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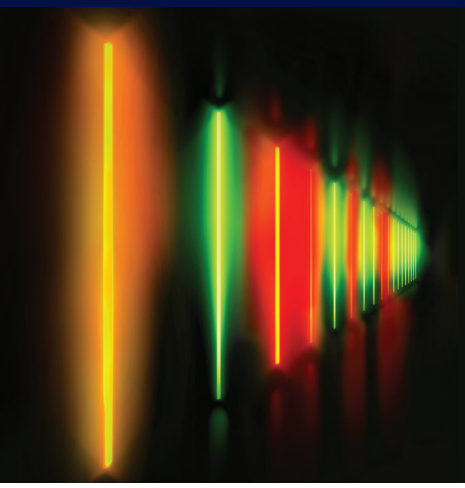
APPLICATION NOTE



STELLAR NIR AND IR MODULES ON JESS FOR SIMPLE WESTERN

A NEW FRONTIER IN FLUORESCENCE DETECTION SENSITIVITY

SIMPLE WESTERN BRINGS THE WESTERN BLOT INTO THE 21ST CENTURY

Simple Western™ from ProteinSimple, a Bio-Techne brand, is a rapidly growing protein analytical platform that automates the detection and quantification of low abundance proteins in complex samples.[1] As a fully automated capillary-based immunoassay housed entirely in a benchtop instrument, Simple Western overcomes the well-known limitations of traditional Western blot while providing unparalleled flexibility for protein analysis. For example, Simple Western is equipped with multichannel immunodetection using standard Western blot antibodies in chemiluminescence, infrared (IR), and near-infrared (NIR) fluorescence channels, enabling channel-based and size-based multiplex protein characterization. Simple Western also offers RePlex™ which performs two serial immunoassays in the same capillary to detect even more protein targets or to normalize expression data by total protein content, which is proven to be more reliable than the use of housekeeping controls. With these advantages, Simple Western users get the most data out of tiny sample volumes (as little as 3 µL) with quick time to results (as little as 3 hours). Featured in over 1200 publications and counting, Simple Western is a proven technology that not only streamlines workflows, but also generates quantitative, reproducible, and publication-ready results that raise the bar on scientific rigor in protein analysis.

NOW WITH INDUSTRY-LEADING FLUORESCENCE SENSITIVITY FOR WESTERN BLOT ANALYSIS

As the current flagship of the Simple Western product line, Jess™ provides users with the most flexibility in protein detection with sensitive chemiluminescence and NIR/IR fluorescence channels. Now NIR/IR fluorescence detection on Jess is even better with Stellar™ NIR/IR Detection Modules (**FIGURE 1**), which provides industry-leading fluorescence sensitivity at detection levels below 1 pg, along with excellent reproducibility and a 4-log dynamic range. With this leap in sensitivity, Stellar fluorescence detection rivals the widely recognized sensitivity of chemiluminescence detection and demolishes the fluorescence detection of the closest competing traditional Western blot imaging technology, which requires at least 50 pg.

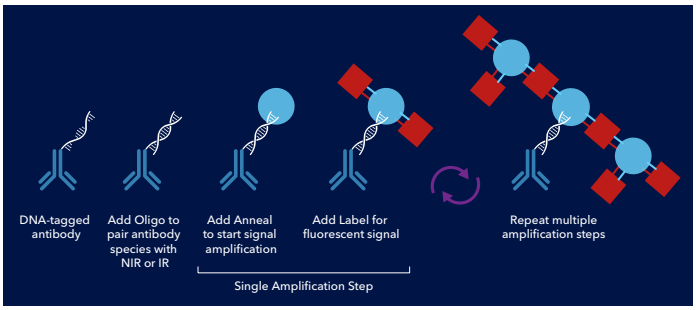


FIGURE 1. The sensitivity of Stellar fluorescence detection is achieved by multiple signal amplification steps tethered by DNA to the detection antibody.

