Easy Transfer of Your Traditional Western Blot to Wes

Introduction

The Western blot has been around in essentially the same form since the late 1970s. A proteomics workhorse, Westerns are a key part of protein analysis in many labs. That said, the continuous manual intervention to run a Western blot is labor intensive and introduces many opportunities for error.

Wes™ builds on over a decade of Simple Western development and makes running a Western even more simple. Wes eliminates all the repetitive processing by automating all manual processes. Loading the "gel", monitoring the separation, "blotting", blocking, antibody incubations, washing, detection and even analysis of data is all done automatically. Assay kits come with a pre-filled microplate, so the only reagents you need to pipette are your samples, your antibodies, and your molecular weight ladder, everything else needed to run the assay is already in the plate. Wes also uses a capillary cartridge that is fully self-contained and stores all assay waste inside. At the end of the run, just toss the used plate and cartridge and you're done. The Compass software used to run Wes also allows you to run standard curves and back-calculate protein quantitation values for unknown samples. Wes not only gives you a fully automated instrument to run your Westerns on for up to 24 samples (plus a molecular weight ladder), he also gives you more from that data than ever before.

So, how do you design an assay for your protein of interest? Let's walk through the process using the protein kinase AKT as an example.

Methods

IDENTIFY YOUR ANTIBODIES

The first step in migrating a traditional Western assay to Wes is to select primary antibodies for testing. Assuming the protein target is already part of your research, it's likely you have primary antibodies in your freezer for Westerns and other applications. Whether you have your own antibodies or not, we recommend you search the Simple Western Antibody Database at proteinsimple.com to find out what other researchers have already learned about running Simple Western assays with their specific antibodies. While the Wes workflow and data output provide the same molecular weight data as a Western blot, the performance of any particular antibody may not be the same. You may also discover other antibodies that can offer advantages in terms of sensitivity, dynamic range, or specificity when used in a Simple Western assay compared to your traditional Western assay.

The Simple Western Antibody Database is an open repository containing hundreds of different antibodies with proven results for Simple Western assays. Over the last several years, countless users have provided these antibody conditions to help each other be successful and efficient at developing assays on Simple Western platforms.

