Blue Native Electrophoresis Protocol

For use with products:

MS603	mAb cocktail against all 5 OXPHOS complexes
MS111	Complex I BNPAGE mAb (NDUFA9)
MS105	Complex I BNPAGE mAb (20 kDa)
MS103	Complex I BNPAGE mAb (Grim-19)
MS204	Complex II BNPAGE mAb (70 kDa)
MS304	Complex III BNPAGE mAb (Core 2)
MS404	Complex IV BNPAGE mAb (COX I)
MS507	Complex V BNPAGE mAb (α subunit)

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I. REQUIRED REAGENTS AND EQUIPMENT

Reagents:

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

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

II. 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 mAbs for the detection of all 5 OXPHOS complexes simultaneously (mAb cocktail MS603) or each of the OXPHOS complexes individually (see front cover).

It is always recommended to isolate mitochondria from cells before analysis. Protocols for mitochondrial isolation can be found at www.mitosciences.com/PDF/mitos.pdf. It is possible to probe whole tissue or cell extract but this may result in a weaker signal.

- 0.4 mg of sedimented mitochondria should be resuspended in 40 μl of 0.75 M aminocaproic acid, 50 mM Bis-Tris, pH 7.0. Add 7.5 μl of 10% n-dodecyl-β-D-maltopyranoside. Mix and incubate for 30 minutes on ice. 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). Collect supernatant and discard pellet. To supernatant add 2.5 μl 5% solution/suspension of Commassie blue G in 0.5 M aminocaproic acid. Protease inhibitors should be added e.g. 1 mM PMSF, 1 μg/ml leutpeptin and 1 μg/ml pepstatin (see section VI).

III. 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 the a 10x gel BioRad Mini-PROTEAN II multicasting chamber is detailed below when using a two chamber gradient former.

BioRad MiniProtean II gel

Typical gradient former casting chamber (165-2950)

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 pH7	16 ml 1M aminocaproic acid pH7
1.9 ml 1M Bis-Tris pH 7	1.6 ml 1M 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

Once poured cover the gels in 50% isopropanol solution. Once all 10 gels have set pour off the isopropanol, rinse with water and remove gels from casting chamber. 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 7.0
2.5 ml 1 M aminocaproic acid pH7
40 μl 10 % APS
10 μl TEMED
Total volume 5 ml

- 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 BNPAGE anode and cathode electrophoresis running buffers are described in section VI.

IV. 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 section V. As an alternative the mitochondrial complexes can be further resolved into their protein subunit in a second (denaturing) dimension. To do this:

- Cut each gel lane out of the first dimension gel and soak in SDS denaturing buffer (see section VI). Each lane should be turned 90° 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 (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). Electroblotting proceeds as described below.

V. 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 BNPAGE gels. The recipes for all buffers are detailed in section VI. Also highly recommended is the use of a PVDF membrane such as Immobilon rather than nitrocellulose membrane.

• After electrophoresis is finished the gel should be soaked in transfer buffer for 30 minutes before assembling the transfer sandwich detailed in Figure 1.

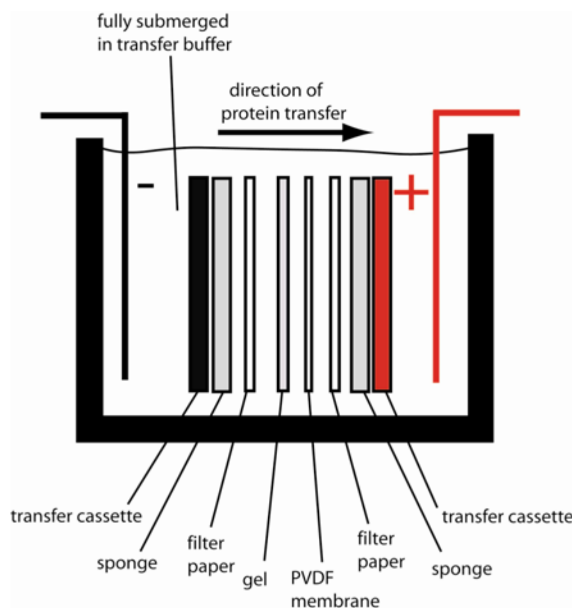


Figure 1. Assembly of Electroblotting sandwich in submerged transfer apparatus.

• 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.

* It is also possible to use a semi-dry system such as Hoefer TE-70 however results are not guaranteed.

• Membranes should be blocked for at least 3 hours in 5% milk/PBS solution, though overnight at 4°C is recommended. Then the membrane should be washed for 10 min in PBS 0.05% Tween-20.

- The membrane can now be incubated with the **primary** MitoSciences BNPAGE **mouse** monoclonal antibody/ies. 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 cm^2 membrane and constant rocking/agitation/rolling is recommended.

- The membrane should now be washed in PBS 0.05% Tween-20 solution for 5 minutes. Repeat this step twice.

- The membrane can now be incubated 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). 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.

- The membrane should now be washed in PBS 0.05% Tween-20 solution for 5 minutes. Repeat this step twice.

