume.
22. Pipette the samples evenly into a rehydration tray. Don't make bubbles! Note the order of the samples.
23. Using tweezers, remove plastic cover from a 3-10NL IPG strip. Gently place the strip gel-side down onto the first sample. Again, don't make bubbles!
24. Repeat procedure for the other sample.
25. Pipette 1.6ml mineral oil onto each strip. Cover tray with lid overnight.

Isoelectric Focusing

26. Cover electrodes of the focusing channels with paper wicks. Wet each paper with 8ul water.
27. Holding the IPG strips vertically, drain oil for 10 seconds.
28. Place the strips gel-side down in the channels, with the + mark on the right. (plus/acidic side on the left)
29. Cover each strip with 1.6 ml mineral oil and place tray into IEF cell.
30. Focus strips with this program:
    1. 250V, linear, 30 minutes
    2. 500V, linear, 1 hour
    3. 8,000V, linear, 2.5 hours
    4. 8,000V, quick up
    5. 35,000 Volt-hours
31. When finished, draining the oil, move strips to a clean tray, and noting the order.
32. Wrap with saran and store in freezer until it's time to run the gels.

Running the gel

33. Take out IPG strips to defrost. Drain oil once more and place gel-side up in a clean well.
34. Add 2ml Equilibration Buffer I to each strip and shake for 10 minutes.

    (In the meantime, prepare and wash two gels under running distilled water. Give them a final rinse with MilliQ water. Using filter squares, blot water from the gel plates. Avoid touching the gel itself.)
35. Move strips to clean wells and add 2ml Eq Buffer II to each. Shake for 10 minutes.

    (In the meantime, prepare 1L of 1X running buffer from 100ml 10X buffer and 900ml MilliQ Water. Also, microwave overlay agarose for 15 seconds each time until melted.)
36. Dip the strips in SDS running buffer and place in clean wells.
37. Position the first strip gel-side up on the back plate with the + side next to ladder lane.
38. Propping the gel up, pipette agarose into the lane (about 1.5ml), letting it spill over into the ladder lane.
39. Quickly, pipette 5ul ladder into the very bottom of the ladder lane. Don't spill!
40. Using tweezers or a spatula, push strip into agarose and into contact with the main gel. Avoid bubbles at the interface. LABEL GEL.
41. Place gel in the box, repeat for other sample, and wait 5 minutes for the gels to set.
42. Pour 1X running buffer over both gels.
43. Run gels at 50V for 15 minutes. Then run at 180V until the blue line runs off the gels. (a little over one hour)
44. Pry open the gel case slowly, allowing the gel to settle on one side.
45. Lift gel by two bottom corners into a clean lidded tray. Label tray.
46. Repeat three times: Cover gel with MilliQ water and shake for 5 minutes. Drain water.
47. After the last drain, add 50 ml Coomassie stain and shake for at least one hour.
48. Drain off stain and wash with water several times.
49. Take an image of the gels on the scanner.

Gel staining, imaging and picking

Staining of 2D gels by comassie blue

1. Wash your gel 3 times with MilliQ water, each time 4 min
2. add 50 ml comassie blue (G-250) solution
3. put it on a robitory shaker and let it shake for more than 30 min
4. decant comassie blue
5. rinse briefly with MilliQ water
6. add approximately 50 ml MilliQ water and put on the shaker
7. shake for 1 hour
8. change water two or three times

Imaging 2D gels

1. wipe the surface of Epson 1600 flatbed scanner
2. lay 2D gel on the top of the flatbed scanner, make sure that there is no air bubble trapped underneath
3. Open the Adobe Photoshop software
4. go to file, select import function
5. select silverfast option
6. choose gray 16 bit first time
7. choose a spatial resolution of 300 dpi
8. prescan to locate your gel and mark the outline of the gel
9. click on Scan function
10. scan one more time as color image, choose color 48 bit the second time
11. repeat step 7-11
12. close Adobe Photoshop and save the images onto a disk

Spot picking

1. use a scaple to cut a 1 ml pipette tip to make the tip opening approximately the size of the spot of interest.
2. aspire about 100 ul-200 ul water and hold the water column above the tip.
3. punch on top of the spot into the gel and take out the gel plug.
4. transfer to an eppendorf tube by pushing the water out of the tip. Close the tube.