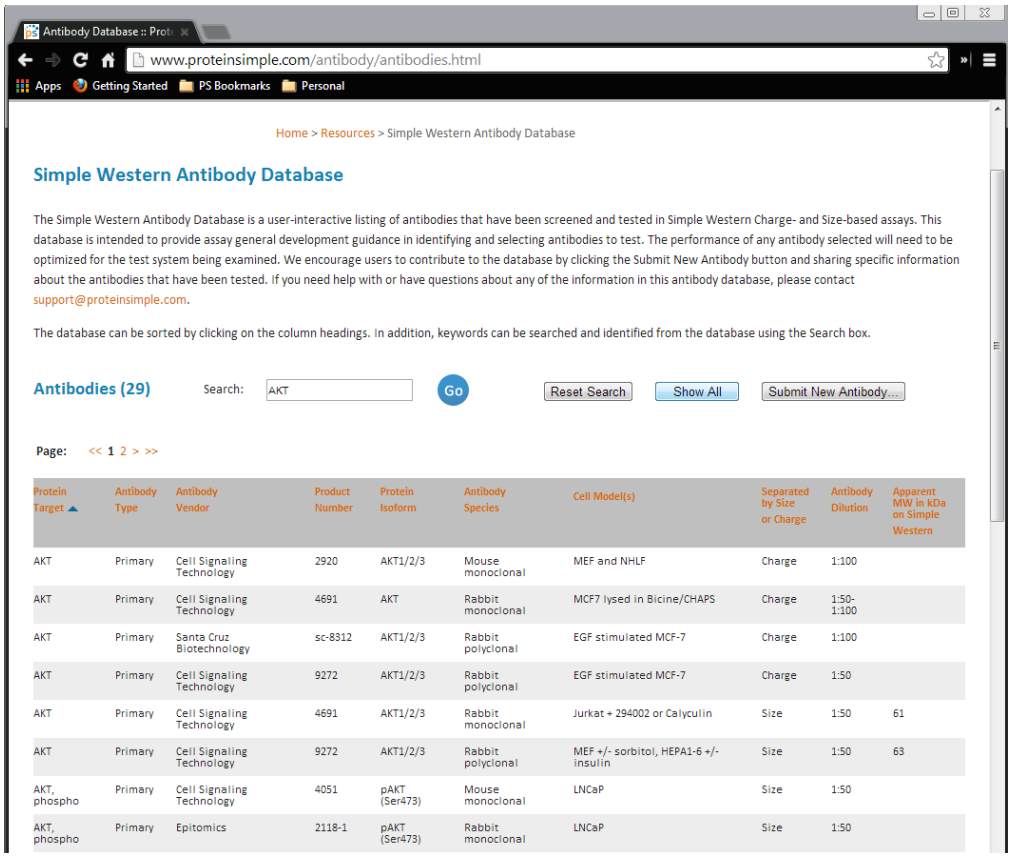

FIGURE 1. Simple Western Antibody Database query for AKT.

You can access the database via the **Resources** menu on proteinsimple.com, or at www.proteinsimple.com/antibody/antibodies.html. Access to this information is open to everyone. If you'd like to add antibody performance information to the database, please register on the ProteinSimple website.

To search for AKT-related antibodies, type AKT in the database **Search** field and click **Go** (**Figure 1**).

IDENTIFY YOUR MODEL SYSTEM

Generally, you will have a model system in mind to evaluate the assay consisting of a positive and negative control for activation or expression. If not, control lysates can be purchased from a number of biological supply companies. In this case, Jurkat lysate positive and negative controls for AKT phosphorylation were purchased from Cell Signaling Technology® (CST, P/N 9273).

SCREEN YOUR ANTIBODIES

The first experiment in optimizing a new Wes assay is the initial antibody screen. The goal of the antibody screen is to evaluate the antibodies in terms of signal and specificity to the protein target. This allows you to choose the most promising antibodies to use throughout the rest of the optimization process. After checking the antibody database, we identified two total AKT antibodies (CST, P/Ns 9272 and 4691) and three phospho-S473 antibodies (CST, P/Ns 4058 and 4060, and Abcam® P/N Ab81283).

The initial screen should be done with an antibody dilution of 1:50 (a mere 4.8 µL for 24 wells), as no optimization has been performed yet. This leaves the antibody concentration high, while still allowing sufficient dilution of the storage buffer. For lower affinity antibodies and low abundance proteins, a 1:25 dilution may be used.

In this example, the positive control for phosphorylation (calyculin-treated cells) was used so that total AKT and

