One-Blot Western Optimization Using the MPX™ Blotting System

LI-COR Biosciences

Abstract

The independent channels of the LI-COR MPX (Multiplex) Blotter make it possible to optimize blocking buffer, primary antibody dilution, and secondary antibody dilution on a single Western blot. Western blotting procedures that generate a blot of 7.0 x 8.5 cm are easily adapted to the MPX format. The process fits into any laboratory's standard Western blot workflow. Both home-made and pre-cast gels can be used to generate blots.

Electrophoresis and transfer to nitrocellulose membrane are performed under standard conditions. Clamping the blot into the MPX Blotter creates up to 24 independent channels, allowing different conditions to be tested in each channel. The range of usable channels per sample is relative to comb size. For Western blot optimization, a single-well gel ("prep gel") is all that is needed. For this application, any detection method can be used, including near-infrared (NIR) fluorescence and chemiluminescence. This document presents general guidelines for use with the Odyssey® family of Infrared Imaging Systems.

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Guidelines

Gel Preparation

- A wide variety of gel matrices are compatible with the MPX Blotter system.If you are pouring your own gels, your gel casting system can be used with a single-well comb such as the LI-COR Single Marker/One Lane Comb (921-00200, 1 mm thickness). Alternatively, pre-cast gels can be purchased and used. Table 1 lists several suggested types of pre-cast gels, and indicates the number of usable ports that each gel will provide for use with the MPX Blotter.

Table 1. Single-sample pre-cast gel options for use with the MPX Blotter.

Vendor	Well Designation	Sample #	MW Marker Well	Usable Ports
Invitrogen	2D	1	Yes	19
Bio-Rad	2D/Prep	1	Yes	21
C.B.S. Scientific	1 Well	1	No	23

Molecular Weight Marker

- It is important to have a molecular weight marker that is visible to the eye because the marker is the primary tool used to align the blot in the MPX Blotter. Odyssey® One-Color Marker or Chameleon® Duo Protein Ladder is recommended.

Electrophoresis

IMPORTANT: The maximum length of the separating gel should not exceed 50 mm—the length of the channels on the MPX Blotter.

Alignment in MPX Blotter

- Detailed instructions for use of the MPX Blotter are found in the MPX Blotter Multiplex Western Blotting Accessory User Guide.

Imaging

- Visual inspection of images with Image StudioTM software or Odyssey application software can

be used to determine which blocking buffer works best for the primary antibody you are testing.

- View all blots together in a single image, with uniform image display settings, to compare membrane background levels and band intensity.

- Individually adjust the image display settings for each blot to get the “best” image.

- Evaluate non-specific banding in each blocking buffer condition.

- Look for blocking buffer conditions that provide strong signals for the expected band(s), low membrane background, and few non-specific background bands from the primary antibody.
• Tradeoffs may be necessary. Blocking conditions that yield very strong bands might also have higher membrane background or non-specific banding.
- The “best” blocking conditions depend on the antigen-antibody pair you are using. Some primary antibodies are dramatically affected by blocking conditions. An inappropriate blocker can alter binding specificity, affecting the intensity of target bands and increasing non-specific banding. The pattern of non-specific bands may also be affected.
- Choose the blocking conditions that are most appropriate for the context and goals of your experiment.
- Quantitative analysis of specific bands on each blot will indicate if signal intensity (after background subtraction) is significantly different between blocker types.

Before start

Developed for:

Aerius, and Odyssey® Family of Imaging Systems

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model

Materials

Odyssey Protein Molecular Weight Marker 928-40000 by LI-COR

Blotted nitrocellulose 926-31090/926-31092 by LI-COR

Odyssey® Blocking Buffer (PBS) 927-40000 927-40100 by LI-COR

Odyssey Blocking Buffer (TBS) 927-50000 927-50100 by LI-COR

IRDye® 800CW, 680RD, or 680LT by LI-COR

Blocking Buffer Optimization Kit 927-40040 by LI-COR

Casein Blocking Buffer (PBS) 927-40200 927-40300 by LI-COR

Chameleon® Duo Pre-stained Protein Ladder 928-60000 by LI-COR

NewBlot IR Stripping Buffer, 5X 928-40028 by LI-COR

MPX Membrane Cushion 921-00120 by LI-COR

4X Protein Sample Loading Buffer 928-40004 by Licor

Protocol

Gel Electrophoresis and Transfer

Step 1.

