



Chapter 17

Preparation of Samples for a Mass Spectrometry-Based Method to Identify Allergenic Proteins

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Abstract

Western blotting is an immunological technique that has been combined with mass spectrometry analysis, to create a high-throughput method for protein identification. Western blotting using serum allows us to identify a protein within an allergenic extract that specifically binds to a serum antibody, immunoglobulin E. This specific IgE binding protein can then be detected with a highly sensitive chemiluminescence detection substrate.

Proteins detected by western blotting can be analyzed by mass spectrometry following an in-gel digestion protocol. The protein band of interest is excised from the gel and digested with trypsin to form peptides. Mass spectrometry will almost certainly have a pre-chromatographic step in which these peptides are separated before becoming ionized and entering a mass analyzer. It is in the mass analyzer that peptides are identified according to their mass-to-charge ratio, compiled into a mass spectrum which is compared to mass spectra held within online protein databases.

Key words Allergen, Mass spectrometry, Gel electrophoresis, Western blotting

1 Introduction

Mass spectrometry (MS) is a powerful analytical technique used to measure the mass of ionic species within a sample. It has become an invaluable tool within many scientific disciplines, including the field of proteomics. In the last two decades, MS-based proteomics has seen significant advancements and is now the method of choice to identify and characterize biological proteins [1]. This method is now used in the identification of allergens as well as their isoforms, variants, and posttranslational modifications [2, 3]. In this chapter we describe how MS can be combined with the traditional Western blotting method, to provide a selective immuno-proteomic tool for the identification of unknown allergens [2].

1.1 Western Blotting

In western blotting, the proteins (containing unknown or putative allergens) are first separated by gel electrophoresis (either according to molecular weight, isoelectric point, or both). The protein is then transferred onto a membrane made of nitrocellulose or polyvinylidene difluoride (PDVF) and probed with a primary and secondary antibody [4]. The primary antibody binds specifically to the protein of interest. For this, serum from an allergic patient containing specific immunoglobulin E can be used. The secondary antibody binds specifically to the primary antibody, and is also labeled with an enzyme, such as horseradish peroxidase or alkaline phosphatase. The enzyme label reacts with a chemiluminescent substrate, producing light as a by-product, which acts as the detectable protein signal. This technique has often been used in immunological studies to analyze the presence of relevant biological markers [5, 6]. Different western blotting detection methods have varying sensitivities. Chemiluminescence is the most sensitive, where light produced from the enzyme-substrate reaction is captured using charge-coupled device (CCD) cameras [7].

1.2 In-Gel Digestion

Using an optimized in-gel digestion protocol [8], proteins can be excised after gel electrophoresis and prepared for MS analysis. The recipe applies to 1- or 2-dimensional gels stained with Coomassie and comprises four main steps: (1) destaining, (2) reduction and alkylation, (3) proteolytic cleavage, and (4) peptide extraction.

1. To destain the excised gel pieces, a solution of ammonium bicarbonate and acetonitrile is used to break the bonds between the protein and Coomassie dye. For mass spectrometry analysis, Coomassie dye is the more commonly used staining method as glutaraldehyde used in silver staining impairs MS analysis.
2. Reduction of cysteine-disulfide bonds is performed to unfold the tertiary structure of the protein. This allows cleavage sites to be more available for the digestive enzyme. Agents such as tris-2-carboxyethyl phosphine (TCEP) or dithiothreitol (DTT) are used in reduction. Since cysteine-disulfide bonds cannot reform, alkylation is performed in combination with reduction. This is done using iodoacetamide (IAA), which adds an acetamide group to the sulfhydryl groups of cysteine residues, hence blocking bonds from reforming.
3. The unfolded proteins are then cleaved into peptides with a proteolytic enzyme. Trypsin is by far the most widely used enzyme for protein digestion. It is a serine protease that specifically cleaves peptide bonds at the carboxyl-terminal of lysine and arginine residues.
4. After digestion, the peptides generated are extracted from the gel matrix into solution using trifluoroacetic acid (TFA) and

acetonitrile. ZipTip®, a 10 µL pipette tip, is often used in this step as it contains a chromatography media for purifying and concentrating the extracted peptides in solution.

