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Analysis of Membrane Protein Complexes by Blue Native PAGE

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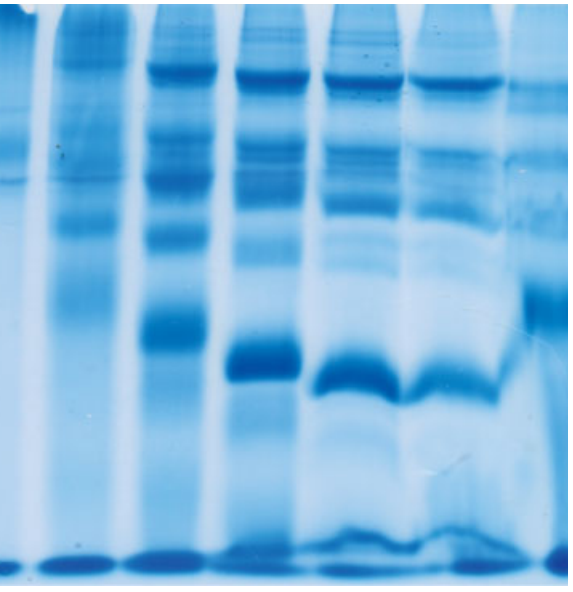

Blue native polyacryamide gel electrophoresis is a special case of native electrophoresis for high resolution separation of enzymatically active protein complexes from tissue homogenates and cell fractions. The method is powerful between 10 and 10 000 kDa. Also membrane protein complexes are separated well after solubilization of complexes with mild neutral detergents. The separation principle relies on binding of Coomassie blue G250 which provides negative charges to the surface of the protein. During migration to the anode, protein complexes are separated according to molecular mass and/or size and high resolution is obtained by the decreasing pore size of a polyacrylamide gradient gel. The principles of 2-dimensional blue native sodium dodecyl sulfate polyacrylamide gel electrophoresis are presented here together with a practical step-by-step guide to performing the method in the laboratory.

"Today, BN-PAGE is well-established as an excellent choice when analysis of protein interactions between native membrane proteins is required at high resolution and high throughput."

Introduction

In recent years, separation of protein complexes by blue native polyacrylamide gel electrophoresis (BN-PAGE) has proven to be a powerful method for identification of functional disorders in total tissue homogenates, cells and cell fractions [1–4]. The method has been used to determine the molecular weight of protein complexes in the range between 10 and 10 000 kDa [5]. Especially the membrane intrinsic electron/proton transfer complexes in mitochondria and chloroplasts have been analyzed with success [6, 7]. The method has been combined with other techniques to determine the oligomeric state, stochiometry, enzymatic activity and the molecular structure of multiprotein complexes [8–10]. As many samples may be separated in parallel in one electrophoretic run, a direct comparison of protein complexes readily allows the identification of disorders and direct further functional analysis. In proteomics, high resolution separation of proteins is generally achieved by two-dimensional (2-D) separation according to the proteins isoelectric point (IEF-PAGE) and molecular mass (SDS-PAGE). This denaturing technique separates hundreds to thousands of proteins in complex samples [11]. However, hydrophobic membrane proteins are hardly detectable after 2-D IEF/SDS-PAGE [12]. Therefore, global analysis of membrane proteomes has not been started, despite the importance of membranes for the living cell. Here, BN-PAGE presents an alternative strategy to separating membrane proteins with high resolution and maintaining their enzymatic function [13]. The method is called native, as most separated protein complexes retain enzymatic functions and blue native, since electrophoretic separation relies on binding of the dye Coomassie blue G250 to protein. Blue native PAGE was first described in 1991 for the separation of membrane protein complexes from the respiratory chain of human mitochondria [14]. Today, BN-PAGE

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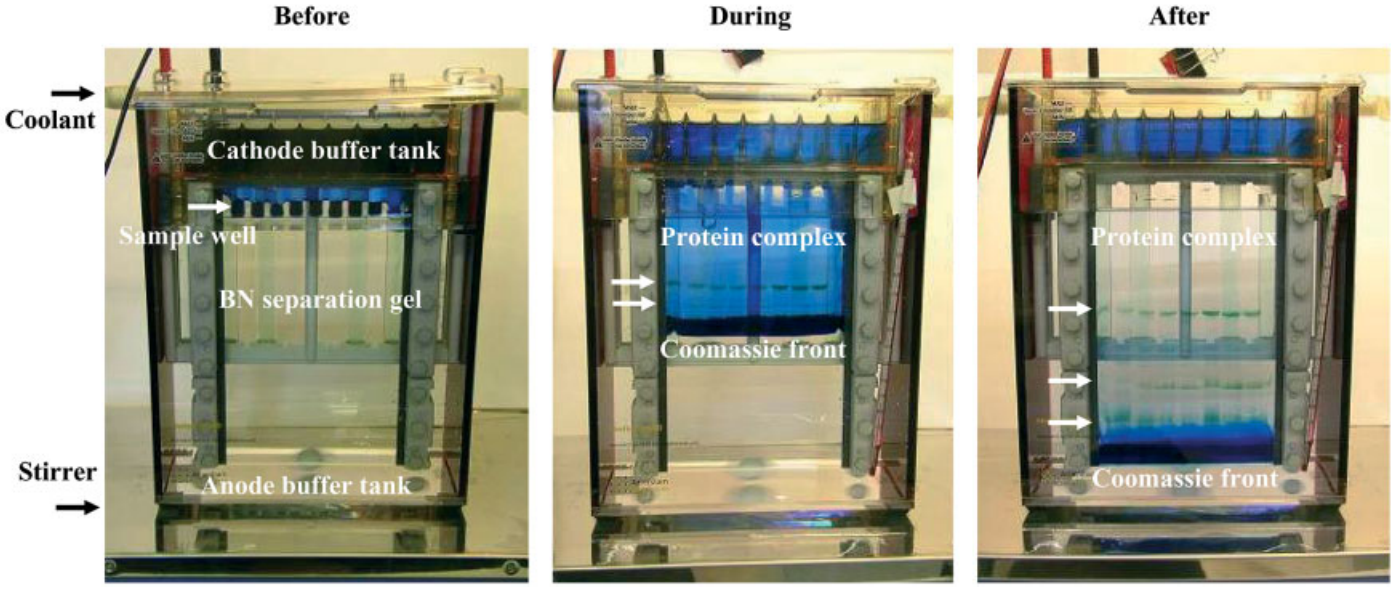