Other gel staining methods

SYPRO Ruby:

5. gel fixing solution: 10% methanol, 7% acetic acid.
6. wash gels for 30 min in the gel fixing solution.
7. remove the fixing solution and add 50 ml SYPRO Ruby stain (for Criterion gel).
8. stain the gel with gentle agitation for at least 3 h. Gels can be left overnight.
9. rinse the gel with the fixing solution for 30 min to 1 h.
10. wash gel in MilliQ water before imaging.

Silver staining (Sigma ProteoSilver Plus kit):

1. **Solutions**:
    - Fixing solution: 50% ethanol, 10% acetic acid in ultrapure water, 100 ml
    - 30% ethanol in ultrapure water, 100 ml
    - Sensitizer solution: add 1 ml of ProteoSilver Sensitizer to 99 ml of ultrapure water (**use within 2 h**).
    - Silver solution: add 1 ml of ProteoSilver Silver to 99 ml of ultrapure water (**use within 2 h**).
    - Developer solution: add 5 ml ProteoSilver Developer 1 and 0.1 ml ProteoSilver Developer 2 to 95 ml ultrapure water (**use within 20 min**).
2. **Procedure:** all steps are at room temperature and on a shaker at 65 rpm.

1) Fix the gel in a clean tray with 100 ml Fixing solution (for Criterion gels) for 40 min to overnight.
2) Decant the fixing solution, and wash with 100 ml 30% ethanol for 10 min.
3) Decant the ethanol solution, wash the gel with 200 ml water for 10 min.
4) Decant the water and incubate the gel with 100 ml Sensitizer solution for 10 min.
5) Decant the sensitizer and wash the gel twice with ultrapure water, each time with 200 ml water for 10 min.
6) Decant the water, and equlibrate the gel with 100 ml Silver solution for 10 min.
7) Decant the silver solution and wash the gel for 1 to 1.5 min with 200 ml ultrapure water (Do not exceed 1.5 min).
8) Decant water and develop the gel in 100 ml Developer solution for 3 to 7 min until the desired staining intensity is achieved.
9) Add 5 ml of ProteoSilver Stop solution to the developer solution to stop the developing reaction and shake for 5 min.
10) Decant the Developer/Stop solution and wash the gel with 200 ml ultrapure water for 15 min.
11) Store the gel in fresh ultrapure water.

In-gel digestion of proteins separated by SDS-PAGE

Stage1

Coomassie/Sypro stained bands

- It is particularly important during this procedure to avoid contaminating your gels or your spots with protein. Keratin, a human skin protein, is a common contaminate. To avoid contamination you should wear gloves when handling any instruments or containers that will come into contact with your sample or solutions. Also tie back your hair and avoid getting dust on your experiment.
- For each spot/band you intend to cut, label a 1.5 mL tube and fill it with 300 uL of MilliQ water. Carefully transfer the gel on to a clean glass plate. Excise gel band or spot using a clean scalpel as close to the band/spot as possible.
- Move the excised band/spot to clean area on the plate, cut into 1 mm square pieces with scalpel and then transfer these pieces into its labelled 1.5 mL tube. To transfer the pieces to the tube you may use the scalpel or a clean pipette tip (though you should not suck the gel pieces inside the tip!). In between cutting each spot, you must wash the scalpel (shake vigorously in water, then **carefully** wipe it with a KimWipe. Caution—it's very sharp!). Also, make sure to cut up each band in a different area of the plate so not to cross contaminate the spots with other proteins.
- After you have cut each spot and placed it in a tube, remove the water by pipetting. Take the 1000 uL pipetteman, attach the 1000 uL tip, and then attach the 10 uL tip to the 1000 uL (the small opening of the 10 uL tip will make it easier to avoid sucking up the gel pieces). Check the tip to make sure gel pieces have not stuck to it before you discard the water. Change tips for each sample so not to cross-contaminate.
- Add 300 µl CH_3CN to each tube. Place it in the shaker or vortex for 15 min. **CAUTION**: Acetonitrile can be very harmful if absorbed though the skin. If you get some on your skin, wash it off immediately. Wear **nitrile** gloves rather than latex, and if some is spilt on your gloves, change them. Wash you hands before leaving the lab. If you spill acetonitrile, let your instructor know and he will clean it up. Acetonitrile evaporates quickly, and the vapours are harmful, so keep the lid on solutions that include it.

