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Simple Western
Automated Solutions

Bringing the Western Blot into the 21st Century with Automated Solutions

Evaluating the performance advantages of the Simple Western platform

The traditional Western blot has been around since 1979 and hasn't changed much since its introduction. A few steps have been streamlined, such as pre-cast gels and faster blotting devices. However, it's still a lengthy and laborious analytical process that can introduce many opportunities for error. You can expect at least six hours from start to finish and sometimes up to two days of work.

About 14 years ago, ProteinSimple, a Bio-Techne brand embarked on a mission to modernize the Western blot with **Simple Western™ technology**—an automated capillary western that uses capillary electrophoresis for protein separation followed by immunoassay-based detection within a fully enclosed system. This innovative system simplifies and automates the traditional western workflow, significantly reducing the processing time to about three hours with minimal hands-on time. In contrast to Western blots, which are often qualitative and poorly reproducible, Simple Western delivers quantitative and highly reproducible data with a dynamic range of up to 6 logs and CVs consistently below 15%. Moreover, Simple Western requires only a fraction of the sample needed for analysis, as little as 3 µL, further enhancing its efficiency.

The **Jess™** system, powered by Simple Western Technology offers versatile multiplex detection strategies in chemiluminescence and fluorescence channels. With the Simple Western™ **Stellar™** NIR/IR assays, Jess can offer industry-leading detection sensitivity that is 100X more sensitive than traditional fluorescence Western blot. Additionally, Simple Western's **RePlex™** assay enables sequential immunoprobing or **total protein** detection in the same capillary, eliminating the strip and re-probe method of traditional Western blotting. With samples covalently immobilized to the capillary wall, RePlex assays ensures complete and reproducible removal of antibodies between probing cycles. This feature prevents signal interference or sample loss between probing cycles.

A Simple Western Comparative Analysis

In this article, we examine the Simple Western platform by comparing the Simple Western workflow to a traditional Western blotting workflow. Then, we'll dive into a few application examples. We'll start with a quick review of how to set up the Simple Western platform and run the process. First, reagents are prepared just like they would be for a traditional SDS-PAGE and Western blot. Next, our plate is filled with samples, antibodies, detection reagents, and wash buffer. Lastly, the capillary cartridge and reagent plate are inserted into the Simple Western instrument, and the Start button is activated. It's that easy.

Now, let's look at what happens inside the instrument after we initiate our run in the Simple Western platform. Both protein separation and immunoassay detection take place in a capillary. Your sample, primary and secondary antibodies, as well as separation and running buffers, are automatically loaded from a specially designed plate. Next, your protein sample is loaded, and capillaries are lowered to contact the running buffer. Voltage is applied to **separate proteins by molecular weight**. Once the separation is complete, UV light immobilizes the protein on the capillary wall. With proteins immobilized and separation materials clear from the capillaries, Simple Western starts the immunoprobing process. Samples are first incubated with a primary antibody, and then in the case of chemiluminescence, with an HRP-conjugated (horseradish peroxidase) secondary antibody, and finally, a chemiluminescence substrate. A CCD camera records the chemiluminescence reaction in a series of images over time. The simple protocol means less hands-on time and a more efficient workflow. Once you hit start on a run, there's no need for additional supervision or manual intervention. The **Compass™ Software** for Simple Western™ platforms allows for data curation in one program. The Simple Western platform has completely automated and streamlined the Western blot, which means faster time to results.

Simple Western doesn't sacrifice data quality to achieve its speed advantage. Simple Western provides excellent reproducibility, not just within a run but also across multiple users and days. Figure 1 shows the same **Simple Western assay** run on Monday (in blue) and Tuesday, Wednesday, and Thursday, each with a different user. In this example, we measured the average ERK1 area daily in Hela Lysates. The percent CV of the ERK1 average area is set on each bar graph. As you can see, the day-to-day average of the ERK1 area doesn't change significantly, and overall, this group generated a CV for all ERK1 data points across four days under 10%. This sort of user-to-user and day-to-day consistency means Simple Western can confidently be adopted where traditional Western blots falls short, like in analytical development groups. In addition to its exceptional reproducibility, Simple Western offers superior sensitivity, which helps reduce sample volume requirements. Figure 2 compares traditional Western and Simple Western for GSK 3 Alpha signal detected in HEK293 lysates. Traditional Western blot (shown in blue squares) can only detect GSK3 Alpha in as low as 10 micrograms per mil of HEK293 lysates. In contrast, Simple Western (shown in blue circles) using the same lysate can detect the same target down to 100 picograms per mil of HEK293 lysate. As this example shows, using chemiluminescence detection, Simple Western has over 4 logs of dynamic range while traditional Western blot has only 2.5 logs.

