

WESTERN BLOT PROTOCOL FOR LICOR ODYSSEY SCANNER (HAKE'S LAB)

WESTERN BLOT FOR ANALYSIS ON LICOR ODYSSEY SCANNER.

- 1) The Licor Odyssey protein marker is optimal as it is visible on channel 700 (2ul is enough for a lane in a 1mm of 10% SDS-Polyacrylamide gel).
- 2) After transfer to a PVDF membrane, use a 1:1 mixture of Licor blocking buffer and 1X PBS for the blocking solution. DO NOT use tween or BSA to block the membrane when used for the Odyssey scanner as it will show background color on the membrane.
- 3) Incubate the PVDF membrane in the blocking solution for 1hr at room temperature on the rocker. This step can also be done overnight on the rocker in the cold room. If you do it overnight, the container must be tightly sealed to avoid any evaporation of the solution. This can be done by completely covering the container with plastic wrap, or putting the container inside a tupperware container with a tight fitting lid. Alternatively, the membrane can be blocked (and other steps performed) by sealing the membrane in a seal-a-meal bag. This is the most certain way of ensuring no loss of fluid by evaporation.
- 4) Make the solution for primary antibody. The solution is made of blocking solution (step 1) plus 0.01% of Tween 20. We strongly recommend optimization of your particular primary and secondary antibody combinations.
- 5) Add the proper volume of the primary antibody to reach the final concentration wanted.
- 6) Incubate the membrane in primary antibody for 1 hr at room temp, or overnight at 4°C in seal-a container, preferably a sealed meal bag.
- 7) Wash the membrane 4x 5 min on the rocker with a washing solution made of 1X PBS + 0.01% tween 20.
- 8) Incubate in secondary antibody for 1hr at room temp on the rocker. The antibody should be diluted in fresh solution (as in step 3).
- 9) Wash 4x 5min in washing solution.
- 10) Wash 2x 5min with 1X PBS only to get rid of the tween (avoiding background).

SETTING UP ON THE ODYSSEY SCANNER.

- 1) Open the Odyssey scanner and add the membrane facing down on the glass and on the bottom left side of the grid.
- 2) Orient the membrane being careful to place it above the arrowhead drawn on the grid.
- 3) Cover the membrane with the thick rubber square that is either placed on the shelf above the scanner or on the left of the scanner.

- 4) With the roller, press and roll over all the air bubbles that are located under your membrane.
- 5) Memorize the height and width extensions of your membrane. You will need these numbers to use in the program.

SCANNING AND ANALYZING YOUR WESTERN BLOT.

- 1) Open the Odyssey program by clicking on the "Odyssey" icon on desktop.
- 2) On the menu "File" click open project.
- 3) Select your folder and click open.
- 4) If your project is still unopened, scroll sideways to your last file and double click it.
- 5) Now that your project is opened, on the toolbar click on the blue icon with a blue arrow.
- 6) Another window will open. With the mouse, double click on the grid that is drawn.
- 7) With the mouse, drag your drawing of the squared space where your membrane is in the scanner (use the numbers that you memorized when looking at the scanner grid).
- 8) Name your file before scanning it, as the program will automatically save your scan with the name written in the top of the scan window.
- 9) Select the channel in which you want to scan your membrane. For antibodies labeled with Alexa Fluor or IR 680, select channel 700 and uncheck channel 800. For antibodies labeled with IR 800, select channel 800 and unselect channel 700. Both channels can be selected at once, but the scanner will scan slower. Two channels are selected mostly when two antibodies with different fluorescent labels were added to the membrane.
- 10) Select the intensity of the laser for the selected channel. To start, I normally use intensity 5. If my antibody is old or not too efficient, I try a higher intensity.
- 11) Click "scan" and wait for the scanner to start. It generally makes a running noise, indicating that it is running. A window will show the scanner progress and you can see your picture while it is scanning. You can cancel or stop your scanning at any time by clicking in the appropriate buttons at the bottom.
- 12) Once the scan is done, you can have the option to name your analysis (it will name it the previous analysis by default) and to change your picture by flipping it or to show it in black and white only (ideal for printing in the 452 room printer).

GENERAL HINTS AND TIPS (BY ODYSSEY)

Two-color detection requires primary antibodies raised in different host species, e.g.: rabbit and mouse.

- Do NOT add Tween to blocking buffer until after blocking.
- Blocking can be performed using LI-COR Blocking Buffer (BB; recommended) or milk. Milk works well with nitrocellulose, but not so well with PVDF. LI-COR BB results in better sensitivity.
- Do not use BSA for blocking. It may decrease sensitivity and increase background.
- Adding Tween to antibody dilutions is recommended. Typically between 0.05% and 0.2% is adequate. 0.1% is most common.
- The addition of 0.01% - 0.02% SDS to the secondary diluent (in addition to Tween) is recommended as it can help reduce background, particularly with PVDF membranes. Do NOT add SDS to blocker or primary antibody.
- Dilute antibodies in LI-COR BB (if used to block) or PBS/TBS.
- Dilute primary antibodies to the dilution you typically use.
- Dilute secondary antibodies to 1:2000 to 1:10000. To start, I typically recommend 1:5000. Based on the results, optimum dilution can be tweaked. Incubation is for 1 hour at room temp.
- Keep in mind that stock Alexa 680 antibodies are at 2 mg/mL and IRD800 antibodies are at 1 mg/mL.
- Handle membranes carefully and with forceps. This is particularly important when using the secondaries. A quick rinse of the forceps in ethanol after being used in secondary dilutions is recommended.
- I recommend a dilution volume of up to 10 mL for the secondary.
- Use pencil to mark membranes. Blue or black ink can run which will give background problems.
- Blue-stained MW markers are visible in the 700 channel; load about 1/3 to 1/5 of what is typically loaded. Load markers on BOTH sides of the gel if needing to determine MW.
- Gels require enough dilution volume to cover the gel.
- Gels require higher antibody dilutions. Start at double the concentration normally used.
- Gels can benefit from having the primary incubated overnight at 4°C.
- Gels do not require blocking, but do require isopropanol/acetic acid treatment for about 10-15 minutes.
- Gels do not yield equivalent sensitivity as their membrane counterparts.

