

2-DE in a Nutshell

**Manuel Mayr, MD, PhD
Cardiac & Vascular Sciences
St. George's Hospital Medical School
Jenner Wing, Room 1.234
Tel: 020 8725 5925
E-mail: m.mayr@sghms.ac.uk**

Introduction

Biomedical research in the post-genomic era will be carried out against the backdrop of an information explosion about the approx. 30,000-40,000 genes whose sequences have been completed for mouse and humans. The importance of measuring protein levels has become increasingly clear, as it has become obvious that the genomic sequence or the transcriptional profile cannot be directly correlated with actual protein levels or protein function. The correlation between RNA transcription and protein expression is poor and transcriptomics reveals no data regarding posttranslational modifications, which are known to be instrumental in the development of many diseases. In contrast, most activities performed by a cell may be better reflected in their proteome.

By analogy to the genome, the proteome is defined as the composition of all proteins expressed by the genome of an organism at a given time-point. The term “proteomics” is the simultaneous analysis of complex protein mixtures like cell lysates and tissue extracts, to look for quantitative changes of protein expression levels. The dynamic range of protein expression and modification makes the identification of the entire proteome a far bigger and more complex challenge than the sequencing of the genome. Hence, proteomics is a large scale-study of gene expression at the protein level including areas such as determination of protein function, characterization of post-translational modifications, studies of protein interactions and protein expression analysis.

Unlike gene sequencing and expression analysis, which can be performed with high throughput and in an automated manner, proteomics suffers the absence of technologies that can deliver fast and parallel quantitative analysis of complex protein distributions in an automated fashion. To date, two-dimensional (2-D) gel electrophoresis is still the preferred method to resolve and array authentic proteins from cellular mixtures. Combined with high-throughput mass spectrometry (MS) techniques, 2-D gels allow the simultaneous analysis of hundreds of protein species. Protein identification made from peptide mass fingerprints can be confirmed by using post source decay (PSD) MALDI-MS or tandem mass spectrometry (LC-MS/MS) fragmentation of individual peptides. These techniques allow the characterization of contemporaneous changes in protein expression as well as post-translational modifications.

Protein expression profiling *per se*, however, provides no data regarding their functional state unless additional experimental steps are included.

Advantages of Two-dimensional electrophoresis (2-DE)

2-DE gels are a classic technology (*O'Farrell, J. Biol. Chem. 250, 4007-4021, 1975*) that has enjoyed a renaissance in the past 10 years. It separates proteins according to their isoelectric point (pI) in the first dimension and molecular weight (Mw) in the second dimension. Conventional one-dimensional SDS PAGE and one-dimensional IEF are each capable of resolving only approximately 100 of the most abundant proteins in a heterogeneous sample. Employing both techniques in a two-dimensional separation allows the theoretical separation of the product of the number of proteins separated by each technique, since protein charge and molecular weight are independent properties. Because of this highly parallel nature of the technique, hundreds to thousands of proteins can be visualized simultaneously, i.e. a single large-format 2-DE gel will resolve 1000 to 3000 different polypeptide spots. Hence, 2-DE gels are high-resolution protein arrays suitable for the **resolution of complex protein mixtures**. A 2-DE gel is also an **array of authentic proteins**, i.e., charge, molecular weight, and posttranslational modifications are preserved during electrophoresis. This allows direct analysis of protein isoforms that may be involved in particular metabolic or disease processes. In addition, the **quantitative** differences between proteins in mixtures can be determined from 2-DE gel images. This allows the direct detection of differentially expressed gene products.

Proteome data analyses suggest that only a fraction of the genes is switched on in a given cell type at any given time. The most recent estimates place the number of genes in the human genome at approximately 30,000-40,000, far less than had been previously predicted. Even with this revised estimate of a "low" number of genes, the number of proteins will still be too many to see on a single gel. If a given cell expresses between 5,000 to 10,000 genes at a given time, between 15,000 to 30,000 distinct cellular proteins must be expected as a result of mRNA splicing and post-translational modifications. Consequently, some reduction in the complexity of samples is needed to display the majority of proteins on gels. Otherwise, low abundance proteins (which might be the most interesting functionally) tend to be masked by housekeeping proteins present at several orders of magnitude higher levels and are invisible because of sensitivity detection limits.

