



# **Western blotting transfer and detection procedure**

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## I. REQUIRED REAGENTS AND EQUIPMENT

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### Reagents:

- PVDF membrane e.g. 0.45µm Immobilon-P (Millipore IPVH00010)
- Blocking buffer – 5% fat free milk in PBS
- Phosphate buffered saline, PBS (recipe see section VI)
- MitoSciences' **primary** monoclonal 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)
- Tween-20 (Aldrich 27,434-8)
- Double distilled water
- Methanol
- CAPS (Sigma C2632)

### 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

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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](http://www.mitosciences.com/PDF/mitos.pdf)

It is possible to probe whole tissue or cell extract but this may result in a weaker signal and/or additional bands resulting from non-specific cross-reactivity to non-mitochondrial proteins. In our experience such non-specific cross-reactivity is limited.

Samples should be solubilized in the SDS-PAGE loading buffer detailed in section VI. After solubilization the sample should be spun at 13000 rpm for 5 minutes. The supernatant should be collected and loaded onto the gel. In most cases the sample need not be heated/boiled in this sample buffer prior to loading. However sample heating may be incorporated into this protocol if necessary, see section VIII.

MitoSciences' antibodies have been optimized for the detection of mitochondrial proteins over a wide range of antigen concentrations however suggested concentrations are presented in Table 1 providing the user with the opportunity of producing the best signal. Using these suggested sample amounts should yield a linearly proportional signal allowing quantitation.

**Table 1. Suggested protein loading concentrations.** Using these loading amounts as simple guidelines in conjunction with the recommended electrophoresis, transfer, and immunodetection methods detailed in this manual should yield optimal, reproducible and quantitative Western blotting results with MitoSciences monoclonal antibodies.

<b>Sample</b>	<b>Loading</b>
Heart mitochondria	1-10 µg/lane
Muscle mitochondria	2-10 µg/lane
Brain mitochondria	5-20 µg/lane
Other tissue mitochondria	5-20 µg/lane
Muscle tissue extract	3-30 µg/lane
Non-muscle tissue extract	20-50 µg/lane
Cultured cell mitochondria	10-20 µg/lane
Cultured cell extract	20-50 µg/lane

### III. ACRYLAMIDE GEL PREPARATION AND ELECTROPHORESIS

Most commercially available pre-cast gel systems are suitable e.g. Invitrogen NOVEX NuPAGE and BioRad Ready gel/Criterion systems. Electrophoresis using pre-cast systems such as these should always be performed according to the manufacturer's instructions.

Alternatively acrylamide gels can be poured by hand. While it is possible to use a single acrylamide concentration such as a straight 15% gel, we highly recommend the use of a linear acrylamide concentration such as 10-20% which will give optimal resolution of all proteins between 10 kDa and 100 kDa. A recipe for pouring 10-20 % acrylamide gels in the a 10x gel BioRad Mini-PROTEAN II multicasting chamber is detailed in Table 2 when using a two chamber gradient mixer.



BioRad MiniProtean II gel



Typical gradient former casting chamber (165-2950)

**Table 2. Recommended acrylamide – BioRad 30% Acrylamide/Bis Solution 37.5:1 (161-0158).**

<b>10% acrylamide</b>	<b>20% acrylamide</b>
10.6 ml 30% acrylamide	21 ml 30% acrylamide
12 ml ddH <sub>2</sub> O	1 ml ddH <sub>2</sub> O
180 µl 10% SDS	180 µl 10% SDS
9 ml 1.5 M Tris pH 8.6	9 ml 1.5 M Tris pH 8.6
180 µl 10% APS	180 µl 10% APS
18 µl TEMED	18 µl TEMED
<b>Total volume 32 ml</b>	<b>Total volume 31.4 ml</b>

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.

**Table 3. Stacking gel**

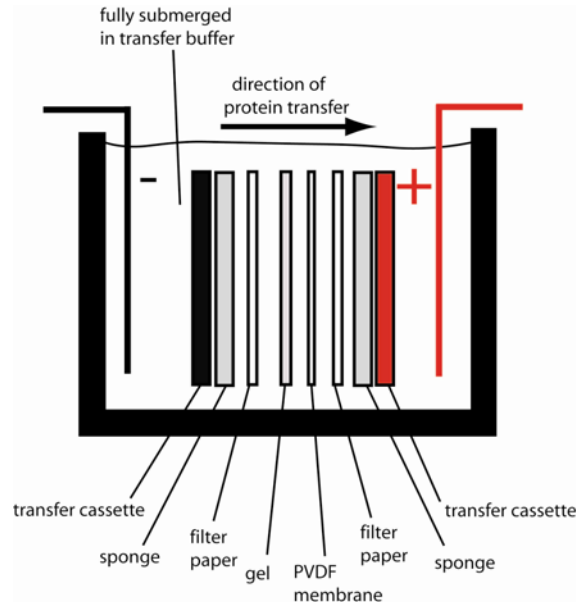
<b>pH 6.8 Stacking gel</b>
0.8 ml 30% acrylamide
3.65 ml ddH <sub>2</sub> O
28 µl 10% SDS
0.5 ml 1 M Tris pH 6.8
28 µl 10% APS
5 µl TEMED
<b>Total volume 5 ml</b>

Samples should be loaded into wells and it is highly recommended to load at least one well with prestained molecular weight standards such as Invitrogen Multimark (LC5725) or BioRad Kaleidoscope (161-0375) markers. Electrophoresis conditions vary, however the samples should be separated at 150V for approximately 2 hours or until the sample buffer, bromophenol blue dye, has almost run off the bottom of the gel. A recipe for electrophoresis running buffer is described in section VI.