- The dyes are stable. Therefore, membranes can be prepared and then stored either dry or wet at 4°C for scanning at a later time. If possible, do not wrap membranes in saran wrap.
- Drying membranes after secondary incubation, particularly nitrocellulose membranes, will yield higher sensitivity, but you will not be able to strip the membranes.
- Be aware that the labeled antibodies are light sensitive. Therefore keep them covered when incubating (by covering the dish with foil or a box).
- Consider how your current research can benefit from a two-color approach. For example:
 1. Look at two targets that run very closely to one another;
 2. Look at different forms of the same protein, e.g.: phosphorylated and non-phosphorylated protein.
- When looking at SENSITIVITY, consider a dot blot-type experiment. This will avoid the problem of loss of protein during transfer and will give you the best sensitivity for your system that is achievable with the Odyssey.
- When looking at LINEARITY, consider a normal Western with a system that has been well characterized in your hands. Prepare two-fold serial dilutions of the protein starting at a concentration suitable for that system. Keep in mind that it is typical to see an extra few dilutions (better sensitivity) when using the Odyssey system compared to ECL. Therefore prepare a few more dilutions than you normally would for ECL.
- A good compromise for evaluating both SENSITIVITY and LINEARITY in one experiment is to perform a normal Western blot with a system that works well in your hands.
- Either of the above blots can be used for evaluating the Odyssey's QUANTITATION capabilities. Keep in mind though that due to the potential loss of protein when transferring during a normal Western, the dot blot may yield somewhat more accurate results.
- Prepare an identical experiment for use with ECL when preparing your experiments for the Odyssey. This will give you the best direct comparison between the two systems.
- Prepare fresh membranes for all of the above experiments, including those used for ECL. Use of stripped blots is not recommended due to loss of protein and potential background issues due to previous treatment.

ANTIBODIES TESTED BY HAKE'S LAB

Antibody Dilutions

Primary	Dilution	Secondary HRP	Secondary dye linked
rabbit anti-Gef	1:4,000	anti-rabbit 1:20,000	anti-rabbit alexa680/IR800 1:20,000
rabbit anti-CPEB	1:4,000	anti-rabbit 1:20,000	anti-rabbit alexa680/IR800 1:10,000
guinea pig anti-CPEB	1:4,000	anti-guinea pig 1:7,000	anti-Guinea pig IRDye 800 1:10,000
goat anti-GST	1:2,000	anti-goat 1:7,000	
mouse anti-HA	1:2,000	anti-mouse 1:12,000	anti-mouse alexa680 1:20,000

Secondary dye linked antibodies tested

Antibody	Company	Product #	Concentration	Amount	Price	Did it work?
alexa fluor 680 goat anti-rabbit	Molecular Probes	A-21076	2mg/mL	0.5mL	\$118	yes
alexa fluor 680 donkey anti-goat	Molecular Probes	A-21084	2mg/mL	0.5mL	\$118	yes
alexa fluor 680 goat anti-mouse	Molecular Probes	A-21057	2mg/mL	0.5mL	\$140	yes
IRDye 800 goat anti-guinea pig	Rockland	606-132-129	0.5 mg	0.5mg	\$185	yes
IRDye 800 goat anti-rabbit	Rockland	611-132-122	0.5mg	0.5mg	\$185	yes
cy 5.5 goat anti-guinea pig	Rockland	606-113-129	0.5mg	0.5mg	\$195	NO

Other products used during testing

Product	Company	Product #	Size	Amt.	Price
Immobilon-P (PVDF)	Fisher	IPVH304F0	26x26cm	10sheets	202.3
Schleicher & Schuell Protran Nitrocellulose	VWR	28151-862	20x20cm	5 sheets	102.9
Odyssey Blocking Buffer (can diluted 1:1 in 1xPBS)	Li-Cor Biotechnology	927-40000	1 bottle	500mL	130
Odyssey Protein Marker (1ul per lane can be used)	Li-Cor Biotechnology	928-40000	0.5mL	0.5mL	\$145

Results after testing milk vs. Odyssey blocker with several different antibodies:

	PVDF	Nitrocellulose
Odyssey Blocker		
700nm	sometimes works	good
800nm	sometimes works	good
5%Milk in TBS		
700nm	sometimes works	good
800nm	sometimes works	good

- PVDF seems to work better when scanned at 800nm but this has only been tested with the IRDye800 anti-rabbit 2^o antibody.
- Anti-Ha 1^o with alexa fluor 680 anti-mouse 2^o, works using both PVDF, NC, and either of the blocking buffers. PVDF has slightly higher background.
- IRDye 800 anti-rabbit 2^o, also works using PVDF, NC, and either of the blocking buffers. PVDF has a slightly higher background.
- Alexa fluor 680 anti-rabbit 2^o, works the best using NC and Odyssey blocker. Milk in TBS can also be used, however there is a slightly higher background. This antibody has worked using PVDF with either of the blocking solutions, however it sometimes does not work.