

Blue Native Electrophoresis

10-10

DESCRIPTION

This protocol for Blue Native Electrophoresis is designed for use with the following MitoSciences products:

- MS603** MitoProfile® Total OXPHOS Blue Native WB Antibody Cocktail
- MS111** Complex I subunit NDUFA9 monoclonal antibody
- MS105** Complex I subunit NDUFB8 monoclonal antibody
- MS103** Complex I subunit GRIM-19 monoclonal antibody
- MS204** Complex II subunit 70 kDa Fp monoclonal antibody
- MS304** Complex III subunit Core 2 monoclonal antibody
- MS404** Complex IV subunit I monoclonal antibody
- MS507** ATP synthase (Complex V) subunit α monoclonal antibody

ADDITIONAL MATERIAL REQUIRED

Reagents:

- MitoSciences (primary) BN-PAGE antibody/ies
- Secondary anti-mouse antibody (typically goat anti mouse) which should be conjugated appropriately for the detection method of choice (MitoSciences MS901-MS908)
- Electrophoresis and Western blotting reagents
- n-dodecyl- β -D-maltopyranoside (MitoSciences MS910)
- 6-aminocaproic acid (Sigma A2504), Bis-Tris (Sigma B9754), Tricine (Sigma T0377)
- Coomassie blue G (Sigma B0770)

Equipment:

- Vertical acrylamide electrophoresis unit-BioRad mini Protean series recommended
- Electroblotting unit-fully submerged BioRad mini Protean series recommended
- pH meter, weighing balance and other standard lab equipment

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. MitoSciences offers monoclonal antibodies for the detection of all five OXPHOS complexes simultaneously (MS603 kit) or each of the OXPHOS complexes individually.

When performing Blue Native Electrophoresis, it is always recommended to isolate mitochondria from cells before analysis.

Protocols for mitochondrial isolation can be found at www.mitoscience.com/PDF/mitos.pdf. 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 minutes on ice.
4. Centrifuge at 72000 g for 30 minutes. The Beckman Optima benchtop ultracentrifuge is recommended for small sample volumes (however at a minimum, a benchtop microfuge at maximum speed, usually around 16000 g should suffice).
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-Page 5).

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, MitoSciences highly recommends the use of a linear acrylamide concentration such as 6-13%. A recipe for pouring these native acrylamide gels in a 10-gel BioRad Mini-PROTEAN II multicasting chamber when using a two chamber gradient former, is detailed below.



BioRad MiniProtean II gel



Typical gradient former casting chamber (165-2950)

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	0.2 mL dd water
19 mL 1M aminocaproic acid pH 7	16 mL 1 M aminocaproic acid pH 7
1.9 mL 1M Bis-Tris pH 7	1.6 mL 1 M Bis-Tris pH 7
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 on Page 5.

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 Page 5).
2. Each lane should be turned 90° and loaded onto the top of an SDS-PAGE 10-20% acrylamide gel. **Note:** This gel should be a wider to accommodate the first dimension gel strip (For details of gel recipes and electrophoresis see section III of the accompanying blotting manual which can be found at www.mitosciences.com/PDF/western.pdf).
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.* MitoSciences recommends using the Tris-Glycine transfer method for blotting BN-PAGE gels. The recipes for all buffers are detailed on Page 5. 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.

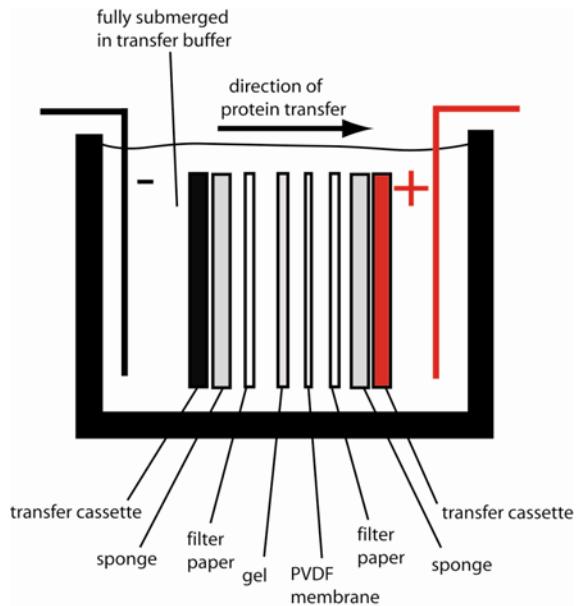


Figure 1: Assembly of Electroblotting sandwich in submerged transfer apparatus.

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 MitoSciences primary BN-PAGE monoclonal antibodies. **Note: 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 an anti-mouse antibody. This antibody should also 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).

9. 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.
10. Wash the membrane in PBS 0.05% Tween-20 solution for 5 minutes. Repeat this step twice.
11. Rinse the blot in PBS to remove any Tween-20 which may be inhibitory to the detection method.
12. The blot is now ready for development.

Blot Development With An Alkaline Phosphatase Conjugated Secondary Antibody.

Membrane should be incubated in AP color development buffer supplemented with 1% v/v BCIP and 1% v/v NBT (all three solutions are supplied by BioRad as product #170-6432). Develop until satisfactory signal achieved. Terminate by rinsing blot in water. *For more details see manufacturer's instructions.*

Blot Development With A Horseradish Peroxidase Conjugated Secondary Antibody.

Membrane should be incubated in HRP color development solution. We highly recommend ECL + system (Amersham product #RPN2132) where solution is 40:1 reagent A:B. Incubate for 2 minutes. Then cover 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 manufacturer's instructions.*

BUFFER RECIPES

**Phosphate buffered saline solution
(PBS)**

1.4 mM KH₂PO₄8 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 (Sigma L7626)

1 mg/mL leupeptin (Sigma L2884)

1 mg/mL pepstatin (Sigma P4265)

**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 (Aldrich 27,434-8)

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 Sigma-D4551

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 on page 4 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

MitoSciences primary antibodies are of the highest quality and should be used at the recommended concentration. 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 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	<p>Do not use azide in secondary antibody solution because inhibits HRP development. Similarly Tween-20 may inhibit alkaline phosphatase blot development.</p> <p>Increase antibody amount used.</p> <p>Extend incubation times.</p> <p>Check primary and secondary mAbs.</p> <p>Longer exposure.</p> <p>Increase sample amount.</p> <p>To check transfer, stain the blot after transfer with Ponceau red. Also pre-stained markers confirm good transfer.</p> <p>Over transfer or 'blow through' may occur. Reduce transfer current or time, or use membrane with smaller pore size or put second membrane behind first as precaution.</p>

FLOW CHART

This guide is for quick reference only. Be completely familiar with the previous details of this document before performing the assay.

