A Fully Automated Capillary Electrophoresis System for Western Analysis Using On-Line Stacking as an Easy Tool for the Enhancement of Sensitivity and Resolution

I. Kazakova PhD, U. Nguyen, J. Dermody PhD, F. Ramirez, T. Yang, R. Gavin, A. Boge PhD

ProteinSimple, 3040 Oakmead Village Drive, Santa Clara, California 95051

Abstract

Size-based characterization of proteins has been predominately performed by either SDS-PAGE/Western blot analysis or by capillary electrophoresis (CE). Each technique has advantages – Western blotting exploits high sensitivity as well as specificity of antibody binding, and CE offers high resolution and reproducibility. The sensitivity and resolution of the results obtained from either method is often challenged by the ability to pre-concentrate or stack enough protein sample before separation. Simon, a new size-based separation platform that runs Simple Western assays, combines for the first time the advantages of both Western blotting and CE into a single automated workflow. The work presented illustrates the dependency of stacking efficiency upon the plug length of the sample and stacking matrix. Optimization of stacking conditions resulted in a significant sensitivity and resolution increase using the Simple Western assay.

Conclusions

- On-line stacking optimization during Simple Western assay development achieved a 10-fold increase in signal, while resolving proteins with a 10% or less differential in molecular weight.
- These experiments determined default loading conditions that can be used to achieve reliable results for most proteins.
- Users can further optimize assay conditions to improve the sensitivity and resolution for their specific target applications.

Simple Western Assay Principles

Simon is a bench top instrument capable of running 12 samples 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). Simon allows for the automation of the entire Western blot procedure, which results in increased reproducibility and significant time savings.

FIGURE 1. Simon

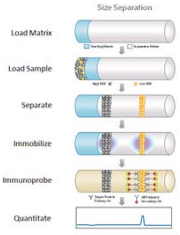

FIGURE 2. Steps of a Simple Western Assay

Workflow Comparison

Traditional Western	Simple Western
Load samples in PAGE	Load plate in Simon
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	

Stacking Principles

Protein/SDS complexes achieve high velocity when subjected to a low conductivity/high electric field stacking matrix. Upon entering the separation matrix, protein velocities decrease and are concentrated into a narrow band which is known as field amplified stacking. The pre-concentrated proteins then enter the separation matrix and are separated based on their sizes.

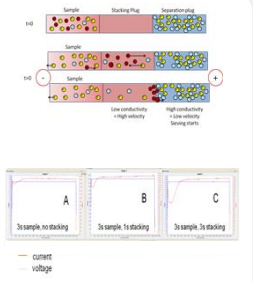

FIGURE 3. On-line sample stacking principle. Separation matrix, stacking matrix and sample are introduced sequentially into the capillary and voltage is applied. The composition of separation and stacking matrices were optimized to create a differential in conductivity in order to subject sample ions to non-uniform electric fields.

FIGURE 4. Current and voltage in non-stacking and stacking configurations. **A**: Sample loaded for 3 seconds, no stacking matrix. The small dip in current is due to sample loading. **B,C**: Stacking matrix loaded for 1 or 3 seconds respectively followed by loading of the sample for 3 seconds. A large decrease in the current which is proportional to stacking matrix load times is displayed. Data was obtained using a model system with uniform separation/running matrix compositions.

Immunodetection of Targets in a Stacked Sample

Stacking efficiency can be controlled by varying the time in which the stacking matrix is loaded in the capillary. An 8 second stacking matrix load time followed by a 6 second sample load results in complete stacking of the sample. Overloading the stacking matrix results in loss of resolution due to insufficient separation distance (Figure 5).

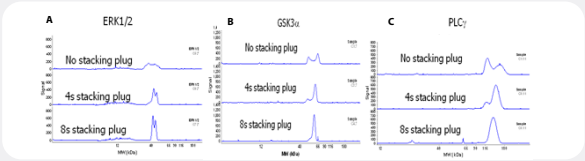

FIGURE 5. Examples of protein stacking. HeLa cell lysate was mixed with 10X Sample Buffer, supplemented with Fluorescent Standards and DTT (all ProteinSimple), denatured 5 minutes at 95 °C, and 1 mg/mL was loaded into capillaries for 6 seconds with no stacking matrix and then for 4 and 8 seconds with stacking matrix. Capillaries were incubated with the following antibodies, diluted 1:50 in Antibody Dilution Buffer (ProteinSimple): **A**) α-ERK1/2, ProteinSimple #040-474; **B**) α-GSK3α, Cell Signaling #4818; **C**) α-PLCγ, Cell Signaling #2821.

