PROTOCOL

MitoSciences
1850 Millrace Drive, Suite 3A
Eugene, Oregon 97403
www.mitosciences.com

Western Blotting Transfer and Detection Procedure

02-11

DESCRIPTION

Western Blotting Transfer and Detection Procedure

ADDITIONAL MATERIALS REQUIRED

Reagents:

- MitoSciences' primary antibody(ies)
- Secondary antibody
- Phosphate buffered saline, PBS (recipe see Page 6)
- Blocking buffer : 5% fat free milk in PBS
- PVDF membrane e.g. 0.45µm Immobilon-P (Millipore IPVH00010)
- Tween-20 (Aldrich 27,434-8)
- CAPS (Sigma C2632)
- Methanol
- Double distilled water

Equipment:

- Vertical acrylamide electrophoresis unit (BioRad mini Protean series recommended)
- Electroblotting unit-fully submerged (BioRad mini Protean series recommended)
- pH meter
- Weighing balance
- Other standard lab equipment

SAMPLE PREPARATION

1. For performing Western blotting transfer and detection, 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 and/or additional bands resulting from non-specific cross-reactivity to non-mitochondrial proteins. In the experience of MitoScience scientists, such non-specific cross-reactivity is limited.

2. Samples should be solubilized in the SDS-PAGE loading buffer (preparation on Page 6).

3. After solubilization the sample should be spun at 13,000 rpm for 5 minutes.

4. The supernatant should be collected and loaded onto the gel. **Note:** 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 desired, see Page 8.

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 an optimal signal. Using these suggested sample amounts should yield a linearly proportional signal that allows for quantitation.

Sample	Loading
Heart mitochondria	1-10 µg/lane
Muscle mitochondria	1-10 µg/lane
Brain mitochondria	5-20 µg/lane
Other tissue mitochondria	5-20 µg/lane
Muscle tissue extract	5-20 µg/lane
Non-muscle tissue extract	10-30 µg/lane
Cultured cell mitochondria	10-30 µg/lane
Cultured cell extract	10-30 µg/lane

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 will yield optimal, reproducible, and quantitative Western blotting results with MitoSciences monoclonal antibodies.

ACRYLAMIDE GEL PREPARATION AND ELECTROPHORESIS

COMMERCIALLY AVAILABLE GELS

Most commercially available pre-cast gel systems are suitable for use with this protocol. As an example excellent pre-cast Tris-glycine gels in acrylamide single step or gradient are available from Invitrogen and Biorad (examples – EC61355BOX and 161-1124, respectively). These Tris-glycine gels can be used with the electrophoresis buffers detailed in this guide. Other buffered gel systems may require specific electrophoresis buffers. Electrophoresis using pre-cast systems such as these should always be performed according to the manufacturer's instructions.

POURING GELS BY HAND

Alternatively, if desired acrylamide gels can be poured by hand. While it is possible to use a single acrylamide concentration such as a straight 15% gel, MitoSciences highly recommends 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 Mini Protean II gel

Typical gradient former casting chamber (165-2950)

Telephone: 1-800-818-6436 or 1-541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

10% acrylamide	20% acrylamide
10.6 mL 30% acrylamide	21 mL 30% acrylamide
12 mL ddH_20	1 mL ddH_20
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 l 10% APS
18 µL TEMED	18 µL TEMED
Total volume 32 mL	**Total volume 31.4 mL**

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

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.

pH 6.8 Stacking gel
0.8 mL 30% acrylamide
3.65 mL ddH_20
28 µL 10% SDS
0.5 mL 1 M Tris pH 6.8
28 µL 10% APS
5 µL TEMED
Total volume 5 mL

Table 3. Stacking gel

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 Page 6.

ELECTROBLOTTING

Electroblotting should be performed with a fully submerged system such as BioRad Mini Trans-blot system*. Additionally, MitoSciences highly recommends using the CAPS buffer transfer system**. The recipes for all buffers are detailed in Page 6. 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.