1.3 Nano Liquid Chromatography (LC) Coupled to Tandem Mass Spectrometry (MS/MS)

Prior to MS analysis, peptides will almost certainly be separated by a chromatographic method. The two most commonly used are gas chromatography (GC) for volatile compounds and liquid chromatography (LC) for biological samples. High-performance LC (HPLC) is commonplace in MS-based proteomics and relies on high-pressure pumps to pass the peptide sample in a solvent solution (mobile phase), through a column with an adsorbent material (stationary phase), typically silica particles. It is the interaction between the mobile and stationary phase that determines the pattern of peptide separation.

The coupling of LC with MS was made possible for mainstream clinical research only after the development of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). The interfaces between LC and MS available before this were pricey and often unreliable. Developed by Fenn et al. [9], ESI works by pumping liquid samples through a needle to be nebulized at the tip, forming a fine spray of charged droplets. These droplets are rapidly evaporated using heat and dry nitrogen and charged sample ions are released from the droplets that are free from solvent. The ionized sample is then transferred into a high vacuum and then through a small aperture into the mass analyzer. MALDI was developed by Tanaka et al. [10] and is less used in complex proteomics. Briefly, the liquid sample is mixed with organic material (matrix) and applied to a metal plate, positioned in the MS high-vacuum source. A pulsed laser beam irradiates the sample causing desorption from the plate followed by desolvation. The sample is then ionized by protonation/deprotonation and speeds toward the vacuum for entry into the mass analyzer.

A commonly used and trade-marked mass analyzer is the Orbitrap, developed by Hu et al. [11]. An Orbitrap consists of an outer barrel-shaped electrode and an inner spindle-like electrode which together trap ions by “electrodynamic squeezing.” The ions oscillate with different rotational frequencies that represent their mass-to-charge (m/z) ratio. After separating ions by their m/z ratio (MS_1), precursor ions can be selected for further fragmentation (MS_2). This is known as tandem mass spectrometry (MS/MS). Collision-induced dissociation (CID) is a popular fragmentation method because of its reproducible fragmentation of the peptide bond. During CID an inert gas (such as helium or argon) is introduced into the ion trap; this induces collision of precursor ions, fragmenting them into n-terminal and c-terminal peptide fragments (termed b and y product ions). This pattern of fragment ions and their mass provides information on the peptide sequence—this is a mass spectrum. The experimental mass spectrum data is

then compared against theoretical spectra in protein databases to determine the best match. Software packages are then used to generate reports showing protein matches. The matches will be accompanied by information on the number of peptides/spectra identified, match score, sequence coverage, and other factors that affect the protein identification probability.

1.4 Application of MS to Allergens

It has already been shown that MS can identify and measure specific allergens in addition to assessing variability between allergen extracts. Since allergen proteomics has developed, online directories have become available such as Allergome and WHO/IUIS Allergen nomenclature that contain allergen sequence information. MS has been applied to measure food allergens [3], house dust mite extracts [12], and variability among pollen allergens [13]. The use of MS with other commonly used immunoassays is thought to facilitate allergen standardization and development of new immuno-therapeutics [14].

We provide a protocol to separate, prepare, and identify allergenic proteins by combining western blotting with MS analysis.

2 Materials

2.1 Gel Electrophoresis

2.1.1 Equipment

Vortex mixer.

Glass vial.

Heating block.

NuPAGE™ 4–12% Bis-Tris Protein Gel (manufactured by Invitrogen: Paisley, UK).

Pasteur pipette.

XCell SureLock™ Mini-Cell (with gel tension wedge, empty cassette, and gel knife) (manufactured by Invitrogen: Paisley, UK).

PowerEase® 90 W (manufactured by Invitrogen: Paisley, UK).

2.1.2 Reagents

Allergen extract.

Ultrapure water (UPW).

NuPAGE™ LDS sample buffer (4×) (manufactured by Invitrogen: Paisley, UK).

Precision Plus Protein™ Dual Color Standards (manufactured by Bio-Rad Laboratories: Hemel Hempstead, UK).

2.1.3 Running Buffer

Add 50 mL NuPAGE™ MES SDS running buffer (20×) (manufactured by Invitrogen: Paisley, UK) to 950 mL ultrapure water and invert the solution ten times to mix.

2.2 Western Transfer Buffer

2.2.1 Equipment

Gel knife.

Plastic container (at least 1.6 cm length × 11.5 cm width, e.g., Scienceware[®] antibody saver trays, manufactured by Sigma: Schnelldorf, Germany).

Pasteur pipette.