- Place tubes in minicentrifuge and spin briefly so that the pieces will fall to the bottom of the tubes. Remove the supernatant (in the same way as you removed the water).
- Add 300 µl 100 mM NH_4HCO_3 / CH_3CN (1:1 v/v). Place it in the shaker or vortex for 15 min.
- Spin in centrifuge. Remove the supernatant.
- Repeat the NH_4HCO_3 / CH_3CN washes until the coomassie blue stain is completely gone. For sypro stained spots, wash in NH_4HCO_3 / CH_3CN a total of three times.
- After spinning and removing the supernatant from the last wash, add 200 µl CH_3CN. Incubate for 5 min, remove the supernatant.
- Place tubes, open, in the Speedvac. Turn on the cold trap. Spin 1-3 minutes; just until the gel pieces are dry.
- For 1D gel bands, you may need to reduce/alkylate your proteins. If so, proceed to Stage 2, **otherwise begin Stage 3**.

Stage 2 (Optional)

Reduction/Alkylation of band pieces

- Add 50 µl/sample of 10 mM DTT in 100 mM NH_4HCO_3.
- Incubate at room temperature for 30 min, remove the supernatant.
- Add 50 µl/gel sample of 55 mM fresh iodoacetamide in 100 mM NH_4HCO_3.
- Incubate at room temperature for 30 min, remove the supernatant.
- Wash the gel pieces with 300 µl 100 mM NH_4HCO_3 for 15 min.
- Remove the supernatant.
- Wash the gel pieces with 300 µl 20 mM NH_4HCO_3/CH_3CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 µl CH_3CN to dehydrate the gel pieces for 5 min.
- Remove the supernatant; dry the gel pieces in a Speedvac for 2-3 min.

Stage 3

Digestion of Proteins

- To each tube, add 25 µl digestion buffer [6 ng/ul promega trypsin in 50 mM NH_4HCO_3 (20 ug trypsin + 200 µl 50 mM acetic acid makes 100 ng/µl; then 5µl 100 ng/ul trypsin + 40 µl water + 40 µl 100 mM NH_4HCO_3)]. If the gel pieces are not covered by liquid, consult your instructor. Make sure the tubes are completely closed to avoid evaporation. Put samples in the 37 degree C oven on the top shelf. Leave up to 12 hours. We will remove the samples and freeze them in –20 C freezer until you can return for extraction. **Ask your instructor for instructions on how to clean up your experiment and dispose of hazardous waste**.
- Remove samples from –20, spin briefly in centrifuge, add 30 µl 1% formic acid/2% CH_3CN to the digest.
- Incubate at 30°C for 30 min on shaking platform, or vortex for 10 min. Meanwhile prepare a clean tube for each sample. Label them accordingly, but also write 'tryptic' on each to indicate the proteins have been digested.
- Spin down. Use 1000 uL pipetteman with 10 uL tip on a 1000 uL tip to transfer the supernatant of each sample to its new tube (DO NOT DISCARD SUPERNATANT!) leaving behind the gel pieces in the old tubes. Try not to lose any liquid (example, try to recover the liquid that gets trapped between the 1000 uL and 10 uL tips). Remember to use different tips for each sample!
- Add 24 µl 60% CH_3CN **to the gel pieces**, Vortex for 10 min.
- Spin down. Remove the supernatant from each sample and transfer it to its 'tryptic' tube, adding it to the supernatant that you previously removed from the gel pieces. Discard the gel pieces, unless the instructor tells you otherwise.
- Place tubes, open, in the SpeedVac. With the cold trap on, spin them until the liquid has evaporated leaving a tiny pellet (about an hour).
- Add 2 uL 1% formic acid/2% CH_3CN to each sample. Vortex for a minute, then spin down briefly to pull all the droplets to the bottom of the tube, then let incubate for at least 15 minutes.
- **Ask your instructor for instructions on how to clean up your experiment and dispose of hazardous waste**.
- The samples can now be store in –20 degree C freezer, prepared for MALDI analysis, or ZipTiped.