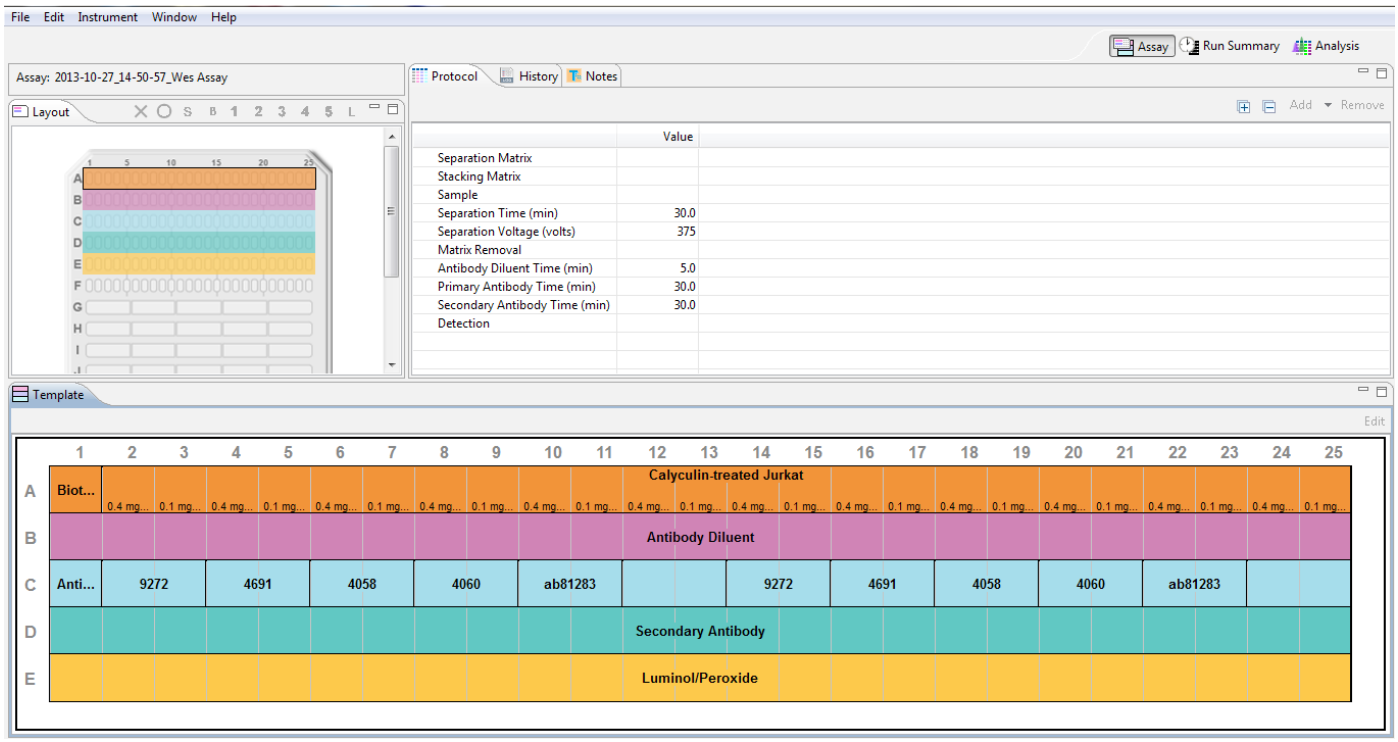

FIGURE 2. Wes assay microplate template in Compass for antibody screening experiment.