XCell *SureLock*[™] Mini-Cell (with gel tension wedge, empty cassette, and gel knife) (manufactured by Invitrogen: Paisley, UK).

PowerEase[®] 90 W (manufactured by Invitrogen: Paisley, UK).

XCell II[™] Blot Module (with sponge blotting pads and roller) (manufactured by Invitrogen: Paisley, UK).

Nitrocellulose pre-cut blotting membranes, 0.2 µm pore size (manufactured by Invitrogen: Paisley, UK).

Tweezers.

2.2.2 Reagents

Methanol.

Ultrapure water (UPW).

2.2.3 Transfer Buffer

Add 50 mL NuPAGE[™] transfer buffer (20×) (manufactured by Invitrogen: Paisley, UK) to 100 mL methanol and 850 mL ultrapure water. Invert the solution ten times to mix.

2.3 Western Blotting

2.3.1 Equipment

Plastic container (at least 1.6 cm length × 11.5 cm width, e.g., Scienceware[®] antibody saver trays, manufactured by Sigma: Schnelldorf, Germany).

Shaker.

2.3.2 Reagents

Patient serum with positive specific IgE.

Control serum without positive specific IgE.

Goat anti-human IgE secondary antibody, HRP (manufactured by Invitrogen: Paisley, UK).

2.3.3 Blocking Buffer

Phosphate-buffered saline (PBS) pH 7.5, 1% bovine serum albumin, and 0.05% Tween-20.

2.3.4 Wash Buffer

Phosphate buffer pH 7.5 and 0.05% Tween-20.

2.4 Western Detection

2.4.1 Equipment

Tweezers.

Parafilm[®] M.

ChemiDoc[™] Imaging System (incl. UV sample tray) (manufactured by Bio-Rad Laboratories: Hemel Hempstead, UK).

2.4.2 Reagents

Pierce[™] ECL Western Blotting Substrate (incl. detections, reagents 1 + 2) (manufactured by Invitrogen: Paisley, UK).

2.5 Coomassie Staining

Plastic container (at least 1.6 cm length × 11.5 cm width, e.g., Scienceware® antibody saver trays, manufactured by Sigma: Schnelldorf, Germany).

2.5.1 Reagents

Ultrapure water.

2.5.2 Equipment

ChemiDoc™ Imaging System (incl. white sample tray for colorimetric imaging) (manufactured by Bio-Rad Laboratories: Hemel Hempstead, UK).

2.5.3 Fixing Solution

Methanol (46%) and acetic acid (7%) mixed in ultrapure water.

2.5.4 Staining Solution

Coomassie® R-250 (0.1%), methanol (46%), and acetic acid (7%) mixed in ultrapure water.

2.5.5 Destaining Solution

Methanol (5%) and acetic acid (7.5%) mixed in ultrapure water.

2.6 Image Laboratory Analysis

USB device.

Computer.

2.6.1 Equipment

2.6.2 Software

ImageLab (Version 5.2.1, manufactured by Bio-Rad Laboratories).

2.7 In-Gel Digestion

Scalpel.

2.7.1 Equipment

Eppendorf.

Centrifuge.

Heating block.

2.7.2 Reagents

ProteoExtract All-in-One Trypsin Digestion Kit (incl. wash buffer, digest buffer, D-buffer reagent, reducing agent, blocking reagent, trypsin) (manufactured by Calbiochem: San Diego, CA).

Trifluoroacetic acid.

Acetonitrile.

Milli-Q-grade water.

2.8 Desalt by ZipTip® and Mass Spectrometry Analysis

SpeedVac®.

Eppendorf.

Microcentrifuge.

2.8.1 Equipment

C18 ZipTip pipette tips (manufactured by Merck: Darmstadt, Germany).

pH paper.

Sonication bath.

Mass spec vial.

Monolithic nano-HPLC column.

Q Exactive Orbitrap mass spectrometer.

- 2.8.2 *Reagents* Trifluoroacetic acid.
 Acetonitrile.
- 2.8.3 *Software* Chromeleon.
 Xcalibur.
 PEAKS Studio 8.

3 Methods

3.1 *Gel Electrophoresis*

The protein content ($\mu\text{g}/\mu\text{L}$) in the allergen extract dissolved in ultrapure water (UPW) should be determined prior to electrophoresis using a quantification assay (e.g., *DC*[™] Protein Assay Kit (Bio-Rad Laboratories, Hertfordshire, UK)), following the manufacturer's instructions.

