Analysis of Proteomes Using the Molecular Scanner

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INTRODUCTION

The molecular scanner offers a flexible and powerful visualization tool that can create a fully annotated 2D gel electrophoresis map. Proteins separated by 2D gel electrophoresis are simultaneously digested while undergoing electrotransfer from the gel to a membrane. The peptides are subjected to peptide mass fingerprint (PMF) analysis to identify proteins directly from the PVDF membranes by MALDI-TOF-MS scanning. An ensemble of dedicated tools is then used to create, analyze, and visualize a proteome as a multidimensional image. The molecular scanner method reduces to a minimum the sample handling prior to mass analysis and decreases the sample size to a few tens of micrometers, that is, the size of the MALDI-TOF-MS laser beam impact. The process can be divided into four parts: separation and digestion of proteins, acquisition of PMF data, processing of the MS data and protein identification, and creation of multidimensional proteome maps.

RELATED INFORMATION

A schematic of the molecular scanner is shown in Figure 1.
predefined masses and generate an intensity image in which the gray levels represent the total intensity of the mass peaks found at each \((x, y)\) position. This image can be smoothed if needed.

**MATERIALS**

**Reagents**

- 2D equilibration solution (optional; see Step 9)
- 2D gel rehydration solution (optional; see Step 9)
- 2D SDS-PAGE gels (optional; see Step 9)
- 2D Equilibration solution (optional; see Step 9)
- Acetonitrile (preparative HPLC grade, 50% and 80%)
- N-\(\alpha\)-Tosyl-L-arginine methyl ester (TAME) (10 mM)
- Amido black (0.5% [w/v]) (optional; see Step 21)
- Buffer for gel electrophoresis (according to need, see Step 9)
- \(n\)-Butanol (optional; see Step 9)
- Capping reagent, 4°C
- Coomassie brilliant blue R250 (0.1% [w/v])
- Destain solution
- First-dimension sample loading buffer (optional; see Step 9)
- HCCA solution
- HCl (1 mM)

High-vacuum grease

- IPG strips, ready-made, 7-cm or 18-cm (Amersham Biosciences) (optional; see Step 9)
- Overlay solution, 70°C (optional; see Step 9)
- Paraffin oil (optional; see Step 9)
- PBS-Tween (pH 7.4)
- Phosphate-buffered trypsin
- Polyacrylamide gels, 12% T, 2.6% C, 1-mm thick, linear (optional; see Step 9)
Protein sample to be analyzed

- Reducing buffer (optional; see Step 9)
- Sodium thiosulfate (5 mM) (optional; see Step 9)
- Sulfhydryl blocking solution (optional; see Step 9)
- TAC buffer
- Transblotting buffer
- Tris-buffered trypsin
- Tris-Cl (460 mM, pH 8.1) containing 11.5 mM CaCl₂

**Equipment**

Electroblotting apparatus

Gel electrophoresis system (1D minigel system [e.g., Mini-Protean II Cell; Bio-Rad], 2D minigel system [e.g., Multiphor II; Amersham Biosciences], or large electrophoresis system [e.g., Protean II Cell; Bio-Rad])

Immobilon AV membrane (Millipore)

_The IAV membrane is a modified PVDF matrix presenting activated carboxylic groups able to react with nucleophiles such as amine groups of proteins._

IPG strips rehydration chamber (optional; see Step 9)

MALDI sample plate (modified)

Mass spectrometer and its acquisition software

PVDF membrane

Rotating hybridizer (e.g., model HB-2D; Techne)

Sample cups (Amersham Biosciences)

UV-visible spectrophotometer (Ultrospec III; Amersham Biosciences)

Water bath or heating block, 95°C

Water bath or incubator, 35°C and 70°C (optional; see Step 9)

**METHOD**

**Preparation of IAV-Trypsin Membranes**
1. Submerge a 10 x 12-cm² IAV membrane in phosphate-buffered trypsin. Incubate in a rotating hybridizer for 3 hours at room temperature.

2. Wash the membrane briefly three times with agitation in 10 ml of PBS-Tween.

3. Incubate the membrane in 10 ml of capping reagent for 3 hours at 4°C to block the remaining free carboxylic groups.

4. Repeat Step 2.

5. Gently wash the membrane twice for 30 minutes each in 10 ml of PBS-Tween. *Membranes can be stored for 2-3 years at 4°C in TAC buffer.*

**Testing the Activity of the Immobilized Trypsin**

6. Add 1 cm² of IAV-trypsin membrane to a mixture of:
   - 2.6 ml of 460 mM Tris-HCl (pH 8.1) containing 11.5 mM CaCl₂
   - 0.3 ml of 10 mM TAME
   - 0.1 ml of 1 mM HCl

7. Stir the mixture for 40 seconds. Measure the absorbance of the solution at 247 nm with a UV-visible spectrophotometer.