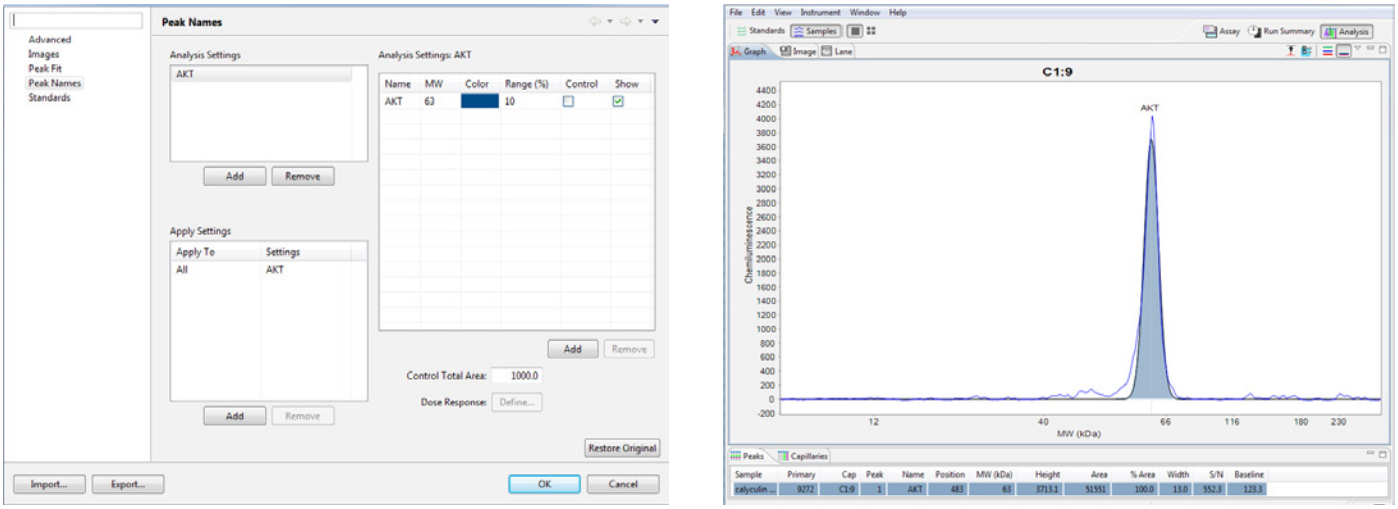

FIGURE 3. Peak naming and molecular weight table (left) and data peak labeling (right) in Compass.

pS473 antibodies could be evaluated with the same lysate. Two concentrations of lysate, 0.4 mg/mL and 0.1 mg/mL, were chosen for testing. The assay was set up in Compass software as shown in **Figure 2**. We then loaded the assay microplate and capillary cartridge into Wes and started the assay.

Once the run is complete, you can easily define peak names in the **Analysis** tab in Compass software by entering a peak name and molecular weight in the **Peak Names** table. Compass will label bands and tabulate peak areas. In our sample experiment, Compass labeled a band at 63 KDa "AKT" (**Figure 3**).

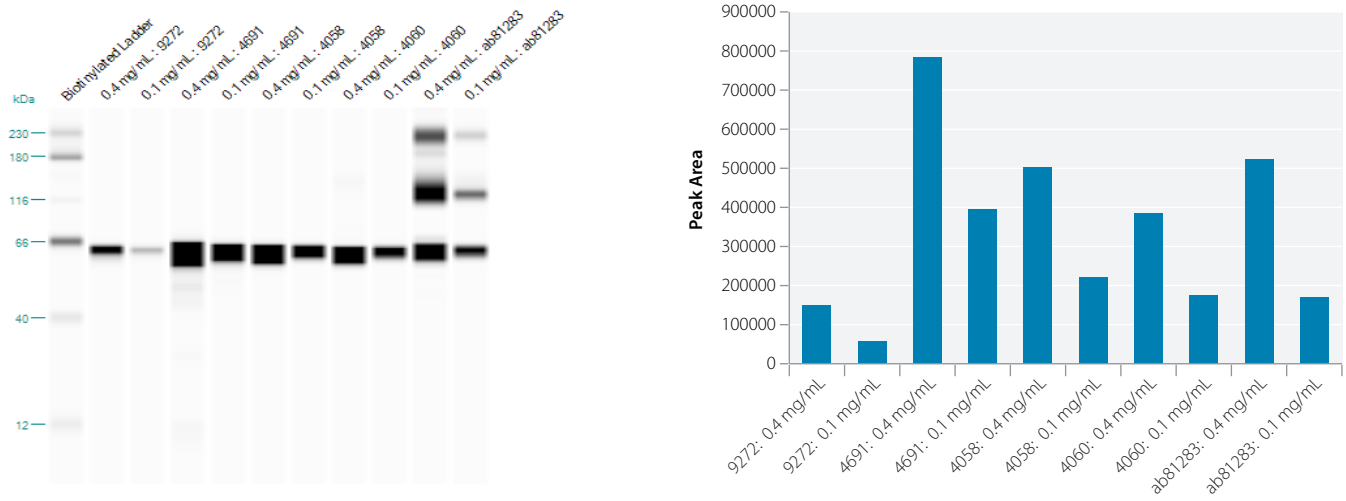

FIGURE 4. Choosing an antibody. Lane view of our antibody screen (left). Total AKT antibodies (CST, P/Ns 9272 and 4691) and phospho-S473 antibodies (CST, P/Ns 4058 and 4060, and Abcam® P/N Ab81283) peak area (right).