In this Application Note, we use cell signaling pathways as an example application to show how Stellar detection on Jess enables highly sensitive multiplex protein detection of total and phosphorylated protein isoforms and simultaneous Stellar Total Protein normalization for accurate protein quantification. To top off the Stellar release, the latest version of Compass for Simple Western comes with a Lane View Annotation tool, so your Stellar results are on the fast track to publication.

MATERIALS AND METHODS FOR STELLAR PROTEIN ANALYSIS

The materials and reagents used in this study are listed in TABLE 1. All reagents were prepared as described in the respective product inserts for the separation and detection modules listed below.

ITEM	VENDOR	PART NUMBER
Fluorescence 12-230 kDa Separation Module	Bio-Techne	SM-FL004
Stellar Anti-Mouse IR Detection Module	Bio-Techne	DM-015
Stellar Anti-Rabbit NIR Detection Module	Bio-Techne	DM-014
Stellar Anti-Rabbit IR Detection Module	Bio-Techne	DM-013
Stellar Total Protein Detection Module	Bio-Techne	DM-TP03
RePlex Module	Bio-Techne	RP-001
Standard 12-230 kDa Separation Module	Bio-Techne	SM-W004
Rabbit Anti-Phospho AKT (pAKT) Antibody	Bio-Techne	AF887
Rabbit Anti-RNase A Antibody	Bio-Techne	NBP1-69256
Ribonuclease A from bovine pancreas	Sigma	R5250
Mouse Anti-Total AKT Antibody	Cell Signaling Technology	2920
Jurkat + Calyculin A Cell Lysate	Santa Cruz Biotechnology	sc-2277
Jurkat Whole Cell Lysate	Santa Cruz Biotechnology	sc-2204

TABLE 1. Materials and reagents used in this study.

STELLAR NIR/IR AND CHEMILUMINESCENCE LIMIT OF DETECTION (LOD)

Recombinant purified RNase A was prepared in a 2.5X serial dilution series from 5 ng/mL to 0.128 ng/mL, and a 0 ng/mL blank was included as a negative control. A volume of 3 µL from each sample dilution was loaded on Jess for analysis by chemiluminescence and Stellar NIR/IR, corresponding to 15 pg, 6 pg, 2.4 pg, 0.96 pg, and 0.38 pg of RNase A per well. RNase A was detected with the rabbit anti-RNase A antibody at a dilution of 1:25 in Antibody Diluent 2. Calibration curves were generated by plotting RNase A peak height by RNase A concentration to perform linear regression analysis. Signal noise was measured using the raw data from the no sample capillaries and a standard deviation for noise was calculated. LOD is defined as the concentration at which the detected protein peak height is 3 times the standard deviation of signal noise.

STELLAR NIR/IR MUTIPLEX REPRODUCIBILITY

Lysate from Jurkat cells treated with calyculin A was loaded at a 0.2 mg/mL final sample concentration in 24 wells for analysis on Jess with the 1st well containing the biotinylated ladder. Total AKT was detected with the mouse anti-total AKT antibody at a 1:50 dilution in Antibody Diluent 2 in the Stellar IR detection channel, and pAKT was simultaneously detected with the rabbit anti-pAKT antibody at a 1:25 dilution in Antibody Diluent 2 in the Stellar NIR detection channel. The intra-assay reproducibility was determined by calculating the coefficient of variation (CV) and standard deviation (Std. Dev.) of the average peak area across all 24 capillaries for both detection channels. To calculate the inter-assay reproducibility, this analysis was repeated on a separate Jess instrument.

STELLAR NIR/IR MULTIPLEX WITH STELLAR TOTAL PROTEIN DETECTION

Lysate from untreated Jurkat cells was analyzed at a 0.25 mg/mL final concentration and lysate from Jurkat cells treated with calyculin A was analyzed at a 0.15 mg/mL final concentration. For each lysate, total AKT was detected with the mouse anti-total AKT antibody in the Stellar IR channel, and pAKT was detected with the rabbit anti-pAKT antibody in the NIR channel, as described above. Both lysates were probed with each antibody alone and multiplexed together with Stellar Total Protein Detection performed in the chemiluminescence channel.