1. Calculate the volume needed (up to 22.5 μL) of the allergen solution to run between 2 and 50 μg of protein. (Prior optimization experiments using gel electrophoresis and Coomassie staining may be necessary to determine which protein mass (μg) of the allergen extract is optimal for protein detection.)
2. Pipette this volume in 2.5 μL of NuPAGE[™] LDS sample buffer ($\times 4$) and make up volume to $<25 \mu\text{L}$ with ultrapure water, in a clean glass vial (*see Note 1*).
3. Heat the sample at 70 °C for 10 min in a heat block.
4. Remove the NuPAGE[™] protein gel from its pouch and using a Pasteur pipette rinse the gel with prepared running buffer. Peel off the tape at the bottom of the gel and gently pull the comb out of the top of the cassette. Rinse the sample wells three times with prepared running buffer and invert the gel to empty the wells (*see Note 2*).
5. Place the gel into the XCell *SureLock*[™] Mini-Cell so the taller side of the gel faces outwards and place the empty cassette in the opposite side. Lock the gel into place using the gel tension wedge.
6. Pour prepared running buffer into the inner chamber, ensuring that there is no leak to the outer chambers, and then continue until just exceeding the level of the wells.
7. Using a pipette, load 5–10 μL of the heated sample into a well of the gel and 2 μL of Precision Plus Protein[™] Dual Color Standards into another well (*see Note 3*).
8. Fill the outer chamber with running buffer to reach the same level as the inner chamber and remove any bubbles with a Pasteur pipette (*see Note 4*).

9. Place the lid on the Mini-Cell securely, connect the electrodes to the PowerEase®, and turn on the power supply. Using the keypad on the power supply input a constant voltage of 200 V, current of 80 mA, and time of 35 min.
10. Press start (ensure that bubbles appear at the bottom) and run electrophoresis for at least 35 min, or until the dye front from the sample buffer has reached the bottom of the gel.
11. Once complete, press stop, turn off the power, disconnect electrodes, and remove the lid.
12. Remove the gel from the cell, then separate the three sides of the cassette using a gel knife, and remove the top cassette plate.
13. Proceed to either western transfer or Coomassie staining.

3.2 Western Transfer

1. Take the gel cassette with top plate removed and cut off the wells with gel knife (*see Note 5*).
2. Place four sponge blotting pads in a plastic container and submerge in prepared transfer buffer (*see Note 6*).
3. Prior to use also place two pieces of filter paper in prepared transfer buffer and in another container using tweezers place the pre-cut nitrocellulose membrane.
4. Place one piece of presoaked filter paper on top of the gel, leaving the gel foot uncovered, and use a roller to remove any trapped air bubbles.
5. Carefully turn over the plate so the gel and filter paper are facing downwards, and then using the gel knife push the gel foot out of the plate slot, so the gel and filter paper fall off the plate.
6. Cut off the gel foot and using a Pasteur pipette wet the gel with transfer buffer.
7. Using tweezers place the presoaked nitrocellulose membrane on top of the gel, and then another piece of presoaked filter paper on top of the membrane. Remove any trapped air bubbles with roller.
8. Place two presoaked sponge blotting pads into the cathode core (–) of the blot module (the larger part of the module). Then pick up the gel-membrane assembly and place on top of the blotting pads. Place another two presoaked sponge blotting pads on top of the assembly.
9. Place the anode core (+) on top, pick up the whole blot module together, and place it into the inner buffer chamber of the Mini-Cell so that the (+) sign is in the upper left-hand corner and the gold post on the right fits into the blot module. Secure the blot module with the gel tension wedge.

10. Fill the blot module with transfer buffer until the blotting sandwich is covered but is not filled to the top of the blot module. Then fill the outer chamber with ultrapure water until it reaches approximately 2 cm from the top and remove any bubbles with a Pasteur pipette (*see* **Note 7**).
11. Place the lid on the Mini-Cell, connect electrodes, and turn on the power supply as before.
12. Input a constant voltage of 30 V, current of 120 mA, and time of 1 h. This will enable the proteins to transfer from the gel to the membrane.