Currently, there are no stains that can span the approximately 7 or 8 orders of magnitude dynamic range of cellular proteins. However, improved methods of protein detection include the use of fluorescent dyes. They offer a broader dynamic range and linear quantitative range of detection than silver staining (which is non-linear). SYPRO Ruby protein stain (Molecular Probes) is a fluorescent ruthenium-based stain that binds non-covalently to proteins in gels. This stain has a linear dynamic range of 2 to 2000 nanograms, spanning the ranges of both Coomassie and silver stains. The most recent development is difference in gel electrophoresis (DIGE): Two samples are labelled with different fluorescent probes (Cy3 and Cy5) and run on the same IPG strip and 2-DE gel, offering the opportunity for direct comparison and avoiding gel-to-gel variations inherent to comparative gel analysis. The use of an internal standard labelled with Cy2 will further improve the accuracy of quantitative comparisons in differential display experiments.

Disadvantages of Two-dimensional electrophoresis (2-DE)

Routine 2-DE analysis of complex mixtures is **biased towards long-lived abundant proteins**. For complex protein mixtures pre-fractionation is essential to facilitate the identification of low-abundance proteins. Sample pre-fractionation techniques aim to reduce the diversity and complexity of protein mixtures, thus increasing the number and concentration of distinct subsets of proteins that can be resolved in a series of complementary narrow pH-range 2-DE gels. Sequential extraction of proteins from cells or tissues can be used to pre-fractionate proteins based on their relative solubility in a series of buffers. Pre-fractionation can also be based on different properties such as net charge, mobility, size, hydrophobicity and affinity. Subcellular fractionation is another useful approach that aims to enrich functionally related proteins based on their colocalization within the cell, which is equally useful for the study of protein translocations. In general, pre-fractionation methods should be kept as simple as possible, target different molecular properties and employ downstream compatible solutions to minimize sample loss and the chance of degradation and/or introduction of artifactual protein modifications.

Unfortunately, **standard 2-DE gels do not reflect** a true representation of **hydrophobic, highly insoluble, very basic, as well as very small and very large, proteins**. 2-DE is unsuitable for analysis of very high molecular weight proteins, which should be separated using one-dimensional SDS-PAGE, preferably following sample pre-fractionation to enrich the targeted proteins. Improved second-dimensional separation of very low molecular weight proteins can be achieved if the Tris-glycine buffer, which is usually used, is substituted by the Tris-tricine-SDS buffer system in combination with high-percentage-gradient gels. The process of solubilization and subsequent separation of hydrophobic proteins in immobilized pH gradient (IPG) strips have been improved by combining more effective reducing agents such as the uncharged agent tributyl phosphine with more powerful chaotropes such as thiourea and surfactants such as linear sulfobetaine-type zwitterionic detergents containing a carboxyamido group to improve urea tolerance. The development of basic IPGs up to pH 12 will facilitate easy, reproducible analysis of alkaline proteins as soon as the problem of reverse electroosmotic flow (anode-directed water flow) is fully solved.

Questions about the limitations of 2-D gels with respect to the resolution of low-abundance or hydrophobic proteins have prompted the search for methods that are unbiased in these respects. As an **alternative to the 2-D gel approach**, initial efforts have focused on multidimensional liquid chromatography to decrease sample complexity coupled with mass spectrometry for protein identification (Lopez and Melov 2002). These approaches have all suffered from the disadvantage that they are not quantitative, hence not useful for differential display analyses. In addition, most of these approaches fall short of the resolution achieved with 2-D gels and are still biased with respect to pI and Mw. This is because very acidic proteins will be underrepresented in the mixture due to fewer tryptic cleavage sites, and low molecular weight proteins will be underrepresented due to production of fewer peptides. A clear advantage is that most of these methods are very automation-friendly. However, exhaustive analysis of the sequence data obtained demands powerful computing facilities.