STELLAR NIR/IR MULTIPLEX VERSUS REPLEX WITH CHEMILUMINESCENCE

Lysates from Jurkat cells untreated and treated with calyculin A were analyzed at 0.2 mg/mL final concentrations by Stellar NIR/IR multiplex detection and by chemiluminescence detection using RePlex. For Stellar analysis, each lysate was probed with the mouse anti-total AKT in the Stellar IR channel and the rabbit anti-pAKT antibody in the Stellar NIR channel, as described above. Both lysates were probed with each antibody alone and multiplexed together. For RePlex analysis, the rabbit anti-pAKT antibody was used for detection in Probe 1 and the mouse anti-total AKT was used for detection in Probe 2 for both lysates using the same antibody dilution factors.

RESULTS OF STELLAR PROTEIN ANALYSIS

HOW BRIGHT IS STELLAR? DETERMINING THE LIMIT OF DETECTION

To determine the sensitivity of Stellar protein detection on Jess for Simple Western, we prepared a serial dilution series of purified RNase A, and each dilution was loaded on Jess and analyzed with an anti-RNase A antibody in chemiluminescence and Stellar NIR/IR detection channels. These results show a signal corresponding to RNase A that decreased with decreasing sample concentration (**FIGURE 2A**). As expected, we did not observe a signal in the 0 pg blank control. Next, we plotted RNase A peak height by RNase A concentration to generate calibration curves that showed a highly linear relationship, with R^2 values >0.99 in all three detection channels (**FIGURE 2B**). Then, we used these calibration curves to calculate the limit of detection (LOD) in each detection channel. This analysis resulted in an LOD of 1.1 pg, 0.4 pg, and 0.7 pg in chemiluminescence, Stellar NIR, and Stellar IR channels, respectively (**TABLE 2**). Furthermore, Stellar fluorescence detection demonstrated 100X greater sensitivity than the closest competing traditional Western blot fluorescence imaging system, which required at least 50 pg for detection (data not shown). Finally, we measured a 4-log dynamic range of RNase A detection in Stellar NIR/IR and chemiluminescence channels (data not shown).