[**Gel Preparation**] A wide variet of gel matrices are compatible with the MPX Blotter system. See guidelines for specifications.

Gel Electrophoresis and Transfer

Step 2.

[**Sample Preparation**] When using a single-well gel, a larger volume of sample is required. Prepare your protein sample so that the sample volume and concentration is equivalent to running all the lanes on a standard 10-well gel.

NOTES

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Example: 5 µg of lysate per lane = 50 µg in a total volume of 100 - 150 µL, including loading buffer.

Gel Electrophoresis and Transfer

Step 3.

[**Sample Preparation**] The following procedure is suggested:

Dilute the sample 1:4 in 4X Protein Sample Loading Buffer with β-Mercaptoethanol. Heat the sample at 95 °C for 5 minutes.

TEMPERATURE

95 °C Additional info:

REAGENTS

4X Protein Sample Loading Buffer 928-40004 by Licor

Gel Electrophoresis and Transfer

Step 4.

[**Molecular Weight Marker**] It is important to have a molecular weight marker that is visible to the eye because the
marker is the primary tool used to align the blot in the MPX Blotter. Odyssey® One-Color Marker or Chameleon® Duo Protein Ladder is recommended.

Gel Electrophoresis and Transfer

Step 5.

[**Electrophoresis**] IMPORTANT: The maximum length of the separating gel should not exceed 50 mm—the length of the channels on the MPX Blotter.

Gel Electrophoresis and Transfer

Step 6.

[**Transfer**]

- Always use clean forceps when handling membranes.
- Nitrocellulose membranes are recommended for this procedure.
- Once electrophoresis is complete, transfer proteins to Odyssey Nitro-cellulose Membrane using standard transfer procedures.

REAGENTS

Blotted nitrocellulose 926-31090/926-31092 by LI-COR

Gel Electrophoresis and Transfer

Step 7.

[**Transfer**] Mark the outside corners of the gel and sample wells with a pencil before separating the transferred gel from the membrane. The marks will help correctly align the membrane when it is placed in the MPX Blotter.

IMPORTANT: Use a pencil to mark the blot! Ink from most pens will fluoresce on the Odyssey Imager and cause increased membrane background.

NOTES

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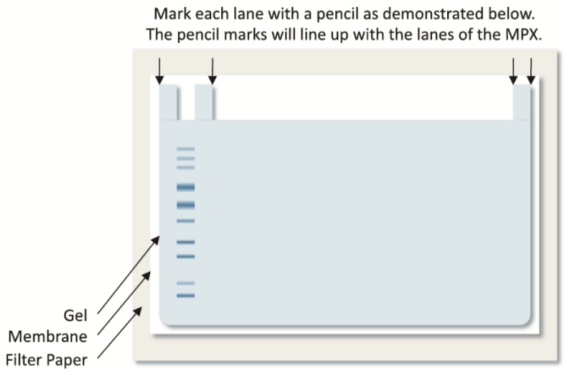

Figure 1. Mark the membrane with pencil, for later alignment into the MPX Blotter.

Gel Electrophoresis and Transfer

Step 8.

[**Transfer**] Allow the membrane to dry for a minimum of one hour before proceeding with the detection.

Membrane Blocking

Step 9.

[**Membrane Preparation**] To prepare the membrane, cut it into four individual blots, as shown in the figure below.

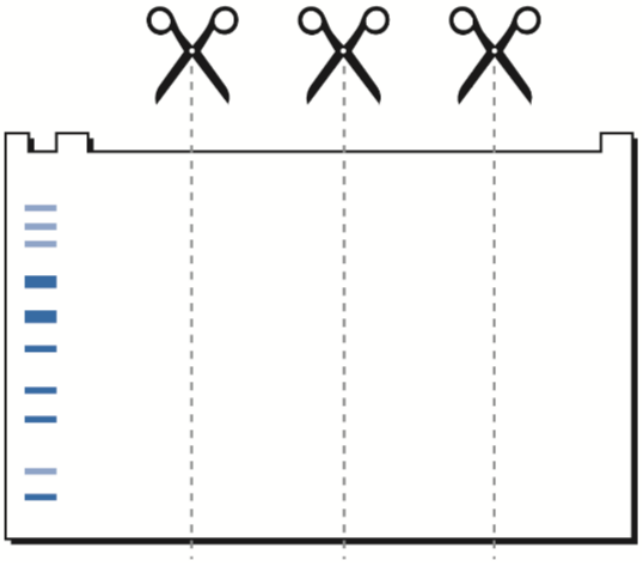

Figure 2. Cut the membrane into four individual blots for blocking buffer optimization.