Mini-reverse phase protein and peptide cleanup

Mini-reversed phase column chromatography-ZipTip is useful for cleanup and concentration of samples. Standard ZipTip C_{18} has 0.6 µl bed volume, good for peptides, small proteins and oligonucleotides. Micro ZipTip C_{18} has 0.2 µl bed volume, better for automation. ZipTip C_4 good for protein and large nucleic acid samples. Other types of ZipTips, e.g. Metal Chelating (MC) for concentration of phosphopeptide are available.

Solutions:

4. Acetonitrile (ACN)
5. 0.1% trifluroacetic acid (TFA)
6. 50% ACN / 0.1% TFA

Procedures:

11. **Condition:** wash ZipTip with 10 µl ACN, then 10 µl 50% ACN / 0.1% TFA, and then then 10 µl 0.1% TFA
12. **Load:** pipet 10 µl sample up and down several times and discard the liquid.
13. **Wash:** wash with 10 µl 0.1% TFA 3 to 5 times to remove buffers and salts.
14. **Elute:** the sample can be eluted with 50% ACN / 0.1% TFA or with matrix solution directly, minimal volume of 2 or 3 µl can be used.

MALDI-TOF MS data acquisition and protein identification by peptide mass fingerprinting

MALDI-TOF MS data acquisition

- Open **Voyager Instrument Control Panel** by clicking on the icon on the desktop. Turn on the high voltage (lightening bolt icon) 20 min before you plan to use the MALDI so the machine can warm up.
- Create a folder. Go to D Drive/Voyager Data/Chen/Services/WashU course2005, then create a folder for your data there.
- Have the instructor change the instrument settings and then go to file, save instrument settings as, and then save this .bic file in your folder.

The following steps are **VERY IMPORTANT**. If the plate is **inserted improperly** it will result in **very costly repairs** and the MALDI will be out of service to those who need it.

- At the opening where you insert the target plate, make sure that the black slit covering is centered in the way that your instructor shows you. If the ejector arm were to hit this covering when the arm is extending, it could damage the machine.
- Click on the hand icon so to extend the ejector arm. If a plate is presently inside the MALDI, you will see the wells move across the TV screen, then the machine will depressurize, and then the ejector arm will extend from the opening.
- Gently hold the ejector arm while sliding the plate out of the ejector arm in the way that your instructor has demonstrated. Hold the ejector arm while carefully sliding the plate into the grooves. Do not force the plate; it will slide in smoothly if you have positioned it properly. You will feel it click into place when it has been inserted far enough. Check the black slit covering to make sure that it is still centered around the ejector arm. DO NOT LEAVE THE EJECTOR ARM EXTENDED for more than a few minutes. This will affect the vacuum.
- In the dialog box that has appeared on the computer screen, make sure to change the plate ID. Choose **PS1 for plates with 96 wells**, and plate1 or plate2 for plates with 100 wells. Then click ok and the ejector arm will go back inside the MALDI.
- Turn on the high voltage if it is turned off during sample loading.
- When the machine has finished aligning the plate, the TV screen will stop moving and the laser will be in position A1_a. On the computer screen there is a map of the plate. Click on the well where your first calmix is located or select this spot from the drop down menu.
- Make sure the mass range has been changed to 900 to 3000.