8. After 3 minutes of constant stirring, take a second absorbance reading. *The value of ΔA_{247}/minute is used to calculate the equivalent amount of active trypsin expressed per unit of surface area.*

**Protein Separation by Gel Electrophoresis**

9. Separate the proteins by either 1D or 2D gel electrophoresis. *The choice of method will depend on the complexity of the sample and the abundance of the protein(s) of interest.*

   **For 1D gel electrophoresis:**
   i. Dilute proteins to the appropriate amount in reducing buffer.

   ii. Heat the samples for 5 minutes at 95°C. Load the samples onto the gel. *12% T, 2.6% C, 1-mm-thick linear polyacrylamide gels are typically used.*

   iii. Separate the proteins at 200 V for 45 minutes.

   **For 2D minigel electrophoresis:**
   i. Add 1 mg of the protein sample to 150 µl of 2D gel rehydration solution.

   ii. Apply the entire diluted protein sample to 7-cm ready-made IPG strips.

   iii. Peel off the protective cover sheets from the IPG strips. Position them in the rehydration chamber such that the gel of the strip is in contact with the sample.
iv. Cover the IPG strips with low-viscosity paraffin oil. Allow them to rehydrate for at least 6 hours (or overnight) at room temperature.

v. Remove the rehydrated IPG gel strips from the chamber with tweezers. Rinse strips with H$_2$O. Place the strips gel-side up in the electrophoresis running tray according to the manufacturer’s instructions.

vi. Increase the voltage linearly from 300 to 3500 V over a 10-minute period, followed by 1 additional hour at 3500 V.

vii. Equilibrate the strips for 12 minutes in the rehydration tray using 3 ml per groove of 2D equilibration solution. Discard the equilibration solution. Replace it with 3 ml per groove of sulfhydryl blocking solution. Incubate the strips for 5 minutes.

viii. After equilibration, cut the IPG gel strips to size.

ix. Overlay the second-dimension gels with overlay solution equilibrated to ~70°C.

x. Immediately load the IPG gel strips through the overlay solution.

xi. Run the gels at 200 V (constant) for 30 minutes at 12°C using a minigel electrophoresis system.

For high-resolution 2D gel electrophoresis:

i. Separate the proteins by IEF in a multipurpose flatbed electrophoresis unit.

Use IPG strips that are 3 mm x 18 cm. Rehydrate the strips in the absence of the protein sample, which should be applied using sample cups. Mix 4 mg of protein sample with 60 µl of first-dimension sample loading buffer. Separate the proteins using a linear voltage gradient that increases from 300 to 3500 V over 3 hours, followed by 3 more hours at 3500 V. Then increase the voltage to 5000 V. Separate the proteins by electrophoresis overnight for a total of 100,000 volt-hours.

ii. Prepare the 2D SDS-PAGE gels.

The gel dimensions should be 160 x 200 x 1.5 mm. Each gel is 9%-16% T/2.6% C acrylamide and bisacrylamide. Add sodium thiosulfate (5 mM) to acrylamide/bisacrylamide to initiate/catalyze polymerization. Pour the gels to within 0.7 cm from the top of the plates. Overlay them with n-butanol. Allow the gels to polymerize for ~2 hours. Remove the butanol. Overlay the gels with H$_2$O. Incubate them overnight; wrap the gel well with plastic wrap to prevent the gel from drying out.

iii. Prepare the proteins for the 2D separation by SDS-PAGE.

Equilibrate the strips for 12 minutes with 100 ml of 2D equilibration solution. Block all sulfhydryl groups for 5 minutes with 100 ml of sulfhydryl blocking solution.

iv. Cut the IPG gel strips to size so that they fit atop the SDS-PAGE gels. Remove 6 mm from the anodic end and 14 mm from the cathodic end.
v. Layer each SDS-PAGE gel with overlay solution heated to 70°C.

vi. Immediately apply the IPG gel strips to the tops of the SDS-PAGE gels.

vii. Electrophorese the gels at 40 mA/gel under constant current for 5 hours at 8°C-12°C using a large electrophoresis system.

