Maximizing TNF-alpha signaling pathway characterization with Simple Western

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Abstract

Aberrant expression and signaling of multiple proteins in the NF-κB pathway are commonly associated with inflammatory and stress-induced diseases, including many cancers. Understanding how NF-κB 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 for the first time results generated on Sally, the new Simple Western 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 low inter- and intra-assay variability. This suggests the potential to characterize whole signaling pathways with as little as 5 µL of sample.

Targets from the NF-κB pathway, including IκB, NF-κB subunits c-Rel, p65, and p50/p105 from both whole cell and nuclear lysates, were screened on the Simple Western platform in response to TNF-α treatment. Statistically significant changes in signal and localization were clearly observed for each of the key targets. 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.
- 16 data points from one 5uL sample from 2 runs on consecutive days
- 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.

Assay Principles

Sally is a bench top instrument that runs Simple Westerns, which are size-based assays equivalent to SDS-PAGE (Figure 1). Sally simultaneously runs 12 samples per cycle and up to 8 cycles per experiment. Samples 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 runs up to 96 samples in one experiment and can generate up to 16 data points from the same microliter volume of lysate in two experimental runs. Sally automates the entire Western blot procedure which results in increased reproducibility and significant time savings.

Figure 1: Sally

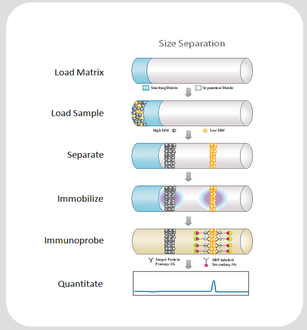


Figure 2: Steps of a Simple Western Assay

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 Specs

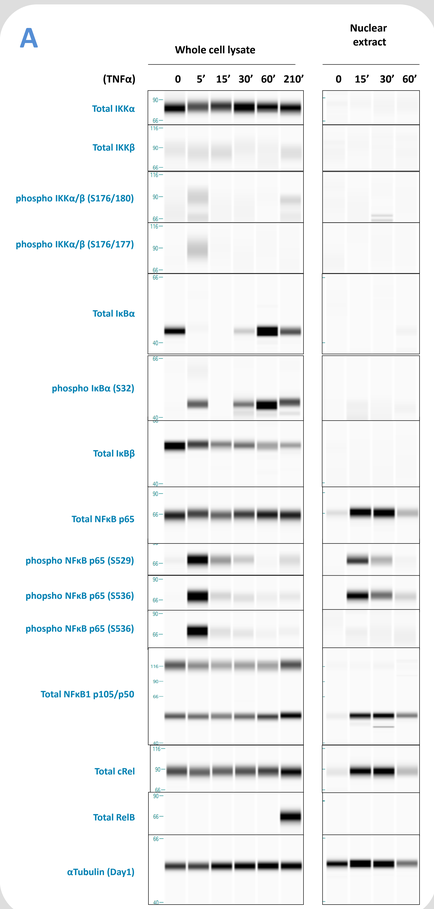
Description	Specifications
Sample required	~3–5 µg
Samples per cycle	12
Number of cycles	Up to 8
Sample throughput	Up to 96
Run time	Up to 19 hours
Sizing range	15–150 kDa
Sizing accuracy	+/– 20%
Sizing CV	10%
Resolution	+/– 10% difference in molecular weight
Quantitation CV	≤ 20%
Dynamic Range	3 logs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

Figure 4: Simple Western Assay Specifications

NFκB Pathway Mapping

The NF-κB pathway plays a key role in immune and stress signaling and apoptotic response via activation of NF-κB transcription factor. In the non-activated state, NF-κB is bound to IκB-α inhibitory protein and retained in the cytoplasm. TNF-α stimulation leads to the recruitment and activation of the cytoplasmic IKK complex, comprised of IKK-α catalytic subunits and scaffolding proteins. The IKK complex phosphorylates IκB-α leading to it's degradation by the proteasome. The NF-κB dimer complex is then released and translocates to the nucleus and activates transcription. Multiple members of the NF-κB family can affect transcription in different ways. The protein p65 (RelA) and cRel contain C-terminal transcriptional activation domains which enable them to activate target gene expression. In contrast, p50 (detected here by antibodies reactive both to precursor and processed protein) may repress transcription as homodimers.