FIGURE // 01

Excellent Reproducibility Across Multiple Users & Days

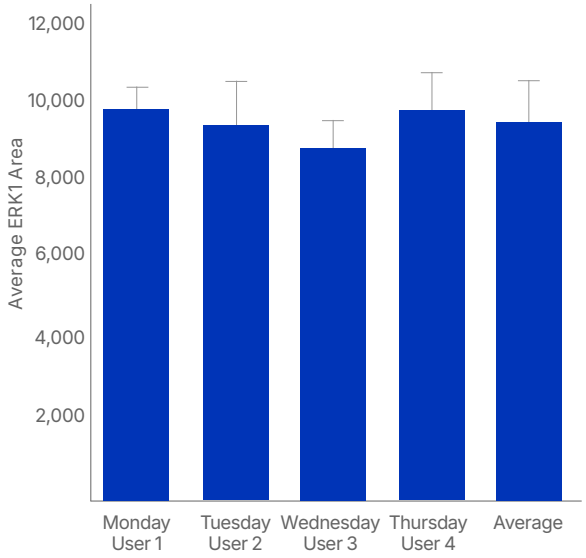


Figure 1. ERK1 expression levels measured using a Simple Western assay by multiple users on separate days. The graph displays all recorded ERK1 data points across four days and shows that the coefficient of variation (CV) for ERK1 levels is less than 10% between users.

FIGURE // 02

Superior Sensitivity Means Less Sample

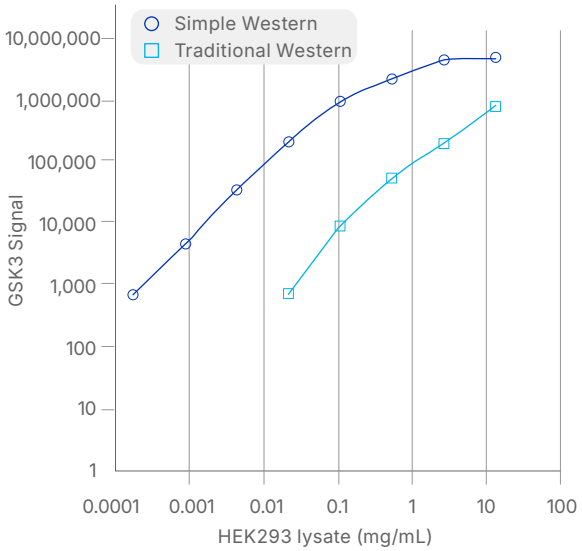


Figure 2. Comparison of GSK3 signal detection in HEK293 lysates by Simple Western and traditional Western blot methods. The graph demonstrates that Simple Western assay has a greater than 4 logs dynamic range for detecting GSK3 signal, whereas traditional Western blot methods using chemiluminescence detection have only a 2.5 logs dynamic range.

