

Blue native electrophoresis protocol

Our blue native electrophoresis protocol is used to determine the size, relative abundance and subunit composition of mitochondrial protein complexes.

This protocol for blue native electrophoresis is designed for use with the following products:

- [Total OXPHOS blue native western blot antibody cocktail \(ab110412\)](#)
- [Complex I subunit NDUFA9 monoclonal antibody \(ab14713\)](#)
- [Complex I subunit NDUF8 monoclonal antibody \(ab110242\)](#)
- [Complex I subunit GRIM-19 monoclonal antibody \(ab110240\)](#)
- [Complex II subunit 70 kDa Fp monoclonal antibody \(ab14715\)](#)
- [Complex III subunit core 2 monoclonal antibody \(ab14745\)](#)
- [Complex IV subunit I monoclonal antibody \(ab14705\)](#)
- [ATP synthase \(complex V\) subunit alpha monoclonal antibody \(ab14748\)](#)

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Reagents and equipment

- Primary BN-PAGE tested antibody
- Secondary antibody, which should be conjugated appropriately for the detection method of choice
- Electrophoresis and western blotting reagents
- 10 % lauryl maltoside solution (n-dodecyl- β -D-maltopyranoside, [ab109857](#))
- 6-aminocaproic acid, Bis-Tris, Tricine
- Coomassie blue G
- Vertical acrylamide electrophoresis unit
- Electroblotting unit- – fully submerged
- pH meter, weighing balance and other standard lab equipment

Buffer recipes

Phosphate buffered saline solution (PBS)

1.4 mM KH_2PO_4
8 mM Na_2HPO_4
140 mM NaCl
2.7 mM KCl, pH 7.3

Protease inhibitor stocks (each is 1000x)

1 M phenylmethanesulfonyl fluoride (PMSF) in acetone
1 mg/mL leupeptin
1 mg/mL pepstatin

First dimension electrophoresis cathode buffer

50 mM Tricine
15 mM Bis-Tris
0.02% Coomassie blue G
Check pH and adjust to 7.0.

First dimension electrophoresis anode buffer

50 mM Bis-Tris
Check pH and adjust to 7.0.

Second dimension electrophoresis running buffer

25 mM Tris
192 mM glycine
0.1% SDS

SDS-PAGE denaturing buffer

10% glycerol
2% SDS
50 mM Tris, pH 6.8
0.002% Bromophenol blue
50 mM dithiothreitol

Tris/glycine or Towbin electroblotting transfer buffer

25 mM Tris
192 mM glycine
10% methanol
0.1% SDS

Membrane washing buffer

PBS plus 0.05% Tween 20

Membrane blocking buffer

PBS plus 5% non-fat milk powder

Alkaline phosphatase color development buffer

0.1 M diethanolamine (DEA)
5 mM MgCl_2
100x NBT stock 50 mg/mL in 100% DMF
100x BCIP stock 50 mg/mL in 70% DMF
DMF dimethylformamide

Buffer A

0.75 M 6-aminocaproic acid, 50 mM Bis-Tris/HCl, pH 7.0

1 µg/mL leupeptin

1 µg/mL pepstatin

1 mM PMSF

Stock leupeptin: 1 mg/mL (water)

Stock pepstatin: 1 mg/mL (ethanol)

Stock PMSF: 0.3 M (ethanol)

LM: n-dodecyl-β-D-maltoside

Sample preparation

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is performed essentially as described by Schägger and von Jagow (1991), *Analytical Biochemistry*, 199, 223–31.

First, solubilized samples are stained with a charged (Coomassie) dye. The intact mitochondrial complexes are then separated by electrophoresis based upon how much dye was bound, which is proportional to their size.

This first dimension gel can be immediately western blotted, or alternatively, the protein components of the resolved complexes can be further separated in a second dimension after soaking the gel in denaturing SDS buffer. Abcam offer monoclonal antibodies for the detection of all five OXPHOS complexes simultaneously ([ab110412](#)) or each of the OXPHOS complexes individually.

