

## Immunoproteomics

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### Abstract

Two-dimensional electrophoresis results in an adequate resolution of the proteome of microorganisms to allow the detection and identification of specific antigens after blotting on membranes and overlaying the protein pattern with patient's sera. The complement of all identified antigens presents the immunoproteome of a microorganism. All the antigens specific for a microorganism or even for a disease are identified by mass spectrometry. For identification, peptide mass fingerprinting is used, and post-translational modifications are detected by mass spectrometry MS/MS techniques. High-resolution two-dimensional electrophoresis and unambiguous identification are prerequisites for reliable results. After statistical analysis, the resulting antigens are candidates for diagnosis or vaccination and targets for therapy.

**Key Words:** Two-dimensional electrophoresis; immunoproteome; diagnostics; therapy; vaccination; mass spectrometry; peptide mass fingerprinting; proteome database.

### 1. Introduction

For about 50 yr scientists have tried to elucidate the protein composition of biological compartments. The first two-dimensional electrophoretic separation resolved 15 proteins from human serum (1). Ribosomes with about 60 protein components were the first completely resolved organelles (2). For more complex compartments, the resolution power had to be improved. This goal was reached by the combination of isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for two-dimensional electrophoresis (2-DE) (3,4). With this improvement, several hundred proteins were separated within one 2-DE gel. The method was further optimized for high resolution, and at present more than 10,000 protein species may be separated within large (30 × 40 cm) gels (5). In 1995 the term proteome was defined: the *proteome* refers to the total protein complement of a genome (6).

The identification of antigens within gels was not possible for a long time. Therefore, blotting of proteins to nitrocellulose membranes was an important milestone for

From: *Methods in Molecular Medicine*, vol. 94: *Molecular Diagnosis of Infectious Diseases*, 2/e  
Edited by: J. Decker and U. Reischl © Humana Press Inc., Totowa, NJ

the detection of antigens (7,8). After transfer of proteins from gels to membranes, antibodies can interact and bind to the antigens for which they are specific. To detect the antigen-antibody complex, a second antibody is overlaid with specificity against the class of the first antibody (e.g., IgG). The secondary antibody is coupled with a detection system. This principle was used early for identification of proteins in 2-DE gels (9). Overlaying blots of gels from microorganisms with sera from patients infected with these microorganisms reveals the immunoproteome of the microorganism under investigation. For many years one-dimensional (1D) gels were used to detect antigens for diagnostics. Because of the high complexity of the proteome, the assignment of a 1D band to a distinct protein is very difficult, and 2D separation is required for unambiguous identification of antigens. *Borrelia garinii* and *Helicobacter pylori* are two of the first immunoproteomes analyzed by 2-DE and mass spectrometry (MS) (10,11). Direct immunodetection within the gels avoiding blotting has been reported recently (UnBlot In-Gel Chemiluminescent Detection Kit, Pierce, Rockford, IL). The future will show whether this procedure can substitute for immunoblotting.

A view of the immunoproteome may also be obtained by enzyme-linked immunosorbent assay (ELISA) tests and immunoprecipitation. ELISA tests do not differentiate between the different proteins, and immunoprecipitation requires larger amounts of antibodies and suffers from problems with removal of antibodies before identification of the antigens. Therefore, at present the 2-DE/MS approach reveals the most complete view of the immunoproteome.

The major steps of immunoproteomics are as follows:

1. Two-dimensional electrophoresis.
2. Semidry blotting.
3. Immunodetection.
4. Data analysis.
5. Antigen identification.

### 1.1. Two-Dimensional Electrophoresis

The smallest unit of a proteome is the protein species (12), which is defined by its chemical structure. Therefore, a myosin phosphorylated at position  $x$  and a myosin phosphorylated at position  $y$  are two different protein species of one protein.

A proteome of a microorganism with 3000 genes may comprise 9000 or more protein species. Even if not all of the genes are represented by proteins in a certain biological situation, several thousands of protein species may be expected to be present. Indeed, about 1800 protein species were detected for *H. pylori*, which contains a genome of about 1600 genes (13). Therefore, high-resolution 2-DE techniques are a prerequisite to resolve this complexity. Resolution may be improved by increasing the gel size or by the production of several gels with different separation ranges. The  $pI$  range of a gel may be modified by the use of different ampholyte or immobililine gradients. The molecular weight ( $M_r$ ) range depends on the porosity of the gel matrix, which itself may be modified by different acrylamide and crosslinker concentrations. The strategy of using large gels has the advantage that the complete information is contained in one gel. High quality of 2-DE gels, as shown in **Fig. 1**, is a necessary prerequisite for successful immunoproteomics.