Tissue and Cell Lysis

In order to achieve a well-focused first-dimension separation, sample proteins must be completely disaggregated and fully solubilized. Complete denaturation ensures that each protein is present in only one configuration and that aggregation and intermolecular interaction is avoided. The compounds used in the buffer must not increase the ionic strength of the solution, to allow high voltages to be applied without producing high currents.

Generally, **2DE lysis buffer** always includes urea (minimum concentration 8M) and one or more detergents. Therefore, **repeated freeze-thawing and heating above 30°C must be avoided!**

1. **Urea**, a neutral chaotrope, is used as the denaturant in the first dimension of 2DE. Urea solubilizes and unfolds most proteins to their fully random configuration, with all ionizable groups exposed to solution. Recently, the use of thiourea has been found to further improve solubilization, particularly of membrane proteins.
2. A **non-ionic or zwitterionic detergent** is always included to prevent aggregation through hydrophobic interactions. Originally, either of two similar non-ionic detergents, NP40 or Triton X, was used. Subsequent studies have demonstrated that the zwitterionic detergent CHAPS is often more effective and is less likely to be a source of contamination in MALDI-MS. New detergents include SB 3-10 and ASB-14.
3. **Reductants** such as DTT are also frequently added to enhance sample solubility. DTT can become ionized above its pK of 8 and migrate towards the anode during IEF in basic pH gradients leading to horizontal streaking from spots in the basic area.
4. **Carrier ampholytes** improve the solubility of proteins considerably by substituting ionic buffers and counteracting the insufficient salt concentration.
5. **Protease inhibitors** are needed because some proteases are even active in the presence of high concentrations of urea.

The easiest way for cell disruption is osmotic lysis of cultured cells using the lysis buffer. Tissues must be snap frozen in liquid nitrogen and grinded with mortar and pestle. About 1 ml lysis buffer is added per 100 mg tissue. Leave sample at room temperature for 30 min, vortex and spin for 15 min at 12000 g. It is important to be aware of the fact, that the disruption method used is influencing the 2D pattern. Therefore, it has to be employed in a reproducible way and be described in the analysis protocol. Cell culture medium should be removed by cell washing. The washing solution must contain enough osmoticum to avoid cell lysis i.e. sucrose. PBS is not recommended because it contaminates the cell surfaces with salt.

Modified Protein Assay

Additives like urea, detergents and reductants interfere with the Bradford assay. Quantification becomes more reliable if samples are acidified with 0.1M HCl.

BSA standard, stock solution of 5mg/ml in lysis buffer

To prepare Standards:

[BSA] (μg)	Vol. BSA (μl)	Lysis buffer (μl)	0.1M HCl (μl)	ddH ₂ O (μl)
0	0	10	10	80
5	1	9	10	80
10	2	8	10	80
20	4	6	10	80
40	8	2	10	80
50	10	0	10	80

Prepare the above into cuvettes and add 3.5ml of Bradford dye (BioRad) to each cuvette. Mix well.

N.B. To prepare Bradford dye, mix 1 part dye to 4 parts water.

Samples:

2.8 μl sample

2.8 μl 0.1M HCl

22.4 μl ddH₂O

Prepare the above for samples in cuvettes, add **1ml dye** and mix well.

Read standards and samples @595nm in a spectrophotometer.

Determine protein amount. Calculate the volume of sample and rehydration buffer you need to add. The following volume is required for complete reswelling of the IPG strip:

7cm IPG strip: 150 μl

18cm IPG strip: 450 μl

24cm IPG strip: 600 μl

The ratio of lysis: rehydration buffer should never exceed 1:3.

Two-Dimensional Electrophoresis

Checklist

First Dimension (IEF)

Multiphor: Temperature set to 20°C

Protein sample in Lysis buffer (100 µg for analytical gel, 400 µg for preparative gel)

Vortex mixer

Fine-ended forceps

P200 pipette

P100 pipette

Reswelling tray

Rehydration solution (see appendix)

Dry strip cover fluid

IPG dry strips

Second Dimension (SDS-PAGE)

Ettan tank: Temperature set to 10°C

DTT

Iodoacetamide

SDS equilibration buffer (see appendix)

SDS electrophoresis running buffer

Large format gels

Agarose sealing solution (see appendix)