3.3 Western Blotting

1. When the transfer is complete, press stop and turn off the power, disconnect the electrodes, remove the lid, and take out the blot module.
2. Take off the anode core (+), remove the sponge blotting pads, and discard the filter paper.
3. Using tweezers pick up the nitrocellulose membrane and place it into a plastic container with prepared blocking buffer (enough to cover and allow the membrane to freely move). Leave at room temperature shaking at 60 rpm for 90 min (*see* **Note 8**).
4. Prepare the primary antibody solution using patient serum diluted in blocking buffer (recommended dilution is between 1:5 and 1:10, serum:blocking buffer). The total volume will depend on how much is needed to cover the membrane in the container you are using (*see* **Note 9**).
5. Replace the blocking buffer with the 1:5–1:10 diluted primary antibody solution and leave the membrane shaking overnight at 2–8 °C (*see* **Note 10**).
6. Remove the antibody solution and wash the membrane at least five times using wash buffer at room temperature, shaking at 60 rpm.
7. Prepare the secondary antibody solution using goat anti-human IgE secondary antibody, HRP diluted in blocking buffer at a 1:5000 dilution. As before, the total volume will depend on the container you are using. Mix by pipetting the antibody in solution several times to ensure that the antibody is dispersed in the buffer.
8. Replace the wash buffer with the secondary antibody solution and leave the membrane for 30 min shaking at room temperature.
9. Remove the antibody solution and wash the membrane at least five times using wash buffer.

3.4 Western Detection

1. Leaving the membrane in wash buffer, cut out a piece of Parafilm® M slightly larger than the membrane and stick on a lab bench. Using tweezers place the membrane onto the Parafilm® M (*see Note 11*).
2. Prepare the substrate working solution immediately before use, by mixing 1 mL each of Pierce™ ECL Western Blotting Substrate detection reagents 1 and 2. Pipette the solution onto the membrane and leave at room temperature for 5 min.
3. During this time, switch on the ChemiDoc™ Imaging System and ensure that the UV sample tray is placed in the imaging stage.
4. Once the substrate incubation is over, immediately place the membrane onto the sample tray, push the transilluminator drawer in, and close the front door of the ChemiDoc.
5. Using the touch screen select “camera view” and specify the image size so the whole membrane is captured on the screen.
6. Press “Application,” and then “Blots,” and select “Chemiluminescence.”
7. Press “Exposure,” and then “Manual,” and select “Configure Signal Accumulation Mode.”
8. For exposure times enter 30 s in the “First Image” box and 300 in the “Last Image” box and in the “Images” box enter 5 as the total number of images. Then tap the Camera icon.
9. Image the dual color standards by selecting “Colorimetric” under “Blots” and “Application.” Then tap the Camera icon as before to acquire the image.
10. Once the images are acquired proceed to Image Lab analysis.

3.5 Coomassie Staining

For Coomassie staining all steps should be carried out at room temperature using enough solution to cover the gel.

1. After gel electrophoresis briefly rinse the gel in ultrapure water and place in a plastic container with prepared fixing solution shaking for 1 h.
2. Replace fixing solution with prepared staining solution and leave shaking for 1 h.
3. After staining, briefly rinse the gel with ultrapure water and place in prepared destain solution shaking overnight. Destain solution should be changed at regular intervals to wash off background staining.
4. Once the background is destained, briefly wash the gel in ultrapure water, place the gel onto the white sample tray, and place in the imaging stage of the ChemiDoc Imaging System (*see Note 12*).

5. Turn on the imaging system, push in the transilluminator drawer, and close the drawer of the imaging system.
6. Using the touch screen select “camera view” and specify the image size so that all of the gel is captured on the screen.
7. Press “Application,” and then “Protein gels,” and select “Coomassie.”
8. Press “Exposure,” and then select “Faint bands.”
9. Then tap the Camera icon to acquire the Coomassie-stained gel image.

3.6 Image Laboratory Analysis

1. Export images from the ChemiDoc Imaging System using a USB device.
2. On a computer device open these image files (Type: Image Lab Image Document) on Image Lab (version 5.2.1).
3. Use the left-hand “Analysis Tool Box” to annotate and edit the image in the following ways.
4. For blots, choose the chemiluminescence image with the optimum exposure of the samples and the colorimetric image of the standards. Click on “Image Tools” and select “Merge.”
5. For both blots and protein gels, crop the image, so that all the standards and samples are showing.
6. From the “Analysis Tool Box” click on “Lanes and Bands,” manually input the number of lanes, and adjust these for your blot. From “Bands,” use the “Add” tool to click on the ten dual color standards.
7. Return to the “Analysis Tool Box” and click on “MW Analysis Tools.” Select the “Bio-Rad Precision Plus” Standard and tick the standards lane. Select “no” to redetecting the bands.

