PROTOCOL

Fluorescent Western Blotting

Quantitative protein identification

Azure Imaging System

Model 400, 500, or 600

NIR RGB CHEMI UV BLUE LIGHT TRUE COLOR TRANS WHITE

Contents

I. Materials and reagents

Material / Reagent	Recommended
SDS-PAGE Gel Electrophoresis System	Azure Aqua Quad Mini-Cell (Azure Biosystems, Catalog #AC4200)
Transfer Cell System	Azure Aqua Transfer Cell (Azure Biosystems, Catalog #4201)
Power Supply	Azure Aqua Power Supply (Azure Biosystems, Catalog #AC4202)
SDS-PAGE Gel	User-provided
Sample Protein	User-provided
Protein Dye	User-provided
Molecular Weight Protein Ladder	User-provided
Running Buffer	User-provided
Transfer Buffer	Azure Transfer Buffer (Azure Biosystems, Catalog #AC2127)
Methanol, 100%	User-provided
Transfer Membrane	PVDF Membranes, 9x7cm mini vertical gels (Azure Biosystems: AC2105)
Forceps	Plastic (not metal) forceps strongly recommended
Blotting Paper	Blotting Paper for Western Blotting (Azure Biosystems, Catalog #AC2111)
Incubation Trays	Opaque Incubation Trays (Azure Biosystems, Catalog #AC2120–AC2123)
Rotary or Rocking Platform	Rocking is recommended over orbital shaking
Blocking Buffer	Azure Fluorescent Blot Blocking Buffer (Azure Biosystems, Catalog #AC2190)
Washing Buffer	Azure Fluorescent Blot Washing Buffer (Azure Biosystems, Catalog #AC2145)
Primary Antibody	User-provided
Secondary Antibody	AzureSpectra Fluorescent Secondary Antibodies (Azure Biosystems, Catalog #AC2128–AC2139, AC2156–AC2171)
Plastic Folder	Blot Development Folders (Azure Biosystems, Catalog #AC2126)
Quenching Sheets	Quenching Sheets (Azure Biosystems, Catalog #AC2144)
Imaging System	Azure Imaging System, Model 400 (Azure Biosystems, Catalog #AZI400-01) Azure Imaging System, Model 500 (Azure Biosystems, Catalog #AZI500-01) Azure Imaging System, Model 600 (Azure Biosystems, Catalog #AZI600-01)

II. Technique

Fluorescent antibodies are attached to specific proteins on a low-fluorescence polyvinylidene fluoride (PVDF) membrane, allowing for their visualization and quantification through an imager with fluorescent compatibility such as the Azure 600 Imaging System.

III. Context and relevance

Western blotting is a technique in which proteins are first separated by size through polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to a membrane. From there, the membrane would previously be treated with chemiluminescent substrates to identify a protein of interest, but technological advances have allowed for a more advanced methodology in fluorescent labeling.

Fluorescence detection uses the combination of a primary antibody that specifically binds a protein of interest followed by a secondary antibody conjugated to a fluorescent dye molecule designed to bind to the specific species of immunoglobulin that the primary antibody was raised in. Through this combination, the protein of interest is bound to a fluorescent dye molecule so that it can be identified by an imager capable of fluorescent imaging. Therefore, a huge advantage of fluorescent labeling is the ability to identify multiple proteins of interest on the same membrane. A technique termed multiplexing involves using primary antibodies derived from different animal species to probe for multiple proteins of interest simultaneously. Through this, several proteins on the same membrane can be labeled and identified with species-specific, differently-colored secondary antibodies.

IV. Objective

Identification and quantification of one or more proteins of interest through antibody-specific fluorescent tagging.

V. Experimental protocol

General guidelines are provided as a reference for experiment-dependent protocol optimizations.