DryStrip cleaning solution

P1000 pipette

Fine-ended forceps

Loading tips

Fixation solution

IEF

1. Handle everything with gloves to avoid keratin contamination!
2. Remove rehydration stock solution from storage at -80°C and thaw at room temperature.
3. Place reswelling tray onto a levelled surface.
4. Dissolve $100\ \mu\text{g}$ of protein into $450\ \mu\text{l}$ of rehydration buffer and mix by vortexing. This provides enough volume to rehydrate an 18 cm IPG strip.
5. Remove required number of 18 cm DryStrips from storage at -20°C . Freeze the remaining strips immediately!
6. Pipette $450\ \mu\text{l}$ ($100\ \mu\text{g}$) of protein solution into the reswelling tray at a central point. Ensure removal of any residual air bubbles from solution.
7. Peel away foil backing from DryStrips from the anode end of the strip. Starting at the basic end might damage the usually softer basic gel surface.
8. Holding strip with forceps immerse into rehydration buffer, gel side down with the anodic end of the strip pointing towards the pointed end of the reswelling tray.
9. To ensure even rehydration along the length of the DryStrips gently lift and lower the strip and slide the strip back and forth along the surface of the solution.
10. Apply gently pressure on top of the DryStrips to remove trapped air bubbles from under the strip.
11. Pipette 2-3 ml of DryStrips cover fluid dropwise into one end of the strip holder until one half of the IPG strip is covered, then pipette the remainder of the cover fluid dropwise into the other end of the strip holder until the entire IPG strip is covered.
12. Close cover of rehydration chamber.
13. Rehydration occurs overnight at room temperature (minimum 12h with sample, 6h without sample for cup loading).
14. After rehydration transfer the reswollen DryStrips onto the Multiphor.
15. Make sure the electrode bars and the plastic aligner are clean and dry.
16. Pipette 5 ml of silicone oil on the centre of the cooling plate of the flat pad and position the tray on top of it.
17. Verify the contact between the tray and the cooling plate.
18. Connect red and black electrode leads of the tray to the Multiphor unit.
19. Pipette 10 ml of DryStrip Cover Fluid on the centre of the tray and place the plastic strip aligner, grooves facing up, on top of the cover fluid.
20. Cut 2 paper electrode wicks to a length corresponding to the number of strips you will run. Soak them by adding single drops of double distilled water.
21. Never put excess of water, you could create horizontal streaking. Remove excess water by pressing the electrode wick strongly and several times onto a tissue paper.
22. Transfer the strips from the reswelling cassette to the adjacent grooves of the plastic strips aligner.
23. Place the acidic end (+) at the top of the tray near the cooling tubes and the red electrode, gel side up.
24. Place the moistened electrode wicks cross on top of the gel ends.
25. Position the electrode bars and press them down on top of the moistened electrode papers. Make sure the marked side of the electrode bar corresponds to the tray giving electrical contact.
26. Cover the strips by pouring DryStrip cover fluid in the centre of the tray.
27. Close the lid and connect the unit to the power supply.

28. Gradually increase voltage from 400V to 3500V during 3 hours. Isoelectric focussing will take approximately 9-21 hrs.
29. Clean the rehydration chamber immediately using a toothbrush and a detergent, you must not allow any remaining fluid to dry out!

IEF Protocol for Heart and Smooth Muscle cells

Analytical gel (100 µg), gradient mode

Steps	Time	Voltage (V)
1	0:01	400
2	0:20	500
3	0:15	750
4	0:25	1200
5	0:10	2000
6	0:07	2500
7	0:07	3500
8	18:00	3500

Total: 64.6 kVhrs

Preperative gel (400 µg), gradient mode

Steps	Time	Voltage (V)
1	0:05	400
2	0:45	500
3	0:45	750
4	0:45	1200
5	0:30	2000
6	0:20	2500
7	0:10	3500
8	18:00	3500

