Three–Color Western Blots with AzureSpectra Antibodies

Visualize Three Proteins at Once – with AzureSpectra labeled antibodies

Classic western blotting techniques using chemiluminescent detection are typically useful for detecting one protein at a time. To evaluate multiple proteins of interest using chemiluminescence, stripping and re-probing the blot or splitting the samples into separate assays is usually required.

Visualizing multiple proteins of interest within the same sample simultaneously – or multiplexing – is possible with fluorescent antibodies. Antibodies carrying different fluorophores (with non-overlapping spectra) can be detected simultaneously with the right imaging system. With a digital imager like the Azure c600 you can visualize three proteins at once.

Why would you need to visualize multiple proteins at once? Convenience is an obvious benefit to multiplexing, as loading controls are often used for Western blot data. By using different fluorescently labeled secondary antibodies for the protein of interest along with the loading control, clear and quantifiable results are ready to be analyzed immediately, even if the proteins are close in molecular weight. Additionally, quantitation is increasingly desirable when publishing protein expression data. What if you have two different proteins of interest, such as phosphorylated and non-phosphorylated version of a protein? In order to include a loading control for accurate comparison of different lanes, a third color would be necessary. Conveniently, 3-color western blotting is possible and easy with AzureSpectra labeled antibodies and the Azure c600 system.

The Azure c600 imaging system is designed for multicolor fluorescent Western blotting, with two nearinfrared (NIR) and 3 visible fluorescent channels. This application note demonstrates a procedure to obtain three color Western Blots using the Azure c600 and Azure's broad selection of fluorescently labeled secondary antibodies. In addition to pre-labeled antibodies, Azure also offers an Antibody Labeling Kit that provides everything you need to fluorescently label your primary or secondary antibodies. The non-overlapping emission spectra of the fluorophores in each of the available dyes means you have the opportunity to easily perform multiplexing assays.

Materials and Methods

Run and transfer gel

HeLa cell lysates were prepared and electrophoresed on a 4-15% Mini-Protean TGX gel, using 1, 2, 5, 10, and 20 µg of lysate for each lane, respectively. After electrophoresis and separation, proteins were transferred to a low fluorescence PVDF membrane using Azure transfer buffer.

Western blotting

After transfer, the membrane was blocked for 10 minutes then probed with 5 µg of mouse anti-GAPDH, 5 µg of rabbit anti-beta-actin, and 5 µg of rat anti-tubulin for 1 hour. The blot was rinsed twice and then washed three times for 5 min each with 25 mL of Azure IR wash buffer. Next, the blot was incubated for 1 hour with the labeled secondary antibodies: 2 µg of goat anti-mouse-550, 4 µg goat anti-rabbit-700, and 4 µg goat anti-rat-800. After incubation, the blot was washed as before in Azure IR wash buffer followed by a 5 min rinse in 25 mL of PBS.

Step	Materials	Part number
Run Gel	SDS Polyacrylamide Gel	
Transfer	PVDF Membrane	AC2105
	Azure Transfer Buffer	AC2127
Block Blot	AzureSpectra Fluorescent Blot Blocking Buffer	AC2190
Probe Blot	Primary Antibodies	Specific for proteins of interest
	Azure IR Wash Buffer	AC2145
	Goat-anti-rat 800	AC2138
	Goat-anti-rabbit 700	AC2128
	Goat-anti-mouse 550	AC2159
	PBS	

Table 1. Materials and product numbers.

Image Western blot

After rinsing in PBS, the blot was allowed to dry before imaging on the Azure Biosystems c600 digital imager.

Results and Conclusions

The Azure c600 and AzureSpectra antibodies allow for quick and easy detection of three different proteins in the same samples on the same western blot. Accurate quantitative analysis of your fluorescent western blot can be performed immediately after imaging.

Figure 2 shows the digital image of the western blot labeled with three different primary antibodies (anti-tubulin, anti-beta actin, and anti-GAPDH) and fluorescently labeled secondary antibodies. The individual channels are shown in grayscale and all three are merged in the three-color image (upper left). The ability to visualize three proteins at once increases the potential information that can be obtained from individual western blot experiments. With more information, better quantitative analysis can be performed – strengthening your data and your science.

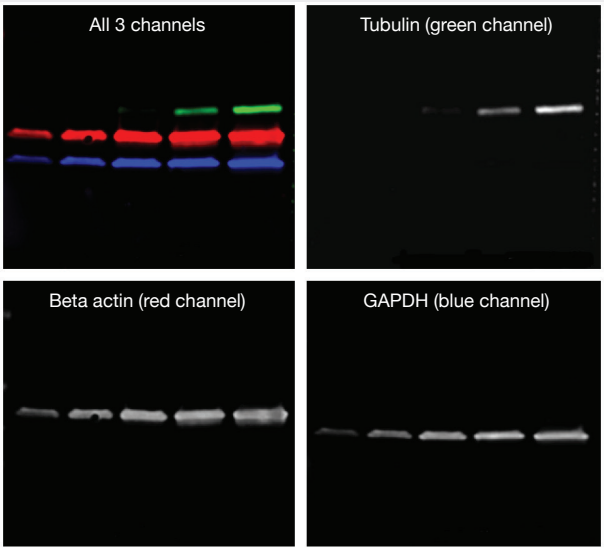

Figure 2. Digital image of 3-color western blot using Azure Biosytems c600 imager. Lanes (from left to right) loaded with 1, 2, 5, 10, 20 µg HeLa cell lysate. Probed for tubulin (top), beta actin (middle) and GAPDH (bottom). The following settings were used: Light sources 6/7/4; Exposure time 1 s/13 s 204 ms/677 ms; Filter positions 6/7/4; Aperture 6400; Focus 5000/5250/5000; bin level 1x1.

Primary Antibody	Secondary Antibody	Excitation/ Emission	Azure Imaging Channel
Rat anti-Tubulin	AzureSpectra Goat-anti-rat 800	785 nm/ 815 nm	IR800
Rabbit anti-beta-actin	AzureSpectra Goat-anti-rabbit 700	660 nm/ 720 nm	IR700
Mouse anti-GAPDH	AzureSpectra Goat-anti-mouse 550	551 nm/ 565 nm	Cy3

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