Exceeding the limitations of a traditional Western Blot

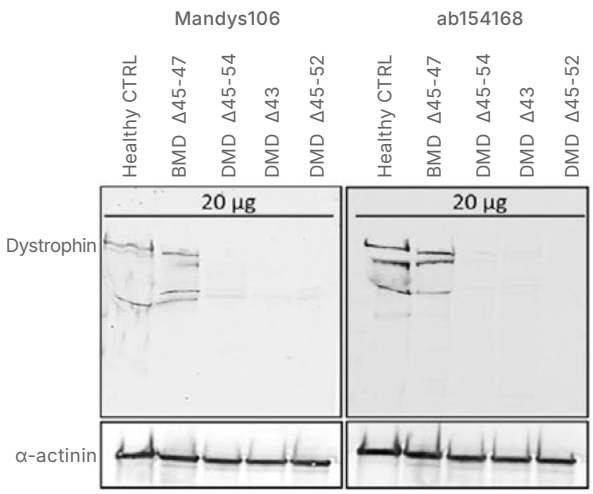
Let's take another look at a real-world example to drive this point home (Figure 3). In this example, traditional Western blot struggles to provide measurable results, whereas Simple Western provides a more robust analysis. In this application, researchers looked at a series of healthy control samples and **Duchenne and Becker muscular dystrophy** (DMD and BMD) samples, which have lower levels of dystrophin expression.[1] Twenty micrograms of protein were loaded per lane, (Figure 3). As you can see for the traditional Western, for the full-length dystrophin peak, it's difficult to measure expression levels in the DMD samples, and it's equally challenging to compare the DMD and healthy controls for any differences. Compare that with the Simple Western analysis below, which shows significantly better banding patterns for dystrophin and 20-fold less protein, which is critical when dealing with precious samples like these.

FIGURE // 03

Traditional Western vs Simple Western

Traditional Western

A



Simple Western

B

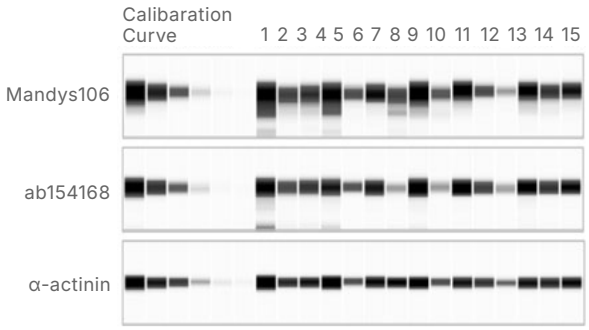


Figure 3. Dystrophin protein expression was assessed using two different methods: traditional western blot (A) and Simple Western (B). In the traditional western blot analysis, 20 µg of protein was loaded per sample lane. For the Simple Western analysis, 1.25 µg of protein was loaded per sample lane for DMD samples and 0.25 µg for healthy controls.

Another important feature of Simple Western is its quantitation capabilities. Simple Western provides two levels of quantitation. First, let's look at relative quantitation (Figure 4). We looked at AKT signaling in a previous **application note** on Stellar™ protein analysis.[2] Shown on the left is an example of Jurkat cell lysates from cells treated with calyculin A phosphatase inhibitor. Here, we're looking at a multiplex of total AKT, shown in green, and phospho AKT in red. In the background, shaded in gray, is the

total protein signature of the cell lysate, which is used for normalization between samples. On the right is an example of the fold change between untreated and calyculin A-treated Jurkat cells. In this case, a 9 and 14-fold increase in AKT phosphorylation is observed with no change in total levels. As the graph illustrates, two sets of blue and light blue bars correspond to the raw immunoassay data and cellular protein normalized data, respectively.