Assay Sensitivity, Linearity and Dynamic Range

Sample stacking results in up to a10-fold improvement of sensitivity. Linearity is also improved, possibly by mitigating protein absorption at lower concentrations. Dynamic range of the assay after sample stacking is >2 orders of magnitude.

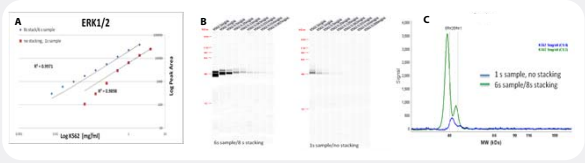

FIGURE 6. Sensitivity, linearity and dynamic range with and without stacking for ERK1/2 (MW 42/44 kDa). **A**) Titration curve. **B**) Simon software Lane View image. **C**) Electropherograms at 1 mg/mL total protein concentration. K562 lysates were diluted from 3 mg/mL to 0.01 mg/mL and prepared as described in Figure 5. In the non-stacking configuration, sample is loaded for 1 second without stacking matrix. In the stacking configuration, sample is loaded for 6 seconds followed by an 8 second load of stacking matrix. The Simple Western default assay procedure was used for these experiments.

Signal and Resolution Optimization

A default stacking matrix load of 6 seconds with an 8 second sample load is suitable for a wide range of targets resulting in robust signal while maintaining high resolution. The flexibility of Simple Western assays allow the user to further optimize stacking conditions for optimal resolution and detection.

An optimal balance for ERK1/2 resolution (5% difference in MW) and signal intensity was achieved using default conditions.

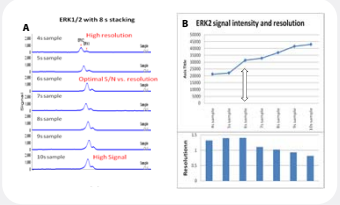

FIGURE 7. ERK1/2 stacking optimization (double peak at 42/44 kDa). **A**) Electropherograms of samples using various sample and stacking matrix load times. **B**) ERK1/2 signal intensity and resolution. K562 cell lysate was prepared as described in Figure 5 to a final concentration of 1 mg/mL and loaded for 4-10 seconds. The stacking matrix load time was maintained at 8 seconds.

Longer sample and stacking matrix loading times can be used to increase sensitivity for single peak detection. The highest GSK3α signal was achieved using a 10 second sample load with a 12 second stacking matrix load. A noticeable loss of resolution occurred when the stacking matrix was overloaded (>20 seconds).

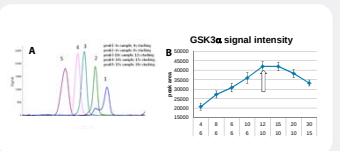

FIGURE 8. GSK3α stacking optimization (single peak, 51 kDa). **A**) Electropherograms of samples using various sample and stacking matrix load times. **B**) GSK3α signal intensity after stacking. Samples were prepared as described in Figure 5. Sample was loaded for 6-15 seconds, while the stacking matrix load time was varied from 4-30 seconds.

A higher stacking to sample loading ratio results in improved resolution for high MW targets. The optimal PLCγ signal was achieved using a 6 second sample load and a 12 second stacking matrix load.

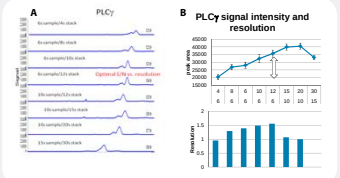

FIGURE 9. PLCγ stacking optimization (double peak, 130/150 kDa). **A**) Electropherograms of samples using various sample and stacking matrix load times. **B**) PLCγ signal intensity and resolution after stacking. HUT78 cell lysate was prepared as described in Figure 5 to a final concentration of 1 mg/mL and loaded for 6-15 seconds. The stacking matrix load was varied from 4-30 seconds.