Comprehensive pathway mapping from 5µL lysate



Inter-assay variability

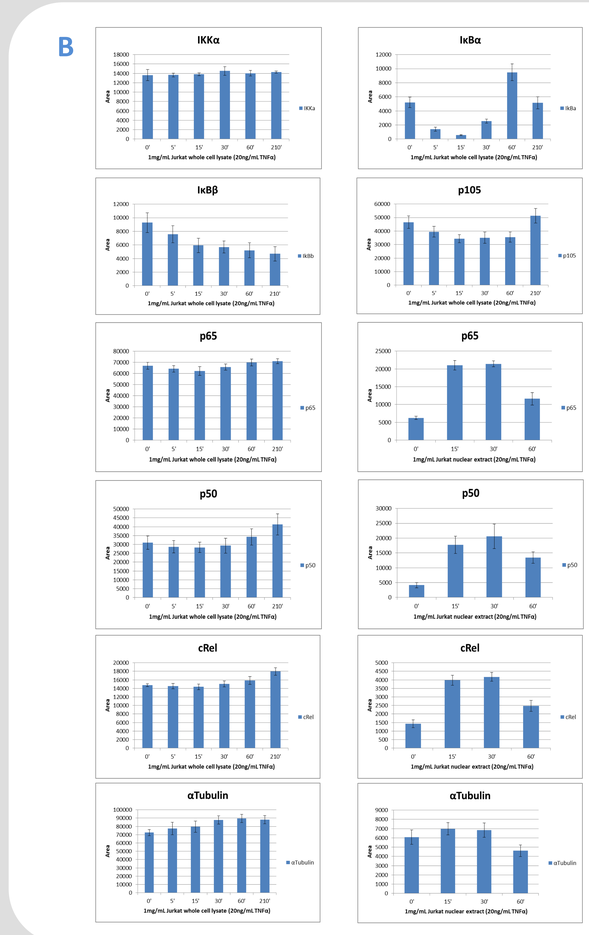


Figure 5: NF-κB pathway targets detected using Jurkat cells treated with 20 ng/mL TNFα (R&D Systems, 210-TA) in a time dependent manner. A) Using only 5 µg of total protein in a volume of 5 µL, a total of 14 different NF-κB pathway target antibodies along with a loading control (αTubulin) were screened in two consecutive runs. Upon completion of the first experimental run, fresh reagents were pipetted into a new assay plate and the lysate preparation was transferred to this plate and assayed. B) Using the same lysate, 5 µg of total protein in a volume of 5 µL of either whole cell or nuclear extract, Jurkat lysate preparation was added to the assay plate and screened with 7 different NF-κB pathway target antibodies along with a loading control. Each bar represents the average of 3 data points, each generated on 3 separate days from individual capillaries.

Eleven different antibodies were purchased from Cell Signaling Technology (IKKα: p/n 2682, IKKβ: p/n 2684, phospho IKKα/β: p/n 2697 (Ser176/180), 2078 (Ser176/177) (1:100), IκBα: p/n 4814, NF-κB p65: p/n 4764, phospho NF-κB p65: p/n 3033, NF-κB1 p105/p50: p/n 3035 cRel: p/n 4727, RelB: p/n 4922 (1:200), α-tubulin: p/n 2125 (1:100)), all were run at 1:50 unless specified. Two antibodies were purchased from Epitomics: phospho IκBα: p/n 2798-1 (1:400), IκBβ: p/n 3696-1 (1:100). Two antibodies were purchased from Active Motif: phospho NF-κB p65: p/n 39692 (Ser529) (1:50), p/n 39676 (Ser536) (1:1500).

Intra-assay Data Reproducibility

High throughput screening applications rely on strong intra-assay reproducibility with data collection. To demonstrate this, a Jurkat lysate preparations was probed for p105 and p50 proteins. Both p105 (running at 116 kDa) and p50 (running at 55 kDa) were detected in the experiment. Moreover, the standard errors over the entire run of 96 samples was less than 7% for both p105 and p50 proteins highlighting the strong reproducibility of results obtained with Sally.

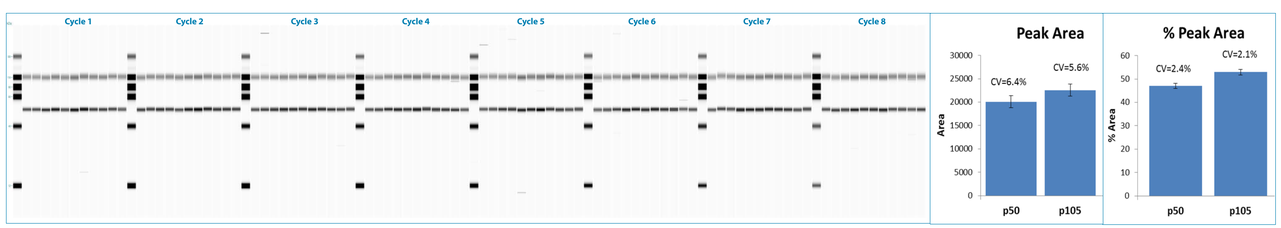


Figure 6: Jurkat Whole Cell Lysate (In-house) was diluted with ProteinSimple Sample Buffer including Fluorescent Standards and DTT to 1 mg/mL total protein and heat denatured for 5 minutes at 95 °C. 5 µL of sample at 1 mg/mL was added per sample well. NFκB1 p105/p50 antibody (Cell Signaling Technology, p/n 3035) was used at a 1:50 dilution. Complete 8 cycle run.