FIGURE // 04

Relative Quantitation: Fold Change Between Samples/Treatments

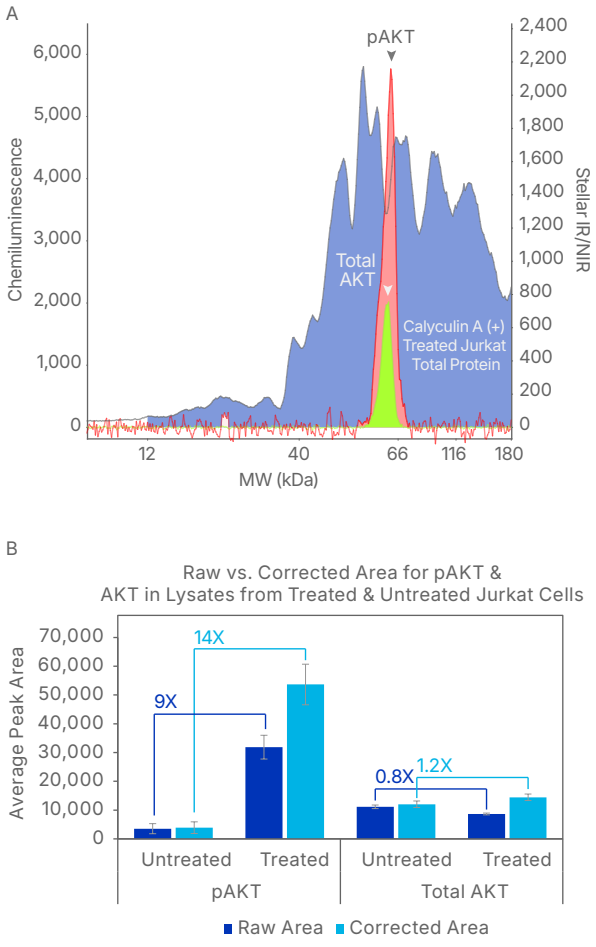


Figure 4. Multiplexed analysis of total AKT and pAKT with total protein normalization using the Stellar Total Protein Assay, all in the same sample. (A) Overlaid electropherograms of Jurkat cells treated (+) with calyculin A and multiplexed for pAKT (red peak) and total AKT (green peak) detection with simultaneous Stellar Total Protein detection (blue peaks). (B) 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.

These results provide a compelling example of how researchers use the platform to measure changes between samples and treatments.

For many applications, relative full-scale analysis is sufficient and ample Simple Western data is published using that approach. However, Simple Western can also provide a deeper level of quantitation. This next **example** shows how Simple Western can provide absolute target concentration in a **complex sample**.[3] For this, we need to start with a recombinant protein standard. Here (Figure 5), we are looking at a titration of a Recombinant PD-1 detected with Anti PD-1 antibody, both from Novus Biologicals, a Bio-Techne brand. The PD-1 protein, which runs at approximately 116 kDa, was titrated from 10 ng/mL to 0.16 ng/mL. Building a standard curve based on the Recombinant PD-1 area under the curve, we can appreciate a highly linear relationship between PD-1 concentration and area, including an R-squared value greater than .997.

FIGURE // 05

Recombinant Protein Standard Curve

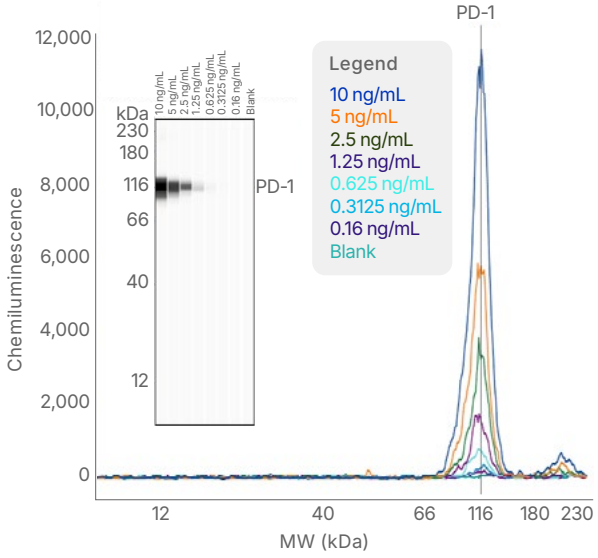
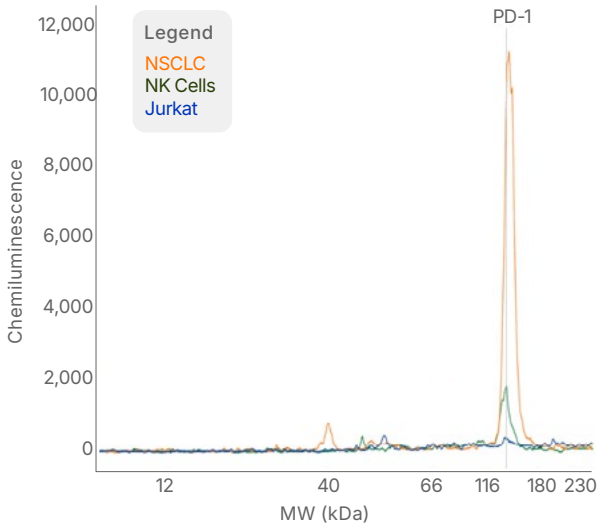


Figure 5. Simple Western detection and quantification of PD-1. Overlaid electropherograms of the 2X serial dilution series of recombinant PD-1.

