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TECHNICAL NOTE

FULL SIGNALING PATHWAY ANALYSIS IN A SINGLE RUN WITH SIMPLE WESTERN

THE IMPORTANCE OF UNDERSTANDING CELL SIGNALING PATHWAYS

Signaling pathways are the means by which important internal cellular communication occurs, and the understanding of signaling networks has significant potential to advance therapies in cancer and regenerative medicine. However, gaining an accurate quantitative understanding of complex signaling networks as a whole is crucial and remains a daunting challenge. The limitations of traditional methods like Western blot in this regard are significant because they lack throughput, multiplexing efficiency, sensitivity, and quantitation to gain a holistic view of cell signaling.

SIMPLE WESTERN TAKES THE COMPLEXITY OUT OF CELL SIGNALING

Have you ever wanted to analyze a full signaling cascade in each of your samples in a single experiment? Because signaling networks are complex, a high-throughput method is often needed to measure all protein targets involved, including phosphorylated and non-phosphorylated protein forms of each protein in the signaling cascade. Traditional Western blots are commonly chosen for signaling studies over other immunoassay approaches since the provided molecular weight information paired with immunodetection increases assay specificity, which can be a challenge when looking at phosphorylated protein isoforms or other post translational modifications. Nonetheless, traditional Western blots are typically limited in their throughput and ability to profile more than 1 or 2 proteins in the signaling pathway at a time.

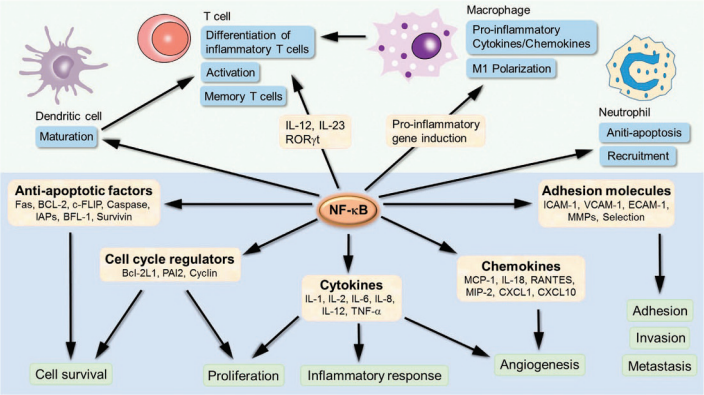

FIGURE 1. NF-κB target genes involved in inflammation development and progression. Figure adapted with permission from Liu *et al.* (2017) *Signal Transduction and Targeted Therapy*. (CC BY 4.0).[1]

Simple Western™ assays are capillary-based automated immunoassays for the high throughput and sensitive detection of proteins. Simple Western generates reproducible and fully quantifiable Western blot data, and it is poised to replace the traditional Western blot in workflows to study cell signaling. Simple Western can analyze up to 96 samples in a single overnight run using only 5 µL of sample, which is ideal for gaining a comprehensive overview of many targets together with their phosphorylated isoforms in a complex signaling pathway in a single experiment.

In this Technical Note, we show how the high throughput and low sample volume requirements of Simple Western work together to comprehensively characterize complex signaling pathways in one simple workflow. The Nuclear Factor-κB (NF-κB) signaling pathway is found in nearly all cell types and it regulates a large array of genes involved in different processes of the immune and inflammatory responses (**FIGURE 1**).[1] Using NF-κB as a reference example, we describe the utilization of Simple Western in two independent studies. In both studies, Simple Western provided advantages in throughput, time to results, and sample size requirements unmatched by traditional methods like Western blot.

COMPREHENSIVE SIGNALING PATHWAY STUDIES WITH SIMPLE WESTERN: TWO CASE STUDIES

To generate 96 data points per run, Simple Western performs 8 cycles of automated analysis with 12 samples per cycle, resulting in 96 total data points. Users may also choose to analyze the same 12 samples in each cycle. In this manner, each 5 µL sample may be interrogated up to 8 times to detect up to 8 distinct protein targets per sample.