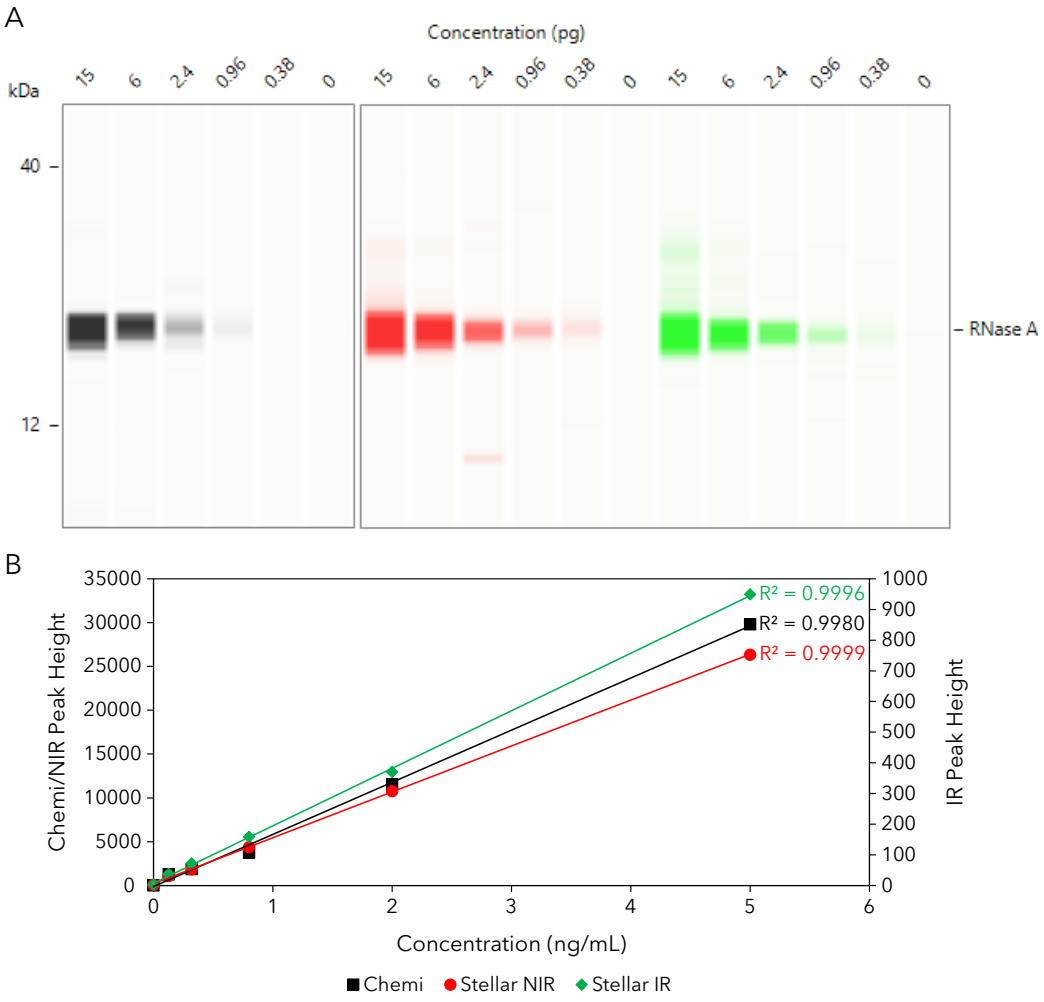


FIGURE 2. Stellar NIR/IR fluorescence offers comparable sensitivity to chemiluminescence. (**A**) Lane view of RNase A analysis by chemiluminescence detection (left lanes, black bands), Stellar NIR (middle lanes, red bands), and Stellar IR (right lanes, green bands). (**B**) Linear regression analysis in all three detection channels.

	JESS CHEMILUMINESCENCE	JESS STELLAR NIR	JESS STELLAR IR	COMPETING FLUORESCENCE WESTERN BLOT
LOD of RNase A	1.1 pg	0.4 pg	0.7 pg	>50 pg

TABLE 2. LODs of RNase A detection on Jess by chemiluminescence, Stellar NIR, and Stellar IR, and compared with a leading competitor Western blot fluorescence imaging system.

REPRODUCIBLE MULTIPLEX ANALYSIS OF TOTAL AND PHOSPHORYLATED PROTEIN ISOFORMS WITH STELLAR

Encouraged by the sensitivity and large dynamic detection range of Stellar, we next tested if Stellar fluorescence detection can be used for simultaneous multiplex analysis of total and phosphorylated protein isoforms in the same capillary. To test this, we loaded lysates from Jurkat cells that were treated with calyculin A, a potent activator of protein kinase B (AKT),[2] across all 24 capillaries on Jess, with the 1st capillary containing biotinylated ladder. Then, we probed each sample with mouse anti-total AKT antibody in the IR channel and rabbit anti-pAKT antibody in the NIR channel. This analysis revealed a green band corresponding to total AKT in the IR channel and a red band corresponding to pAKT in the NIR channel in each capillary (FIGURE 3). When this analysis was performed on 2 separate Jess instruments, we calculated the inter-assay and intra-assay reproducibility of Stellar fluorescence detection, which generated <13% CVs for pAKT and <6% CVs for total AKT (TABLE 3). Collectively, these results show that Stellar NIR/IR modules for Jess enable the detection of multiple targets within the same capillary with excellent reproducibility.

NAME	PEAK AREA	CV	STD. DEV.
pAKT (Jess #1)	60666	9.6%	5799
pAKT (Jess #2)	73277	7.2%	5305
pAKT (Overall)	66972	12.6%	8416
Total AKT (Jess #1)	15405	4.8%	737
Total AKT (Jess #2)	14985	5.6%	837
Total AKT (Overall)	15195	5.3%	808

TABLE 3. Inter-assay and intra-assay reproducibility of Stellar fluorescence detection. Total AKT was detected by Stellar Mouse IR and pAKT was detected by Stellar Rabbit NIR.