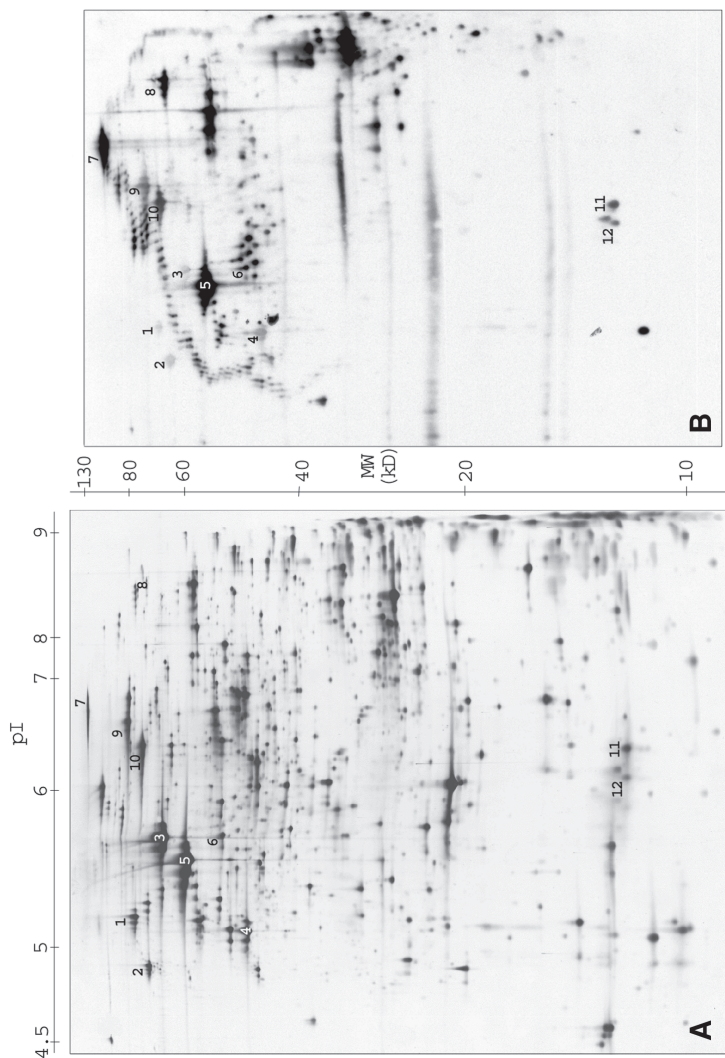


Fig. 1. Comparison of a 2-DE gel and a 2-DE gel immunoblot. (A) Silver-stained large 2-DE gel (23 × 30 cm) of *H. pylori* lysate containing about 1800 spots. This gel is the standard gel in the 2D-PAGE database (<http://www.mpiib-berlin.mpg.de/2D-PAGE/EBP-PAGE/index.html>). (B) Large 2-DE immunoblot (23 × 30 cm) of *H. pylori* lysate probed with a human serum. About 400 spots are recognized. Twelve of the spots are numbered in A and B. These spots contain the following proteins: 1, elongation factor G; 2, heat shock protein 70; 3, urease  $\beta$ -subunit; 4, elongation factor TU; 5, GroEL; 6, glutamine synthetase; 7, cag26; 8, not yet identified; 9, N-methylhydantoinase; 10, hydantoin utilization protein A; 11 and 12, GroES.

### 1.2. Semidry Blotting

Towbin et al. (7) blotted the proteins within a tank, where the blot sandwich was surrounded by large volumes of buffer. Potential impurities from the buffer are avoided by the use of semidry blotting, which is also easier to perform (14). The critical point of the blotting mechanism is the time point at which the SDS is stripped off from the protein (15). If SDS is removed from the protein still in the gel, the protein cannot be transferred to the membrane. If SDS is not stripped off from the protein at the moment the SDS-protein complex reaches the membrane, the protein cannot bind to the membrane and moves through the membrane to the anode. High- $M_r$  proteins tend to lose SDS early and remain in the gel. Low- $M_r$  proteins tend to lose SDS too late and do not bind to the membrane. Improving the SDS-protein binding by addition of SDS to the cathode buffer improves the blotting efficiency for large proteins. A better blotting efficiency for low- $M_r$  proteins is obtained by improving the hydrophobic interaction between membrane and protein by increasing the ionic strength of the blotting buffer.