## IV. ELECTROBLOTTING AND BLOCKING

Electroblotting should be performed with a fully submerged system such as BioRad Mini Trans-blot system\*. Additionally, we highly recommend using the CAPS buffer transfer system\*\*. 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 a nitrocellulose membrane.

After electrophoresis is finished the gel should be soaked in CAPS 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 2 hours. Good electrophoretic transfer is indicated by the complete transfer of prestained molecular weight markers below 100 kDa. Prestained markers of greater than 100 kDa may not transfer completely 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.

\*\* The possibility also exists to use a Tris-glycine or Towbin buffer for electroblotting however results with individual antibodies may vary from the established CAPS transfer method.

## V. IMMUNODETECTION

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- 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' **mouse** monoclonal antibody(ies). Antibodies should be diluted to the recommended concentration in a 1% milk/PBS incubation solution (**NB.** Except for ANT mAb, MSA02, which is provided with its own incubation solution). 5 ml of antibody solution should be enough to cover a 50 cm<sup>2</sup> membrane in a sealed bag with constant rocking/agitation/rolling for 2 hours is recommended.
- The membrane should now be washed in PBS 0.05% Tween-20 solution for 5 minutes. Repeat this step twice more.
- The membrane can now be incubated with the **secondary** antibody which should be an **anti-mouse** antibody for 2 hours. 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 more.
- 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 (now GE Healthcare) 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**

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### Phosphate buffered saline solution (PBS)

1.4 mM  $\text{KH}_2\text{PO}_4$

8 mM  $\text{Na}_2\text{HPO}_4$

140 mM NaCl

2.7 mM KCl, pH 7.3

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### Membrane washing buffer

PBS plus 0.05% Tween-20 (Aldrich 27,434-8)

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### Membrane blocking buffer

PBS plus 5% non-fat milk powder

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### CAPS electroblotting transfer buffer

10 mM 3-[cyclohexylamino]-1-propane sulfonic acid (Sigma C2632) pH

11 with NaOH

10% methanol

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### Tris/Glycine or Towbin electroblotting transfer buffer

25 mM Tris

192 mM glycine

10% methanol

0.1% SDS

No pH adjustment necessary

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### 2x SDS page sample buffer

20% glycerol

4% SDS

100 mM Tris pH 6.8

0.002 % Bromophenol blue

optional – 100 mM dithiothreitol

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### Electrophoresis running buffer

25 mM Tris

192 mM glycine

0.1% SDS

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### Alkaline phosphatase color development buffer

0.1 M diethanolamine (DEA)      100xBCIP stock 50 mg/ml in 70% DMF

5 mM  $\text{MgCl}_2$                               100xNBT stock 50 mg/ml in 100% DMF

No pH adjustment necessary      DMF dimethylformamide Sigma-D4551

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## **VII. OPTIMIZATION STEPS AND GENERAL TIPS**

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### **Sample concentration**

It is always recommended to optimize sample concentration. Additionally more than one amount loaded per sample will improve quantitative analysis of immunoblotted proteins. Samples may also be supplemented with fresh reducing agent such as dithiothreitol or  $\beta$ -mercaptoethanol. Heating the sample may also be optimized prior to electrophoresis.

### **Gel acrylamide concentrations and transfer**

Many different gel systems are available commercially. Alternatively the acrylamide concentrations given on page 5 can be adjusted to optimize separation of proteins of interest. Also altering electroblotting current and duration may improve transfer of some proteins.

### **Antibody concentration**

MitoSciences primary monoclonal 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.

### **Blot development**

Many options are available for development in addition to the alkaline phosphatase (NBT/BCIP) and horseradish peroxidase (ECL) methods described on page 8. For example more sophisticated methods including direct fluorescence are available giving improved quantitative results particularly when analyzed by laser densitometer such as the BioRad FX imager or GE Healthcare (Amersham) Typhoon. Image analysis software is provided by these manufacturers. Also available are non-commercial software applications such as NIH Image and Scion Image for the quantitative analysis of scanned Western blots.