- The blot should be rinsed in PBS to remove any Tween-20 which may be inhibitory to the detection method. The blot is now ready for development.

- 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 1 mM EDTA solution. *For more details see manufacturer's instructions.*

- 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 min. 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.*

VI. 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 1000 x)
1 M phenylmethanesulfonyl fluoreide (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)	100xBCIP stock 50 mg/ml in 70% DMF
5 mM $MgCl_2$	100xNBT stock 50 mg/ml in 100% DMF
No pH adjustment necessary	DMF dimethylformamide Sigma-D4551

VII. 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.

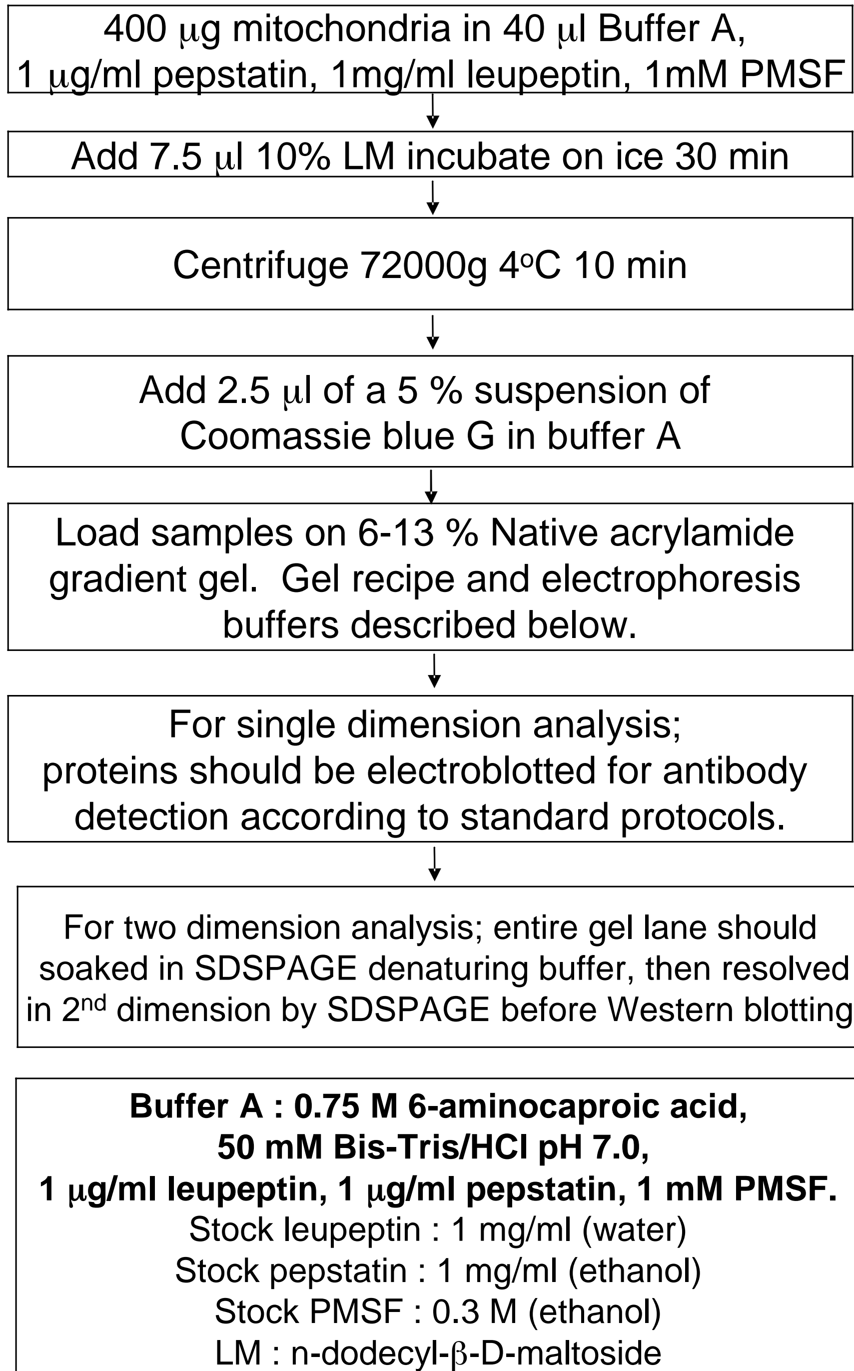
Antibody concentration
MitoSciences primary antibodies are of the highest quality and should be used at the recommended concentration. However when using low sample loads or particularly when analyzing alternative species as a source of material, some optimization may be necessary (usually involving 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.

VIII. 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 secondary antibody solution because inhibits HRP development. Similarly Tween-20 may inhibit alkaline phosphatase blot development.
	Increase antibody amount used
	Extend incubation times
	Check primary and secondary mAbs
	Longer exposure
	Increase sample amount
	To check transfer stain the blot after transfer with Ponceau red. Also prestained markers confirm good transfer
	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

IX. FLOW CHART

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



X. NOTES

www.mitosciences.com
Phone: 1 800 910-6486
Fax: (541) 284-1801