FIGURE // 06

Absolute Quantitation: PD-1 Expression in Cell Lysates



Cell Lysate	Average	Std. Dev	% CV
Jurkat	5,812	187	3.2%
NSCLC	128,405	4,576	3.6%
NK Cells	19,816	1,172	5.9%

Figure 6. PD-1 detection in tumor cells and immune cells. Shown are overlaid electropherograms of PD-1 detection in Jurkat, NSCLC, and NK cells. The table shows the average area, standard deviation, and % CV of the peak area from 3 replicates.

Next, we examined samples to measure PD-1 expression (Figure 6). These included non-small lung cancer cells (NSCLC) shown in orange, NK cells in green, and Jurkat cells in blue. The area under the curves was calculated for each PD-1 peak. Using the standard curve, we can interpolate the PD-1 peak areas for both non-small cell lung cancer samples, again in orange, and the NK sample in green. These data show that the non-small cell lung cancer lysate loaded at 1 mg/mL contains 5.21 ng/mL of PD-1, while NK cells only contain 0.68 ng/mL of PD-1. This example shows how Simple Western can provide absolute target concentration in a complex sample like cell lysate while also providing size information about the target of interest.

In this next example, we showcase Simple Western's **multiplexing** feature. In a recent **application note**, we looked at signaling iPSCs and differentiated cells into mesoderm and ectoderm lines on Simple Western using antibodies from Cell Signaling Technology (CST).[4] These antibodies make three target pairs for total phospho-AKT, total phospho-ERK, and total phospho-RS6 protein. The data in Figure 7 are from a single Simple Western run where we leverage multiplexing with Stellar Fluorescence Modules. We simultaneously ran for both total and phospho-AKT (shown on the top left), total phospho-ERK (shown in the middle left), and total phospho-RS6 (shown on the bottom left). The total protein signatures for each cell sample are set in blue behind each multiplex detection series. The total protein is measured automatically in the same capillary as the immunoassay and is ideal for normalizing data between treatments.

We went a step further to also look at treatments for each lineage, where we compared either the addition of small molecules from our Tocris brand, including CHIR 99021, Noggin, and SB 431542, or time points of differentiation in the case of endodermal cells. The total target signals are in blue, and the phospho-signals are in green, showing either activation or inactivation of these three signaling molecules. Overall, we can see the analysis of eight samples for six targets simultaneously in one run with total protein analysis. Imagine experimenting with traditional Western blotting, where you would need several gels and blots and stain each membrane with Ponceau S for total protein before your Western blot experiment even starts.