The results shown in **FIGURE 2** show the Simple Western characterization of 8 different protein targets per sample, 7 of which are involved in the NF-κB signaling pathway and 1 loading control. For each target, 2 different cellular fractionations were analyzed (whole-cell and nuclear extracts) with 2 different biological treatments (-/+ TNFα treatment) performed in duplicate. In total 64 data points were generated, which is less than the 96 total data points that Simple Western is capable of analyzing per run. Thus, this experiment is not even using the Simple Western run at full capacity. For each cycle, a different antibody was used to detect the target of interest. Clear biological response, as well as localization of the protein with treatment, can be observed. The experiment demonstrates the power and flexibility of Simple Western to be able to screen different targets or lysates and the ability to use that 5 µL lysate/well to comprehensively characterize signaling pathways.

In a study published by a team from Janssen BioTherapeutics, Simple Western was instrumental in revealing how the TIM-3 receptor inhibits T cell activation.[2] TIM-3 serves as a negative regulator of T cell function by suppressing anti-CD3/CD28-induced T cell receptor (TCR) mediated activation, but the mechanism of suppression had remained elusive. To address this lack of understanding, the authors relied on Simple Western to perform a comprehensive analysis of the TIM-3 signaling cascade (**FIGURE 3**). Their results identified a very interesting signaling complex involving TIM-3 and several kinases known to be involved in TCR signaling. In the unactivated state, they identified the association of Vav-1, Akt, SLP-76, ZAP-70, Syk, P85α-PI-3K, Fyn, and the adaptor proteins 3-BP2 and SH2D2A (T cell-specific adaptor protein) with TIM-3 (**FIGURE 3**, left lane). Conversely, in an activated state, TIM-3 no longer associated with those kinases, but associated instead with the Src family kinase, Lck, as well as enhanced association of PLC-γ1. Furthermore, they did not see an association of TIM-3 with P85α-PI-3K subunit in the activated state (**FIGURE 3**, middle lane). Therefore, the sequestration of both Lck and PLC-γ1 by TIM-3 could serve as an inhibitory mechanism in which TIM-3 negatively regulates NF-κB/NFAT signaling pathways.

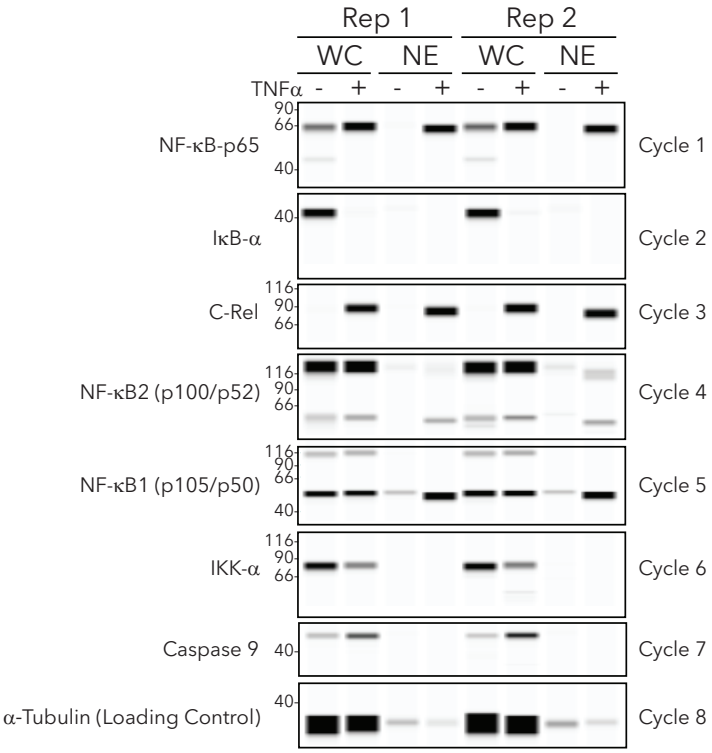

FIGURE 2. Simple Western analysis of the NF-κB pathway in TNFα-treated (+) and untreated (-) HeLa cells (5 µg in 5 µL) of whole-cell (WC) or nuclear extract (NE) cellular fractionations. Each sample was screened with 7 different antibodies and 1 loading control (α-Tubulin).

