Simple Western analysis of Signaling cascade proteins

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Abstract

Proteins in the AKT signaling cascade are associated with tumor cell survival, proliferation, and invasiveness. The activation of AKT is also one of the most frequent alterations observed in human cancer and tumor cells. Understanding how AKT signaling impacts disease progression is important to the development of novel therapeutics. Cell signaling events are routinely assessed using traditional Western blot analysis. The Western blot technique is very labor intensive and generally yields results that are semi-quantitative. The Simple Western platform described here completely automates the manual steps involved in traditional Western blot protocols and can analyze up to 96 samples in a single experiment. Because Simple Western protocols consume only microliter sample volumes, reproducible and quantitative results can be generated from precious or quantity-limited samples.

We present results generated on Sally, the higher throughput platform from ProteinSimple. Sally is able to run up to 96 data points in a single experiment, addressing the needs for higher throughput applications in screening signaling pathways. Data generated on Sally demonstrates high reproducibility and intra-assay variability of less than 10%.

Seven targets from the AKT pathway, were screened with both anti -total and anti-phospho antibodies on the Simple Western platform from a total volume of 5 µL/treatment. In response to Insulin treatment, significant and expected signal changes were clearly observed for each of the key targets. This suggests the potential to characterize whole signaling pathways with as little as 5 µL of sample. Results and workflow comparisons indicate a distinct advantage of the Simple Western when compared to traditional Western methods.

Conclusion

- The Simple Western permits the rapid and quantitative high throughput analysis of signaling pathways at a level of efficiency and reproducibility not obtainable by traditional Western blots.
- The platform's ability to sample and generate multiple data points from extremely small sample volumes enables new approaches to the characterization of biomarker targets involved in the progression of disease states.

Figure 1: Sally

Assay Principles

Sally is a bench top instrument capable of running up to 12 samples per cycle simultaneously with Simple Western assays, which are size-based assays equivalent to SDS-PAGE (Figure 1). Samples for Simple Western assays are treated with SDS/DTT and heat denatured. Samples are then loaded into capillaries, separated by size and immobilized to the capillary wall via a proprietary UV capture method. Target proteins are immunoprobed with an antibody followed by HRP-amplified chemiluminescent detection (Figure 2). Sally is able to run up to 96 samples in one experiment with the ability to generate 8 data points from the same microliter volume of lysate in a single experiment.. Sally automates the entire Western blot procedure which results in increased reproducibility and significant time savings.

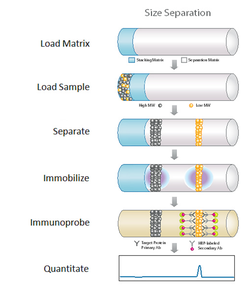

Figure 2: Steps of a Simple Western Assay

Assay Workflow

Traditional Western	Simple Western
Load samples in PAGE	Load plate in Sally
Transfer sample to membrane	Start instrument
Block membrane	Integrated data analysis
Incubate with primary antibody	
Wash (3x 5-10 min)	
Incubate with secondary antibody-HRP	
Wash (3x 5-10 min)	
Incubate with enzyme substrate	
Expose	
Manual data analysis	

Figure 3: Workflow comparison for Traditional Western and Simple Western

Simple Western Assay Specifications

Description	Specifications
Sample required	~3–5 µg
Samples per cycle	12
Number of cycles	Up to 8
Sample throughput	Up to 96
Run time	Up to 19 hours
Sizing range	15–150 kDa
Sizing accuracy	+/– 20%
Sizing CV	10%
Resolution	+/– 10% difference in molecular weight
Quantitation CV	≤ 20%
Dynamic Range	~3 logs
Sensitivity	Low ng

Figure 4: Simple Western Assay Specifications

AKT Pathway Mapping Using Small Sample Volumes

Sally enables mapping of the AKT pathway signaling cascade using only 5µL sample per treatment condition as shown in Figure 5.

Insulin and other growth factors activate the PI3K pathway which leads to the phosphorylation and activation of AKT. In turn, AKT phosphorylates PRAS40, preventing it from inhibiting mTORC1. AMP-activated kinase (AMPK) can also have a regulatory role in the mTORC1 pathway. When the AMP:ATP ratio increases, AMPK phosphorylates TSC2 and RAPTOR, leading to inhibition of mTORC1. p70S6 kinase is a well characterized target of mTORC1. mTORC1 phosphorylatesS6K1 on a threonine residue (T389). This event stimulates the subsequent phosphorylation of S6K1 byPDK1. Active S6K1 can in turn stimulate the initiation of protein synthesis through activation of S6 Ribosomal protein (a component of the ribosome) and other components of the translational machinery. cRAf is mainly known as a member of the Raf-MEK-ERK pathway, but has been shown to be directly phosphorylated by AKT, an event that induced proliferation.