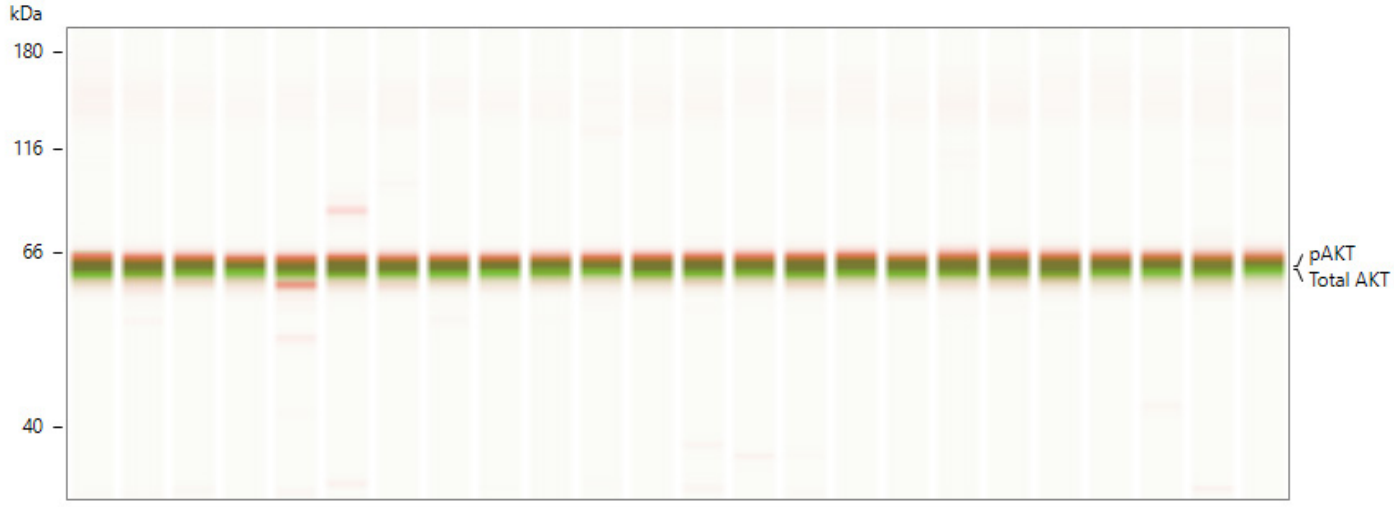


FIGURE 3. Multiplex analysis of total and phosphorylated protein pairs by Stellar IR and NIR fluorescence detection, respectively. Lysate from Jurkat cells treated with calyculin A and probed using mouse anti-total AKT with Stellar IR (green bands) and rabbit anti-pAKT primary antibodies with Stellar NIR (red bands). Overlapping signals are displayed as black bands. This analysis was performed on two Jess instruments and the inter-assay and intra-assay reproducibility values were calculated (TABLE 3).

PAIRING STELLAR NIR/IR DETECTION WITH STELLAR TOTAL PROTEIN DETECTION

Historically, the norm in protein normalization has been to use a housekeeping protein as a reference to quantify target protein expression. However, it is becoming increasingly clear that housekeeping proteins are unreliable controls that can change in response to cell stimuli, which is particularly problematic for studying cell signaling pathways, to name just one example. As a result, scientific journals are elevating their standards to require total protein normalization in place of housekeeping proteins to improve the quality and reproducibility of published results.[3]

When it comes to normalizing protein expression data on Simple Western, Jess users can rely on the Protein Normalization Assay Module (DM-PN02) for fluorescence-based total protein assays, or on RePlex assays with the Total Protein Detection Module for chemiluminescence-based total protein assays (DM-TP01). With the release of Stellar, Jess users now have the option to combine the Stellar NIR/IR Detection Modules with the new Stellar Total Protein Detection Module (DM-TP03) that performs chemiluminescence-based total protein assays simultaneously with Stellar NIR/IR detection of target proteins.

