Multiplexed Fluorescent Westerns on Jess

Introduction

Dissecting the complexity of any one aspect of human biology requires researchers to obtain information about multiple proteins at once to truly make meaningful hypotheses and conclusions. In fact, the intertwined, cross-talking networks of receptors and intracellular protein components combined with fluctuating post-translational modifications that drive, maintain or halt biological processes and disease states demand these types of analyses. Along with this demand comes the need for increasingly sensitive, quantitative and reliable multiplexing Western blot systems. Sure, multiple targets can be detected at once using ultrasensitive chemiluminescence-based Western blotting approaches, but they require your proteins of interest to not comigrate and instead have well-differentiated apparent molecular weights.

Researchers interested in, for example, phosphoproteins, have long been frustrated by the drawn-out strip and reprobe steps that make quantitative analysis difficult and perpetuate reproducibility problems. But because phosphorylation is an important post-translational modification that regulates a large number of cellular activities and responses, phosphoproteins will remain popular targets in Western blotting for life science researchers across the board. Multiplexing, by way of fluorescent detection, is an attractive solution that may provide a reliable, quantitative means for obtaining accurate information about phosphorylated proteins of interest from each experiment performed.

Jess, our newest member of the Simple Western family, meets even your most challenging multiplexing needs with both fluorescent and chemiluminescent detection capabilities, giving you more data points out of each sample analyzed. The process is easy, quick and quantitative. In this technical note, we'll walk you through fluorescent multiplexing on Jess and provide you with guidance for developing an assay efficiently.

Overview: Fluorescent Multiplexing with Jess

Tired of stripping and reprobing? Jess's fluorescence detection lets you maximize the data you get in one shot by enabling two-color protein detection for multiplexing. During the immunoprobing process, samples are first incubated with the chosen primary antibodies, followed by infrared (IR) and near-infrared (NIR) fluorescent-tagged secondary antibodies. The emission signal is recorded by a charge-coupled device camera in a series of images over time. In just three hours, you'll have multiplexed, quantitative, size-based data ready for analysis. At the end of your run, use the lane view in Compass for Simple Western software to compare band intensities and to quickly view phosphorylated versus total protein signals—simultaneously and in the same lane! Or, dive deeper into the electropherogram view to make quantitative fold change conclusions. Compass completely automates analysis, allowing you to compare changes in protein expression or size, as well as protein isoforms.

Materials & Methods

Table 1 lists the commercially available cell lysate samples used in this technical note.

PRODUCT	VENDOR	PRODUCT NUMBER
Human Whole Cell (IP) Lysates		
A-431 epidermoid carcinoma cells	Santa Cruz Biotechnology	sc-24781
A-431 epidermoid carcinoma cells induced with EGF	Santa Cruz Biotechnology	sc-24782

TABLE 1. Product samples used in this technical note.

ANTIBODY PREPARATION

The Mouse Monoclonal p-EGFR (Santa Cruz Biotechnology, PN sc-57542) and Rabbit Monoclonal EGFR (Cell Signaling Technology, PN 2646S) were each diluted at a ratio of 1:10 with ProteinSimple Milk-free Antibody Diluent (PN 043-524) and prepared either in separate vials or as a cocktail in the same vial for multiplexing. Similarly, the Anti-Mouse NIR (ProteinSimple, PN DM-009) and Anti-Rabbit IR (ProteinSimple, PN DM-008) secondary antibodies were prepared either separately or as a cocktail, according to their respective product inserts.

SAMPLE PREPARATION

For linearity assays, titrations of A-431 cell lysate induced with EGF were prepared using two-fold serial dilutions at final sample concentrations of 1.5 mg/mL down to 0.012 mg/mL, and a 2 mg/mL sample was also included when detecting p-EGFR. For multiplexing assays, A-431 cell lysate samples were prepared at final concentrations of 0.75 mg/mL. All samples were prepared following the recommended protocol found in the 12-230 kDa Jess Separation Module (PN SM-W003) product insert. Finally, samples were denatured and reduced for 5 minutes at 95 °C, then run using the default fluorescent assay conditions and settings for Jess in Compass for Simple Western software.

Getting Started

For instructions on primary and secondary antibody preparations, refer to the Jess Detection Module for Fluorescence. You can also follow the "Pipette your plate" and "Start Jess" sections located in the Separation Module for further guidance on setting up your assay and getting started in Compass for Simple Western software.