- Fire (On the joy stick box, there are two buttons, **fire** is the button that is further from the power cord.)
- Wait until the machine has finished acquiring, then check to make sure that the intensity is between 8000 and 6E4 (60,000). If it isn't, move the laser within the well using the joystick and acquire again. If after several tries in different locations, you cannot get data in the recommended range, ask you instructor for help.
- When you have a good spectrum, accumulate it. This is done by first clicking on the spectrum window to highlight it, then clicking the accumulation icon (∑).
- Fire again. You may or may not need to move the laser. If the spectrum is good, accumulate again. Accumulate a total of 3 spectra. It will note how many spectrum are accumulated above the accumulated spectrum window, check to make sure you have three.
  Note: the noise level is decreased by accumulating more spectra.
- Click on the accumulation window to highlight it. In the data storage window, change the directory to your folder and the filename to calmix. In order for this spectrum to be saved, you must then click on the burning disk icon.
- Minimize Voyager. Open Data Explorer by clicking on the icon on the desktop. Go to File, Open, then select your folder, highlight the calmix file, click Add, and then Finish. The spectrum you saved should open.
- Go to Process, Mass Calibration, Manual Calibration. A dialog box will appear, do not close it but move it out of the way. Left click and drag over the first set of peaks. This will zoom in. Highlight the monoisotopic peak, which will always be the first in the series. Do this by right click and dragging over it. A dialog box will appear with a list of peaks and one will be hightlighted. Check to make sure the highlighted peak is the one whose reference mass is closest in value to the one that you have selected from the spectrum. Also make sure that the highlighted peak is specified as "resolved" rather than "averaged". Once you have made sure that the correct peak is highlighted, click okay. This peak will be added to the peaks matched list in the manual mass calibration dialog box.
- Move the dialog box out of the way, zoom in on the next set of peaks, highlight the monoisotopic peak, etc. Repeat these steps for all of the peaks that are displayed for Calibration Mixture 1 and 2 (see peptide standard spectra and masses diagram). You will not be able to match peak 3660.22 or 5734.6 because we have only acquired data in the 900 to 3000 mass range.
- In the manual mass calibration dialog box, click Plot, Apply Calibration, then Export. Export will save this calibration.
- Minimize Data Explorer and open Voyager again. In the calibration window, where it says external file, select the calmix file that you just saved in Data Explorer. This step applies the calibration.
- Now you will acquire the spectrum for your first sample. On the map of the plate, click on the well where your sample is located. The laser will change positions. Fire and accumulate 3 spectrum as before.

- Save the accumulated spectrum: Highlight the accumulated spectrum by clicking, then, in the file name box, change the name from calmix to sample1 (or whatever you'd like to name it). Make sure that your folder is still the one open in the directory. Then click on the burning disk. This data is now saved. You will use it to search for your protein's ID later by peptide mass fingerprinting.
- Go on to the second calibration spot. Follow the same calibration procedure as before. When you reach the manual mass calibration dialog box you can bypass highlighting each monoisotopic peak by choosing 'match peaks'. The peaks used in the previous calibration will appear. Check to see if they are all present. Click on 'plot', 'apply calibration', 'export'.
- Return to Voyager. Acquire data for the second sample in the same way as you did with the first. (But don't forget to apply the new calibration file before acquiring!)
- After you have finished acquiring spectrum for all of your samples, we will search their mass lists in the database.

Peptide mass fingerprinting (PMF)

- Open data file using Data explorer software
- Run **Macro 1** function to do baseline correction (BC), noise reduction (NR) and deisotoping (DI) (isotope Carbon 12)
- Use **Peaks** function, selection **Peak detection**, choose a baseline. Signals above the baseline are going to used for fingerprinting. For spectrum with a uneven basline, **Advanced settings** need to be chosen so that different baseline threshold within the spectrum can be specified.
- Use **Edit** function, **copy**, **mass list** (peptide fingerprints)
- Go to moleculardetective website: http://www.moleculardetective.org
- Click on Protein Search Engine, past the **mass list** in the **Data Paste Area**
- Select databases, enzyme used, organism, species, cysteine modifications and other potential modifications, MW range, pI, mass tolerance and other parameters.
- Start search
- Evaluate protein identification quality
- Change and play with baseline levels of the data file, change and play with database searching parameters, especially the mass tolerance, observe and note down the effects on protein identification process and results. Learn the criterion for judging the confidence of protein identification by peptide mass fingerprinting.