Using the molecular weights of the standards, determine which bands on the Coomassie gel also appear on the chemiluminescence blot. Use these bands for in-gel digestion.

3.7 In-Gel Digestion

1. Using a clean scalpel excise the bands from the Coomassie-stained gel that match those detected in the chemiluminescence blot (*see Note 13*).
2. Cut each band into 2 mm² pieces and transfer into an Eppendorf.

For the following steps use reagents from the ProteoExtract All-in-One Trypsin Digestion Kit, according to the manufacturer’s instructions.

3. Wash the gel pieces three times with 80 µL of wash buffer (acetonitrile) at 37 °C in a heating block.
4. Dry the gel pieces by heating to 90 °C for 15 min.

5. Prepare digest buffer by using 0.5 mL of digest buffer solution to dissolve the lyophilized D-buffer reagent.
6. Prepare reducing agent by addition of 120 μL of ultrapure water to make stock solution. Before use, take 1 μL of stock solution and dilute in 9 μL of ultrapure water.
7. Add 20 μL of digest buffer to the gel pieces, mix in 2 μL of diluted reducing agent, and incubate at 37 °C for 10 min. Remove the Eppendorf from the heat block and cool to room temperature.
8. Prepare blocking reagent by addition of 120 μL of ultrapure water to make stock solution. Before use, take 1 μL of stock solution and dilute in 9 μL of ultrapure water.
9. Mix 2 μL of diluted blocking agent into gel pieces and incubate at room temperature for 10 min (*see Note 14*).
10. Prepare trypsin by addition of 50 μL of ultrapure water to the lyophilized trypsin to make stock solution. Before use, take 1 μL of stock solution and dilute in 3 μL of ultrapure water.
11. To the gel pieces, add 0.5 μL of diluted trypsin and mix. Incubate in trypsin, shaking for 3–5 h at 37 °C. The gel pieces must be fully submerged (*see Note 15*).
12. Centrifuge the sample for 10 min at $10,000 \times g$, and then transfer the supernatant to a new Eppendorf.
13. Add 10 μL of 0.1% trifluoroacetic acid (TFA) to the gel pieces and shake at 37 °C for 10 min. Centrifuge this sample and combine the supernatants.

Proceed with ZIPTIP extraction or samples can be stored at $-20\text{ }^{\circ}\text{C}$.

3.8 Desalt by ZipTip®

1. Dry the gel pieces by placing in a SpeedVac for 10 min.
2. Check the acidity of the sample by addition of 13 μL of 1% TFA and pH paper (should turn red) (*see Note 16*).
3. Using a ZipTip_{C18} tip, depress pipette plunger to a dead stop and using a 13 μL volume setting aspirate acetonitrile (100%) into the tip. Dispense, discard, and repeat.
4. Then aspirate 0.1% TFA, dispense, discard, and repeat.
5. Depress pipette plunger to a dead stop, then aspirate, and dispense the peptide sample in 7–10 cycles in the Eppendorf.
6. Aspirate 0.1% TFA, dispense, discard, and repeat.
7. Prepare elution solution as 0.1% TFA and 50% acetonitrile.
8. Dispense 13 μL of elution solution into a clean Eppendorf, then aspirate, and dispense the eluent through the ZipTip pipette three times, without introducing any air bubbles.
9. Dry the sample in a SpeedVac for 10 min.

10. Dissolve the sample in 6 μL of 0.1% TFA and place in a sonication bath for 5 min.
11. Briefly centrifuge the sample and then transfer to a mass spectrometry specialist vial.

The sample is now ready for mass spectrometry analysis. The following is based on using an automated EASY Nano Liquid Chromatograph 1000 coupled to an electrospray ionization Orbitrap mass spectrometer. Chromeleon Chromatography Data System and Xcalibur software should be used to program the instruments. For chromatography, the flow rate should be selected as 200 nL/min over 60 min using a 75 μm column with a linear gradient of acetonitrile in 0.1% formic acid. For mass spectrum scans, a data-dependent switching mode and Top20 collision-induced dissociation fragmentation method should be used.