Figure 1. Time course of an electrophoretic run. The pictures represent three characteristic time points during performance of blue native PAGE. The electrophoretic apparatus consists of the anode (bottom) and the cathode buffer tank (top, filled with blue cathode buffer), the gel glass sandwich and a temperature exchange module to cool the gel *via* the anode buffer to 4°C during electrophoresis. The anode buffer is stirred for temperature control. Before electrophoresis is started, blue cathode buffer is filled in the buffer tank and samples are underlayed into the sample wells (white arrow, sample well, Before). During electrophoresis, charged samples enter the stacking gel and after some time Coomassie which is moving with the buffer front has reached half of the separating gel. At this point, protein complexes begin to be visible in the separation gel and the blue cathode buffer is exchanged for colorless cathode buffer (white arrows, During). Blue native electrophoresis may be stopped when the Coomassie stained buffer front has reached the end of the separating gel and the separation of the complexes is finished (white arrows, After).

is well-established as an excellent choice when analysis of protein interactions between native membrane proteins is required at high resolution and high throughput [5,15]. But why is it of importance in biology to know about protein interactions?

Proteins express the function of genes in all organisms that exist on earth. Proteins are the functional molecules which operate metabolic, developmental and regulatory pathways in a cell, tissue or an organism. Multiple proteins are integrated in complexes in order to organize multi-enzymatic functions. Protein complexes therefore reflect the cell's macromolecular organization state which is a fundamental basis for understanding cellular functions. In practical proteomics, the goal is to identify all proteins present in a defined developmental state of an organism, tissue, cell or their subfractions and to characterize their qualitative and quantitative changes in response to environmental changes [17–19]. Although protocols have been worked out in general to start proteomic analysis in the many domains of life, systematic proteome analysis awaits the development of adequate technology. In this respect, BN-PAGE is one of the candidates to standardize the first step during the sequential high resolution analytical fractionation of the proteomes [20]. In particular, the natural organization of living systems has offered this opportunity to trace the function of native protein complexes after biochemical separation in BN-PAGE. Due to the association of a large number of single proteins in multi-enzymatic protein complexes, the level of complexity is maintained at a low level. This is the basis for every successful technological analysis of dynamic developmental and metabolic states and organization levels. With BN-PAGE, a simple technology for separation and identification of protein complexes, and especially of membrane protein complexes, has been established for a high number of developmental states over recent years. In the following, allow me to explain the principles of BN-PAGE and show you how to get started with the technology (Fig. 1).

Materials

Sample Preparation for BN-PAGE

1. Sample buffer BN: 750 mM ε- aminocaproic acid,
   50 mM Bis-Tris-HCl pH 7.0,
   0.5 mM EDTA-Na_2.
2. Detergent solution:
   - Dodecylmaltoside solubilization buffer:
     10 % (w/v) *n*-dodecyl-β-D-maltoside.
   - Digitonin solubilization buffer:
     30 mM HEPES pH 7.4,
     150 mM potassium acetate,
     10 % (v/v) glycerol,
     5 % (w/v) Digitonin.
3. Loading buffer: 750 mM ε-aminocaproic acid,
   5 % (w/v) Coomassie G 250.

Casting of Gradient Gels for BN-PAGE

1. BN gel buffer (6 ×): 3 M ε-aminocaproic acid, 0.3 M Bis-Tris-HCl pH 7.0.

2. Acrylamide solution: 30% (w/v) acrylamide/ bis acrylamide solution (37.5:1, 2.6%C).
3. Glycerol (100%).
4. TEMED: *N,N,N,N*`-tetramethyl-ethylenediamine.
5. APS: ammonium persulfate: 10% (w/v) solution.
6. Water-saturated isobutanol: 50% (v/v) isobutanol.

BN-Electrophoresis

1. BN running buffer cathode (10 x):
   a. Blue cathode buffer: 500 mM Tricine, 150 mM Bis-Tris-HCl pH 7.0, 0.2 % Coomassie G250.
   b. Colorless cathode buffer: 500 mM Tricine, 150 mM Bis-Tris-HCl pH 7.0.
2. BN running buffer anode (10 x): 500 mM Bis-Tris-HCl pH 7.0.