### 1.3. Immunodetection

After blotting, the proteins are immobilized in the membrane. Sera of patients are overlaid to detect the antigens against which the patients have produced antibodies. Here one has to be aware of two causes of variability. First, the genetic variability of the microorganism and second, the variability of the immunological response of the host. This response depends on the strain of the microorganism the host is infected with and its own genome and environment. Because individual medicine is only a vision at the moment, for the development of diagnostics, therapeutics, and vaccines at present we have to search for proteomic signatures independent of the strain and individual host. Therefore large series of patient sera with one or several common strains of the microorganisms have to be searched for antigens; only those antigens common for a majority of them are potential candidates for diagnosis, therapy, and vaccination. Attempts were also made to correlate antigen composition with certain disease manifestations (10,11,16). For each microorganism, its own rules for acceptance of immunologically relevant candidates have to be delineated from the immunoproteomes obtained.

With the standard procedure, primary antibody/secondary antibody coupled with peroxidase or alkaline phosphatase, a better sensitivity than with silver staining is already mostly obtained. When chemiluminescence is applied, a further enhancement of sensitivity may be reached. For optimal sensitivity, the blocking reagents and washing procedures play an important role to avoid background staining.

### 1.4. Data Analysis

After blotting and immunostaining, a pattern of spots arises, which normally is completely different compared with the silver-stained 2-DE pattern. The assignment of spots between these two patterns is easy if highly intense characteristically formed spots are found in both patterns. However, assignment becomes more and more complicated the lower the number of spots in the immunostained pattern and the higher the number of antigens not stained in the silver-stained pattern. Several strategies are used

for unambiguous assignment. One is replica blotting. Here, during the blotting procedure, the proteins are blotted to both sides of the gel by changing the direction of the electric field strength during the blotting procedure (17). One blot is immunostained, and the other is stained by Coomassie Brilliant Blue (CBB) or by more sensitive stains like Aurodye. Another strategy is to counterstain the membrane with CBB after immunostaining (18). This is possible because the surface-bound blocking proteins are removed from the membrane during the washing procedures, and the blotted proteins, which are bound within the membrane, remain in the membrane during washing.

Because of the potentially high variance, each immunostaining experiment has to be repeated at least three times. The resulting spot patterns are spot detected and matched by commercial image processing software. Within a virtual master gel, the spot intensity differences of all the tested sera can be visualized (Fig. 2), and nonspecific reactions may be eliminated by comparison with control sera.

### 1.5. Antigen Identification

If highly specific antibodies are available, antigens may be identified by them after stripping of the antibodies from the serum directly from the same membrane used for the serum tests. Protein chemical identification is more reliable and may also lead to identification at the protein species level. Here MS is the method of choice. Peptide mass fingerprinting (19) after tryptic digestion from spots out of preparative gels stained with CBB G-250, results in secure identification, if the genome of the microorganism is already completely sequenced (Fig. 3). Sequence information by MS/MS techniques gives information about post-translational modifications and also of genes not described before (20). For identification of an antigen, it is important to show by MS that the immunostained spot contains only one protein. Because of the high sensitivity of the immunostaining, minor components of a spot may also be detected.

## 2. Materials

### 2.1. Two-Dimensional Electrophoresis

The 2-DE procedure is beyond the scope of this chapter; see Subheading 3.1.

### 2.2. Semidry Blotting

1. Polyvinylidene difluoride (PVDF) blotting membrane (Immobilon-P Transfer Membrane, Millipore, Bedford, MA).
2. Filter paper (GB003 Gel-Blotting-Papier, Schleicher & Schuell, Dassel, Germany).
3. Blotting buffers: *methanol is toxic by inhalation—prepare and use solutions under a hood!*
  - a. For the high- $M_r$  part of the gel (30–150 kDa):  
Cathode buffer: 50 mM boric acid, 10% methanol, 5% SDS; add NaOH to adjust to pH 9.0.  
Anode buffer: 50 mM boric acid, 20% methanol; add NaOH to adjust to pH 9.0.
  - b. For the low- $M_r$  part of the gel (4–30 kDa):  
Cathode and anode buffers: 100 mM boric acid, 20% methanol; add NaOH to adjust to pH 9.0.
4. Blotting chambers (Hoefer Large SemiPhor, semidry transfer unit, Amersham Pharmacia Biotech, San Francisco, CA).