Membrane Blocking

Step 10.

[**Membrane Preparation**]

- Each individual blot will be processed with a different blocking buffer, and that blocking buffer will also be used for dilution of antibodies.
- TBS or PBS buffer systems may be used for blocking.
- During washing steps, rinse and wash each blot with an appropriate wash buffer that matches the buffer system used for blocking.
- Pre-wet each membrane with TBS or PBS buffer as appropriate (see note).

NOTES

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Blot 1:	Odyssey Blocking Buffer (TBS)
Blot 2:	Odyssey Blocking Buffer (PBS)
Blot 3:	Casein Blocking Buffer
Blot 4:	Blocking buffer of your choice (milk, BSA, etc. in TBS or PBS)

Membrane Blocking

Step 11.

[**Blocking]**

- Place the membranes into 4 different incubation boxes.
- In each box, cover the entire membrane with blocking buffer (approximately 0.4 mL/cm^2), using

a different blocking buffer for each membrane.
- Block the membrane for 1 hour at room temperature with gentle shaking.

NOTES

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Blot 1:	Odyssey Blocking Buffer (TBS)
Blot 2:	Odyssey Blocking Buffer (PBS)
Blot 3:	Casein Blocking Buffer
Blot 4:	Blocking buffer of your choice (milk, BSA, etc. in TBS or PBS)

Alignment in MPX Blotter

Step 12.

Place the four blocked membranes into the MPX Blotter so that there are at least 4 channels available for use on each membrane, as shown in figure below.

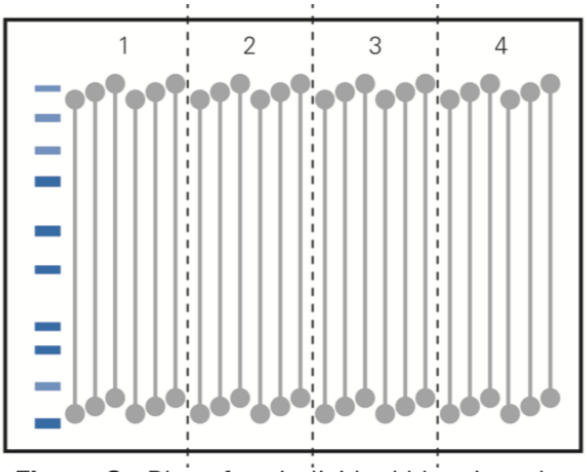

Figure 3. Place four individual blots into the MPX Blotter as shown.

Detailed instructions for use of the MPX Blotter are found in the MPX Blotter Multiplex Western Blotting Accessory User Guide at http://www.licor.com/mpxuserguide.

Primary & Secondary Antibody Application

Step 13.

[**Primary Antibody Preparation**] Two dilutions of primary antibody should be made for each blocking buffer that is tested. Suggested starting dilutions are 1:500 and 1:1,000. You may wish to modify these dilutions, based on vendor recommendations.

NOTES

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The correct working range for antibody dilution depends on the characteristics of your primary

anti- body. Start with the dilution recommended by the primary antibody vendor for Western blot applications.

Primary & Secondary Antibody Application

Step 14.

[**Primary Antibody Preparation**] 700 µL of each dilution will be needed.

Dilute the primary antibody in the appropriate block- ing buffer (see following table) with 0.2% Tween® 20.

Blot	Blocker	Primary Antibody Dilutions	
1	Odyssey® Blocking Buffer (TBS)	1:500	1:1,000
2	Odyssey Blocking Buffer (PBS)	1:500	1:1,000
3	Casein Blocking Buffer	1:500	1:1,000
4	Blocking buffer of your choice (milk, BSA, etc.)	1:500	1:1,000

AMOUNT

700 µl Additional info: of each dilution

Primary & Secondary Antibody Application

Step 15.