What makes the Stellar Total Protein Detection Module (DM-TP03) different from the traditional Total Protein Detection Module (DM-TP01)? First, the formulation of the Stellar total protein streptavidin-HRP detection antibody is unique to the Stellar assay for compatibility with the Stellar secondary antibodies. Second, Stellar total protein includes 2X luminol and 2X peroxide formulations to account for the different plate setup required for Stellar assays with total protein detection. As a result, the Stellar Total Protein Detection Module allows users to perform total protein normalization at low concentration ranges (0.005-0.2 mg/mL) simultaneously with sensitive detection of target proteins in Stellar NIR/IR channels. Because the Stellar Total Protein Detection and Stellar NIR/IR Detection Modules are separate from the Fluorescence Separation Modules, users have the flexibility to pair any Stellar Detection Module with any Fluorescence Separation Module with the desired molecular weight range (12-230 kDa, 2-40 kDa, or 66-440 kDa). It should be noted that the Stellar Total Protein Detection Module requires the use of a Stellar NIR and/or IR Detection Module in the same run.

To demonstrate how Stellar NIR/IR detection can be paired with Stellar Total Protein Detection, we loaded lysates from Jurkat cells that were either untreated or treated with calyculin A on Jess. For both sample types, we probed for pAKT and total AKT protein isoforms with the Stellar NIR/IR Detection Modules (in a similar manner as shown in **FIGURE 3**). In addition, we used the Stellar Total Protein Detection Module for the chemiluminescence-based total protein assay. As expected, total AKT was detected in both treated and untreated Jurkat samples, whereas pAKT was only detected in treated samples (**FIGURE 4A**). Furthermore, Stellar Total Protein Detection revealed the presence of many proteins in both treated and untreated Jurkat whole-cell lysates (**FIGURE 4B**). Representative overlaid electropherograms of treated cells multiplexed for pAKT, total AKT, and total protein Stellar detection are shown in **FIGURE 4C**. When normalizing the pAKT and total AKT expression levels to total protein content and using the untreated sample as a reference, the peak area values for pAKT and total AKT are adjusted accordingly (orange versus blue bars in **FIGURE 4D**). Compared with the raw data values, corrected values provided a more accurate measurement of the change in protein expression of pAKT (9-fold versus 14-fold) and total AKT (0.8-fold versus 1.2-fold) upon treatment. In summary, Stellar NIR and IR channels provide multiplex target protein detection simultaneously with Stellar Total Protein Detection in the chemiluminescence channel, producing more data per sample with accurate protein quantification.

Fluorescence 12-230 kDa Separation Module (SM-FL004)

Stellar Anti-Rabbit NIR Detection Module (DM-014)

Stellar Total Protein Detection Module (DM-TP03)