Solubilization and Transfer of BN Strips

1. BN solubilization buffer: 2% (w/v) SDS, 66 mM Na_2CO_3, 0.67% β- Mercaptoethanol.
2. Overlay solution: 0.5% (w/v) agarose in 1 × SDS running buffer.

Casting of Second Dimension Gels

1. SDS separating gel buffer (8 x): 3 M Tris pH 8.8.
2. SDS stacking gel buffer (2 x): 250 mM Tris pH 6.8.
3. Acrylamide solution: 30% (w/v) acrylamide/ bis acrylamide solution (37.5:1, 2.6%C).
4. TEMED: *N,N,N,N*`-tetramethyl-ethylenediamine.
5. APS: ammonium persulfate: 10% (w/v) solution.
6. Water-saturated isobutanol: 50% (v/v) isobutanol.

"In order to obtain optimal results, the sample preparation is the most critical step so carry out all the steps on ice!"

SDS Electrophoresis

1. SDS running buffer (10 ×): 250 mM Tris, 1.92 M glycine, 1% (w/v) SDS.

Methods

Sample Preparation for BN-PAGE (~ 1 h)

Blue native PAGE separates membrane protein complexes in a native state. In order to obtain optimal results, the sample preparation is the most critical step so carry out all the steps on ice! As the extent of protein complex solubilization and the stability of the solubilized proteins depend on both the nature of the single sample as well as on the detergent type and concentration, these variables have to be tested for the sample of interest.

Solubilization of Protein Complexes

Biological membranes are complex assemblies of lipids and proteins. A lipid is typically composed of two hydrophobic hydrocarbon tails connected to a polar head group. Lipids are mostly arranged in a bilayer structure with the hydrophobic tails sandwiched between the hydrophilic head groups pointing outward. Membrane proteins are embedded in the hydrophobic core region of the membrane bilayer by interaction with the hydrophobic lipids phase. Proteins assemble in multisubunit complexes to carry out cellular functions within the membrane phase.

For an investigation of the subunit composition of specific protein complexes, assemblies have to be extracted from the lipid phase and separated from each other. For BN-PAGE, the goal is to maintain the native structure and subunit composition of all membrane components required for enzyme activity in one complex during electrophoresis. Two steps are required to prepare the sample ready for BN-PAGE.

First, for extraction of native complexes from the lipid phase, typically amphipilic detergents are used. Detergents dissolve the lipid bilayer and solubilize lipids as well as membrane proteins in detergent micelles. Detergents are amphipatic molecules composed of a polar, ionic or non-ionic head group and one hydrocarbon tail. The result of the amphiphlic nature of the detergent is the formation of thermodynamically stable micelles with hydrophobic cores in aqueous media. This hydrophobic core provides an environment that allows for the dissolution of hydrophobic molecules or domains of proteins. Above a critical concentration, which is the maximum monomer concentration (CMC), detergents self-aggregate in micelles *via* the hydrocarbon tails. The lower the CMC, the more stable the micelle and the more slowly molecules are incorporated into or removed from the micelle. The CMC is highly dependent on factors such as temperature, pH, ionic strength, and detergent homogeneity and purity. In the presence of membranes, micelles provide the reservoir for detergent accumulation in the membrane phase, membrane bilayer disruption and lysis. Since detergents partition into the lipid phase on a concentration-dependent basis, lipid-detergent, lipid-protein-detergent, and protein-detergent micelles are formed. The correct detergent type and concentration

are determined experimentally in order to maintain protein-protein interactions and to ensure that one protein complex is solubilized *per* micelle (Fig. 2).

Second, non-solubilized membranes and aggregates are separated away by centrifugation. Protein complexes solubilized in detergent micelles will remain in the supernatant. Now, separation of protein complexes by BN-PAGE may start.

When selecting a detergent for BN-PAGE, the first consideration is usually the non-ionic form of the hydrophilic group, since anionic, cationic, and zwitterionic detergents typically modify protein structure to a greater extent. The degree of modification by ionic detergents varies with the individual protein and the particular detergent. Ionic detergents are also more sensitive to pH, ionic strength, and the nature of the counterion, and can interfere with charge-based analytical methods. Most non-ionic detergents are non-denaturing, but are less effective at disrupting protein aggregation. For BN-PAGE, *n*-dodecyl-β-D-maltoside and digitonin have been proven to be most useful (Fig. 2) [21].

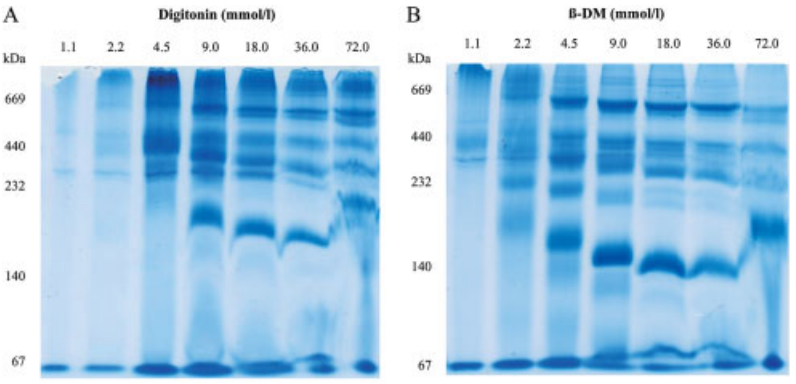


Figure 2. Comparison of two detergents for solubilization and separation of protein complexes by BN-PAGE. Thylakoid membranes (100 µg protein) were solubilized with detergent concentrations between 1.1–72 mmol/l. These concentrations were tested for the two nonionic detergents digitonin (A) and ß-dodecyl-*n*-maltoside (B). After the removal of unsolubilized material by centrifugation, the protein complexes were applied to separation by BN-PAGE (7.5 %). After electrophoresis the gels were stained with colloidal Coomassie. The molecular mass of the complexes was determined using the HMW native protein marker kit (GE Healthcare) (see Fig. 3).