To multiplex the signal of two different proteins in the same capillary, you'll need to probe for both proteins using the same lysate concentration. Ideally, the signals from your multiplexed IR- and NIR-detected proteins are in the same linear dynamic range of their respective detection channels. ProteinSimple recommends running a quick sample titration experiment to assess whether the targets you want to multiplex are in the linear detection window of both Jess's IR and NIR channels at the same lysate concentration.

In **Figure 1**, we performed a titration of the EGF-induced A-431 cell lysate beginning at 2.0 mg/mL, then 1.5 mg/mL, followed by two-fold serial dilutions down from there to detect our two proteins of interest, p-EGFR and total EGFR, a member of the erbB/HER family of receptor tyrosine kinases whose overexpression is known to drive cancer cell proliferation and tumor growth[1-3].

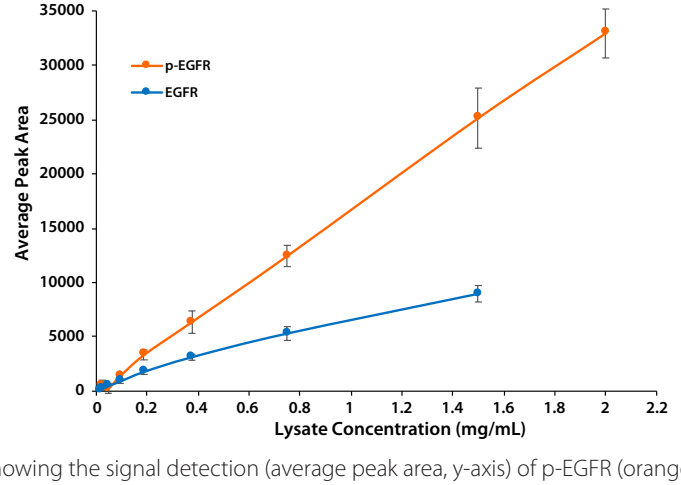

FIGURE 1. Combined graph showing the signal detection (average peak area, y-axis) of p-EGFR (orange line, NIR detection) and EGFR (blue line, IR detection) over a series of EGF-induced A-431 cell lysate concentrations (x-axis). Samples were run in triplicate, and all data points for p-EGFR were generated using a 60 second exposure time, and for EGFR, a 300 second exposure time.

The preliminary sample titration experiment allows you to answer the simple question of whether or not your targets can be multiplexed via IR and NIR signal detection at the same concentration of lysate. To determine the optimal concentration of lysate to use in your assay, apply linear regression analysis to the selected detection range from your titration data. Using Excel and the selected data points from **Figure 1** (from 2 mg/mL down to 0.05 mg/mL), we demonstrate the lysate concentrations within the linear range of detection of each target within their respective NIR and IR channels (p-EGFR, R^2=0.9998; EGFR, R^2=0.9904) in **Figure 2**. However, it is important to note the difference in the linear detection range of concentrations between the two channels for each protein. Looking at our targets independently, we can select anywhere from 0.18 mg/mL to 2 mg/mL of lysate for detecting p-EGFR in the NIR channel (**Figure 2**, orange line), whereas the linear range for total EGFR is at lower concentrations (0.047–0.75 mg/mL) in the IR channel (**Figure 2**, blue line). Considering both targets, the ideal lysate concentration should fall between 0.18 mg/mL and 0.75 mg/mL. We chose to proceed with 0.75 mg/mL of A-431 lysate, a concentration that is comfortably within both detection ranges for loading in our multiplexed fluorescent Simple Western assay on Jess.

Post-Run Data Analysis

Once your run is complete, you can view the results by selecting the **Analysis** perspective in Compass for Simple Western software. Navigating to the **Graph** view pane will display the electropherogram (e-gram) for each channel/target, if individually selected, or as a multiplexed overlay if both NIR and IR channels are selected (**Figure 3**). After running the unstimulated versus EGF-induced A-431 cell lysates on Jess, our results reveal very little p-EGFR detected in unstimulated A-431 cell samples, hence no NIR (red) peak seen in **Figure 3** *left*, whereas a peak for total EGFR can be seen in the IR (green) channel overlay. To illustrate a scenario in which target phosphorylation in response to applied stimuli is the anticipated experimental readout, we used the commercially available EGF ligand-induced A-431 epidermoid carcinoma cells for comparison with the unstimulated control. In **Figure 3** *right*, EGF-induced A-431 cells were analyzed for total EGFR and p-EGFR. Now, in the NIR channel, a clearly present p-EGFR peak is detected and can be compared with total EGFR shown in the IR e-gram multiplexed overlay.