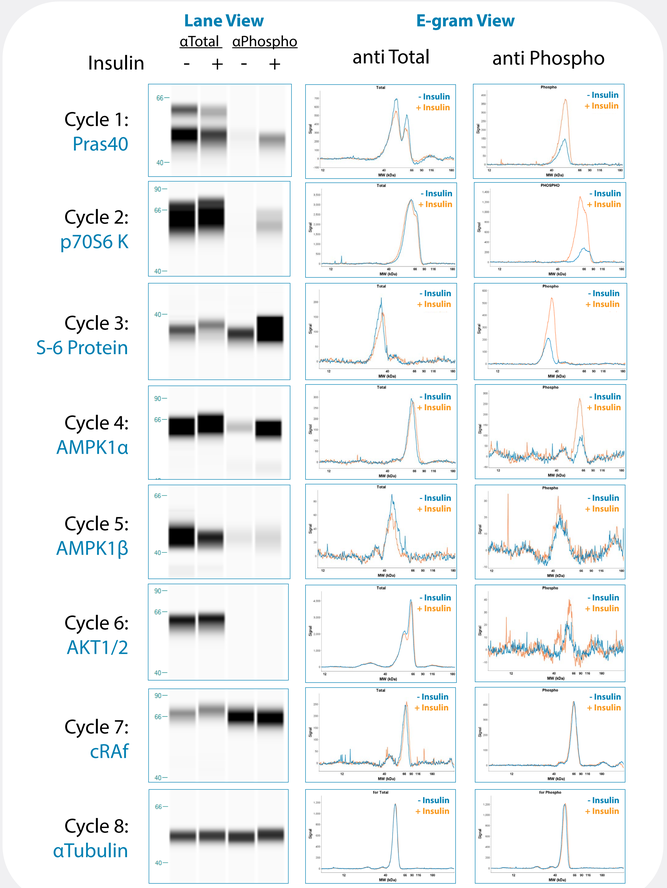

Figure 5: Shown are both lane view and electropherogram images of detected targets from the AKT pathway. The experiment was carried out in a single automated screen using only 5 µg in a volume of 5 µL of MCF7 cell lysate +/- Insulin to collect 8 data points for each target (p70S6K Control Cell Extracts, Cell Signaling #9203). The following total and phospho antibodies were used at a 1:50 dilution in ProteinSimple Antibody Diluent unless stated otherwise (anti-PRAS 40 (Bethyl #301-202, Cell Signaling #2997, anti-p70S6 (Cell Signaling #2708, Millipore #04-393), anti-S6-Protein (Cell Signaling #2217, Cell Signaling #4858), anti-AMPKα (Cell Signaling #2606, Cell Signaling #2537), anti-AMPKβ (Cell Signaling #4178, Cell Signaling #4181), anti-AKT (Cell Signaling #4691, Cell Signaling #4060), anti-cRaf (Cell Signaling #9422, Cell Signaling #9421) anti-Tubulin (Cell Signaling #2125).

Data Reproducibility

High throughput screening applications rely on excellent reproducibility within the instrument, within each set of 12 capillaries, as well as across the 8 cycles. To demonstrate this, a K562 lysate was simultaneously probed with both anti-AKT2 and anti-GAPDH antibodies . The sample was run in all positions in the instrument and the variability was assessed, resulting in standard errors of less than 9% over an entire run of 96 samples. This highlights the strong reproducibility of results obtained with Sally.

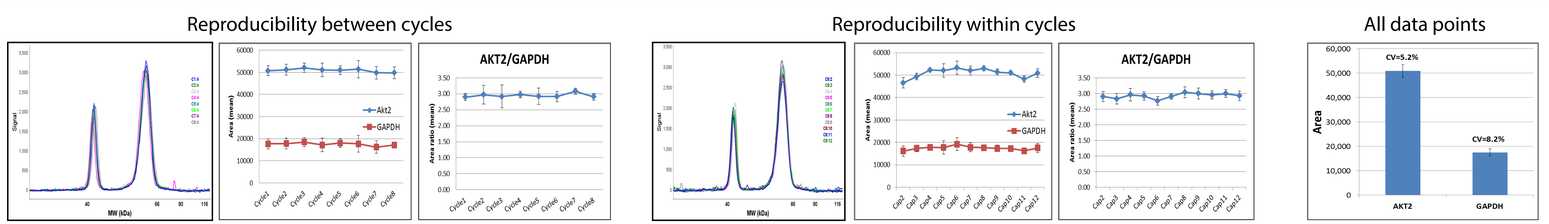

Figure 6: K562 Whole Cell Lysate (in-house) was diluted with ProteinSimple Sample Buffer along with Fluorescent Standards and DTT to 1 mg/mL total protein, and heat denatured for 5 minutes at 95 °C. 5 µL of sample volume was added per sample well. AKT2 and GAPDH (Cell Signaling Technology, p/n 3063 and p/n 2118) were multiplexed by probing with a mix of both antibodies diluted 1:50 each. A complete 8 cycle run was performed.