Required to start:

Hardware: 1.5 mL caps, table top centrifuge, 100 µL pipette, 20 µL pipette, 1 µL pipette, yellow pipette tips, white pipette tips

Solutions: in the refrigerator: membrane fraction, sample buffer, digitonin solubilization buffer, detergent solution, loading buffer

1. Pellet the membrane fraction of your sample (corresponding to 400 µg protein) by centrifugation at 4°C. Remove the supernatant containing all soluble and peripheral proteins. To be sure that all peripheral proteins are removed, repeat this step at least once.
2. Resuspend the pellet in 70 µL of sample buffer for subsequent solubilization with *n*-dodecyl-β-D-maltoside.
3. Add 10 µL of *n*-dodecyl-β-D-maltoside detergent solution and mix. For solubilization with digitonin resuspend the pellet directly in 50µL of digitonin sample buffer.
4. Incubate the sample on ice for at least 10 min to solubilize the membrane protein complexes. Depending on the sample it can be necessary to extend this step to up to one hour to achieve complete solubilization of the membrane protein complexes.
5. Centrifuge for 10 min at maximum speed to pellet the unsolubilized material. Unsolubilized material can affect the subsequent electrophoretic separation of the protein complexes in a negative way.
6. Add 5 µL of loading buffer to the bottom of a new cap and transfer the supernatant. Mix the sample gently.

Preelectrophoretic Labelling of Protein Complexes with CyDyes

Usually, separated proteins are stained with colored or fluorescent dyes for viewing following electrophoresis. Chemical stains with a higher affinity to the protein molecules than to the gel matrix detect proteins based on the differential non-covalent binding of the stain. The result is a local increase in concentration of the dye in the protein bands. The important characteristics for a useful stain are: low matrix background, high sensitivity for protein binding and large linear range for binding to proteins at different concentrations. In BN-PAGE, labelling of proteins for high sensitivity detection can also be achieved before solubilzation of the protein complexes and hence separation of the complexes into subunits. By crosslinking of fluorescent dyes to the protein on the level of the native protein complex, single protein subunits of the native complex are labelled with the dye *via* the lysine side

groups and fluorescence is detected after separation of the complex by BN-PAGE or the corresponding protein subunits by 2-D BN-/SDS-PAGE [22].

Required to start:

Solutions: membrane fraction, TMK buffer pH 8.5, Cy2/5, lysine

1. Resuspend the membrane pellets (200 µg protein) in 35 µL TMK buffer pH 8.5 and follow the instructions for CyDye labeling given by the manufacturer.
2. After labeling, sediment the membranes by centrifugation.
3. Resuspend the pellet of sample 1 in sample buffer or Digitonin solubilization buffer.
4. Transfer the resuspended sample 1 into the cap containing sample 2 and resuspend sample 2.
5. Continue the sample preparation procedure as described above. Proceed at step 3.

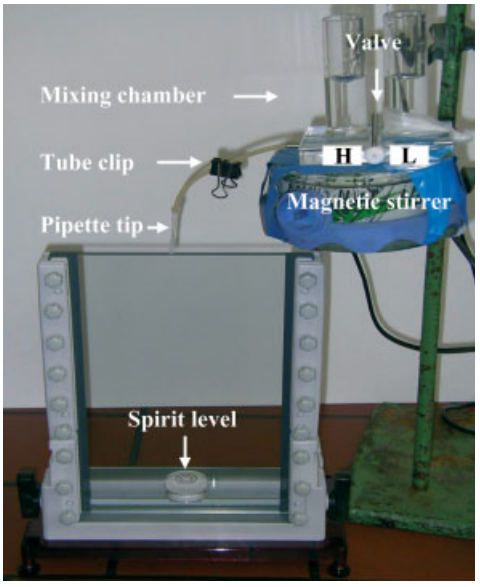


Figure 3. Assembled stand for casting gradient gels. The mixing chamber "H" of the gradient casting stand is placed centrally on a magnetic stirrer. The chambers show a moderate incline towards the gel cassette to achieve a directed flow of the acrylamide solutions from the mixing chamber containing the light solution (L) towards the heavy solution (H). The tube which connects the gradient mixer with the glass plates is closed by a tube clip and is fixed in the middle between the assembled glass plates. The tube ends in a cut pipette tip. First, the valve between the two chambers is closed. Chamber H is filled with a 12 % acrylamide solution and stirred with a magnetic rod. A 6 % acrylamide solution is filled in the chamber L together with a non-magnetic rod to balance the solution levels in the both chambers. The valve between the chambers is opened. Therafter the tube clip is removed and the gel is cast within a few minutes.