From the evaluation of our total AKT antibodies it was clear that CST 4691 had a higher signal compared to CST 9272 (**Figure 4**). Among the pS473 antibodies, the signal was similar in all three tested. Ab1283 generated a large number of non-specific bands. While this could be reduced during optimization, we decided not to move forward with Ab1283 since it would be easier to start with a more specific antibody. To confirm which of the remaining two antibodies (CST 4058 and 4060) to use, we tested both using the negative control lysate (data not shown). Based on results from this assay, we chose to proceed with CST 4060.

RUN A LYSATE DILUTION

Now that a total AKT (CST 4691) and a phospho-specific AKT (CST 4060) antibody have been selected, the next step is to run a lysate dilution experiment to confirm the optimal concentration of lysate. The lysate dilution is necessary to ensure that the protein being interrogated is within its optimal dynamic range on Wes, meaning it must be in a range where an increase or decrease in its concentration will lead to an increase or decrease in material bound to the inside of the capillary.

In our sample experiment, the 1:50 concentration of antibody was used again. This concentration was necessary to make sure the protein would be saturated with antibody, confirming that the chemiluminescent signal is indicative of the amount of protein bound. Once the linear range of protein binding is established, the antibody concentration can be optimized.

The positive control for phosphorylation was also used, so both antibodies could be run using the same lysate. The concentration of Jurkat lysate in our experiment ranged from 0.8–0.003 mg/mL.

Linear regression analysis confirmed that the chemiluminescent signal continued to increase linearly up to 0.1 mg/mL, and then plateaued for both antibodies (**Figure 5**). Based on these results, the final optimization experiment will need to be run with 0.1 mg/mL lysate to maximize signal while staying within the linear range of the protein.

DETERMINE THE OPTIMAL ANTIBODY DILUTION

The final step in our optimization is to determine the optimal antibody concentration. In order to generate comparable data for all samples, it is important that the antibody concentration be sufficient to saturate the protein bound to the capillary wall. This will allow a quantitative comparison of signal between samples.

Once again, in our sample experiment both antibodies were run against the positive control lysate. The antibody dilution range used for this optimization step was from 1:25 to 1:800.