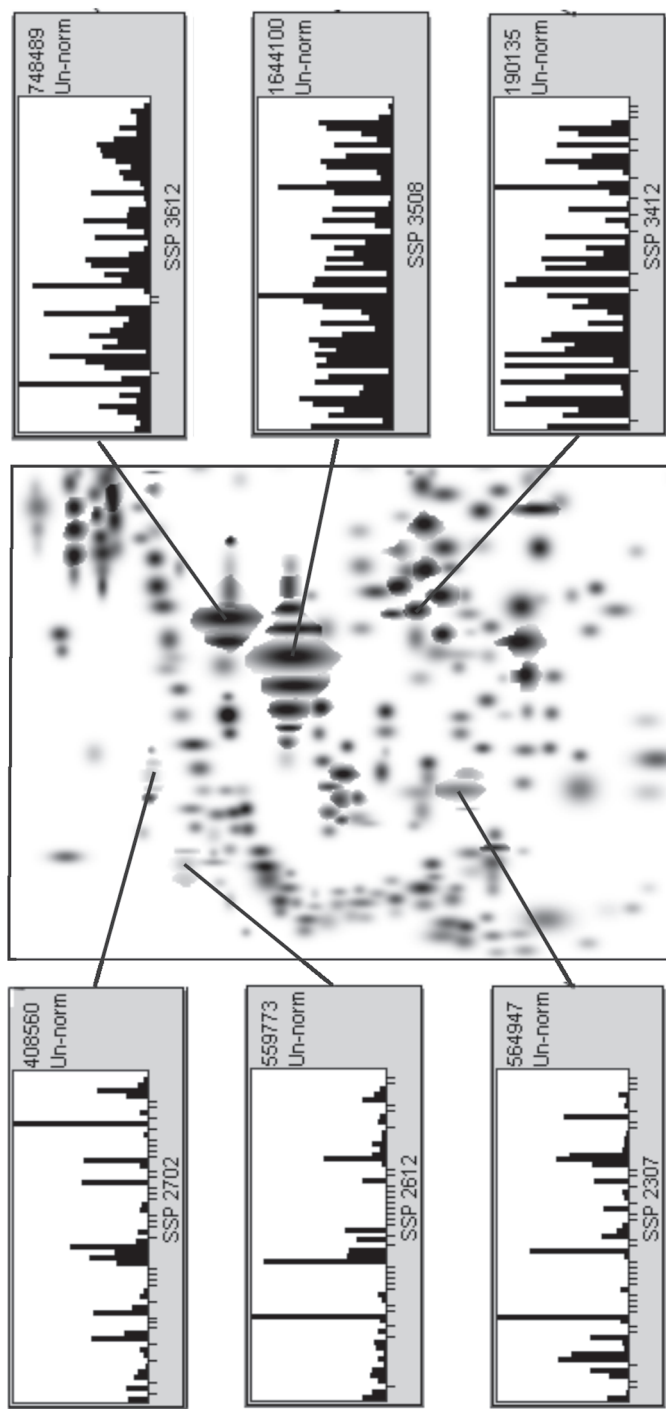


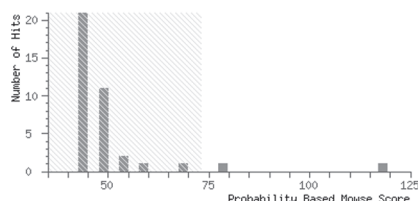
Fig. 2. Result of a data analysis of 2-DE immunoblots using PDQuest software (see **Subheading 2.4**). Upper left section of the master blot, which contains the gaussian fitted spots of all immunoblots in the analysis set. Marked are spots 1–6 from **Fig. 1**. For each spot the columns in the small boxes correspond to the intensity in each immunoblot of the analysis set. The numbers in the upper right corners of the small boxes show the maximum intensity of each spot. SSP numbers are unique spot numbers that are automatically generated by the software.

## *{MATRIX}* *{SCIENCES}* Mascot Search Results

User : MPIIB  
 Email : krah@mpiib-berlin.mpg.de  
 Search title :  
 Database : NCBI nr 20021113 (1231734 sequences; 391905809 residues)  
 Timestamp : 20 Nov 2002 at 10:30:08 GMT  
 Top Score : 118 for gi|15644739, chaperone and heat shock protein 70 (dnaK) [Helicobacter pylori 26695]

### Probability Based Mowse Score

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



### Concise Protein Summary Report

[Switch to full Protein Summary Report](#)

To create a bookmark for this report, right click this link: [Concise Summary Report \(./data/20021120/FtgizTt.dat\)](#)

Re-Search All

Search Unmatched

1. [gi|15644739](#) Mass: 67011 Total score: 118 Peptides matched: 14  
 chaperone and heat shock protein 70 (dnaK) [Helicobacter pylori 26695]

2. [gi|1561171](#) Mass: 67081 Total score: 80 Peptides matched: 11  
 70kDa chaperone [Helicobacter pylori J99]

Fig. 3. Search result of a protein identification by a peptide mass fingerprint using the search machine Mascot (see **Subheading 2.5.**). The search result of spot 2 (see **Fig. 1**). The columns show the number of protein hits with a certain score value. Scores above 73 are considered to be significant (outside the hatched area). Hits that have no significant score must be considered random events. In this case, two significant hits are found. The first is the heat shock protein 70 of *H. pylori* 26695, the strain used in this experiment. The second hit is the corresponding protein in strain J99. This protein has a highly similar, but not identical, sequence in both strains. Links are given in the search result to gain more information about the proteins.