Casting of Gradient Gels

Vertical gels are cast in a cassette made up of two glass plates separated by spacers which run along the sides of the plates. Gradient gels are cast with a higher concentration of acrylamide at the bottom than the top. Casting of gradient gels requires a gradient forming apparatus (Fig. 3). In practical proteomics, the separation distance is of ultimate importance to resolve proteins and protein complexes into single components. As blue native PAGE separates protein complexes depending on the molecular mass and/or size of the single protein complexes, the acrylamide concentration of the gel is responsible for the separation range. In practice a separation of membrane protein complexes in the mass range from 10 to 10 000 kDa is possible. Instructions stated below correspond to a 6–12 % linear gradient separating gel and a 4 % stacking gel. This set up allows the separation of protein complexes in the molecular mass range from ~ 50 kDa to 1000 kDa. The entire experiments presented were carried out in a Hoefer SE 660 (GE Healthcare) which has a gel size of 0.75 × 180 × 240 mm^3 and provides a good compromise between handling efficiency and high resolution for most applications.

Separating Gel for BN-PAGE (~ 2h)

Required to start:

Hardware: 2 glass plates (18 × 24 cm), 2 spacers (0.75 mm), 2 clamps (16 cm), 2 clamps (8 cm), 1 casting stand, spirit level, gradient mixer, magnetic stirrer, filtering flasks, clip, tissues (low fluff), 20 µL pipette, yellow pipette tips.

Solutions: ethanol, glycerol TEMED, isobutanol, in the refrigerator: acrylamide (30/0.8), 6 × gel-buffer BN, APS.

	12 %	6 %
acrylamide (30/0.8)	4.60 mL	2.30 mL
BN gel buffer	1.92 mL	1.92 mL
glycerol	2.30 g	–
ddH_2O	3.29 mL	7.28 mL
Σ	11.5 mL	11.5 mL

1. Clean glass plates and spacers with denatured ethanol (100%), assemble the glass plate sandwich, fix it on the casting stand and adjust the assembled casting stand by a spirit level.
   Place the gradient mixer on the magnetic stirrer and connect the pipette tip with the casting stand.
2. Place a magnetic stirring rod in chamber 1 of the gradient mixer and fill in the 12 % gel solution.

3. Pour the 6 % gel solution in chamber 2 of the gradient mixer and add a non magnetic rod to balance the solution levels in the both chambers.
4. Add 5.5 µL of TEMED and 20 µL of APS to both chambers and mix the solutions gently.
5. Open the valve between the two chambers and remove the tube clip. Allow the solutions to rinse between the glass plates.
6. Overlay the cast gel with water-saturated isobutanol.

Stacking Gel for BN-PAGE

Required to start:

Hardware: Whatman paper, comb (10 wells), filtering flask, 5000 µL pipette, 200 µL pipette, yellow pipette tips, white pipette tips, tissues (low fluff)

Solutions: TEMED, in the refrigerator: acrylamide (30/0.8), 6 × gel buffer BN, APS

1. After polymerization of the separating gel cast the stacking gel.

	4 %
acrylamide (30/0.8)	1.35 mL
BN gel buffer	1.67 mL
ddH_2O	6.98 mL
Σ	10.00 mL

2. Insert a clean 10 well comb in the glass plate sandwich.
3. Add 10 µL of TEMED and 100 µL of APS to the solution and pipette the gel solution quickly between the glass plates up to the top of the glass plate.

Loading the Samples and Electrophoresis (~ 4 h)

The term 'electrophoresis' refers to the transport of charged particles like single proteins and protein complexes along an electric field gradient. In a homogenous electric field (E), proteins will move at constant velocity (v) when the electromotive force of the accelerating electric field is balanced by the constricting frictional resistance (f) and further ionic forces in the separation medium (isotachophoresis). Empirically, the proteins' velocity and hence their separation distance at one point in time are directly proportional to the charge state (q), and the field strength (E), and are inversely proportional to the size (r) and/or mass (m) of the hydrated protein and the viscosity (η) of the separation medium. The frictional resistance of the separation medium can be nicely attenuated by performing electrophoresis in a medium reinforced by a chemically inert matrix called a "gel".

Two materials, agarose and polyacrylamide, have proven to form stable and well-controllable gel-matrices. The pore sizes obtained by polymerization of acrylamide were shown to blend well with separation of proteins and protein complexes leading to the term polyacrylamide based gel electrophoresis (PAGE). For an excellent spatial resolution of PAGE, the density and effective pore size of the gel matrix is of importance. In BN-PAGE, high resolution separation is mainly achieved by the pore size of the gel which decreases with separation distance. Hence, when protein complexes approach their pore size limit, their velocity is gradually decreased to zero whereby high resolution separation is promoted (Fig. 4).

"In BN-PAGE, high resolution separation is mainly achieved by the pore size of the gel which decreases with separation distance."

Resolution is counteracted by two forces. In the electric field, the ionic cloud of the charged proteins induces a counter-movement within the solute molecules of the separation medium (retardation) and the proteins' counter-ionic shell leads to lagging (relaxation). Furthermore, the hydrate shell of the protein will diminish the proteins charge state, increasing its effective volume. These factors may lead to a fuzzy appearance of proteins in the gel-matrix and are responsible for the small amount of structural information which can be drawn from migration of a protein during PAGE.