When performing blue native electrophoresis, it is always recommended to isolate mitochondria from cells before analysis. The following kits can be used:

- [Mitochondria Isolation Kit for Tissue \(ab110168\)](#)
- [Mitochondria Isolation Kit for Tissue \(with Dounce Homogenizer\) \(ab110169\)](#)
- [Mitochondria Isolation Kit for Cultured Cells \(ab110170\)](#)
- [Mitochondria Isolation Kit for Cultured Cells \(with Dounce Homogenizer\) \(ab110171\)](#)

It is possible to probe whole tissue or cell extract but this may result in a weaker signal.

1. Resuspend 0.4 mg of sedimented mitochondria in 40 µL 0.75 M aminocaproic acid, 50 mM Bis-Tris, pH 7.0.
2. Add 7.5 µL of 10% n-dodecyl-β-D-maltopyranoside.
3. Mix and incubate for 30 min on ice.
4. Centrifuge at 72,000 g for 30 min. The Beckman Optima bench top ultracentrifuge is recommended for small sample volumes (however, a bench top microcentrifuge at maximum speed, usually around 16,000 g should suffice, although it is not ideal).
5. Collect supernatant and discard pellet.
6. Add 2.5 µL 5% solution/suspension of Coomassie blue G in 0.5 M aminocaproic acid to the supernatant.
7. Add protease inhibitors (e.g. 1 mM PMSF, 1 µg/mL leupeptin and 1 µg/mL pepstatin, see buffer recipes).

Native acrylamide gel preparation and electrophoresis in the first dimension

Native acrylamide gels can be poured by hand. While it is possible to use a single acrylamide concentration such as a straight 10% gel, we highly recommend the use of a linear acrylamide concentration such as 6-13%. A recipe for pouring these native acrylamide gels in a 10-gelBioRad Mini-PROTEAN II multicasting chamber when using a two chamber gradient former is detailed below.

1. Recommended acrylamide - BioRad 30% Acrylamide/Bis Solution 37.5:1 (161-0158).

For 38 mL	For 32 mL
6% acrylamide	13% acrylamide
7.6 mL 30% acrylamide	14 mL 30% acrylamide
9 mL dd water	0.2 mL dd water
19 mL 1 M aminocaproic acid, pH 7.0	16 mL 1 M aminocaproic acid, pH 7.0
1.9 mL 1 M Bis-Tris, pH 7.0	1.6 mL 1 M Bis-Tris, pH 7.0
200 µL 10% APS	200 µL 10% APS
20 µL TEMED	20 µL TEMED

2. Once poured, cover the gels in 50% isopropanol solution.
3. When all 10 gels have set pour off the isopropanol, rinse with water and remove gels from casting chamber.
4. Now a stacking gel and comb are used.

Stacking gel

For 5 mL:

0.7 mL 30% acrylamide

1.6 mL dd water

0.25 mL 1 M Bis-Tris, pH 7.0

2.5 mL 1 M aminocaproic acid, pH 7.0

40 µL 10% APS

10 µL TEMED

5. Samples between 5–20 µL should be loaded into wells. Electrophoresis conditions vary. However, the samples should be separated at 150 V for approximately 2 h or until the sample buffer blue dye has almost run off the bottom of the gel. A recipe for BN-PAGE anode and cathode electrophoresis running buffers are described in the buffer recipes section.

Electrophoresis in the second dimension

The first dimension gel may be western blotted and the separated mitochondrial complexes probed with antibodies. If so, proceed to the next section. As an alternative the mitochondrial complexes can be further resolved into their protein subunit in a second (denaturing) dimension. To do this:

1. Cut each gel lane out of the first dimension gel and soak in SDS denaturing buffer (see buffer recipes)
2. Each lane should be turned 90° and loaded onto the top of an SDS-PAGE 10–20% acrylamide gel.

This gel should be wider to accommodate the first dimension gel strip.

3. Electroblotting proceeds as described in the next section.

Electroblotting and immunodetection

Electroblotting should be performed with a fully submerged system such as BioRad Mini Trans-blot system. We recommend using the Tris-Glycine transfer method for blotting BN-PAGE gels. The recipes for all buffers are detailed in the buffers section. Also highly recommended is the use of a PVDF membrane such as Immobilon rather than nitrocellulose membrane.