1. Sample Preparation **Note:** *Sample preparation method will differ significantly depending on the experiment.*	a. Homogenize cell cultures while keeping cells at ice-cold temperatures to prevent protein degradation.
	b. Add ice-cold lysis buffer containing a protease inhibitor cocktail, as well as phosphatase inhibitor if working with phosphorylated targets to homogenized cells. Centrifuge to separate lysate supernatant from cell debris and collect the lysate supernatant only.
	c. Measure protein concentration via a protein assay such as BCA or Bradford.
	d. Add sample buffer to the lysate supernatant. Ideally, prepare lysate stocks that are at a final protein concentration of at least 1mg/mL. If reduction of disulfide bonds is desired, include a reducing agent such as DTT, β-mercaptoethanol or TCEP in the sample buffer.
	e. Denature the sample by heating at 98°C for 5 minutes.

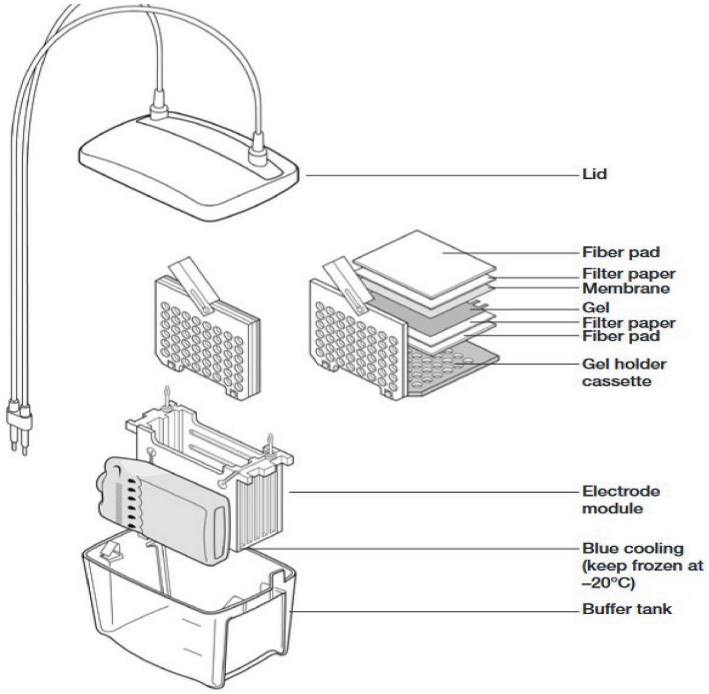
2. SDS-PAGE Gel Electrophoresis	a. Unpack the pre-cast SDS PAGE gel by unwrapping the gel, removing the tape at the bottom of the gel, and slowly removing the plastic comb from the gel lanes.
	b. Place the unwrapped gel with the well's open side facing inward on the Azure Aqua Quad Mini-Cell's gel holder. If an odd number of gels are being run, use a plastic barrier to ensure each gel holder can contain liquid. Push the sides of the gel holder inwards to secure the gels and barriers into place.
	c. Fill the middle of each gel holder with 1x Tris-Glycine SDS PAGE Running Buffer and check for leaks. If buffer leaks out of the bottom of the gel holder, readjust the sides of the gel holder to ensure that both gels or barriers are sealed securely.
	d. Once the middle of each gel holder is filled to the top, fill the body of the Azure Aqua with 1x Running Buffer up to either the "2 Gel" or "4 Gel" lines based on the number of gels being run, rounded up to the nearest multiple of two.
	e. Once the gels are secured and the Aqua filled with running buffer, load the denatured/reduced protein sample and the molecular weight marker into the gel lanes. • Before loading sample, be sure to flush out each well by pipetting up and down a few times in each well. There should be visible streaks that are removed from each well upon doing this.
	f. Set up the Azure Aqua Quad Mini-Cell by connecting the red and black power cable from the lid to the Azure Aqua Power Supply. Place the lid on the Quad Mini-Cell by securely attaching the color-coordinated electrodes to the correct colors on the gel holder. Make sure the color of the electrodes on the gel holder matches the labels on the side of the Quad Mini-Cell as well.
	g. After connecting the electronics securely, turn on the Azure Aqua Power Supply with the switch on the back-right of the instrument. • Set the voltage and preferred run time on the Azure Aqua Power Supply. These settings will differ depending on the running buffer, SDS-PAGE gel used, and expected protein size. Initially, run the gel at a constant voltage of 100V. Once the dye front has concentrated, increase the voltage to 200V and run the gel until the dye front has reached the bottom of the gel.
	h. Once the cable is secured on both ends, press the "Run" option on the Aqua Power Supply to begin the run. When voltage is applied, bubbles should immediately begin to rise in the Running Buffer within the gel holder from the wire at the bottom.