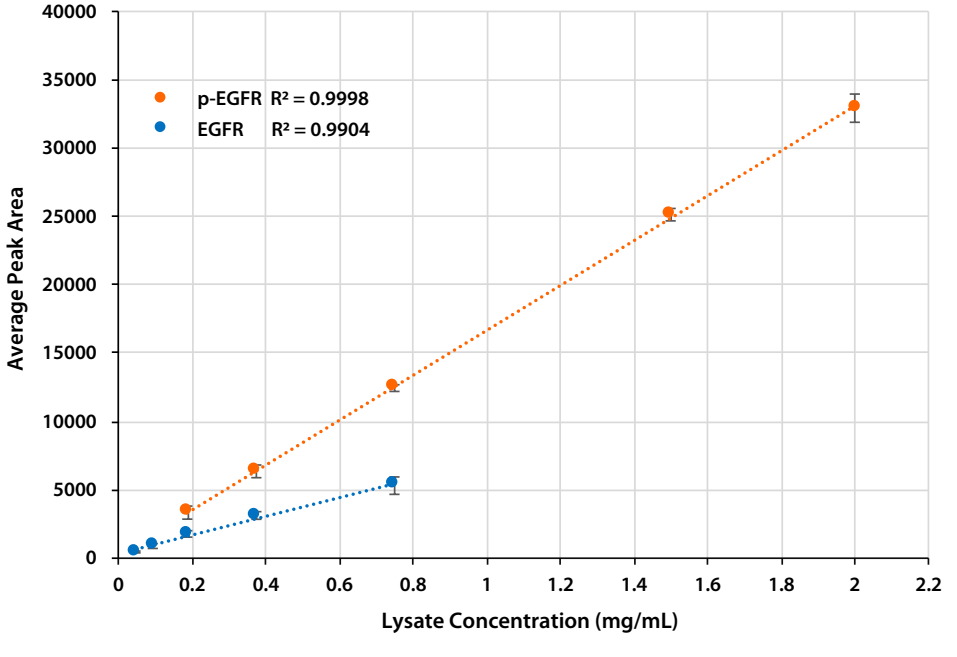

FIGURE 2. Linear regression analysis showing the signal detection range (average peak area, y-axis) of p-EGFR (orange line, NIR detection) and EGFR (blue line, IR detection), as selected from **Figure 1**. The concentration of lysate you load in your multiplex assay should fall within the linear range of detection for both of your targets. In this case, a lysate concentration between 0.18 mg/mL and 0.75 mg/mL will accommodate both targets in their respective fluorescence channels.

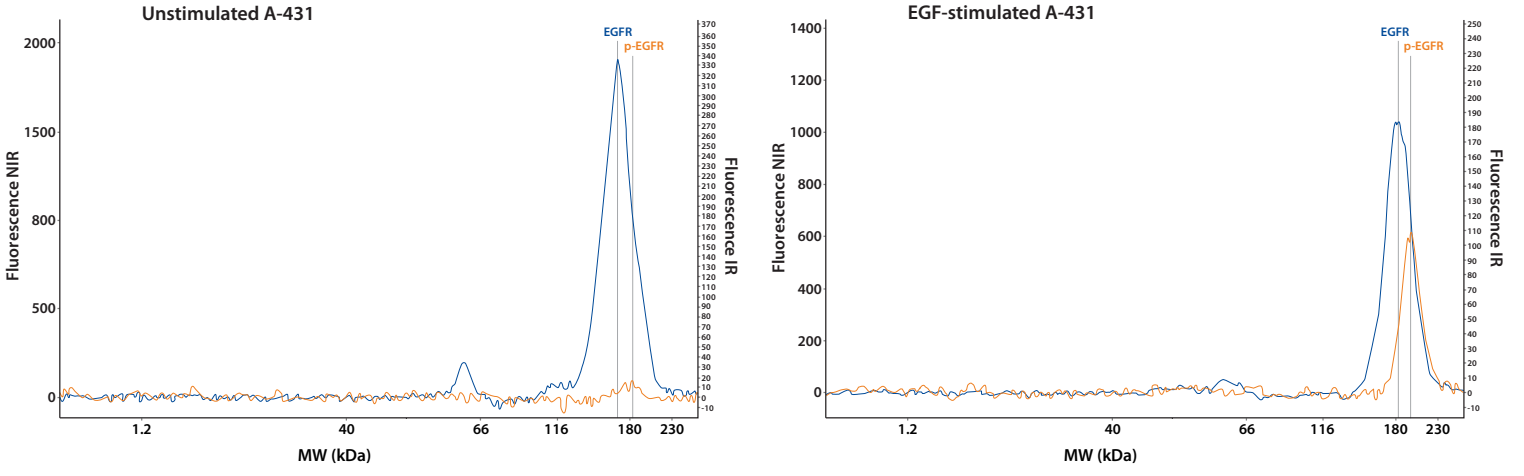

FIGURE 3. NIR/IR e-grams showing the detection of p-EGFR and total EGFR, respectively, using unstimulated A-431 (left) versus EGF-induced A-431 (right) whole cell lysates.