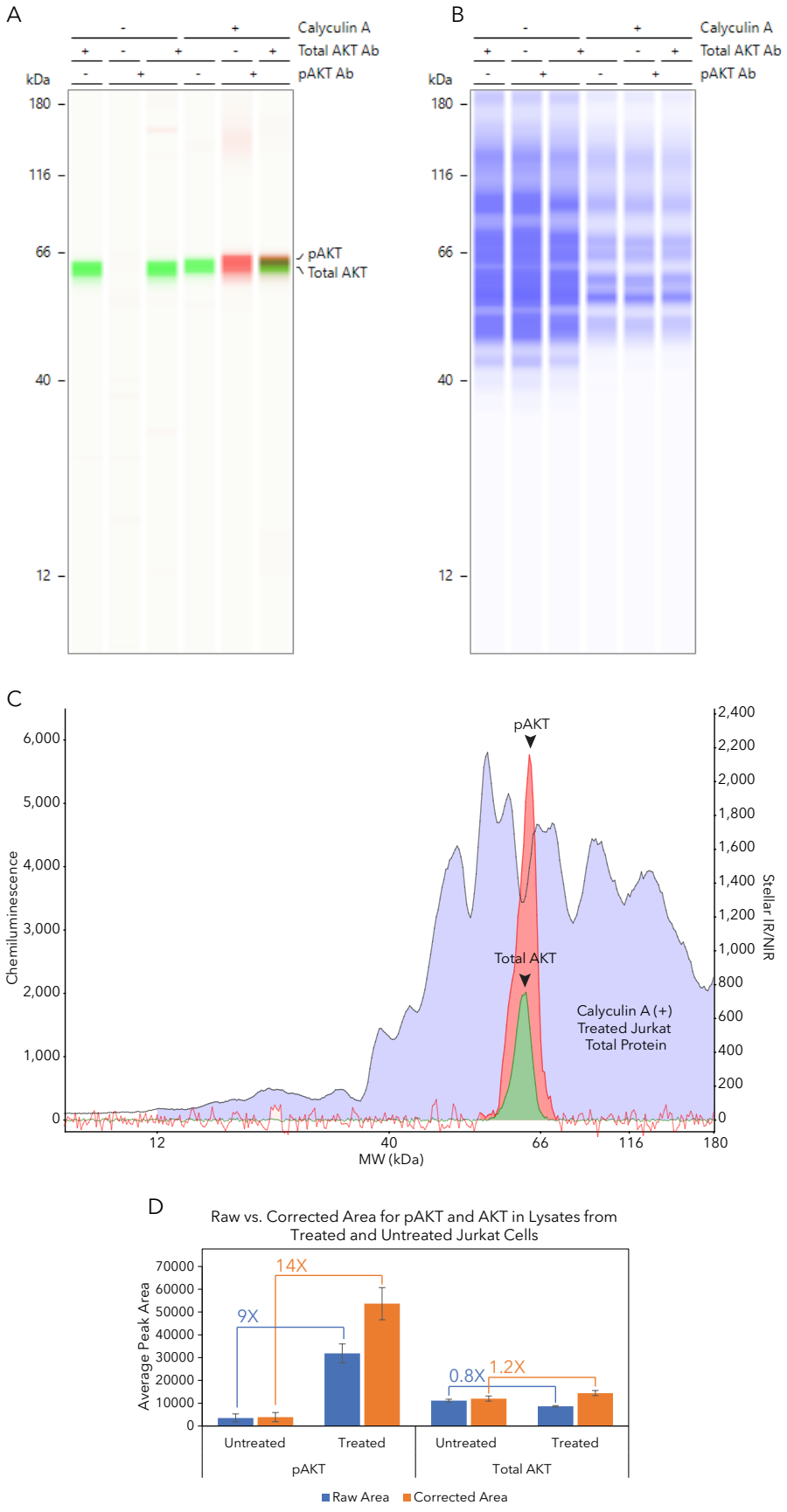


FIGURE 4. Multiplexed analysis of total AKT and pAKT with total protein normalization using the Stellar Total Protein Assay, all in the same sample. (**A**) Lysates from Jurkat cells either untreated (-) or treated (+) with calyculin A were analyzed at 0.25 mg/mL and 0.15 mg/mL final concentrations, respectively. Each lysate was probed for total AKT with Stellar IR (green bands) and pAKT with Stellar NIR (red bands). (**B**) The Stellar Total Protein Assay was simultaneously performed in the same lanes shown in panel A. (**C**) Overlaid electropherograms of Jurkat cells treated (+) with calyculin A and multiplexed for pAKT (red peak) and total AKT (green peak) detection with simultaneous Stellar Total Protein detection (blue peaks). The baseline threshold was set to 1, the window was set to 15, and the stiffness was set to 5. (**D**) Raw and corrected average peak area of pAKT and total AKT. The untreated sample was used as a reference to calculate fold changes in expression.