Total: 66.6 kVhrs

IEF Protocol for Liver and Hematopoietic cells

Analytical gel (100 µg), gradient mode

Steps	Time	Voltage (V)
1	0:01	400
2	1:00	2000
3	0:15	3500
4	7:00	3500

Total: 26.4 kVhrs

Preperative gel (400 µg), gradient mode

Steps	Time	Voltage (V)
1	0:05	400
2	2:00	2000
3	0:30	3500
4	11:00	3500

Total: 42.3 kVhrs

How to Cast 12% Second dimension Gels

1. Take 40% Acrylamid solution 187.5 ml
2. Add 150 ml 1.5 M Tris pH 8.8
3. Add 253.5 ml double distilled water
4. Degas for at least one hour with stirring
5. At the same time, assemble gel caster
6. Keep on stirring, add 6 ml 10% SDS (filtered)
7. Add 252 µl TEMED
8. Add 2.4 ml 10% APS
9. Pour the gel solution without delay till the level has reached 1 cm below the upper edges of glass plates.
10. Overlay with 3~5ml of upper layer of water-saturated 2-Buthanol (H₂O : 2-Buthanol = 2 : 3)
11. After 100 min, discard 2-Buthanol
12. Rinse gel surface with double distilled water
13. Overlay gel top with gel storage solution
14. Complete polymerization at RT overnight
15. Seal in plastic bags and store at 4°C

Casted gels should be used within a week!!

Assembly of gel caster:

Everything has to be perfectly clean!

1. Place the caster flat on the bench for loading.
2. Put the V-shaped rubber insert in place, the plastic separator sheets between the gel and blank cassettes. Start with a separator sheet (offset edges up), place the cassettes alternating with separator sheets.
3. At the end place a separator sheet and fill up with thicker filling sheets.
4. Turn the screws into the bottom wholes.
5. Place the front plate on the caster, apply the clamps and tighten the screws.
6. Place the caster upright and ready for casting gels.

Troubleshooting:

If the polymerization runs too fast, heat is produced in the centre of the multi-caster, leading to thermal convection, which results in curved gel edges. In order to get reproducible gels with a straight edge, more TEMED and less APS should be added. This prevents overheating. The quality of the edge is also influenced by the quality and the age of these chemicals. A straight gel surface is a prerequisite for placing the IPG strip on the gel edge to edge.

SDS-PAGE

1. Upon completion of IEF remove DryStrips with forceps from Multiphor unit and note data supplied on the LCD readout and condition of strips in terms of presence of urea precipitation.
2. Place strips sidewise onto paper towels to drain excessive cover fluids.
3. The strips are now ready for equilibration. Alternatively, strips can be sealed in a plastic bag and stored in a film cassette at -80°C .
4. Remove the required number of vials of SDS equilibration buffer from -20°C freezer and thaw at 30°C .
5. Weigh out 100 mg of DTT and dissolve into 10ml of SDS equilibration buffer, sufficient for 1 DryStrip.
6. Place DryStrip into an equilibration tube with gel surface facing into the tube.
7. Add 10 ml of DTT containing SDS equilibration buffer to the tube and incubate at room temperature on a shaking platform for 15 minutes. Tape tube to surface of platform to avoid tube rolling off platform and to ensure DryStrip remains covered in equilibration buffer during the incubation.
8. Weigh out 480 mg of iodoacetamide and dissolve into 10 ml SDS equilibration buffer, protect from light until use.
9. Replace the DTT solution with the iodoacetamide solution after 15 minutes by pouring out the DTT solution, ensuring the DryStrip remains in the tube!
10. Incubate the DryStrip for 15 minutes at room temperature on a shaking platform in the iodoacetamide SDS equilibration buffer.
11. Remove required number of gels from storage at 4°C and remove from packaging.
12. Rinse gel surface with double distilled water.
13. Heat one aliquot of agarose sealing solution in microwave until it gets liquid.
14. Discard iodoacetamide solution on dry strips and rinse with 15 ml water twice.
15. Pipette 2ml agarose sealing solution into well of gel so that it provides an even layer across the bottom. **The agarose solution should not be hotter than 60°C .** It could cause carbamylation of proteins because of the presence of urea.
16. Place DryStrip onto gel cassette with plastic baking against gel backplate and push the DryStrip at the centre into the agarose sealing solution so that it displaces the agarose sealing solution around the DryStrip. Hold DryStrip in place until agarose is set.
17. Fill remainder of well with excess agarose sealing solution.
18. Allow agarose to set completely for at least 15 minutes.
19. Pre-cool buffer in lower chamber of the Ettan tank to 10°C . Never switch on the pump without liquid in the tank.
20. Place gel cassettes into cassette carrier. Insert blank cassettes into free positions.
21. Slide upper chamber carefully over gel cassettes after wetting the tubing of the buffer seal.
22. Place electrophoresis chamber into electrophoresis tank.
23. Fill upper reservoir with fresh 2x SDS electrophoresis buffer (1x is sufficient for 3 gels or less).
24. Immediately fill outer chamber with 1x SDS electrophoresis buffer to the same level as upper chamber. The hydrostatic balance is necessary to prevent leakage of the upper buffer tank and mixing of the buffers.
25. Attach lid to tank, connect electrodes to power supply and run gel at 2 W per gel for 15 min and 2.5 W per gel for 30 min.

