Multiplexed Western Blotting Redefined: Superplexing on Jess

Introduction

Hours, days, even months of planning and work go into the execution of any one experiment before embarking on the laborious journey that is traditional Western blotting. And in the end, limited sample volumes often limit your ability to perform multiple and/or repeat analyses, amounting to a data collection process that can drag on for years before publication. For researchers performing protein expression analyses by traditional Western blotting, getting more out of less can feel out of reach. What if you could take just 3 µL of your sample lysate and use it as a source of multiple data points, all the while normalizing protein expression to confirm your results? You could simultaneously look at multiple targets and parameters. You could get information about protein abundance, isoform and size all at once. You could tease apart signaling pathways without the fuss of stripping and reprobing a membrane ever again!

Simple Western assays on Jess accomplish all of this at the throughput level you need, in a quantitative manner and with minimal hands-on time. With both fluorescent and chemiluminescent detection capabilities, you can efficiently develop an assay to get more data points out of one sample. In this application note, we simultaneously assess p-Akt and total Akt levels using different detection channels combined with in-capillary protein normalization to demonstrate the superplexing power Jess possesses.

Meet Jess

With Jess, everything happens inside a capillary. The protein electrophoresis step, reagent addition and incubation steps and even signal detection are all fully automated and precisely controlled. You'll only need 3 µL of starting material to get picogram-level sensitivity, and you can choose between capillary cartridges that accommodate 13 or 25 samples to get the throughput you need. Superplexing using Jess's infrared (IR), near-infrared (NIR) and chemiluminescence channels helps you identify and differentiate relative protein expression between samples while built-in protein normalization gives you the confidence you need in your results all in one shot (**Figure 1**). All you have to do is load your samples, antibodies and reagents into a plate, insert the plate and capillary cartridge and press Start! Then enjoy 3 hours of hands-free run time to focus on your science, product development or project deadline. When Jess is done, come back to fully analyzed and quantitative results!

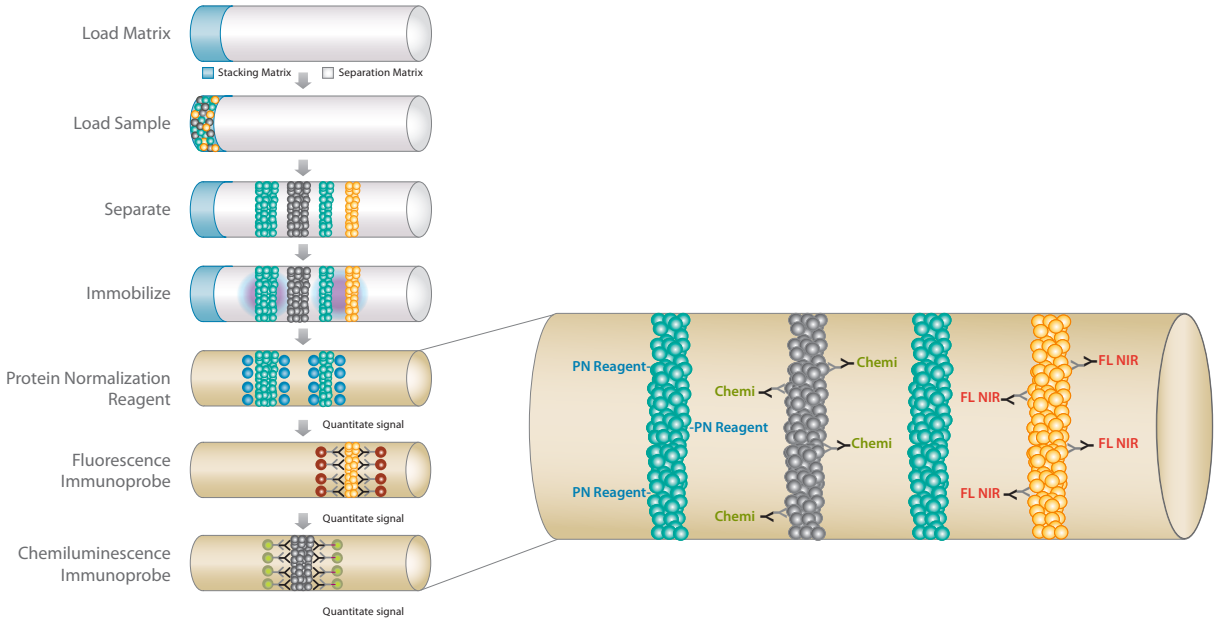


FIGURE 1. Superplexed Simple Western Assay Principle. Your sample, separation matrix, stacking matrix, antibodies and reagents are loaded automatically from a specially designed plate. All steps of a superplexed immunoassay take place in a single capillary. Jess first detects the total amount of protein present in your sample and then probes for your targets of interest in order of fluorescence-based detection first, followed by chemiluminescence. In just 3 hours, you will have multiplexed, normalized, quantitative, size-based data.