**Staining the Gel-Bound Proteins**

10. Submerge the gel in Coomassie brilliant blue R250 (0.1% [w/v]) for 30 minutes.

11. Destain the gel in repeated washes of destain solution.

**Double-Parallel Digestion of Proteins**

12. Soak the gel in H2O for 5 minutes.

13. Repeat Step 12 two more times.

14. Soak the gel in 50% acetonitrile for 20 minutes, followed by 10 minutes in 80% acetonitrile.

15. Air-dry the entire wet gel (or a selected piece) at room temperature.

16. Incubate the gel with Tris-buffered trypsin for 30 minutes at 35°C. *Use a volume of trypsin solution equal to 3-5 times the initial volume of the gel. This step both rehydrates the gel and begins protein digestion.*

17. After 30 minutes of incubation, discard the trypsin solution.

18. Just prior to electroblotting the proteins, equilibrate the IAV trypsin membrane and the PVDF membrane (in separate containers) in transblotting buffer for 5 minutes.

19. Carry out the electrotransfer overnight (12-18 hr) at room temperature in a semidry apparatus by inserting a double layer of IAV-trypsin membrane between the gel and the PVDF membrane. *The migration time of the proteins through the enzymatic membranes can be increased by applying an alternating square-shape voltage during the transfer: +12.5 V for 125 msec followed by -5 V for 125 msec.*

20. Wash the PVDF membrane in H2O for 5 minutes.

**Staining the Membrane-Bound Proteins**

21. Optionally, stain the PVDF membranes with 0.5% Amido black for 1 minute. Destain with repeated washes of H2O.

**Acquisition of PMF Data**

22. Wash the PVDF membrane containing the bound proteins with H2O and air-dry.
23. Spray the membrane surface with HCCA solution until it is completely wetted. Allow the membrane to air dry.

24. Attach a 4 x 4-cm² piece of PVDF membrane onto a modified MALDI sample plate with a very small amount of high-vacuum grease. Make sure that the grease does not penetrate the membrane.

25. Define absolute coordinates on the membrane, and establish a grid that represents positions where mass spectra will be acquired.
For a given experiment, the distance between distinct mass spectrum acquisitions should be defined as a constant ranging between 0.2 and 0.5 mm, the exact distance determined empirically.

26. Insert the MALDI plate into the mass spectrometer. Set the parameters for mass spectra acquisition, according to the manufacturer’s instructions.

Protein Identification

27. Using the mass spectrometer’s acquisition software, set the peak detection threshold to the value optimized for a set of calibrated spectra. Convert the positions on the MALDI sample plate to apparent molecular mass (M_r) and pI values.

28. Combine the PMF data from all of the spectra with calculated pI and M_r, and also with user-defined interrogation criteria, such as peptide mass tolerance and chemical modifications. The combined data are sent automatically to SmartIdent (http://ch.expasy.org/tools/).
The process of identification takes into account weak expression of some proteins but also overlapping protein spots, thus the minimal number of matching peptides for the PMF search should be set as low as possible (i.e., three peptides). Set the number of missed cleavages to 1.

29. Write peak-list and identification results into a text file.

Creation of Virtual Maps

30. Analyze and cluster the identified proteins according to the procedure described by Bienvenut et al. (2001).
Several criteria are defined to trace and exclude false identifications. Identifications that are isolated or spread out over a large portion of the scanned membrane should be discarded. Identifications that require matches with a matrix cluster or impurity masses also should be excluded, as well as weakly expressed proteins that required peptide masses of abundant proteins. Identifications that have a low average score are not considered.

31. Express the identification results as a virtual, annotated 2D electrophoresis map.
The program generates a multidimensional image where x and y coordinates are related to pI and M_r, respectively, and the z axis can be related to numerous data or information associated with the identified protein such as PMF spectra intensity, SmartIdent identification score, number of matching peptides, etc. The user can choose to filter and visualize only particular aspects, as desired.
REFERENCES


Caution

N,N-Dimethylformamide (DMF)

N,N-Dimethylformamide (DMF), HCON(CH₃)₂, is a possible carcinogen and is irritating to the eyes, skin, and mucous membranes. It can exert its toxic effects through inhalation, ingestion, or skin absorption. Chronic inhalation can cause liver and kidney damage. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

Caution

N-α-Tosyl-L-arginine methyl ester hydrochloride (TAME)

N-α-Tosyl-L-arginine methyl ester hydrochloride (TAME) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use only in a chemical fume hood.