After samples have been run, the mass spectrum data can be searched against Uniprot protein database. On PEAKS Studio 8 the search should be selected with a fragment ion mass tolerance of 0.80 Da, a parent ion tolerance of 20 ppm, trypsin as the digestion enzyme, and a false discovery rate (FDR) of 1%. These results can then be exported to Excel and scores ($-10\lg P$), coverage (%), signal intensities, and no. of peptides used to determine the most abundant proteins present in your sample.

4 Notes

1. When pipetting the LDS sample buffer and ultrapure water into the allergen solution, use a mix-pipetting technique, to ensure that the final solution is thoroughly mixed.
2. When pulling out the gel comb, hold the gel with both hands and push the comb out using slight pressure upwards with your thumbs.
3. When loading samples and standards with a pipette, do so slowly and continuously, so as not to introduce any air bubbles.
4. To minimize bubbles, pour the running buffer solution slowly.
5. To cut wells, place gel knife directly below them, apply little pressure, and move the gel knife away from the gel.
6. Squeeze the pads while submerged to ensure that any air bubbles are removed.
7. To minimize bubbles, pour the transfer buffer solution and ultrapure water slowly.
8. Prepare blocking buffer prior to use to ensure that solution is dissolved properly.

9. Use a mix-pipetting technique to ensure that the serum is thoroughly mixed into the blocking buffer.
10. Cover the container with parafilm, to avoid any evaporation of solution.
11. Parafilm can be stuck on a lab bench by pushing down the corners with a blunt object such as a pen or pencil.
12. Fixing, staining, and destaining solution can all be prepared before use and kept for further use.
13. To more accurately see the bands which need to be excised, perform this step on a bright surface.
14. Add reducing and blocking reagent by mix-pipetting technique.
15. This can also be left to incubate overnight.
16. If sample is not acidic, continue adding 1% TFA and retesting.

References

1. Schuchardt S, Sickmann A (2007) Protein identification using mass spectrometry: a method overview. *EXS* 97:141–170
2. Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, Schmid E (1995) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* 270(6):2607–2613
3. Koeberl M, Clarke D, Lopata AL (2014a) Next generation of food allergen quantification using mass spectrometric systems. *J Proteome Res* 13(8):3499–3509. <https://doi.org/10.1021/pr500247r>
4. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76(9):4350–4354
5. Ebner C, Birkner T, Valenta R, Rumpold H, Breitenbach M, Scheiner O, Kraft D (1991) Common epitopes of birch pollen and apples—studies by western and northern blot. *J Allergy Clin Immunol* 88(4):588–594
6. Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF (2004) Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin Exp Allergy* 34(4):583–590. <https://doi.org/10.1111/j.1365-2222.2004.1923.x>
7. Ghosh R, Gilda JE, Gomes AV (2014) The necessity of and strategies for improving confidence in the accuracy of western blots. *Expert Rev Proteomics* 11(5):549–560. <https://doi.org/10.1586/14789450.2014.939635>
8. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1(6):2856–2860. <https://doi.org/10.1038/nprot.2006.468>
9. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science (New York, NY)* 246(4926):64–71
10. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T (1988) Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2(8):151–153. <https://doi.org/10.1002/rcm.1290020802>
11. Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Graham Cooks R (2005) The Orbitrap: a new mass spectrometer. *J Mass Spectrom* 40(4):430–443. <https://doi.org/10.1002/jms.856>. Accessed 13 Dec 2018
12. Harris J, Mason DE, Li J, Burdick KW, Backes BJ, Chen T, Shipway A, Van Heeke G, Gough L, Ghaemmaghami A, Shakib F, Debaene F, Winssinger N (2004) Activity profile of dust mite allergen extract using substrate libraries

- and functional proteomic microarrays. *Chem Biol* 11(10):1361–1372. <https://doi.org/10.1016/j.chembiol.2004.08.008>
13. Fenaille F, Nony E, Chabre H, Lautrette A, Couret M, Batard T, Moingeon P, Ezan E (2009) Mass spectrometric investigation of molecular variability of grass pollen group 1 allergens. *J Proteome Res* 8(8):4014–4027. <https://doi.org/10.1021/pr900359p>
14. Chapman MD, Briza P (2012) Molecular approaches to allergen standardization. *Curr Allergy Asthma Rep* 12(5):478–484. <https://doi.org/10.1007/s11882-012-0282-3>