The **Lane** view pane in Compass will display the signals detected in the NIR and IR channels within each capillary as bands. **Figure 4** shows the individual and multiplexed p-EGFR and EGFR signals, which can be differentiated in one capillary, or "lane." As expected, in our unstimulated A-431 cell lysate control, no basal p-EGFR signal is detected, and hence no NIR signal is observed (**Figure 4**, lane 1). However, clearly present is a basal level of total EGFR, detected by the IR channel at around 180 kDa (**Figure 4**, lane 2). The multiplexed fluorescent overlay (**Figure 4**, lane 3) confirms the individual protein profiles present in our unstimulated lysate, showing no basal p-EGFR and a total EGFR signal matching that of lane 2.

With regard to EGF-induced A-431 cell lysates, now, in the NIR channel, a clearly present p-EGFR signal is detected (**Figure 4**, lane 4) and can be compared with total EGFR, detected by the IR channel in the same EGF-induced sample lysate **(Figure 4**, lane 5). The multiplexed overlay displayed in Compass for Simple Western (**Figure 4**, lane 6) allows for target differentiation and expression signal overlap in the case of two proteins having similar molecular weight, of which p-EGFR versus total EGFR is a great example.

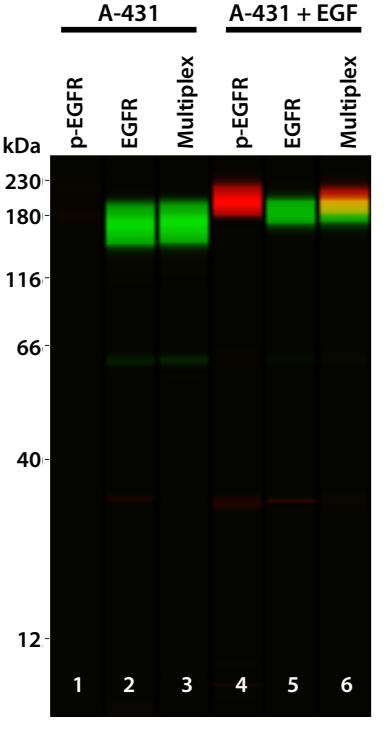

FIGURE 4. Lane view in Compass for Simple Western software showing p-EGFR and EGFR expression levels in unstimulated versus EGF-induced A-431 cells. Lane 6 shows the overlapping signal achievable with multiplexing on Jess.

Exporting Your Results

Making conclusions about a change in target phosphorylation requires additional quantitative analyses. To achieve such results by conventional chemiluminescence Western blotting, chances are you've either had to produce duplicate gels and blots from your samples or strip and reprobe a single blot—both of which complicate technical accuracy, data analysis, and prolong an already tedious experimental workflow to days at a time before recording results. Multiplexing on Jess allows you to directly compare phosphorylated and total protein signals generated by identical experimental conditions all in less than a day's work. Compass for Simple Western software then greatly simplifies data analysis by automatically populating a Peaks Table (**Figure 5**) where you can view the resulting target protein expression values in each capillary, and various assay run parameters and settings.

The Peaks Table shown in **Figure 5** can be exported for further analysis. Using Excel, in **Figure 6** we show example exported data generated from running 0.75 mg/mL of A-431 or EGF-induced A-431 cell lysates immunoprobed for p-EGFR and EGFR as either a singleplex or multiplex assay. The orange bars signify detection of p-EGFR on the NIR channel, whereas the blue bars denote detection of EGFR on the IR channel. Multiplexing within the same capillary was performed using a combined antibody cocktail. Four replicates of each sample were run; these yielded intra-assay CV values less than 11%, attesting to the reproducibility of multiplexing on Jess. These data suggest that the signal detected is similar across singleplex and multiplexed assays and that multiplexed Simple Western assays not only give you more data points in less time but also minimize reproducibility problems that often arise from needing to perform more manual and inaccurate technical steps.