1. After electrophoresis, the gel should be soaked in transfer buffer for 30 min before assembling the transfer sandwich.
2. Electroblotting should be carried out at 150 mA for 1.5 h. Good electrophoretic transfer is indicated by the complete transfer of blue dye from the gel onto the membrane.
3. Membranes should be blocked for at least 3 h in 5% milk/PBS solution, blocking overnight at 4°C is recommended.
4. Wash the membrane for 10 min in PBS 0.05% Tween 20.
5. Incubate the membrane with the primary BN-PAGE monoclonal antibody.

Antibodies should be diluted to the recommended concentration in a 1% milk/PBS incubation solution. 5 mL of antibody solution should be enough to cover a 100 cm² membrane and constant rocking/agitation/rolling is recommended.

6. Wash the membrane in PBS 0.05% Tween 20 solution for 5 min. Repeat this step twice.
7. Incubate the membrane with the secondary antibody, which should be conjugated appropriately for the detection method of choice. Two highly recommended methods are alkaline phosphatase (AP) and horseradish peroxidase conjugated secondary antibodies.
8. Use this antibody at the dilution recommended by the manufacturer in a 1% milk/PBS solution. Include sodium azide as a preservative in this solution or subsequent solutions will inhibit the activity of horseradish peroxidase conjugated antibodies.
9. Wash the membrane in PBS 0.05% Tween 20 solution for 5 min. Repeat this step twice.
10. Rinse the blot in PBS to remove any Tween 20 which may inhibit detection.
11. The blot is now ready for development.

Blot development with an alkaline phosphatase conjugated secondary antibody

The membrane should be incubated in AP color development buffer supplemented with 1% v/v BCIP and 1% v/v NBT. Develop until a satisfactory signal achieved. Terminate development by rinsing the blot in water. For more details see the manufacturer's instructions.

Blot development with a horseradish peroxidase conjugated secondary antibody

The membrane should be incubated in HRP color development solution. We highly recommend the ECL + system where the solution is 40:1 reagent A:B. Incubate for 2 min.

Cover the membrane with a transparent wrap/cling film and expose to X-ray film under appropriate darkroom conditions and film development. For more details see the manufacturer's instructions.

Optimization steps and general tips

Sample preparation

It is always recommended to optimize sample concentration.

Gel acrylamide concentrations and transfer

The acrylamide concentrations given in this procedure can be adjusted to optimize separation of complexes of interest. Also altering electroblotting current and duration may improve resolution and transfer of some proteins.

Antibody concentration

The primary antibody should be used at the recommended concentration provided on the online datasheet. However, when using low sample loads or particularly when analyzing alternative species as a source of material, some optimization may be necessary (usually involving increasing the concentration of the primary antibody). Secondary antibodies also vary and should be optimized for your system. Typically, a 1:1000–10,000x dilution is normal for commercially available enzyme-conjugated secondary antibodies.

Troubleshooting tips

After electrophoresis, the gel or blot has a blue background

Once the first dimension separation is almost complete, the cathode dye containing Coomassie blue G can be replaced by cathode buffer without dye. Further electrophoresis will remove most of the dye from the gel.

Weak or no western blotting signal

- Do not use sodium azide in the secondary antibody solution because this inhibits HRP development
- Similarly, Tween 20 may inhibit alkaline phosphatase blot development
- Increase the concentration of the antibody
- Extend incubation times
- Expose the film for longer
- Increase the sample amount

To check the transfer, stain the blot after transfer with Ponceau Red. Pre-stained markers confirm good transfer. Over transfer or "blow through" may occur. Reduce transfer current or time, or use a membrane with smaller pore size or put a second membrane behind first as precaution.

Protocol summary

For quick reference only. We recommend becoming familiar with previous details of this protocol document before performing the assay.

1. Add 400 µg mitochondria to 40 µL buffer A, 1 µg/mL leupeptin, 1 mM PMSF.
2. Add 7.5 µL 10% LM and incubate on ice for 30 min.
3. Centrifuge 72,000 g at 4°C for 10 min.
4. Add 2.5 µL of a 5% suspension of Coomassie blue G in buffer A.
5. Load samples on 6–13% native acrylamide gradient gel. Gel recipe and electrophoresis buffers described below.
6. For single dimension analysis, proteins should be electroblotted for antibody detection according to standard protocols.
7. For two dimension analysis, the entire gel lane should be soaked in SDS-PAGE de-naturing buffer, then resolved in second dimension by SDS-PAGE before western blotting.