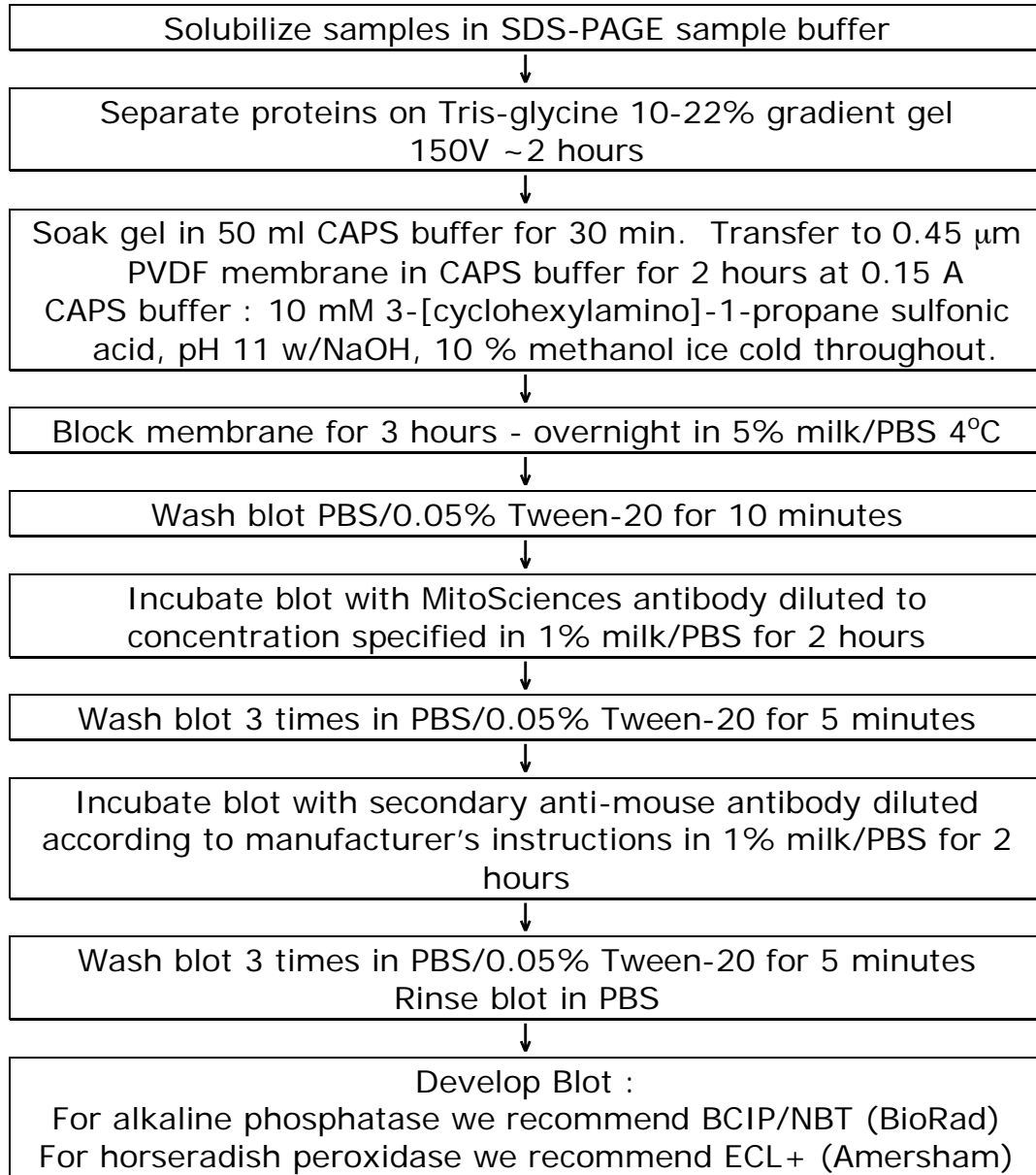
## VIII. TROUBLESHOOTING GUIDE

<b>High background</b>	Increase blocking time
	Change secondary antibody
	Decrease concentration of primary mAb
	Increase washing steps
<b>Blotchy or speckled</b>	Ensure even blocking each membrane
	Do not allow membranes to dry between steps
	Check antibodies are completely in solution and no precipitation has occurred
	Antibody concentration may be too high
<b>Weak or no signal</b>	Handle with forceps
	Do not use azide in secondary antibody solution because it inhibits HRP development
	Increase antibody amounts
	Extend incubation times
	Check primary and secondary mAbs
	Longer exposure
	Tween-20 contamination inhibits AP development
	Increase sample amount
	Check transfer equipment and stain blot after transfer with ponceau. Prestained markers help identify quality of transfer
	Over transfer or "blow through". Reduce transfer current or time, or use membrane with smaller pore size or put second membrane behind first as precaution
<b>Non-specific bands</b>	Isolate mitochondria to higher purity
	Reduce the sample and/or mAb concentration
	Add a reducing agent to the sample e.g. DTT
	Heat sample e.g. 95°C for 5 min or 37°C for 30 min before loading (NB. MS404, COXI, is very sensitive to heating.)

## IX. FLOW CHART

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



## X. NOTES

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