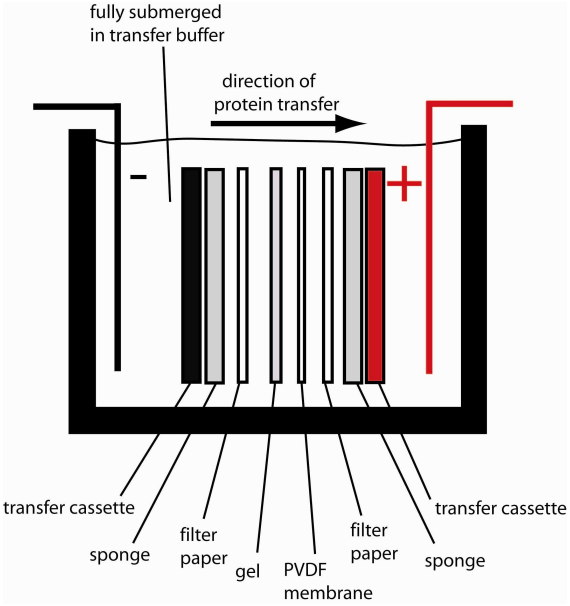

Figure 1. Assembly of Electroblotting sandwich in submerged transfer apparatus.

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.

BLOCKING AND IMMUNODETECTION

1. Membranes should be blocked for at least 3 hours in 5% milk/PBS solution, although blocking overnight at 4^{o}C is recommended.

2. The membrane should be washed for 10 minutes in PBS 0.05% Tween-20.

3. 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 (**Note:** Except for MitoSciences ANT mAb, MSA02, which is provided with its own incubation solution). *An antibody solution of 5 mL should be enough to cover a 50 cm^2 membrane in a sealed bag or tube with constant rocking/agitation/rolling for 2 hours is recommended.*

4. Now wash the membrane in PBS 0.05% Tween-20 solution for 5 minutes.

5. Repeat the above step twice more.

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

6. Now incubate the membrane with the secondary antibody, which should be an anti-mouse antibody, for 2 hours.

Note: 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.

7. Now wash the membrane in PBS 0.05% Tween-20 solution for 5 minutes.

8. Repeat the above step twice more.

Note: The blot must be rinsed in PBS to remove any Tween-20, which can be inhibitory to the detection method.

9. 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.*

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

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

Membrane blocking buffer
PBS plus 5% non-fat milk powder

CAPS electroblotting transfer buffer
10 mM 3-[cyclohexylamino]-1-propane sulfonic acid (Sigma C2632) pH 11 with NaOH
10 % Methanol

Tris/Glycine or Towbin electroblotting transfer buffer
25 mM Tris
192 mM glycine
10% methanol
0.1% SDS
No pH adjustment necessary

2x SDS page sample buffer
20% glycerol
4% SDS
100 mM Tris pH 6.8
0.002 % Bromophenol blue
optional – 100 mM dithiothreitol

Electrophoresis running buffer
25 mM Tris
192 mM glycine
0.1% SDS

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

OPTIMIZATION STEPS AND GENERAL TIPS

Sample Concentration

It is always recommended to optimize the 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 β-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 3 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.

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

TROUBLESHOOTING GUIDE

Band wrong size	Some proteins can migrate at an apparent molecular weight which is different from their theoretical weight. This is often due to hydrophobicity. Well characterized examples are COXI (MS404) theoretical 57 kDa, apparent 39 kDa and COXII (MS405) theoretical 26 kDa, apparent 18-20 kDa.
High background	Increase blocking time Change secondary antibody Decrease concentration of primary mAb Increase washing steps
Blotchy or speckled	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 Handle with forceps
Weak or no signal	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
Non-specific bands	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^{o}C for 5 min or 37^{o}C for 30 min before loading (Note- MS404, anti-COXI, is very sensitive to heating.) Western blotting of immunoprecipitates or samples contaminated with rodent blood may show bands associated with IgG contamination of the sample. Using a secondary antibody which only recognizes the native MitoSciences’ antibody applied to the membrane for blotting is recommended for example Trueblot secondary antibodies from ebiosciences (consult Manufacturer for more details).

FLOW CHART

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