3. Membrane Transfer

Note: *The following process will only be detailed for one gel.*

a. Gather a blot incubation tray, one transfer membrane, two blotting papers, plastic tweezers, a container large enough to fully submerse the stack in buffer, gel roller, and one transfer cassette from the red-and-black transfer case within the Azure Aqua Transfer Cell.

- As the transfer stack is assembled in the following steps, keep the entire stack fully immersed in Transfer Buffer. Remove air bubbles with a gel roller as the stack is assembled to prevent bubble artifacts.



b. Equilibrate the PVDF membrane in 1X Azure Transfer Buffer. First, place enough 200 proof Methanol in a blot incubation tray to completely submerge the blot. Gently agitate for 15 seconds to ensure that the membrane is completely wet. Next, decant the methanol and add ~50mL of high purity water to the membrane. Incubate with rocking for 5 minutes. Decant the water and add ~25mL of 1X Azure Transfer Buffer to the membrane. Incubate with rocking for at least 5 minutes.

c. Open the transfer cassette by sliding the white lock along its track and rotating upwards. Separate the two porous sponges within and place them on opposite sides in a large container containing 1X Azure Transfer Buffer.

d. Using the forceps, fully immerse the four blotting papers in transfer buffer and place two on each sponge within the transfer cassette.

- **Note:** *For the blotting papers and especially the transfer membrane, handle by using the forceps to hold the very corner of the membranes. This gentle and careful handling minimizes the risk of permanent protein contamination.*

e. Remove the gel from its casing by cracking open the casing with a designated metal or hard plastic tool.
   - The very top and bottom of the gel should be cut clean off to make handling and removal easier. Be sure not to cut so much that any of the sample will be removed.

f. Place the gel centered on the soaked blotting paper that is on the black side of the transfer cassette.

g. Place the PVDF membrane that has been equilibrated in 1X Azure Transfer Buffer onto the gel. Ensure that the gel and membrane are exactly the same size and aligned for the best transfer.

h. Close the transfer cassette so that, from the black side to the clear side, the order should now be: outer sponge, soaked blotting papers, SDS-PAGE gel, equilibrated transfer membrane, soaked blotting papers, outer sponge.
   - The transfer cassette will be resistant to being closed, but it is critical to compress it closed and lock it by sliding the white lock on the top. The pressure within the cassette is necessary for the protein transfer process.

i. Place the transfer cassette back into the red-and-black transfer case in the Azure Aqua Transfer Cell such that the clear side of the cassette is towards the red side of the case. Additionally, place a frozen ice pack into the Transfer Cell to maintain a low temperature during transfer.

j. Fill the Azure Aqua Transfer Cell with ice-cold Transfer Buffer to the top line labeled “Blotting” and connect it to the Azure Aqua Power Supply.
   - Securely fasten the lid of the Aqua Transfer Cell with the red and black electrodes matching their respective colors on the red-and-black transfer case.
   - Plug the power cable into the Azure Aqua Power Supply with the matching colors.

k. Set the power supply to 55V and run for 35 minutes.

l. After the transfer process is finished, unplug the Aqua Transfer Cell from the power supply and turn the power supply off.

m. Remove the transfer cassette and open it. Using the forceps, carefully remove the transfer membrane and place it in an Incubation Tray.

n. The rest of the contents of the cassette with the exception of the two external sponges can be discarded in the appropriate disposal now. Wash the sponges thoroughly with high purity water then air dry for next usage.