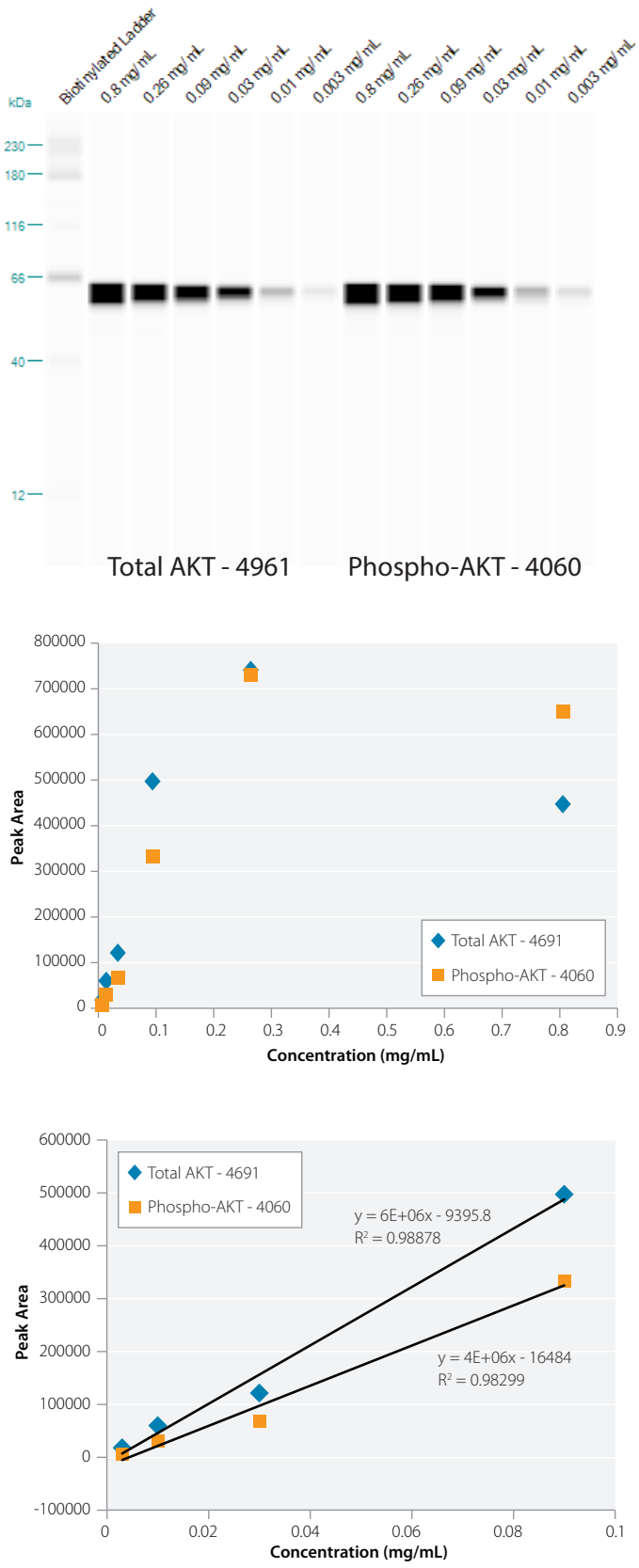

FIGURE 5. Optimizing the lysate concentration.