FIGURE // 07

Quantitative Protein Expression with Multiplexing and Total Protein Detection

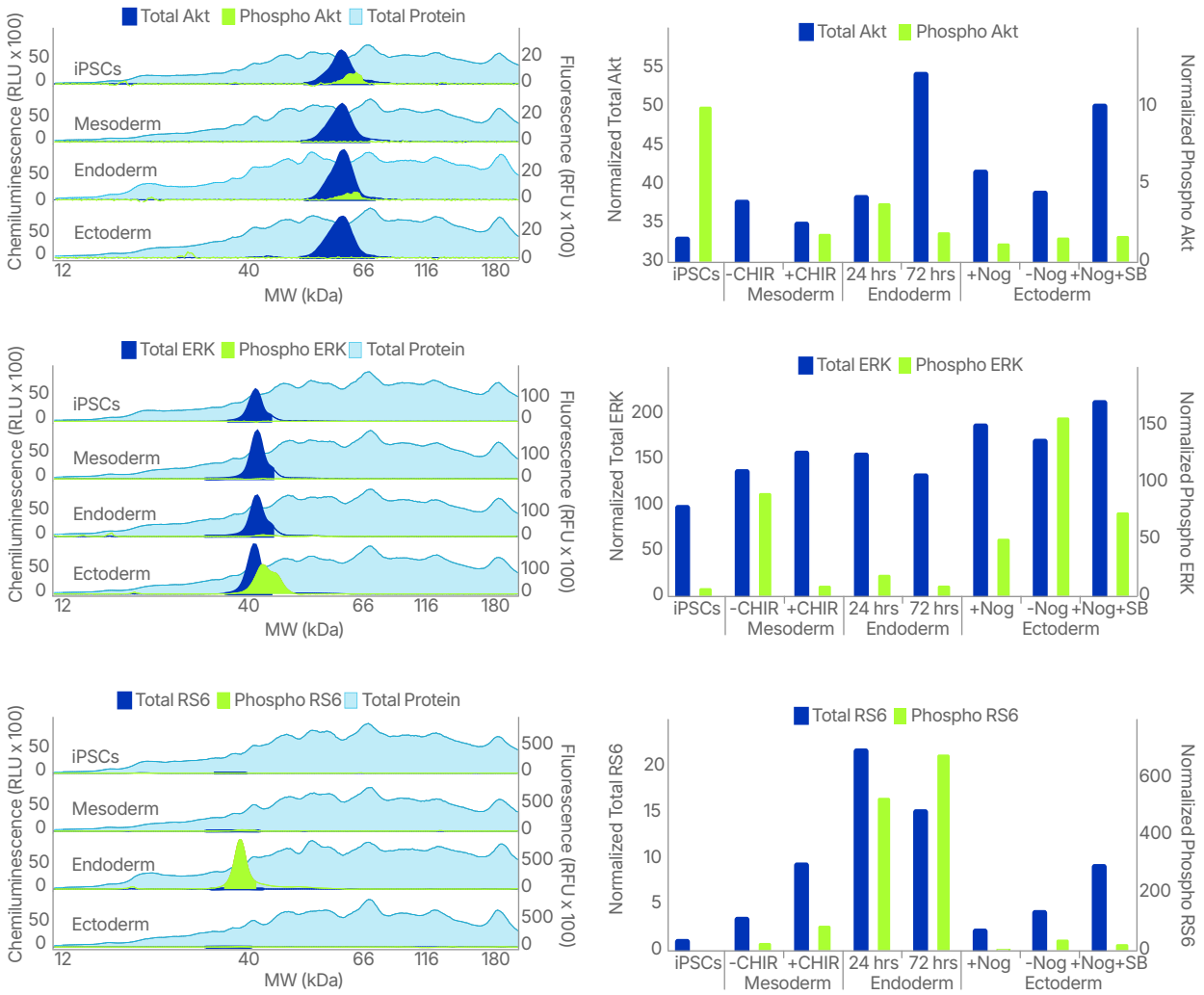


Figure 7. Simple Western analysis of total and phospho-protein isoforms of Akt, ERK, and RS6 across undifferentiated iPSCs and iPSC-derived mesoderm, endoderm, and ectoderm samples. Total and phospho-protein isoforms were detected in Stellar NIR/IR channels, and total protein detection was detected using chemiluminescence. Overlaid electropherograms are shown on the left, and protein expression values normalized to total protein are shown on the right.

Analyzing the performance of popular CST's antibodies on Simple Western platform

Before initiating a recent series of experiments using Cell Signaling Technologies (CST) antibodies on the Simple Western platform, CST worked closely with Bio-Techne to develop a pilot cohort of 100 CST antibodies validated for traditional Western blotting. The antibodies in the pilot cohort represented common protein targets. From the 100 CST antibodies in the pilot cohort, 83 were successfully validated for Simple Western. In this first example, we document the performance of CST's Stat3 Mouse monoclonal antibody on the Simple Western platform. In (Figure 8) we have a traditional Western blotting assay probing various human cell lines using CST's Stat3 Mouse monoclonal antibody. You can see this antibody generates a strong band in human cell lines. Overall, this antibody performs well when used in traditional Western blotting.

Let's compare the results using the same Stat3 Mouse monoclonal antibody on Simple Western. On the right, we have our Simple Western analysis of serum-starved HeLa cells treated with INF-α. The virtual band view on the left shows a strong, clean band for Stat3 at a 1-to-10 and 1-to-50 dilution of the primary antibody. On the right, we calculate chemiluminescence by molecular weight along the capillary. We see a very high signal-to-noise ratio and a very low baseline. The results show the Stat3 Mouse monoclonal antibody performing exceptionally well on the Simple Western system. At the recommended dilutions, you can expect these antibodies to provide an excellent foundation to optimize your assay in the Simple Western platform further. We documented similar results for the CST Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit and Phospho-Stat3 (Tyr705) antibodies, showing very strong performance of these antibodies on the Jess instrument.