## 2.3. Immunodetection

Prepare stackable boxes a little larger in size than the membranes.

1. PBST buffer: add Tween-20 to phosphate-buffered saline (PBS) at pH 7.6 to obtain a concentration of 0.05%.
2. Dry milk (Blotting Grade Blocker, Non-Fat Dry Milk, Bio-Rad, Hercules, CA).
3. Primary antibodies or patient sera.
4. Secondary antibody directed against the primary antibody used (e.g., goat anti-human polyvalent IgG-Peroxidase Conjugate, Sigma; cat. no. A-8400): store at  $-20^{\circ}\text{C}$  and avoid thawing-freezing cycles by freezing in aliquots.



5. Chemiluminescence reagents (Western Lightning Chemiluminescence Reagent NEL-101, NEN, Perkin Elmer, Boston, MA); store at 4°C.
6. Film (Biomax MR, Kodak, Rochester, NY).
7. Film cassette (Hypercassette, Amersham Pharmacia Biotech UK, Buckinghamshire, UK).
8. Photo machine in a dark room.
9. CBB R-250 staining solution: 50% methanol, 10% acetic acid, 0.1% CBB R-250 (Bio-Rad, Hercules, CA). *Methanol is toxic by inhalation!*
10. CBB destaining solution: 50% methanol, 10% acetic acid.

## 2.4. Data Analysis

1. Scanner (Umax Mirage IIse, Taiwan).
2. 2-DE analysis software (PDQuest, Version 7.1, Bio-Rad).

## 2.5. Antigen Identification

Avoid contamination of the buffers by dust and keratin!

1. 2-DE public database for the organism examined (in-house if available or via Internet: World 2D PAGE: <http://www.expasy.org/ch2d/2d-index.html>).
2. Fixing solution for preparative gels: 50% methanol, 2% phosphoric acid.
3. CBB G-250 staining solution for preparative gels: 34% methanol, 17% (w/v) ammonium sulfate, 2% phosphoric acid; 0.66 g/L CBB G-250 is added later (Bio-Rad).
4. Spot destaining solution: 200 mM  $\text{NH}_4\text{HCO}_3$ , 50% acetonitrile.
5. Digest buffer: 50 mM  $\text{NH}_4\text{HCO}_3$ , 5% acetonitrile.
6. Sequencing grade modified trypsin (Promega, Madison, WI) is dissolved to 0.2  $\mu\text{g}/\mu\text{L}$  in resuspension buffer provided by the manufacturer. Freeze in aliquots for storage.
7. Shrink buffer: 60% acetonitrile, 0.1% trifluoroacetic acid.
8. Sample buffer: 33% acetonitrile, 0.1% trifluoroacetic acid.
9. Matrix solution: 50 mg/mL 2,5-dehydroxybenzoic acid dissolved in 33% acetonitrile, 0.33% trifluoroacetic acid.
10. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager Elite DE, Perseptive Biosystems or others; *see Note 19*).
11. Search machines for peptide mass fingerprints, e.g., Mascot (<http://www.matrixscience.com>), ProFound ([http://129.85.19.192/profound\\_bin/WebPro\\_Found.exe](http://129.85.19.192/profound_bin/WebPro_Found.exe)), or MS-Fit (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>).

## 3. Methods

### 3.1. Two-Dimensional Electrophoresis

It would go beyond the scope of this chapter to explain sample preparation and 2-DE in detail. The procedure we are using (as shown in **Fig. 1**) is comprehensively described in refs. **5** and **21**. Briefly, for the first dimension, 150  $\mu\text{g}$  of protein sample was applied to the anodic side of the IEF gel. In the second dimension, after equilibration in SDS-containing buffer, the IEF gel was placed onto a 23  $\times$  30-cm gel and proteins were separated according to their  $M_r$ .

It is important to consider that the quality of the immunoblots strongly depends on the quality of the gels that are used (such as resolution power; *see Note 1*). Depending on the scientific goal, one should only use the gel system (size, sample buffer, detergents, pH gradient) that is able to resolve the proteins of interest. Because experiments



often search for unknown proteins, it is better to use the gel system with the highest resolution power. We recommend the use of large gels for immunoproteomics of microorganisms. The following procedure is used for 23 × 30-cm gels.