26. Then set to 100W (maximum 30W per gel) and continue the run until the dye front has reached the bottom of the gel.
27. Disassemble electrophoresis tank/electrode. Empty upper buffer tank first before lifting the cassette carrier and removing the gels cassettes.
28. When the anodal buffer should be re-used, the upper buffer must be collected and poured into the lower buffer in order to bring the Tris ions back. However, 2x upper buffer has to be diluted to 1x buffer first.
29. Open the cassettes gently with a piece of plastic.
30. Place the gels immediately in fixing solution. Gels are then ready for staining.
31. All the equipment has to be washed thoroughly. Rinse the cathode panels on the safety lid with deionised water; glass plates should be soaked in water with detergent overnight.

Silver Staining Protocol for Proteins
Using PlusOne Silver Staining Kit, Protein (GE Healthcare)

250ml of solutions are needed per 12.5 x 26 cm precast gel. The staining time is 2 hours. All trays and beakers have to be perfectly clean! All steps should be performed with gentle shaking of the staining tray.

1. Fixation overnight
Soak the gel in fixing solution overnight.
2. Sensitizing for 30 min
Remove the fixation solution. Add sensitizing solution and leave shaking for at least 30 minutes.
3. Washing with double distilled water for 3x5 min
Remove the sensitizing solution. Add double distilled water and wash three times for 5 minutes each time.
4. Silver reaction for 20 min
Add silver solution and leave shaking for 20 minutes.
5. Washing with double distilled water for 2x1 min
Remove the silver solution. Rinse twice in double distilled water for one minute each time.
6. Developing for 2-8 min
Add developing solution and leave shaking for 2-8 minutes until protein spots show up and before background become dark.
7. Stopping for 10 min
Remove the developing solution. Add stop solution and leave shaking for 10 minutes.
8. Washing with double distilled water for 3x5 min
Remove the stop solution. Add double distilled water and wash three times for 5 minutes each time.
9. Seal Gel into sample bag with little double distilled water.
10. Store at 4°C cold room

PlusOne Silver Staining kit, Protein

If you want to achieve identification on MALDI-MS:

Use no glutardialdehyde in the sensitizing reaction

Use no formaldehyde in the silver solution

For 1 large format gel:

FIXATION	Methanol Acetic acid Add ddH ₂ O to	100ml 25ml 250ml	overnight
SENSITIZING	Methanol Sodium thiosulfate Sodium acetate Add ddH ₂ O to	75ml 10ml 1 packet 250ml	30 min
WASHING	ddH ₂ O	250ml	3 x 5 min
SILVER REACTION	Silver Nitrate Add ddH ₂ O to	25ml 250ml	20 min
WASHING	ddH ₂ O	250ml	2 x 1 min
DEVELOPING	Sodium carbonate Formaldehyde* Add ddH ₂ O to	1 packet 0.1 ml 250ml	2 - 8 min
STOPPING	EDTA Add ddH ₂ O to	1 packet 250ml	10 min
WASHING	ddH ₂ O	250ml	3 x 5 min

Seal gels in plastic bags with A. bidest (1-2 ml), store in cold room

* added just before developing

Tryptic digestion in polyacrylamide gel slices

Following the selection of the spots of interest, the protein spots have to be excised from the gel. If this step is performed manually, wear powder-free gloves, try to minimize potential keratin contamination and use a clean scalpel blade to cut around the protein spot of interest. Attempt to take as little of the surrounding gel as possible. Our protocol is based on the published method of M. Mann for the digestion of silver stained gels for analysis by nanospray mass spectrometry (*Shevchenko A, et al. Analytical Chemistry 1996; 68: 850-858*). Trypsin specifically hydrolyses peptide bonds at the carboxyl side of lysine and arginine residues.