These results provide a reliable reference point for transitioning from traditional Western blotting to Simple Western using CST's antibodies. Over 300 CST's antibodies have been validated on Simple Western, and more than 700 antibodies are from customer submissions in the Bio-Techne antibody database. Moving forward, CST plans to continue its collaboration with Bio-Techne as it looks to introduce more CST-validated antibodies for the Simple Western platform.

FIGURE // 08

Comparison between Traditional Western & Simple Western Analysis

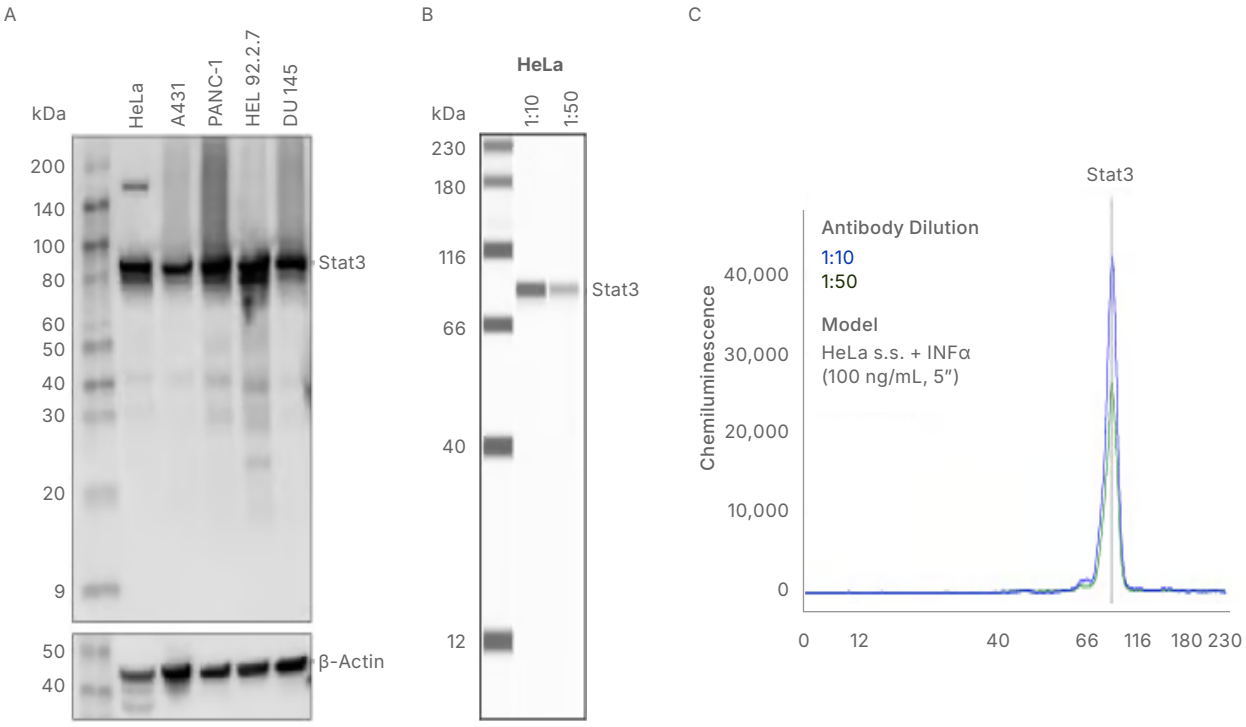


Figure 8. Performance of CST's Stat3 (124H6) Mouse mAb #9139 using a traditional Western blot (A) and on Simple Western (B, C).

References

1. Beekman C, Janson AA, Baghat A, van Deutekom JC, Datson NA. Use of capillary Western immunoassay (Wes) for quantification of dystrophin levels in skeletal muscle of healthy controls and individuals with Becker and Duchenne muscular dystrophy. PLoS One. 2018 Apr 11;13(4):e0195850.

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