### 3.2. Semidry Blotting

1. Prepare two blotting chambers and cut PVDF membrane to 23 × 15 cm (half the size of the gel). Filter papers should be at least 22 × 17 cm. Mark PVDF membrane unambiguously using a pencil (*see Note 2*).
2. Soak filter papers into the blotting buffers, three sheets each in anode and cathode buffers of high- $M_r$  and six sheets in low- $M_r$  buffer (*see Note 3*).
3. Cut the large 2-DE gel into two pieces (high and low  $M_r$ ) right after the run has finished.
4. Build up the following sandwich in both blotting chambers, avoiding any air bubbles: anode (+), three filter papers (soaked in anode buffer), PVDF, gel, three filter papers (soaked in cathode buffer), cathode (−) (*see Note 4*).
5. Apply a constant current of 1 mA/cm<sup>2</sup> for 2 h.
6. Discard filter papers and gel (*see Note 5*).
7. Dry PVDF membranes at room temperature and freeze at −20°C for prolonged storage.

### 3.3. Immunodetection

Volumes of buffers depend on the size of the boxes. Here volumes for half an immunoblot in 20 × 20-cm boxes are given.

1. Thaw PVDF membranes and put each half into separate boxes.
2. Soak membrane in 100% methanol for about 1 min (for PVDF only; *see Note 6*). Do not let membrane dry out from this point until the film is exposed (*see Note 7*).
3. Block membrane in 100 mL 5% milk in PBST buffer for at least 1 h (or overnight at 4°C) using an appropriate shaker (*see Note 8*).
4. Add primary antibody or serum directly to the solution so that an appropriate dilution is reached (from 1:200 for human sera to 1:10,000 for some monoclonal antibodies; *see Note 9*). Shake for at least 1 h or overnight at 4°C.
5. Wash membranes four times for 15 min in 100 mL of PBST buffer.
6. Incubate in secondary antibody solution diluted in 100 mL 5% milk in PBST buffer (e.g., 1:5000) for 1 h.
7. Wash membranes four times for 15 min in 100 mL of PBST buffer.
8. Warm up chemiluminescence reagents to room temperature during this time (*see Note 10*).
9. Freshly mix equal amounts of both reagents and apply to the membranes (up to 50 mL per half blot). Shake for 1 min. Make sure the whole membrane is covered with liquid.
10. Drip off membrane and wrap it into a foil to keep it wet.
11. Quickly go to the dark room as chemiluminescence will drop in intensity after some minutes. (There will be some intensity until about 30 min after mixing the reagents.)
12. Place the membrane and a film on top in the cassette and expose for 1 min. Develop this film to decide on the appropriate exposure time. This can vary from a few seconds up to half an hour, depending on the strength of the signal. The exposure of several films within half an hour after mixing of reagents is possible.
13. Store developed films in a dark and dry place.
14. For quality assessment and spot assignment, stain the PVDF membranes in CBB R-250 staining solution for about 5 min.
15. Destain the background in destaining solution three times for about 2 min.
16. Dry membrane at room temperature and store in a dry place.

### 3.4. Data Analysis

Usually data analysis is performed by comparing spot patterns of immunoblots to search for differences according to the biological state represented by the immunoblots (*see Note 11*). This analysis can only be done by eye when a few immunoblots containing not too many spots are compared. For extensive studies it is necessary to apply a 2-DE analysis software, preferably with implemented statistical tools. One example of such a software is PDQuest.

To perform the computer-aided analysis, it is necessary to scan the films properly. All films from one experiment should be scanned using exactly the same parameters. These parameters strongly affect the following analysis and, therefore, they need to be optimized for resolution (for PDQuest: 150 dpi), contrast, and brightness. Files should be saved as gray-scaled images with at least 8-bit depth (256 gray values per pixel).

It is not possible to explain data analysis in great detail here as there are many different software solutions available. However, to our knowledge there is no 2-DE analysis software currently available that is able to perform the analysis automatically and achieve acceptable results without interactive corrections. We therefore strongly recommend careful review of all results manually (*see Note 12*).

When using PDQuest, spot detection and quantitation are performed first. Then spots in different immunoblots that represent the same protein species are matched. It is now very important to take the time to check and correct the spot detection and matching thoroughly. Depending on the number of immunoblots in the experiment, this can last for up to several weeks—but there is no alternative if reliable results are to be achieved. After this procedure, it is possible to group immunoblots according to their biological state, e.g., each group may contain three or more replicate immunoblots of the same sample. Using statistical tools like the *t*-test or Mann–Whitney test, it is now possible to compare the intensities of all the spots in all the immunoblots in order to find significantly regulated spots (significance level adjustable to 90, 95, or 99%). It is also possible to perform analyses like “show all spots that are up-/downregulated by factor *x*” or spots unique to one of the groups of immunoblots. Using these tools, one will be able to find spots that are reliable antigen candidates (*see Note 13*).