4. Blocking and Staining	a. Transfer the blot to an incubation tray containing ~25mL of high purity water after the transfer is complete and incubate for 5 minutes with rocking.
	b. Discard the water and add 10mL of Azure Fluorescent Blot Blocking Buffer.
	c. Close the lid of the Incubation Tray and rock under mild agitation for one 30–60 minutes.
	d. While the blot is being blocked, prepare the primary antibody solution. Transfer 10mL of Azure Fluorescent Blot Blocking Buffer to a 15mL conical tube then add primary antibody. Typical primary antibody dilutions range from 1:1000–1:5000. • The antibody or antibodies being used as primary antibodies and their proper dilution will differ depending on each individual protocol, so be sure to check those first
	e. After blocking, discard the Blocking Buffer in the container, while being very careful not to pour the transfer membrane out as well.
	f. Cover and rock under mild agitation for 60 minutes.
	g. Wash the blot three times. • Pour off primary antibody solution. If necessary, return to falcon tube and store for re-use. Do not re-use the solution more than 5 times. • Apply ~25mL of 1X Azure Blot Washing Buffer and rock under mild agitation for 5 minutes each time.
	h. During the washing step, prepare a 1:10,000 dilution of secondary antibody solution. Transfer 10mL of Azure Fluorescent Blot Blocking Buffer to a 15mL Falcon tube then add 1µL of secondary antibody-fluorophore conjugate at 1 mg/mL.
	i. After the third wash, decant the washing solution and apply the diluted secondary antibody-fluorophore conjugate.
	j. Cover and rock under mild agitation for one 30 minutes.
	k. Wash the blot three times • Pour off secondary antibody solution. • Apply~25mL of 1X Azure Blot Washing Buffer (or Azure Fluorescent Blot Washing Buffer if using IR dyes) and rock under mild agitation for 5 minutes each time. • Decant the final wash and add ~25mL of 1X PBS and rock under mild agitation for 5 minutes to remove any residual detergent from the blot.

	• Remove the blot from the PBS solution and drain excess wash buffer. Dip the membrane in 200 proof methanol for 30 seconds then place the blot on a dry clean room sheet free of particulates and allow it to completely dry before imaging.
5. Imaging	a. (Optional, but recommended) Prior to imaging, place the membrane on a background Quenching Sheet to reduce background autofluorescence.
	b. Place the membrane inside the Azure 600 Imaging System on the included Black Chemi Tray.
	c. Turn on the Azure 600 by using the power switch on the back-right, then the green power button on the front.
	d. Boot up the Azure Imaging System Software using the  icon on the desktop.
	e. Select the “Fluorescent Blot” option and select the fluorescent dyes on the left that match the Secondary Antibodies used as well as the pseudo-colors they will be visualized as.
	f. Choose between a system-calculated auto exposure with “Auto Image” or input specific desired settings in “Manually Image.”
	g. After the image is taken, it will automatically appear in the Gallery tab. From here, contrast settings can be altered in “Adjustments,” the image can be saved in multiple image formats to the imager’s D drive or an external drive, as well as other options detailed in the Azure Imaging System User Manual.

VI. Results

Images can then be quantitated through Azure Biosystems’ state-of-the-art analysis software AzureSpot Pro or other image analysis programs. Fluorescent quantification excels at identifying sample quantities through relative fluorescent signal intensities. Additionally, multiplexing allows for fluorescent quantifications of different proteins of interest within the same sample simultaneously.

VII. Conclusions

Fluorescent Western blotting allows for more versatile labeling for proteins that have been transferred to a membrane. Unlike chemiluminescent labeling, fluorescent Western blotting allows for multiplexing several proteins of interests with individual fluorescent dye colors. In order to image each protein, the Azure 600 Imaging System is designed to detect multiple fluorescent dyes in both RGB visible and near-Infrared channels.

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