Materials & Methods

All experiments were performed using the products listed in **Table 1**.

PRODUCT	VENDOR	PRODUCT NUMBER
Protein Normalization Assay Module for Jess	ProteinSimple	AM-PN01
Jurkat Whole Cell Lysate	Santa Cruz Biotechnology	sc-2204
Jurkat + Calyculin A Cell Lysate	Santa Cruz Biotechnology	sc-2277
Akt (pan) Mouse Monoclonal Primary Antibody	Cell Signaling Technology	2920
Phospho-Akt (Ser473) Rabbit Polyclonal Primary Antibody	Cell Signaling Technology	9271
Anti-Rabbit Secondary HRP Antibody*	ProteinSimple	042-206
Anti-Mouse Secondary NIR Antibody	ProteinSimple	043-821

TABLE 1. Products used in this application note. *Secondary antibodies are not cross-adsrobed

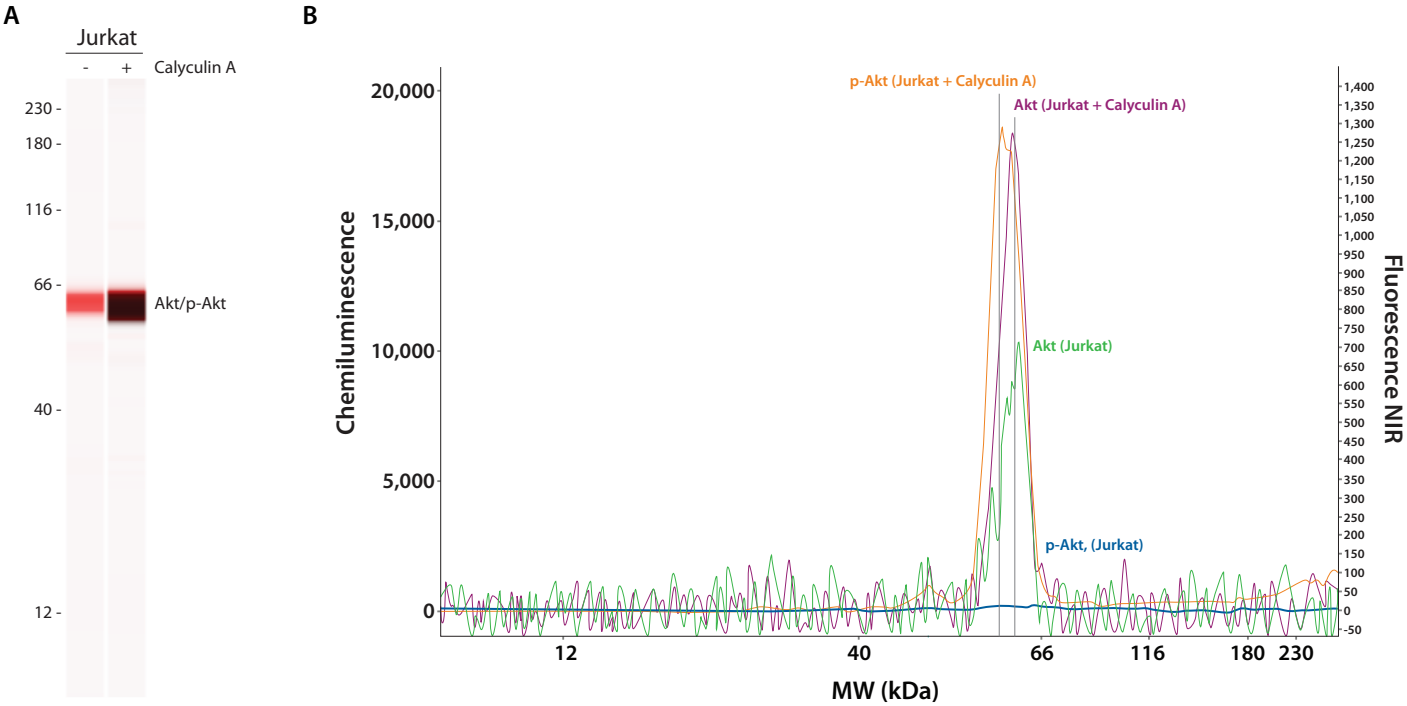
SAMPLE PREPARATION

Jurkat cell lysate samples were prepared at final concentrations of 0.4 mg/mL, 0.6 mg/mL and 0.8 mg/mL. All samples were prepared following the recommended protocol found in the Jess Protein Normalization Assay Module and denatured under reducing conditions for 5 minutes at 95 °C.