All reagents are prepared fresh before use:

1. 100 mM ammonium bicarbonate: 158.12 mg/20 ml ddH₂O
2. 50 mM ammonium bicarbonate: 79.06 mg/20 ml ddH₂O
3. 10 mM DTT: 1.54 mg/ml in 100 mM ammonium bicarbonate
4. 50 mM iodoacetamide: 9.25 mg/ml in 100 mM ammonium bicarbonate
5. Tryptic solution (on ice): 20 ng/μl Promega sequencing grade modified trypsin in 50 mM ammonium bicarbonate
6. Extraction solution: 5% formic acid in 50% acetonitrile

Reagents:

Ammonium bicarbonate	Sigma A-6141 (min 99%); FW 79.06
DTT	Sigma D-9779 (min 99%); FW 154.2
Iodoacetamide	Sigma I-1149 (ultra); FW 185.0
Acetonitrile	Aldrich 27,071-7
Sequencing grade modified trypsin	Promega V5111

Procedure:

Eppendorf tubes are all rinsed with acetonitrile, water and methanol. Thorough washing removes acetonitrile soluble material, which forms a layer on aqueous solutions and interferes with evaporation.

DAY 1:

1. Cut band from gel as closely as possible. Divide into smaller pieces.
2. Wash gel pieces with 100 μ l 100 mM ammonium bicarbonate for 5 min.
3. Discard the washing solution and dehydrate the gel slices in 200 μ l acetonitrile. Gel pieces should turn opaque-white within 5 min. If not, remove acetonitrile and add another 200 μ l of acetonitrile.
4. Discard acetonitrile and evaporate residual acetonitrile in Speed Vac for 5 min.
5. Reduce the gel pieces in 30 to 50 μ l 10mM DTT for 30 min at RT.
6. Remove DTT solution.
7. Alkylate in 30 to 50 μ l 50 mM iodoacetamide for 30 min at RT (in the dark).
8. Remove iodoacetamide solution.
9. Wash with 100 μ l 100 mM ammonium bicarbonate for 10 min.
10. Remove wash.
11. Dehydrate gel slices in 200 μ l acetonitrile.
12. Remove acetonitrile and rehydrate by swelling in 100 μ l 100 mM ammonium bicarbonate for 10 min.
13. Dehydrate gel slices in 200 μ l acetonitrile.
14. Remove acetonitrile and add another 200 μ l aliquot of acetonitrile.
15. Remove acetonitrile.
16. Dry gel pieces in Speed Vac for 5 min.
17. Prepare trypsin: 20 μ g of Promega trypsin in 1ml ice cold 50 mM ammonium bicarbonate (trypsin concentration = 20 ng/ μ l). Keep ice cold.
18. Add 30 to 50 μ l of the trypsin solution to cover the gel pieces and incubate 45 min on ice. Watch that gel pieces appear re-swollen (the idea is to allow the trypsin to move into the gel but not begin digestion)
19. Remove any excess trypsin solution and add 5-20 μ l 50 mM ammonium bicarbonate. React overnight at 37°C.

DAY 2:

20. Extract with 30 μ l of 100 mM ammonium bicarbonate. Vortex. Incubate for 10 min, take supernatant to a clean 0.5 ml tube.
21. Extract the peptides by adding 30 μ l of extraction solution. Vortex. Incubate for 10 min, take supernatant to the same 0.5ml tube in step 20.
22. Repeat the extraction with a second aliquot of extraction solution, combining the extracts in the same 0.5ml tube in step 20.
23. Lyophilize samples, resuspend in 0.1% TFA, and purify peptides by use of a μ C-18 zip-tip (Millipore).
24. Now sample is ready for MALDI-ToF MS.