Taken together, in the directed electric field of an electrophoresis apparatus, the movement of particles can be predicted if the charge to molecular mass and/or size ratio is maintained at a constant throughout separation. Then the mobility (µ) of all particles will be constant and separation will occur as distance travelled *per* time due to decelerating forces reflecting molecular mass and/or size. This means that in BN-PAGE, the charge state of the protein complexes is proportional to the mass and/or size of the protein complexes. How solubilization and the binding of Coomassie influences separation of protein complexes and their corresponding protein subunits can be demonstrated well (Fig. 5).

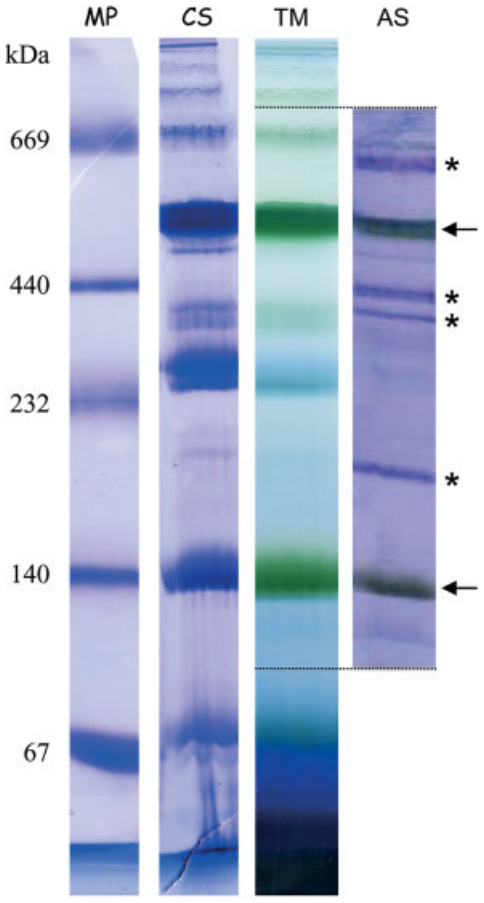


Figure 4. Blue native PAGE of protein complexes from thylakoid membranes. A high molecular weight standard protein (MP) containing thyroglobulin 696, ferritin 440, catalase 232, lactate dehydrogenase 140, and albumin 67 in kDa was separated (GE Healthcare, HMW native protein marker kit 17-0445-01). 1 × 10^8 chloroplasts corresponding to 400 µg protein were isolated from barley chloroplasts, plastids were lysed, and the thylakoid membranes were collected by centrifugation. The membrane-bound proteins were solubilized by dodecylmaltoside and separated by a linear polyacrylamide gradient gel (6–12%) by blue native PAGE (TM). A second lane of the gel was stained with Coomassie blue (CS). To determine the enzymatic diaphorase NAD(P)H:plastoquinone-oxidoreductase activity (asterisk, right-hand side of gel), the first dimension gel (TM) was incubated in 50 mM phosphatbuffer (pH 8.0, 1mM EDTA). Staining was initiated by adding 0.2 mM NADH and 0.5 mg/ml nitrotetrazoliumblue. Incubation was continued for 12 hours. The two chlorophyll-containing protein complexes at 550 kDa (upper signal, PS I-LHC I-complex) and at 120 kDa (lower signal, LHC II-complex) allow molecular mass orientation (arrows).

Most effective in controlling the separation distance are the gels. The gel is mounted between two buffer chambers in such a way that the only electrical connection between the two chambers is through the gel. Here, mostly vertical slab gel systems are used which have direct liquid buffer connections and make the most efficient use of the electric field. Because of the high resistance of the gel, the apparatus should have provisions for cooling.

$$v = E \bullet \mu = E \bullet \{z \bullet e / (6\pi \bullet \eta \bullet r)\}$$

• Electrical field strength	E	Voltage / Distance
• Mobility	µ	[cm2 / V • s]
• Surface charge of molecule	(z • e)	pH-dependent
• Size (mass) and form of molecule	r	Stokes Radius
• Viscosity of the medium	η	buffer medium

Loading the Samples

Required to start:

Hardware: power supply, magnetic stirrer, magnetic stirring bar, thermostatic cooler, microsyringe, electrophoresis apparatus, syringe.

Solutions: in the refrigerator: 1 × cathode buffer BN (blue), 1 × anode buffer BN, 1 × cathode buffer BN (colorless)

1. Assemble the electrophoretic apparatus.
2. Pour the blue 1 × cathode buffer into the upper chamber.
3. As soon as the stacking gel has set, remove carefully the comb from the blue native gel.
4. Underlay the samples into the wells using the microsyringe. Rinse the microsyringe with buffer before applying a new sample.
5. Pour the 1 × anode buffer in the lower buffer chamber.

