Native electrophoresis protocol

Blue native electrophoresis protocol

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

Reagents:

- Primary BN-PAGE tested antibody/ies
- Secondary antibody which should be conjugated appropriately for the detection method of choice
- Electrophoresis and Western blotting reagents
- n-dodecyl-ß-D-maltopyranoside
- 6-aminocaproic acid, Bis-Tris, Tricine
- Coomassie blue G

Equipment:

- Vertical acrylamide electrophoresis unit
- Electroblotting unit-fully submerged

- pH meter, weighing balance and other standard lab equipment

Blue native electophoresis protocol

Sample preparation:

Blue native polyacrylamide gel electrophoresis (BNPAGE) is performed essentially as described by Schägger and von Jagow, Analytical Biochemistry (1991) 199, 223-231.

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 offers monoclonal antibodies for the detection of all five OXPHOS complexes simultaneously 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
- Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer)

- Mitochondria Isolation Kit for Cultured Cells
- Mitochondria Isolation Kit for Cultured Cells (with Dounce Homogenizer)

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-ß-D-maltopyranoside
3. Mix and incubate for 30 minutes on ice
4. Centrifuge at 72000 xg for 30 minutes. The Beckman Optima benchtop ultracentrifuge is recommended for small sample volumes (however a benchtop microfuge at maximum speed, usually around 16000 xg should suffice, although it is not ideal)
5. Collect supernatant and discard pellet
6. To the supernatant add 2.5 µl 5% solution/suspension of Coomassie blue G in 0.5 M aminocaproic acid
7. Add protease inhibitors (e.g. 1 mM PMSF, 1 µg/mL leupeptin and 1 µg/mL pepstatin) (see Buffer Recipes section)

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, Abcam highly recommends 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)

6% acrylamide	13% acrylamide
7.6 ml 30% acrylamide	14 ml 30% acrylamide
9 ml dd water	row 0.2 ml dd water
19 ml 1M aminocaproic acid pH 7	16 ml 1M aminocaproic acid pH 7
1.9 ml 1M Bis-Tris pH7	1.6 ml 1M Bis-Tris pH7

200 µl 10% APS	200 µl 10% APS
20 µl TEMED	20 µl TEMED
Total volume 38 ml	**Total volume 32 ml**

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

0.7 ml 30% acrylamide

1.6 ml dd water

0.25 ml 1 M Bis Tris pH 7

2.5 ml 1 M aminocaproic acid pH 7

40 µl 10% APS

10 µl TEMED

Total volume: 5 ml

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 hours 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 90o and loaded onto the top of an SDS-PAGE 10-20% acrylamide gel

   **This gel should be a 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. Abcam recommends 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 is finished the gel should be soaked in transfer buffer for 30 minutes before assembling the transfer sandwich detailed in Figure 1
2. Electroblotting should be carried out at 150 mAmp for 1.5 hours. 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 hours in 5% milk/PBS solution, though overnight at 4°C is recommended
4. Wash the membrane for 10 minutes 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 minutes. 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 (see below)
8. Use this antibody at the dilution recommended by the manufacturer in a 1% milk/PBS solution Inclusion of 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 minutes. 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 minutes.

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

Buffer recipes:

Phosphate buffered saline solution (PBS)

1.4 mM KH2PO4

8 mM Na2HPO4

140 mM NaCl

2.7 mM KCl, pH 7.3

Protease inhibitor stocks (each is 1000 x)

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 Bi-Tris

0.02% Coomassie blue G

pH 7.0

First dimension electrophoresis Anode buffer

50 mM Bis-Tris

pH 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

No pH adjustment necessary

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 MgCl2

100x NBT stock 50 mg/ml in 100% DMF

100x BCIP stock 50 mg/ml in 70% DMF

DMF dimethylformamide

No pH adjustment necessary

Optimization steps and general tips

Sample concentration

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 - 10000x dilution is normal for commercially available enzyme-conjugated secondary antibodies.

Troubleshooting guide

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 azide in the secondary antibody solution because this inhibits HRP development
- Similarly Tween-20 may inhibit alkaline phosphatase blot development
- Increase the concentration of antibody
- Extend incubation times
- Expose film longer
- Increase sample amount

To check 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 assa.

1. 400 µg mitochondria in 40 µl Buffer A, 1 µg/mL pepstatin, 1 mg/mL leupeptin, 1mM PMSF
2. Add 7.5 µl 10% LM incubate on ice 30 minutes
3. Centrifuge 72000g 4°C 10 minutes
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; entire gel lane should be soaked in SDSPAGE denaturing buffer, then resolved in 2nd dimension by SDSPAGE before Western blotting

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: 1mg/mL (water)

Stock pepstatin: 1mg/mL (ethanol)

Stock PMSF: 0.3 M (ethanol)

LM: n-dodecyl-ß-D-maltoside

Origin: Pioneer Biotechnolgy, Inc of China
Distributor: Pioneer Biotechnolgy,Inc
Contact: Tel 029-88237772 or 技术支持电话：13484545009（微信同号） Fax 029-88237772
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