### 3.5. Antigen Identification

After detection of the spots, which are differently recognized by the antibodies or sera, the question of the identity of these spots arises. Usually one will find information about the protein contained in the spot rather than the protein species (*see Note 14*).

One possible way to determine the protein(s) contained in the spot is to compare the spot patterns produced by the immunoblots with a spot pattern in a database. There are 2-DE databases for many different species available for the public via the Internet (*see Subheading 2.5., item 1*). With a little luck, one can find one's spot of interest already identified. Take care to avoid erroneous assignments, because finding the corresponding spot can be very difficult (*see Note 15*).

Another way to identify the proteins of interest is to run a preparative gel of the sample applying more sample protein than for analytical gels, e.g., 500  $\mu\text{g}$  for 23  $\times$  30-cm gels. These gels are stained with CBB G-250 (22). CBB G-250 is more sensitive than CBB R-250. The spots of interest can be excised for MALDI-TOF analysis (see **Note 16**). In this case, assignment problems as described above will also occur. Take as much care as you can to avoid contamination of the gel or spots with dust or keratin (see **Note 17**).

### 3.5.1. CBB G-250 Staining of 23 $\times$ 30-cm Gels

1. Directly after the end of the gel run, move the gel to 1 L of fixing solution and shake overnight.
2. Wash three times for 30 min in 1 L distilled water.
3. Shake for 1 h in 1 L staining solution (still without CBB G-250).
4. Add 0.66 g/L CBB G-250 to the solution and shake for about 5 d.
5. Rinse with 25% methanol for 1 min. Destaining is not required.
6. The stained gel can be stored shrink-wrapped in distilled water for several weeks at 4°C.

### 3.5.2. Protein Digest

1. Excise spots of interest using a Pasteur pipet that was cut in length to achieve an internal diameter of about 1 mm. Place spot in a clean dust-free tube.
2. Destain spots by shaking in 500  $\mu\text{L}$  spot destaining solution for 30 min at 37°C.
3. Shake spots in 500  $\mu\text{L}$  digest buffer for 30 min at 37°C.
4. Dry spots in Speed Vac for about 30 min at 30°C.
5. Apply 0.25  $\mu\text{L}$  trypsin solution directly to the dried spot and add 25  $\mu\text{L}$  of digest buffer.
6. Incubate overnight at 37°C on a shaker.
7. Spin down and transfer supernatants into new tubes.
8. Shrink spots in 20  $\mu\text{L}$  shrink buffer for 10 min (to recover all the peptides) and combine supernatants in the new tubes.
9. Dry supernatants in a Speed Vac for about 60 min at 45°C.
10. Dissolve peptide pellet in 1.3  $\mu\text{L}$  sample buffer.
11. Apply 0.25  $\mu\text{L}$  sample solution to the MALDI template and add 0.25  $\mu\text{L}$  matrix solution and wait for the drop to be dried (see **Note 18**).
12. Sample solution can be stored at -20°C.

The peptide mass fingerprints can be obtained from different MALDI-TOF mass spectrometers. Therefore, the exact procedure cannot be described here. The parameter settings must be optimized according to the device used.

After measuring the peptide mass fingerprints by MALDI-TOF MS, the protein can be identified by a database search (see **Note 19**). In such a search the “fingerprint” of peptide masses created by the trypsin digest is compared with a list of theoretically digested proteins in a database. Search machines are available to the public via the Internet (see **Subheading 2.5., item 11**), and a protein sequence database can be chosen in the search masks, e.g., NCBI, SwissProt, or OWL. Make sure the protein hit meets reliable identification criteria, e.g., the hit has the highest score value when performing an “all species” search, 30% sequence coverage is achieved, and few modifications are found.

#### 4. Notes

1. It is most important to use 2-DE gels of high resolution. Much experience is required to produce gels of high resolution and quality reproducibly. Remember, the better the quality of the gels, the better the immunoblot quality that can be achieved.
2. Keep dust or chemicals from reaching the membrane at any time because detection with chemiluminescence is extremely sensitive. Use gloves and a lab coat at all times. Never touch the membrane—use tweezers instead.
3. It is possible to use nitrocellulose instead of PVDF membranes. However, these membranes are usually more fragile. Blotting parameters must then be adapted.
4. If there are air bubbles left in between the sandwich layers, it is possible to remove them by carefully rolling a glass pipet over the filter papers. Do not let the filters dry out.
5. To assess the blotting efficiency, it is recommended to stain a test gel with CBB G-250 (as described in **Subheading 3.5.**) after blotting. If too much protein is left in the gel, SDS should be added to the cathodic blotting buffer, and the blotting time may be extended. If low- $M_r$  proteins did not bind to the membrane, the ionic strength of the blotting buffer has to be increased.
6. It is essential to soak PVDF membranes in 100% methanol to prepare the surface for aqueous solutions.
7. Always apply enough solution for the membrane to swim freely. The protein surface should be on top.
8. The quality of the milk strongly influences the background detection. One might try one from the supermarket, but this must be tested in advance.
9. It is always necessary to optimize the dilution of primary and secondary antibodies or sera. Tests should be made using a dilution series. Sometimes milk does not work well as a blocking reagent—there are other blocking reagents available, e.g., bovine serum albumin. Some antibodies may not work properly in PBS—try TBS instead.