[**Primary Antibody Applicaton**] Load the primary antibody/blocker dilutions into the MPX Blotter in the indicated locations for each blocking buffer you are testing.

Primary & Secondary Antibody Application

Step 16.

[**Primary Antibody Applicaton**]

- Apply 2 replicates of each primary antibody dilution, as shown in the figure below.
- Fill the unused channels with the appropriate corresponding blocking buffer.

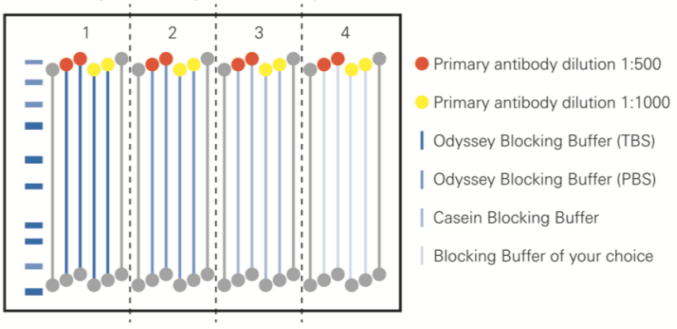

Figure 4. Placement of primary antibody dilutions in the channels of the MPX Blotter.

- Incubate for 1-4 hours at room temperature.

Primary & Secondary Antibody Application

Step 17.

[**Primary Antibody Applicaton**]

- Wash primary antibody from the channels thoroughly according to MPX Blotter manual instructions, using a buffer that matches the buffer system used for blocking.
- Wash buffers should contain 0.1% Tween® 20. **Do not remove blot from MPX blotting manifold during washing.**

Blot 1:	TBS-T
Blot 2:	PBS-T
Blot 3:	PBS-T
Blot 4:	TBS-T or PBS-T, as appropriate

Primary & Secondary Antibody Application

Step 18.

[**Secondary Antibody Preparation**]

Two dilutions of secondary antibody should be made for each blocking buffer that is tested for IRDye® secondary antibodies, we recomment 1:5,000 and 1:10,000 as a starting point.

Dilutions may be modified, based on vendor recommendations.

Blot	Blocker	Secondary Antibody Dilutions	
1	Odyssey® Blocking Buffer (TBS)	1:5,000	1:10,000
2	Odyssey Blocking Buffer (PBS)	1:5,000	1:10,000
3	Casein Blocking Buffer	1:5,000	1:10,000
4	Blocking buffer of choice	1:5,000	1:10,000

Primary & Secondary Antibody Application

Step 19.

[**Secondary Antibody Preparation**]

700 µL of each antibody will be needed. Dilute the secondary antibody in the appropriate blocking buffer with 0.2% Tween® 20.

AMOUNT

700 µl Additional info: of each antibody

Primary & Secondary Antibody Application

Step 20.

[**Secondary Antibody Applicaton**]

- Load the secondary antibody/blocker dilutions into the MPX Blotter in the indicated locations for each blocking buffer you are testing.
- Add the secondary antibody dilutions to the channels previously stained with primary antibody,

as shown in the figure below.

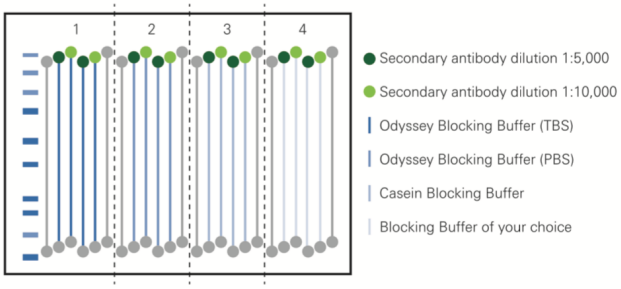

Figure 5. Placement of secondary antibody dilutions in the channels of the MPX Blotter.

Primary & Secondary Antibody Application

Step 21.

[**Secondary Antibody Applicaton**]

- Fill the unused channels with the appropriate corresponding blocking buffer.
- Incubate 1 hour at room temperature. **Protect from light during incubation.**

Imaging

Step 22.

Membranes can be imaged immediately. Image all four blots side-by-side, using standard Western blot imaging settings on any Odyssey® Family Imaging Systems. See guidelines for specifications.

Warnings

See SDS for safety warnings and hazards.