APPENDIX RECIPES**I. LYSIS BUFFER (-80°C)**

	DUNN	100ml	GÖRG
Urea (Mw=60.06)	10M	60g	9M
Chaps (w/v)	2%	2g	2-4%
DTT (MW=154.2) (w/v)	1%	1g	1%
Pharmalyte (v/v)	2%	2ml	2%
Proteinase inhibitor	tablets	2	10mM PMSF
Bromphenol Blue		trace	

II. REHYDRATION BUFFER (-80°C)

	DUNN	100ml	GÖRG
Urea	8M	48g	8M
Amberlite		1g	filter
Chaps (w/v)	0.50%	0.5g	0.5-4%
DTT (MW=154.2) (w/v)	0.20%	200mg	0.40%
Pharmalyte (v/v)	0.5%	0.5ml	0.5%
Bromphenol Blue		trace	

III. EQUILIBRATION BUFFER (-20°C)

	DUNN	500ml	GÖRG
Urea (Mw=60.06)	6M	180.17g	6M
SDS (w/v)	2%	10g	2%
Glycerol (100%) (w/v)	30%	150ml	30%
1.5M TrisHCl pH 8.8	50mM	16.75ml	50 mM
Bromphenol Blue		trace	
Add fresh DTT (w/v)	1%	10mg/ml	1%
Add fresh Iodoacetamide (w/v)	4.8%	48mg/ml	4%

IV. 4x GEL BUFFER (4°C)

	1000ml	200ml	100ml
Tris (MW=121.14)	181.7g	36.34g	18.17g
Adjust pH 8.8 over 3-4h filter (0.45 µm), store at 4°C			

V. GELS 12%

	Amersham (Acryl 12.5% Bis 0.33%)
For 6 Gels	
Acrylamid/Bis 40%/2.6%	187.5ml
Tris 1.5M, pH 8.8	150.0ml
ddH ₂ O	253.5ml
SDS 10%	6.0ml
TEMED	252µl
APS 10%	2.4ml
Total Volume	600ml

VI. GEL STORAGE SOLUTION (20°C)

	200ml
TrisHCl pH 8.8 1.5M	50ml
SDS 10%	2ml

Fill up to 200 ml with ddH₂O

VII. OVERLAY SOLUTION (20°C)

	250ml
Isobuthanol (2-buthanol)	150ml
ddH ₂ O	100ml

Take only upper layer!!

VIII. 1% BROMPHENOLBLUE (20°C)

	10ml
Bromphenol Blue	100mg
Tris base	60mg
ddH ₂ O	10ml

IX. 1% LMP agarose sealing solution (20°C)

	100 ml
Agarose LMP agaros 1%	1g
Bromphenol blue 0.03% (w/v)	30 mg
Running buffer 1x	100 ml

X. Electrode buffer (Amersham) (20°C)

			10x stock
Tris base (MW=121.1)	25 mM	30.4g	605 g
SDS	0.1%(w/v)	10g	2882g
Glycine (MW=75.07)	192 mM	144g	200 g
ddH ₂ O		10L	20L

Do NOT adjust pH, approx. pH 8.3.

XI. Running buffer (20°C)

	DUNN	10x (Invitrogen)
Tris base (MW=121.1)	1.38g	29g
SDS	0.48g	10g
Glycine (MW=75.07)	7.17g	144g
ddH ₂ O	500ml	1000ml

XII. SDS 10% (w/v) (20

	100 ml
SDS (MW 288.38)	10g
ddH ₂ O	100ml
Filter (0.45 µm)	

XIII. APS 10% (w/v) (prepare fresh)

	3ml
APS (MW 228.20)	300mg
ddH ₂ O	3ml

XIV. Sucrose wash buffer (4°C)

		1000ml
Sucrose	350 mM	119.79g
ddH ₂ O		1000ml

XV. Tris-Sucrose wash buffer (4°C)

		1000ml
Sucrose	250 mM	85.57g
Tris (pH=7, 1M)	10mM	10ml
ddH2O		1000ml