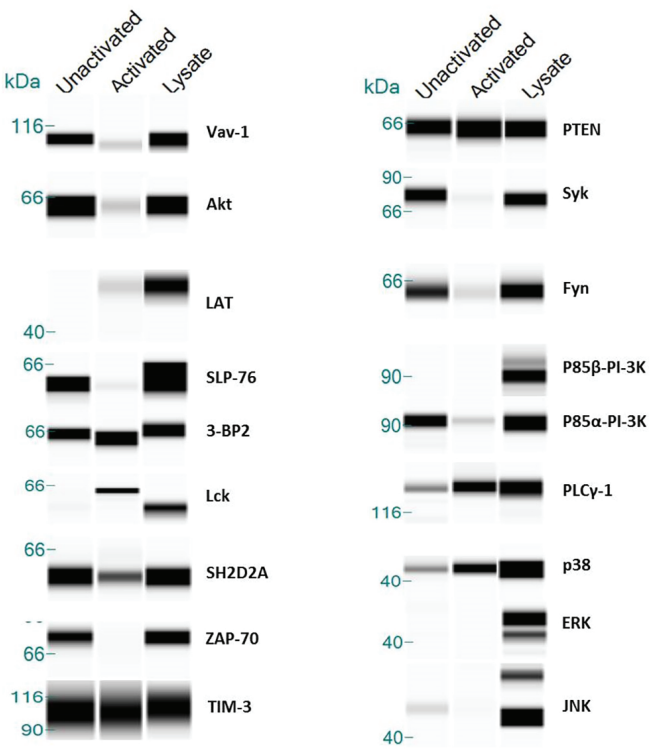

FIGURE 3. Simple Western analysis reveals the TIM-3 signaling web in unactivated and anti-CD3/CD28-activated T cells. Cleared lysate served as a loading control for individual antibody reactivity. Figure adapted with permission from Tomkowicz *et al*. (2015) *PLoS ONE*. (CC BY 4.0)[2]

THE SIMPLE WESTERN ADVANTAGE IN CELL SIGNALING STUDIES

Signaling cascades are often complex and involve many proteins and phosphorylated isoforms. In a single fully automated run, Simple Western can comprehensively characterize complex signaling pathways by generating up to 96 data points overnight, allowing users to capture all proteins in a signaling cascade simultaneously. The Simple Western platforms with the highest throughput like Peggy Sue and Sally Sue require only 5 µL of sample, and the same 5 µL sample may be interrogated up to 8 times. Performing similar experiments by traditional Western blot would take several days of manual labor, use significantly more sample volumes, and the data generated would not be as reproducible or quantifiable.

Multiplex analysis, whereby multiple protein targets are detected in the same sample, can drive cell signaling research even further. Toward this end, Simple Western instruments like Jess and Abby can perform the RePlex assay, which efficiently removes antibodies from the capillary for a second round of interrogation with fresh antibodies. The second round may also be dedicated to Total Protein Detection so that users can normalize protein expression data with confidence. The RePlex assay is also fully automated, generating more data points per sample and reducing the cost per result. And while chemiluminescence detection comes standard with all Simple Western instruments, the Simple Western instrument Jess takes multiplexing even further by also offering protein detection in infrared (IR) and near-infrared (NIR) channels, as well as built-in fluorescence-based Protein Normalization.

REFERENCES

1. NF-κB signaling in inflammation, T. Liu, L. Zhang, D. Joo and S-C Sun, *Signal Transduction and Targeted Therapy*, 2017; **2**:17023.

2. TIM-3 suppresses anti-CD3/CD28-induced TCR activation and IL-2 expression through the NFAT signaling pathway, B. Tomkowicz, E. Walsh, A. Cotty, R. Verona, N. Sabins, F. Kaplan, S. Santulli-Marotto, C.N. Chin, J. Mooney, R.B. Lingham, M. Naso and T. McCabe, *PLoS ONE*, 2015; **10**:1–22.

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