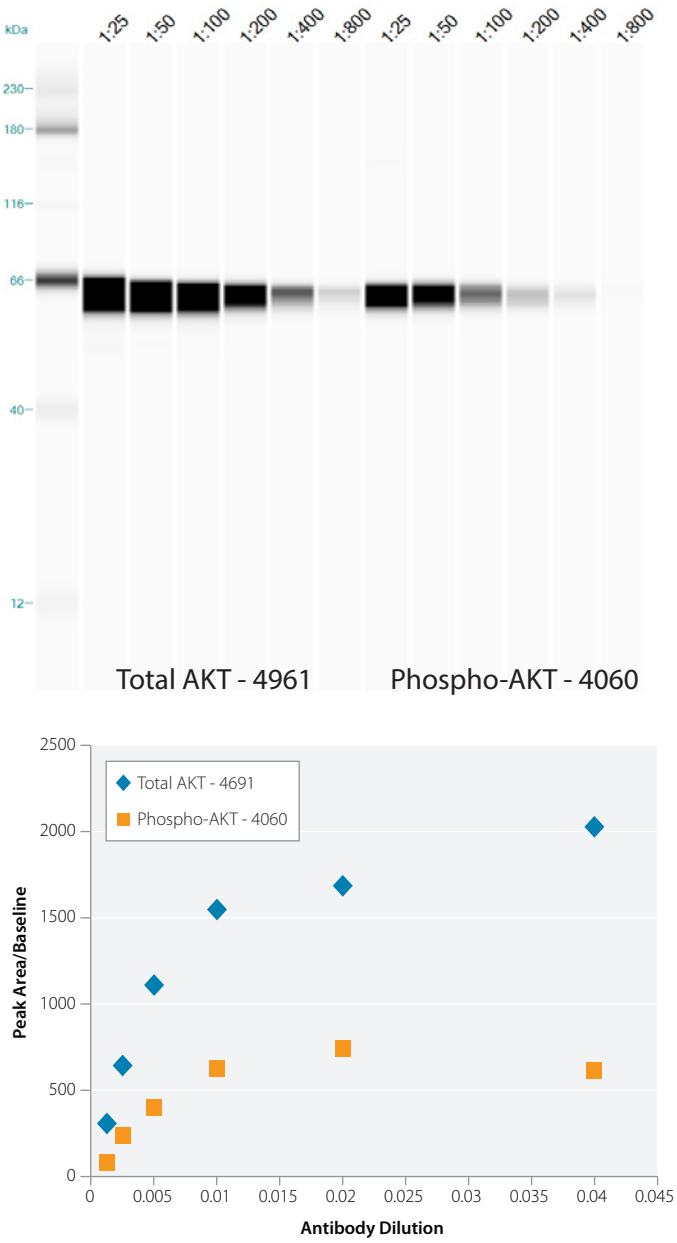

FIGURE 6. Optimizing the antibody concentration.

As the antibody concentration increases, the signal increases until the point of saturation (in this case, at the 1:50 dilution, approximately). To have a reliable, quantitative assessment of protein concentration, an antibody concentration near this saturation level needs to be used. Once that concentration has been identified, our final antibody and lysate condition is optimized and the assay is ready to use with any of our samples.

RUN YOUR OPTIMIZED ASSAY

We used all the information gathered from our previous optimization experiments to set up a final experiment that will look at changes in AKT phosphorylation when Jurkat cells are treated with calyculin. After setting up the assay plate using our optimized conditions and inserting the capillary cartridge and plate into Wes, we ran the assay.

Using a concentration of 0.1 mg/mL for the Jurkat lysates and a 1:50 dilution for the total AKT (CST, P/N 4691) and phospho-AKT (CST, P/N 4060) antibodies, we were able to confirm that treatment with calyculin induces a 280-fold increase in phosphorylation of AKT in Jurkat cells (**Figure 7**).

Conclusion

Wes takes the benefits of Simple Western assays a step further by simplifying their workflow with his pre-filled plate and disposable capillary cartridge. As with all Simple Western assays, those run on Wes dramatically streamline the assay development process compared to cumbersome, traditional Western blot assays. Completing three simple optimization steps over the course of a single day resulted in an optimized assay that was ready to use with any target sample. Simple your Western and enjoy working with Wes.

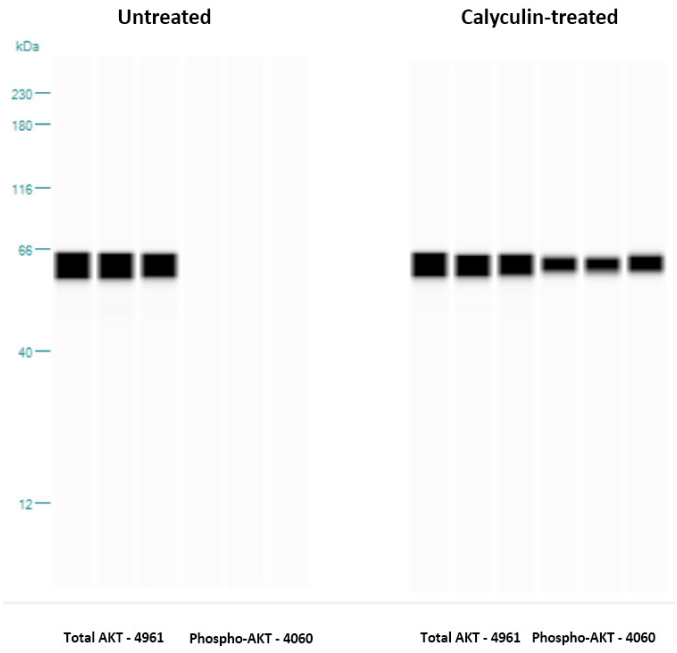

FIGURE 7. Analysis of lysate from untreated (left) and calyculin-treated (right) Jurkat cells using the optimized lysate concentration and antibody dilution.

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