Simple Western analysis of NF-κB signaling cascade proteins

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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 newest member of the Simple Western platform from ProteinSimple. Sally is easy to set-up and runs hands-free up to 96 data points in a single experiment, thus addressing the need for higher throughput. Sally generates 8 individual measures from 5 µL of sample which allows for characterization of whole signaling pathways from one small sample size.

Data generated on Sally from examination of targets in the NF-κB (p100/p50) pathway demonstrate high reproducibility and low intra-assay variability. Response to TNF-α treatment in whole cell and nuclear extracts of IκB and NF-κB subunits (c-Rel, p65, and p50/p105) demonstrates, as expected, statistically significant changes in signal and localization. Results and workflow comparisons indicate a distinct advantage of the Simple Western when compared to traditional Western methods.

Conclusion

- The Simple Western allows for hands-free automation of the complete Western blot workflow with superior reproducibility and equal sensitivity.
- Sally permits processing of 96 Simple Western data points in one run at a level of efficiency and reproducibility unobtainable by traditional Western blots.
- The platform's throughput and ability to generate 8 data points from one extremely small sample volume provides a new approach 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 96 samples in one experiment, simultaneously running 12 samples per cycle for up to 8 cycles (Figure 1). Simple Western assays, performed by Sally, are size-based assays equivalent to SDS-PAGE. 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 generate 8 data points from the same microliter volume of lysate and automates the entire Western blot procedure resulting in increased reproducibility and significant time savings.

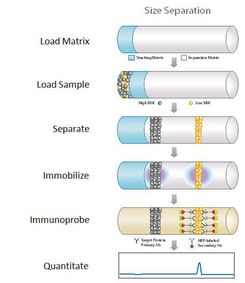

Figure 2: Steps of a Simple Western Assay

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

Pathway Mapping Using Small Sample Volumes

The NF-κB pathway plays a key role in immune and stress signaling as well as 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 (cycle 6) catalytic subunits and scaffolding proteins. The IKK complex phosphorylates IκB-α (cycle 2) leading to it's degradation by the proteasome. NF-κB dimer 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) (cycle 1) and c-Rel (cycle3) contain C-terminal transcriptional activation domains enabling them to activate target gene expression. In contrast, p50 and p52 (cycles 4 and 5) (detected here by antibodies reactive both to precursor and processed protein) may repress transcription as homodimers. Caspase 9 (cycle 7) is one of the downstream targets of the NF-κB cascade acting through expression of apoptotic inhibitor protein cIAP.

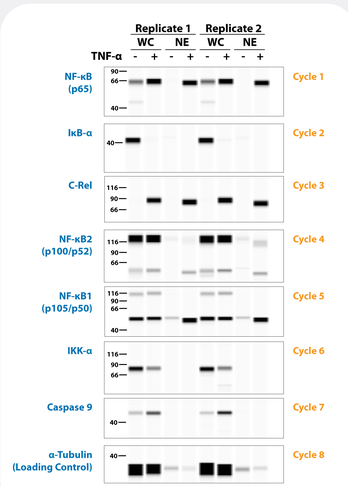

Figure 5: NF-κB pathway targets detected in a single automated screen using HeLa cells. Only 5 µg in a volume of 5 µL of either whole cell (WC) or nuclear extract (NE) HeLa lysate preparation (Santa Cruz Biotechnologies, p/n sc-2200 and sc-2228, and Abcam, p/n 14655 and 14562) was added to the assay plate and screened with 7 different NF-κB pathway target antibodies along with a loading control. Eight different antibodies were purchased from Cell Signaling Technology (IκB: p/n 4814, RelC: p/n 4727, NF-κB1 p105/p50: p/n 3035, NF-κB2 p100/p52: p/n 4882, α-tubulin: p/n 2125, IKK-α: p/n 2682, Caspase 9: p/n 9502) with each antibody used at a 1:50 dilution.

Data Reproducibility

High throughput screening applications rely on strong intra- and inter-assay reproducibility. To demonstrate this, identical lysate preparations were analyzed on Sally on three different days, probing for p50-p105 each time. As expected, both p105 (running at 114 kDa) and p50 (running at 55 kDa) reacted with the antibody used. The standard errors over the entire run of 96 samples were less than 15% for both p105 and p50 proteins in 3 independent runs. Additionally, the normalized ratio of these 2 peaks were within 10% of each other as compared over 3 independent days.

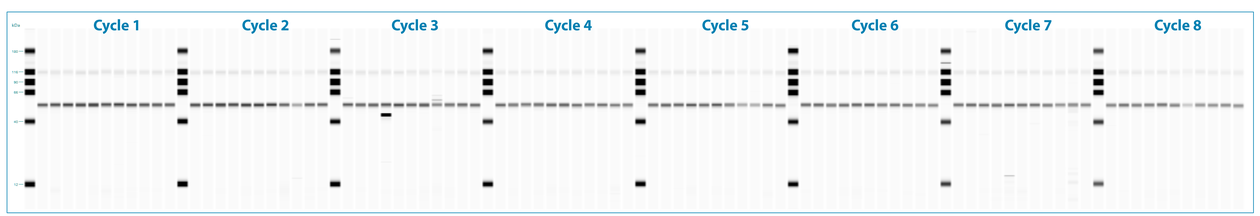

Figure 6: HeLa Whole Cell Lysate (Santa Cruz Biotechnologies, p/n sc-2200) was diluted with ProteinSimple Sample Buffer including Fluorescent Standards and DTT to 1 mg/mL and heat denatured for 5 minutes at 95 °C. 5 µL of sample volume was added per sample well. P105/p50 antibody (Cell Signaling Technology, p/n 3035) was used at a 1:50 dilution. Complete 8 cycle run.

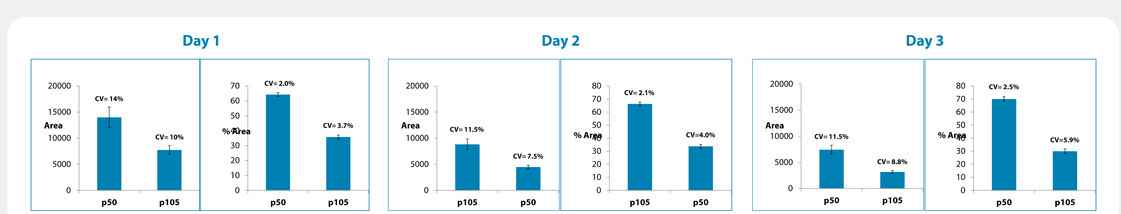

Figure 7: Statistical analysis of the three independent 8 cycle run experiments performed on different days and by different users. For each day, the absolute signal or area under the curve (left graph) and normalized % area signal (right graph) are shown