DETECTION OF MULTIPLE PROTEIN TARGETS BY STELLAR MULTIPLEX VERSUS REPLEX

Jess users seeking detection of multiple protein targets in the same capillary can do so by channel-based and size-based multiplexing, or they can use RePlex to run two sequential immunoassays in the same capillary. Here, we evaluated how Stellar NIR/IR multiplex detection compares with the sequential detection of multiple targets with RePlex. To do so, we loaded lysates from Jurkat cells untreated and treated with calyculin A on Jess and we detected total AKT and pAKT with RePlex, using Probe 1 of RePlex to detect pAKT and Probe 2 of RePlex to detect total AKT. Again as expected, pAKT was only detected in treated lysate samples, whereas total AKT was detected in both treated and untreated lysate samples (**FIGURE 5A**). As before, we also probed treated and untreated lysate samples with mouse anti-total AKT antibody in the Stellar IR channel and rabbit anti-pAKT antibody in the Stellar NIR channel. This Stellar analysis revealed detection of pAKT and total AKT that was virtually identical to the previous Stellar analysis above (**FIGURE 5B** vs. **FIGURE 4A**). Thus, both Stellar fluorescence and RePlex assays detected total AKT and pAKT targets within the same capillary. However, the Stellar fluorescence runs were completed in approximately 3 hours, while RePlex analysis took approximately 5 hours. Nonetheless, compared with traditional Western blot workflows which can span multiple days, these Simple Western methods dramatically reduce the time to generate high-quality multi-target protein expression data.

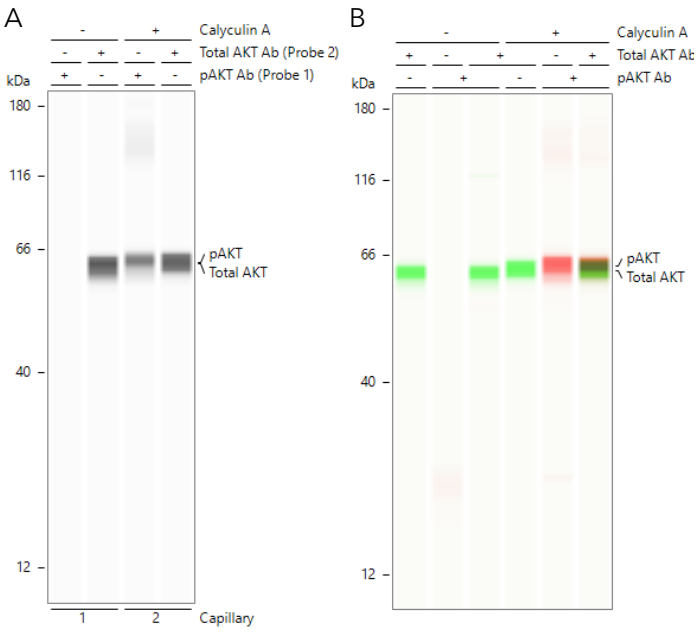


FIGURE 5. Analysis of multiple protein targets by RePlex with chemiluminescence detection and by Stellar IR/NIR multiplex detection. Lysates from Jurkat cells either untreated (-) or treated (+) with calyculin A were probed using mouse anti-total AKT and rabbit anti-pAKT primary antibodies. Targets were visualized using (**A**) RePlex with chemiluminescence detection of total AKT in the first probe and pAKT in the second probe and (**B**) using Stellar multiplex detection by Stellar Mouse IR (green bands) and Stellar Rabbit NIR (red bands).

THE FUTURE LOOKS BRIGHT FOR STELLAR PROTEIN ANALYSIS

In this Application Note, we provide empirical evidence that Stellar on Jess for Simple Western is the new leader in sensitive fluorescence-based detection and quantification of low abundance proteins in complex samples. With LODs as low as 0.4 pg, CVs consistently below 10-15%, and a 4-log dynamic range, we conclude that Stellar fluorescence detection is comparable to the widely recognized and highly regarded chemiluminescence detection. Unlike standard chemiluminescence detection, the multicolor detection flexibility Stellar offers channel-based multiplexing of protein targets, including targets with overlapping molecular weights, such as phosphorylated and total protein isoforms. With a 100-fold improvement in sensitivity over traditional fluorescence-based Western blot detection, the case for Simple Western as a leading protein analytical tool grows ever stronger. While we anticipate that the Simple Western Stellar assay will be broadly applicable in protein biochemical research, here we chose just one example application of Stellar to study cell signaling networks, which enabled the accurate and rapid multiplex quantification of phosphorylated and total protein isoforms in whole-cell lysates. With one example application provided here, it is tantalizing to consider all the other ways Stellar on Simple Western may advance future cutting-edge research.

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