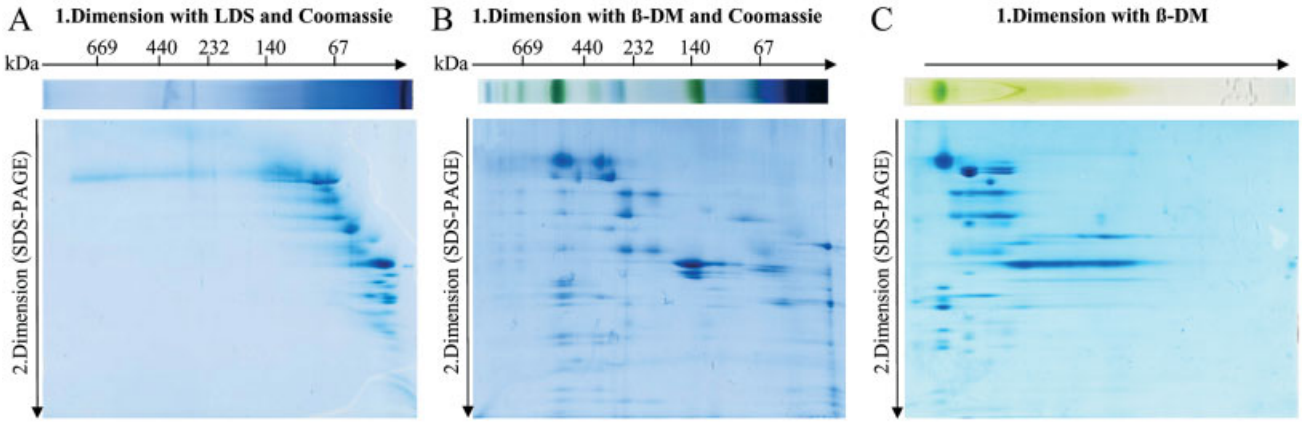


Figure 5. Influence of detergents and Coomassie blue on the separation of proteins by 2-D native/SDS-PAGE. Thylakoid membrane complexes each from 1 × 10^8 chloroplasts were solubilized with lithium dodecyl sulfate (LDS) (A) or n-dodecyl-ß-d-maltoside (DM) (B, C). Solubilized proteins were separated by BN-PAGE (6–12% AA). For samples in "A" and "B", BN-PAGE was carried out as described in [22], for the sample in "C" electrophoresis was carried out in the absence of Coomassie blue as a component in the buffer. The molecular mass of the resolved complexes was determined by the HMW native protein marker kit (GE Healthcare). After electrophoresis, strips of the first dimension gels were cut and protein complexes were denatured. After denaturation, lanes were applied to a second dimension (SDS-PAGE) for the resolution of the complex subunits. After SDS-PAGE, all gels were stained with colloidal Coomassie.

6. Assemble the electrophoresis unit completely and connect to a power supply. The electrophoretic run is carried out at 4 °C.
7. Set the power supply at 12 mA, 1000 V and 24 W and start the electrophoresis.

Exchange of Cathode Buffer

To clear the background of the gel and visualize the blue bands after the first dimension, the blue cathode buffer is exchanged after half of the run. This is particularly advisable if immuno blotting of first dimension strips is planned.

1. As soon as the blue Coomassie dye front has reached half of the separating gel, interrupt the electrophoretic run and replace the blue cathode buffer with colorless blue native cathode buffer in the upper buffer chamber and then continue the electrophoretic run.
2. Stop electrophoresis when the blue cathode buffer front has reached the bottom of the separating gel.
3. Disassemble the buffer chamber assembly and the glass plate sandwich and remove the stacking gel.

The separating gel can be used for different applications. The single complexes can be visualized both by Coomassie or silver staining and by antibody detection after immunoblot [23]. Furthermore the gel can be used for "in gel"- activity assays, *e.g.* for measuring the activity of mitochondrial enzyme complexes [10]. The subunits of protein complexes can be resolved in a subsequent electrophoretic step. In this 2-D approach, the protein complexes are denatured with SDS/β-mercaptoethanol within the first dimension gel and are transferred directly to a standard SDS-PAGE (according to [24]) to separate the protein subunits according to their molecular weights (Fig. 5). After SDS-PAGE, protein subunits can be visualized by Coomassie staining and identified by mass spectrometry [22].

Casting of Standard SDS Gels and Second Dimension SDS-PAGE (~ 3 h)

Required to start:

Hardware: 2 glass plates (20 × 20 cm, 20 × 22 cm), 2 spacers (1 mm), tissues (low fluff), spirit, level, filtering flask, funnel, 1000 µL pipette, blue pipette tips.

Solutions: ethanol, urea, TEMED, displacing solution, isobutanol, in the refrigerator: acrylamide (30/0.8), 8 × gel buffer SDS, APS.

Casting of SDS Separating Gel

A standard gel consists of a homogeneous 12.5 % separating gel and a 4% stacking gel. This set up allows the separation of proteins in the molecular mass range from ~ 10 kDa to 200 kDa. For the second dimension a polyacrylamide gel with dimensions 20 × 20 × 1 mm^3 is used.

1. Clean glass plates and spacers and assemble the casting stand.

	12.5 %
urea	7.21 g
acrylamide (30/0.8)	12.50 mL
SDS separating gel buffer	3.75 mL
ddH_2O	9.00 mL
Σ	30 mL

2. Degas solution after dissolving the urea for 5 min.
3. Add 15 µL TEMED and 50 µL APS and mix the solution.
4. Pour the acrylamide solution between the glass plates.
5. Overlay the cast gel with isobutanol.