Peaks | Capillaries

Sample	Primary	Secondary	Cap	Peak	Name	Position	MW (kDa)	Height	Area	% Area	Corr. Area	Width	S/N	Baseline	Channel
A431+ EGFR	EGFR	Anti-Rabbit IR	18	1	p-EGFR	523	183	37.4	79	100.0	76.7	2.0	3.6	149.8	NIR
A431 Untrea...	p-EGFR	Anti-Mouse NIR	20	1	p-EGFR	521	182	105.1	1025	100.0	822.4	9.2	7.1	211.8	NIR
A431 Untrea...	Multiplex	Multiplex	22	1	p-EGFR	519	184	79.8	868	100.0	710.9	10.2	5.0	204.1	NIR
A431+ EGFR	EGFR	Anti-Rabbit IR	6	1	EGFR	517	182	170.3	3407	100.0	3156.6	18.8	94.1	9.5	IR
A431+ EGFR	Multiplex	Multiplex	7	1	EGFR	518	183	192.6	3784	100.0	3613.4	18.5	131.8	10.9	IR
A431 Untrea...	EGFR	Anti-Rabbit IR	9	1	EGFR	512	171	281.6	6759	100.0	5599.8	22.5	142.7	20.0	IR

FIGURE 5. Peaks Table in Compass for Simple Western software showing quantitative target protein expression data for each capillary.

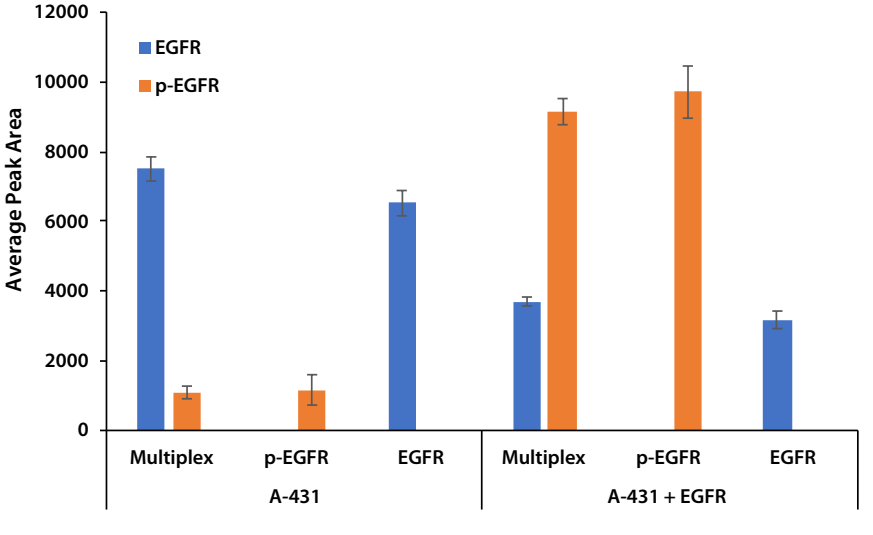

FIGURE 6. Comparative bar graph showing singleplex and multiplex assay signals detected on Jess for p-EGFR (orange bars) and EGFR (blue bars) in A-431 (left) versus EGF-induced A-431 cells (right).

Conclusion

Automated, multiplexed Simple Western assays on Jess have the power to give you more than double the data points per experiment while increasing your confidence level exponentially. To obtain quantitative and meaningful results, you should begin by carefully optimizing the detection of your targets in a sample titration experiment. Next, select the lysate concentration for loading that accommodates your proteins of interest from the plotted linear range of detection. Once your assay is optimized for the detection of your targets, simply follow the primary and secondary antibody preparation steps in the Jess Fluorescence Detection Module, pipette your assay plate according to the Separation Module, load the parameters in Compass and press Start! In this technical note, we walked you through the steps for running a multiplexed fluorescent Simple Western assay on Jess using p-EGFR and EGFR as an example to help you get started. For next-level multiplexing, Jess can also utilize the high sensitivity of chemiluminescence paired with NIR detection for even greater multiplexing capabilities.

References

1. Epidermal growth factor receptor cell proliferation signaling pathways, P Wee, Z Wang, *Cancers (Basel),* 2017; 9:52.

2. Targeting the EGFR signaling pathway in cancer therapy, P Seshacharyulu, MP Ponnusamy, D Haridas, M Jain, AK Ganti, SK Batra, *Expert Opinion on Therapeutic Targets,* 2012;16:15-31.

3. Emerging functions of the EGFR in cancer, S Sigismund, D Avanzato, L Lanzetti, *Molecular Oncology,* 2017;12:3-20.

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