Caution

Acetonitrile

Acetonitrile is very volatile and extremely flammable. It is an irritant and a chemical asphyxiant that can exert its effects by inhalation, ingestion, or skin absorption. Treat cases of severe exposure as cyanide poisoning. Wear appropriate gloves and safety glasses. Use only in a chemical fume hood. Keep away from heat, sparks, and open flame.

Caution

CaCl₂ (Calcium chloride)

CaCl₂ (Calcium chloride) is hygroscopic and may cause cardiac disturbances. It may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety goggles.
Caution

General warning

This material contains hazardous components. Please see recipe for full details.

Caution

HCl (Hydrochloric acid, Hydrochloride)

HCl (hydrochloric acid, hydrochloride) is volatile and may be fatal if inhaled, ingested, or absorbed through the skin. It is extremely destructive to mucous membranes, upper respiratory tract, eyes, and skin. Wear appropriate gloves and safety glasses. Use with great care in a chemical fume hood. Wear goggles when handling large quantities.

Caution

Polyacrylamide

Polyacrylamide is considered to be nontoxic, but it should be treated with care because it may contain small quantities of unpolymerized material (see Acrylamide).

Recipe

2D equilibration solution

50 mM Tris-Cl (pH 6.8)

6 M urea

30% (v/v) glycerol

2% (w/v) SDS

2% (w/v) dithioerythritol (DTE)
Recipe

2D gel rehydration solution

- 8 M urea
- 4% (w/v) CHAPS
- 2% (v/v) Resolyte 4-8
- 65 mM DTE
- A trace of bromophenol blue

Recipe

Amido black (0.5% w/v)

Amido black (0.5% w/v)

- 2-Propanol
- Acetic acid

Dissolve the dye in 25% (v/v) 2-propanol and 10% (v/v) acetic acid.

Recipe

Capping reagent

- 1 M ethanolamine (pH 10.5)

- 1 M sodium bicarbonate buffer (pH 9.5)

Recipe

Coomassie brilliant blue R250 (0.1% w/v)

- Coomassie brilliant blue R250
- Methanol
Acetic acid

Prepare the Coomassie brilliant blue R250 in a 60/30/10 (v/v/v) mixture of H2O, methanol, and acetic acid.

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**Recipe**

**Destaining solution for Coomassie brilliant blue R250**

- Methanol
- Acetic acid

Mix H2O, methanol, and acetic acid in a ratio of 50/40/10 (v/v/v).

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**Recipe**

**First-dimension sample loading buffer**

- 8 M urea
- 4% (w/v) CHAPS
- 40 mM Tris
- 65 mM DTE
- 0.05% (w/v) SDS
- A trace of bromophenol blue

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**Recipe**

**HCCA solution**

- 10 mg/ml α-cyano-4-hydroxy-cinnamic acid (HCCA)
- 70% methanol
- 0.1% TFA
Recipe

Overlay solution

0.5% (w/v) agarose
- 25 mM Tris (pH 8.3)
- 198 mM glycine
- 0.1% (w/v) SDS

Shortly before use, boil overlay solution to melt the agarose. Maintain the solution at ~70°C until use.

Recipe

PBS-Tween (pH 7.4)

- 20 mM NaH₂PO₄
- 140 mM NaCl
- 0.5% Tween 20

Recipe

Phosphate-buffered trypsin

- 2 mg/ml TPCK-treated trypsin (Type IX from porcine pancreas, Sigma Chemical)
- 20 mM NaH₂PO₄ (pH 7.8)

Recipe

Reducing buffer

- 60 mM Tris-Cl (pH 6.8)
- 10% (v/v) glycerol
- 2% (w/v) SDS
3% (v/v) β-mercaptoethanol

Recipe

Sulfhydryl blocking solution

50 mM Tris-Cl (pH 6.8)
6 M urea
30% (v/v) glycerol
2% (w/v) SDS
2.5% (w/v) iodoacetamide
A trace of bromophenol blue

Recipe

TAC buffer

46 mM Tris-Cl (pH 8.1)
0.1% sodium azide
1 mM CaCl₂

Recipe

Transblotting buffer

15.1 g Tris-base
72.1 g glycine
500 ml methanol
0.5 g SDS
Add H₂O to 5 liters.
Recipe

Tris-buffered trypsin

- 0.05 mg/ml TPCK-treated trypsin (Type IX from porcine pancreas, Sigma Chemical)
- 10 mM Tris-Cl (pH 8.2)

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