Required to start:

Hardware: Whatman paper, filtering flask, 5000 µL pipette, white pipette tips

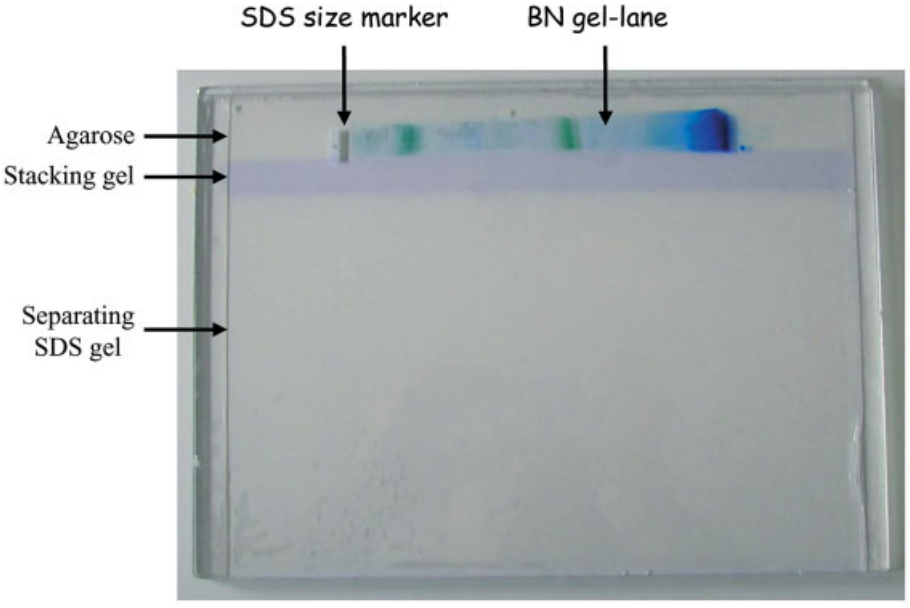
Solutions: agarose, TEMED in the refrigerator: acrylamide (30/0.8), 2 × gel buffer SDS, APS.

Casting SDS Stacking Gels

	4 %
acrylamide (30/0.8)	0.82 mL
SDS separating gel buffer	2.54 mL
ddH_2O	1.64 mL
Σ	5.00 mL

1. Add 5 µL TEMED and 50 µL APS and mix the solution.
2. Pour 5 mL of gel solution on each SDS gel and overlay the gels with isobutanol.
3. After polymerisation of the stacking gel remove the isobutanol by rinsing with water.

Figure 6. First dimension BN-PAGE strip has been loaded onto second dimension gel. A strip of the first dimension gel has been transferred onto a second dimension stacking gel between the two glass plates of the gel cassette. The blue side of the gel (12 % side of the gel strip) is positioned onto the stacking gel first. The strip is positioned centrally on the second dimension after all liquid has been removed from the top of the stacking gel and around the first dimension strip with Whatman paper. A piece of Whatmann filter paper soaked in 10 µl SDS-PAGE standard marker proteins was positioned on the low percentage side (6% side of gel strip) of the first dimension strip and pressed onto the stacking gel. For high transfer yield, air bubbles between the first dimension and the stacking gel have to be avoided. The gel strip and the SDS size marker are fixed with overlay solution containing agarose. Protein subunits released from the first dimension gel are concentrated in the stacking gel and separated in the separating SDS gel.



Equilibration and Transfer of Proteins to the Second Dimension (Fig. 6)

Required to start:

Hardware: casted gels, staining box, Whatman paper, Pencil, 0.75 mm spacer, Pasteur pipette

Solutions: solubilization buffer, SDS marker, overlay solution

1. Separate the strips of the first dimension gel by cutting them with the spacer.
2. Fill 50 mL solubilization buffer in a small staining box.
3. Transfer the strip in the small staining box containing the solubilization buffer.
4. Shake the strip in BN solubilization buffer at room temperature for 20 min.
5. Cut a small piece of Whatman paper.
6. Apply 10 µL SDS marker to the piece of Whatman paper.
7. Position the strip between the glass plates of the SDS gel touching the surface of the SDS stacking gel. Avoid air bubbles between the first and second dimension gel.
8. Place the SDS marker piece according to the sample on the surface of the SDS stacking gel next to the first dimension strip.
9. Overlay the first dimension strip and the marker with overlay solution.

Start of the Second Dimension

Required to start:

Solutions: 1 × running buffer SDS (used), 1 × running buffer SDS (new)

1. Fill 1 × SDS running buffer (used) in the anode tank.
2. Assemble the electrophoresis unit and pour 1 × SDS buffer (new) into the cathode tank.
3. Program the control unit (*per* gel):
   - Time 1: Constant 25 mA, 00:30 h, temperature 15°C
   - Time 2: Constant 5 mA, 24:00 h, temperature 15°C
4. Start the electrophoretic run overnight.

When the SDS-PAGE run is completed, protein subunits of the complexes which were separated during the first dimension BN-PAGE have been separated into single protein spots. All proteins released from one complex have now been separated along the electric field gradient and are positioned among each other according to the proteins' molecular mass forming a straight line. Hence, by identifying one of the protein subunits from the gel by mass spectrometry or gel-blot analysis, the corresponding native protein complex from the first dimension BN gel can be identified. The method is therefore particularly useful in practical proteomics to identify changes in the composition of protein complexes and correlate changes to the functional states of tissue, cell or cell fractions. A detailed description of how data obtained by BN-PAGE approaches can be analyzed will be given in the next issue.

"The method is particularly useful to identify changes in the composition of protein complexes and correlate changes to the functional states of tissue, cell or cell fractions."

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Supplementary Material

Microsoft PowerPoint presentation with animations and more detail see online version