ANTIBODY & REAGENT DILUTION

Akt, also commonly referred to as PKB, plays a critical role in controlling cell survival and apoptosis[1,2]. Activation at Thr308 and Ser473 are the main activating phosphorylation sites. The data shown herein were generated using a phospho-Ser473-specific Akt antibody.

The primary antibody multiplexing cocktails were created by combining a 1:25 dilution of p-Akt and 1:25 dilution of Akt (pan) using Milk-Free Antibody Diluent. To create the secondary antibody multiplexing cocktail, the 20X stock solution of anti-mouse NIR secondary



C

Peaks | Capillaries

Sample	Primary	Secondary	Name	MW (kDa)	Area	Corr. Area	S/N	Channel
Jurkat	AKT (M) + pAKT (R)	NIR (M) +HRP (R)	p-Akt	60	4685	7814.8	15.8	CHEMI
Jurkat + Calyculin A	AKT (M) + pAKT (R)	NIR (M) +HRP (R)	p-Akt	60	318721	481412.1	1212.2	CHEMI
Jurkat	AKT (M) + pAKT (R)	NIR (M) +HRP (R)	Akt	63	8958	14942.7	32.4	NIR
Jurkat + Calyculin A	AKT (M) + pAKT (R)	NIR (M) +HRP (R)	Akt	62	16689	25208.1	59.8	NIR

FIGURE 2. Jurkat cells treated with Calyculin A show increased Akt phosphorylation at S473. Lane view of Akt and p-Akt detection in Compass for Simple Western software using the NIR (red) and chemiluminescence (black) channels, respectively (A). Graph view of e-gram overlays in Compass showing p-Akt (chemi) and Akt (NIR) protein peaks using 0.6 mg/mL of Jurkat whole cell lysate (blue and green trace) and Jurkat + Calyculin A lysate (orange and fuchsia trace) (B). Peaks Table in Compass for Simple Western software showing quantitative target protein expression data for each capillary (C).

antibody was diluted to 1X directly in the ready-to-use anti-rabbit HRP-conjugated secondary antibody. Immunoassay conditions for each target were optimized individually, and no primary controls were tested to verify compatibility of multiplexed antibodies (data not shown). Protein Normalization reagents were diluted and prepared according to the dilution matrix found in Protein Normalization Assay Module for Jess.

Use Way Less Sample, Get Way More Data

Using just 3 µL of our 0.6 mg/mL Jurkat or Jurkat + Calyculin A commercially available lysates, we were able to detect p-Akt, total Akt and the amount of total protein expressed (for normalization) at once, and in the same capillary! In this example, we demonstrate how Simple Western assays on Jess allow you to take advantage of the ultra-sensitive detection properties chemiluminescence affords for phosphorylated proteins, while multiplexing using fluorescence detection for the total amount of target protein expressed. And total protein normalization? It happens in a separate channel, maximizing your multiplexing abilities by letting you keep the IR, NIR and chemiluminescence detection channels for your immunoassays. By collecting your answers all at the same time, you'll not only get more datapoints from one sample, but more consistent results than you may be currently collecting with manual methods. Not to