If human sera or expensive antibodies are used, it is advantageous to reduce the necessary amount of antibody to a minimum. To do this, it is possible to shrink-wrap the membranes. For this purpose, cut a piece of polyethylene foil into an appropriate size, place the two membrane pieces from one gel (faces to the outsides) inside, and shrink-wrap three from four sides. Now the blocking solution can be applied. (Do not forget the methanol at first.) Take care not to catch air bubbles within the package. After the blocking (of membrane and plastic foil!), one side is carefully sliced, antibody is added, and the package is sealed again. Use an appropriate shaker and cover the package using a glass slide. Make sure the glass slide can shake freely; thus the solution can circulate properly within the package. Care must be taken to keep the glass from slipping, e.g., by the use of adhesive tape. By applying this method, the minimum amount of antibody solution can be reduced to as little as 0.1 mL/cm<sup>2</sup> immunoblot area (50 mL for one 23 × 30-cm blot).

10. Use film, chemiluminescence reagents, and cassette only at a certain temperature (room temperature, if not fluctuating), because the light-emitting biochemical reaction is strongly temperature-dependent. Otherwise spot intensities from different immunoblots cannot be compared.
11. Data analysis is as important as your experiments in the lab. For this reason, take a little time and think about the analysis strategy your experiment requires. According to the biological questions that are to be answered, the analysis must be performed in such a way that these are addressed properly. For instance, it is important to think about how many replicate immunoblots must be made to achieve significant results. Also, the selec-

tion criteria for spots of interest should be fixed in advance of the analysis in order to avoid “wishful analyzing.” Do not forget appropriate control immunoblots.

12. Many different 2-DE analysis software packages are available. It is very time-consuming to test all the new software versions that are brought to market continuously. We have chosen PDQuest mainly for two reasons. First, statistical analysis tools are implemented. Second, PDQuest allows the user to correct spot detection and perform manual matching easily. This is a matter of particular interest for blot analysis, as immunoblots usually contain fewer spots compared with gels, which makes it harder for the automated matching procedure to find corresponding spots. Act with caution when software claims to process “every” gel automatically without giving you the chance to correct errors.
13. After exhaustive use of the 2-DE analysis software, it is still possible to export spot intensity data for even further analysis. One possibility is the application of multivariate statistics, e.g., principal component analysis or hierarchical clustering. This can give information on whether whole spot patterns cluster according to the biological state of the sample.
14. Caution should be used since spots do not represent proteins but rather protein species, i.e., proteins as they are found in vivo may have post-translational modifications, may be partly degraded, or might be splicing variants. Additionally, spots often contain several different proteins or protein species. However, when using peptide mass fingerprints for identification, little or no information about modifications or variants can be given. Search results for spots containing more than one protein can be very difficult to interpret.
15. Comparing spot patterns from immunoblots (or in fact films) with spot patterns from the gel in the database is not as easy as one might think. To find the corresponding spot unambiguously, it is important to take account of “local” spot patterns close to the spot of interest. Only by looking at these is it possible to overcome the problem of different running behaviors of proteins under different running conditions. The same assignment problem occurs between immunoblots and preparative gels.
16. The use of CBB G-250 leads to a higher sensitivity compared with CBB R-250. However, methylation of the proteins will occur that must be taken into account for the database search since methylated peptides have a 14 Dalton higher mass.
17. Contamination of spot samples with keratin is a serious problem. It is highly recommended to clean the bench, pipets, boxes, and Speed Vac prior to use. Otherwise keratin peaks will appear in the peptide mass fingerprints and may interfere with the identification.
18. The matrix  $\alpha$ -cyano cinnamic acid can be used instead of 2,5-dehydroxybenzoic acid.
19. For identification, many other mass spectrometers can also be used, e.g., ESI-MS, MALDI-TOF/TOF, or other devices that allow sequence information to be revealed (MS/MS).

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<http://www.springer.com/978-1-58829-221-6>

Molecular Diagnosis of Infectious Diseases

Decker, J.; Reischl, U. (Eds.)

2004, XVI, 464 p., Hardcover

ISBN: 978-1-58829-221-6

A product of Humana Press