Increasing assay efficiency with four-color detection

By Joy DeTorres, Product Manager, Azure

The field of Western blot multiplexing – the ability to probe for multiple proteins on a single blot simultaneously – is developing rapidly. Chemiluminescent assays allowed for the detection of a single protein followed by multiple rounds of time consuming stripping and re-probing, with associated potential loss of protein and corresponding signal.

Early Western blot multiplex imaging systems allowed for the imaging of two spectrally distinct fluorophores on a single blot. Researchers rapidly used this technique to assay for loading controls and proteins of interest on a single blot, to compare two distinct proteins, and to devise many other methodologies which are discussed in other application notes.

We have previously described an improvement on this two-channel methodology by imaging three proteins simultaneously by combining the fluorescent and nearinfrared (NIR) imaging capabilities offered by the Azure Biosystems c600 digital imager.

In this note we discuss a further improvement – four-color Western blot multiplexing using the Azure Biosystems Sapphire™ Biomolecular Imager. The ability to simultaneously image four colors at one time greatly increases Western blot efficiency and the ability to make meaningful quantitative comparisons. Four-color Western blotting is made possible through the use of four spectrally distinct fluorophores and the Sapphire's

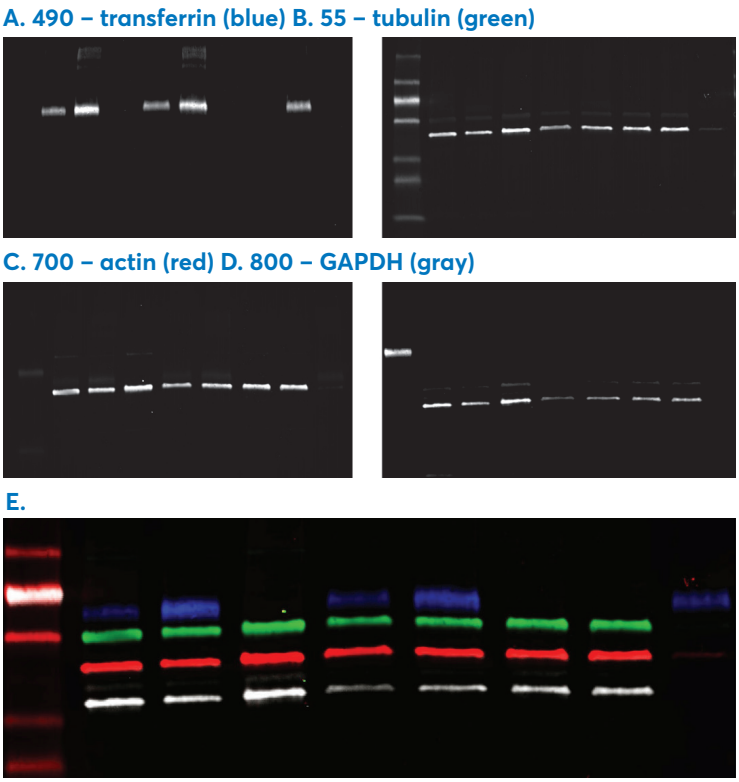

FIGURE 1: **Digital images of 4-color Western blot, captured using Azure Biosystems Sapphire™ Biomolecular Imager.** Transferrin (a), tubulin (b), actin (c) and GAPHD (d) were probed on a single blot using distinct fluorescent and near-infrared targeting antibodies. Images were captured using the Azure Biosystems Sapphire™ Biomolecular Imager at the specified wavelengths and merged into the four-color multiplex image (e). Sensitive and specific detection of all four proteins was possible with no evidence of background auto-fluorescence or bleed between channels.

selective laser based excitation and sensitive PhotoMultiplier Tube (PMT) and Avalanche PhotoDiode (APD) detection systems.

MATERIALS AND METHODS

Run and Transfer Gel

Samples of 1.25 to 5 µg of HeLa cell lysate, some spiked with varying amounts of transferrin were electrophoresed on a Tris-Glycine gel. After electrophoresis and separation, proteins were transferred to a low fluorescence PVDF membrane using Azure Transfer Buffer.

Four-color Western Blotting

Following transfer, the membrane was blocked for 30 minutes with Azure Fluorescent Blot Blocking Buffer then probed with rat anti-tubulin (green), rabbit anti-beta actin (red), and chicken anti-GAPDH (gray) primary antibodies; and anti-transferrin (Blue) which had previously been labelled with AzureSpectra 490 dye using the AzureSpectra Labeling Kit.

Blots were rinsed and washed with Azure Fluorescent Blot Washing Buffer before being incubated with AzureSpectra labeled secondary antibodies – goat anti-rat 550 (green), goat anti-rabbit 700 (red), and goat anti-chicken 800 (gray). After incubation, the blot was washed as before in Azure Florescent Blot Washing Buffer followed by a final rinse in PBS.

Image Four-color Western Blot

After rinsing in PBS, the blot was allowed to dry before imaging on the Azure Biosystems Sapphire™ Biomolecular Imager.

RESULTS AND CONCLUSIONS

In this note a single Western blot was probed for four proteins simultaneously. HeLa cell samples, or HeLa cell samples spiked with transferrin were probed with tubulin (green), beta-actin (red) and GAPDH (gray) antibodies followed by isotype appropriate secondary antibodies; as well as transferrin (blue) antibody previously conjugated with AzureSpectra 490 dye for direct analysis.

The Western blot was imaged using the Azure Biosystems Sapphire™ Biomolecular Scanner and Figure 1 shows the grayscale image captured at each wavelength for transferrin (a), tubulin (b), beta-actin (c) and GAPDH (d); together with a merged, colorized image (e). Together the images demonstrate the high level of sensitivity, specificity and lack of background signal it is possible to achieve using this methodology which allows for rapid and accurate quantitative analysis.

The ability to image four proteins on a single blot greatly increases the efficiency of Western blotting, saving time and precious samples and allows for better quantitative analysis. The methodology also allows for the use of novel assays such as investigations of protein: protein interactions or on-blot total vs phosphorylated protein assays.

Step	Product	Part Number
	4-15% Tris-Glycine gel	N/A
Electrophoresis & transfer	PVDF Membrane	**10147-300**
	Azure Transfer Buffer	**10147-348**
Blocking & antibody labelling	AzureSpectra Fluorescent Blot Blocking Buffer	**75794-864**
	AzureSpectra Labeling Kit – 490	**75794-836**
	Primary Antibodies	Per protein of interest
	AzureSpectra Goat-anti-rat-550	**75794-882**
	AzureSpectra Goat-anti-rabbit-700	**10147-350**
	AzureSpectra Goat-anti-chicken-800	**10147-368**
Probe blot	PBS	N/A

TABLE 1: Material and product numbers.