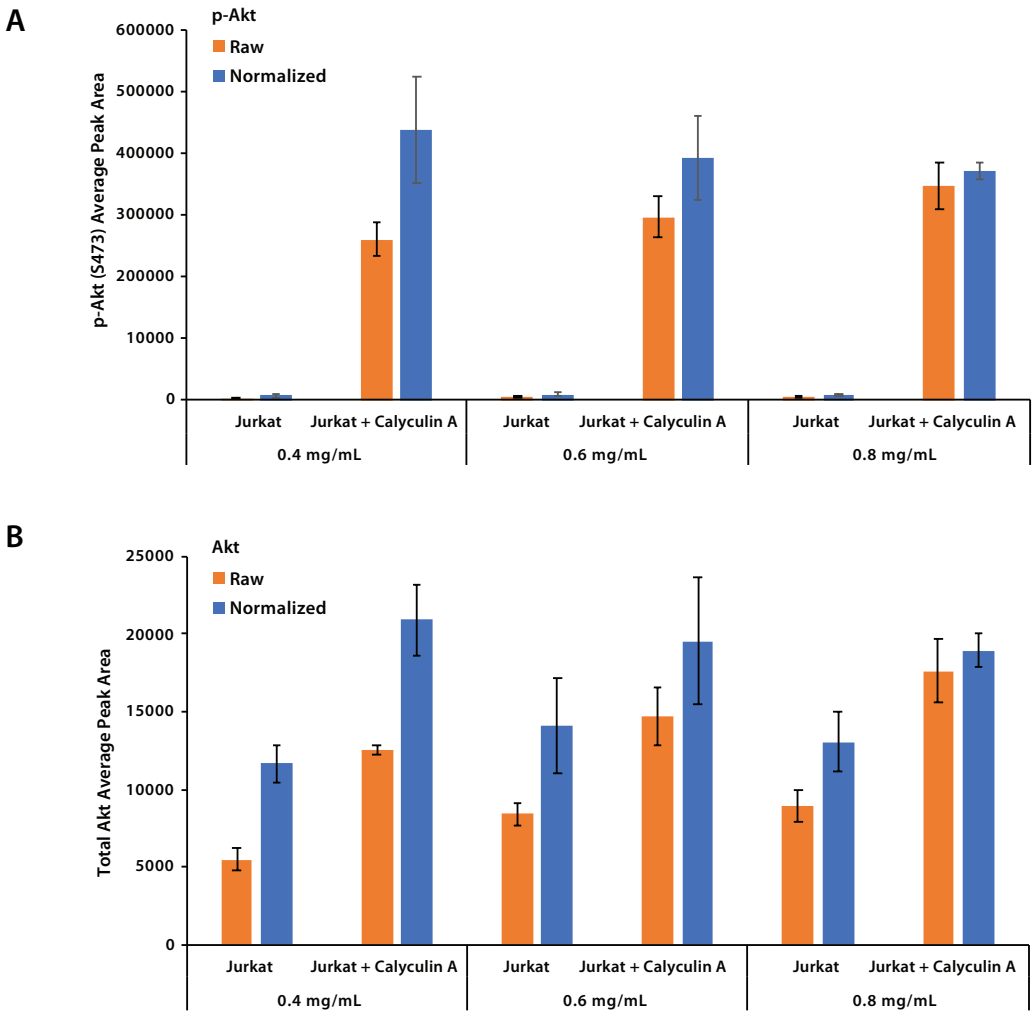


FIGURE 3. Protein normalization reveals comparable p-Akt signals across increasing concentrations of Jurkat + Calyculin A lysate. Comparative data showing p-Akt protein expression detected on Jess's chemiluminescence channel (A) and total Akt detected by the NIR channel (B) as a measure of raw peak area (orange bars) and separate-channel normalization for the normalized peak area (blue bars). As expected, normalization of the raw data results in similar peak area across all three lysate concentrations. Values plotted are mean protein peak areas for samples run in triplicate.

mention, you'll also be saving time and avoiding the hassles of probing, washing and staining blots all day.

Figure 2 illustrates the detection of p-Akt (Ser473) via Jess's chemiluminescence channel using an anti-rabbit HRP secondary antibody while multiplexing for total Akt on the NIR channel with an anti-mouse NIR secondary antibody. The lane view in **Figure 2A** shows the basal total Akt level in Jurkat whole cell lysate in the absence of Calyculin A, a phosphatase inhibitor (left lane), followed by a robust overlapping signal of p-Akt and Akt in the presence of Calyculin A (right lane). The electropherograms (e-grams) in **Figure 2B** mirror these results in quantitative form, denoted by the chemiluminescence (left y-axis) or fluorescence (right y-axis) peak area values automatically recorded by Compass for Simple Western Software for each protein detected.

The number summary is accessible from the Peaks Table (**Figure 2C**), located in the Analysis view within Compass for Simple Western and can be exported for normalization and plotting of your target protein expression to the overall amount of protein present in each capillary.

Now let's look at how Jess's protein normalization capabilities and Compass for Simple Western software take this dataset to the next level. Because raw peak area values are automatically transformed into corrected—or normalized—peak area values, no extra work by you is required. To demonstrate the value of in-capillary normalization for data generation and further analyses, the values recorded in the Peaks Table were exported and analyzed using Microsoft Excel (**Figure 3**). Here, resulting raw (orange bars) and normalized (blue bars) peak area values for various concentrations of Jurkat ± Calyculin A

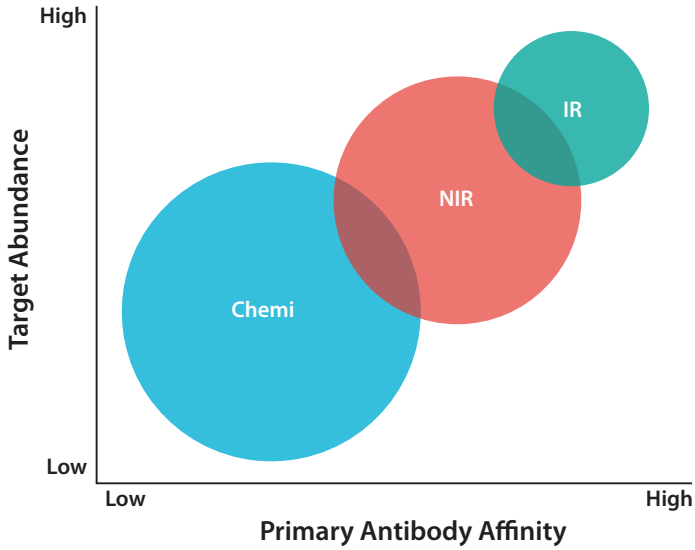


FIGURE 4. When designing a multiplexed assay on Jess, it's important to be mindful of her channel detection sensitivities in addition to your target abundance and/or primary antibody affinity.

whole cell lysates, probed for p-Akt (**Figure 3A**) and total Akt (**Figure 3B**) are shown.

This example highlights the power of protein normalization to account for real-world sample to sample (or prep to prep) variation, where the total amount of lysate loaded per well may be slightly different. Most researchers quantify their lysate protein concentration prior to running a Simple Western assay using, for example, the BCA or Bradford protein assays, allowing for the normalization of total protein loaded across the sample capillaries, or wells. As expected, the raw peak area for p-Akt expression increased as the concentrations of Jurkat + Calyculin A lysate increased (**Figure 3A, orange bars**). However, the normalized values account for the different amounts of lysate loaded per well, and thereby show that a similar level of p-Akt is detected in all three Jurkat + Calyculin A samples run (**Figure 3A, blue bars**). This highlights the importance of using total protein normalization before making conclusions about target expression and fold change that might, in fact, be due to variations in protein loading. The chemiluminescent (p-Akt) and fluorescent (Akt) multiplexed overlay in **Figure 2** confirms the individual target profiles, and the corrected peak area values (summarized in **Figure 3**) led us to more accurately determine fold change for p-Akt and total Akt in this setting. An approximately 51-fold increase in p-Akt phosphorylation (pre-normalized value = 67-fold) with only an approximately 1.5-fold increase in total AKT (pre-normalized value = 2-fold) was observed in response to Calyculin A treatment. Data were generated by running three replicates of each sample concentration, which produced average intra-assay CV values of <13% for both raw and normalized areas reported.

Choosing the Appropriate Detection Channel Combo

When designing your multiplexed assay, you should be mindful of a few things. Specifically, your target abundance, the affinity of your antibody to that target and the suitability of Jess's detection channels for any given protein. If both the protein abundance and antibody affinity are similar for your proteins of interest, then your choice of detection channel is flexible. However, as a general rule, it's best to plan on detecting low abundance proteins and/or those with low-affinity antibodies by chemiluminescence, as we did in this example for p-Akt. To facilitate multiplexing and avoid target loss by traditional stripping and reprobing procedures, go ahead and use Jess's NIR and IR channels in the same experiment to detect other targets of higher abundance and/or those with higher affinity antibodies. Multiplexing requires combining primary and secondary antibodies together, so it is important to examine any potential cross reactivity of your antibodies by running controls. Where available, the use of cross-adsorbed antibodies is recommended to improve multiplexing success. However, when unavailable,

testing different combinations of host species and secondary antibodies is recommended to identify the best multiplexing cocktails to minimize cross reactivity. In this application note, we utilized the NIR channel simultaneously to detect the amount of total Akt in our sample. All the while, normalizing target expression to the total protein present via an entirely separate channel! To help with your strategy for detecting multiple targets by way of channel combination, we've summarized Jess's superplexing capabilities according to detection sensitivity in **Figure 4**.

Conclusion

Jess is unrivaled in her multiplexing abilities. There isn't another who works across chemiluminescence and fluorescence channels while simultaneously analyzing the amount of total protein present in your sample—it's "get more out of less" at its finest. Her demonstrated superplexing capabilities let you maximize the data you get in one shot, all with minimal hands-on time required for experimental setup and cleanup. In this application note, we've shown you how you can apply two entirely different modes of detection to reliably get the target information you need while utilizing same-time total protein detection for normalization. Now, you can effectively normalize protein data, produce accurate and consistent results and mitigate experimental setup and user error!

Welcome to the new frontier in multiplexing: Superplexing!

References

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2. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change, H Yamaguchi and HG Wang, *Oncogene,* 2001; 20:7779-86.

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