Size and Charge Based Analysis of ERK1/2 and 4E-BP1 Using Automated Capillary-Based Systems for Nanoscale Protein Analysis

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Abstract

Aberrant expression and signaling in the EGF signaling cascade is a common occurrence in a variety of cancers including breast cancer. Understanding how EGF signaling impacts disease progression is key to the development of novel therapeutics. Analysis of ERK1/2 and 4E-BP1 expression in cancer samples frequently employs Western blot analysis. In-depth phosphorylation analysis often requires 2D gels which are extremely variable and labor intensive, followed by MS analysis. In this study, novel, capillary-based technologies by ProteinSimple are used to evaluate changes in signaling proteins. Size-based as well as charge-based separation techniques were utilized, each of which is followed by immunodetection.

The Simon system performs size-based separation of proteins. Alternatively, NanoPro systems utilize charge-based separation in capillaries via isoelectric focusing (IEF). Using capillary electrophoresis (CE) modes coupled to an automated workflow, Simon and NanoPro instruments eliminate the need for manual processing of multiple steps. Proteins from a single sample preparation were analyzed on two platforms: either in a native conformation using charge-based separation (NanoPro) or in a denatured state followed by size-based separation (Simon).

Utilizing both platforms allowed for detailed analysis of post-translational modifications (charge-based) and protein abundance (size-based) within a prepared sample. As a validation of synergy between the platforms, we investigated key members of the EGF signaling cascade in different phosphorylation states. We showed that the same cellular lysate preparation can be used for charge- or size-based separation. Advantages of the Simple Western assay over Western blot assays include ease of use, minimal user intervention, automatic analysis and excellent reproducibility.

Simple Western and NanoPro 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. Using NanoPro assays, samples are separated by isoelectric focusing (Figure 1). After separation in either mode, proteins in the sample are immobilized to the capillary walls via a proprietary UV capture method followed by removal of the separation matrix. The proteins of interest are detected via specific primary antibodies along with HRP-amplified chemiluminescent detection. Both Simon and NanoPro platforms provide full automation of the entire separation, immobilization and detection process which results in increased reproducibility and significant time savings.

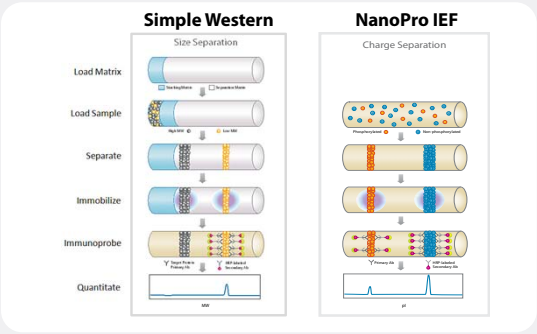


Figure 1: Assay Principles for Simple Western and NanoPro Assays

Assay Workflow

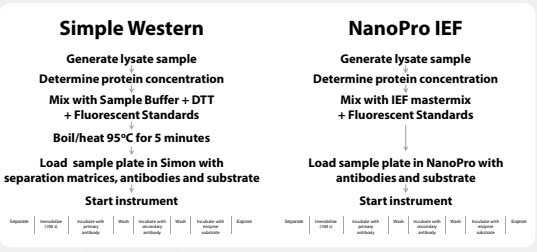


Figure 2: Workflow Comparison for Simple Western and NanoPro Assays

Dynamic Range Comparison

Simple Western and NanoPro assays use the same 5 cm capillaries (100 µm ID). In NanoPro assays, the capillary is filled with sample and then isoelectric focusing takes place. In Simple Western assays, the sample initially occupies about 10% of the capillary length. Hence, the sensitivity differences between the two assays is about 10-fold, as shown in Figure 3.

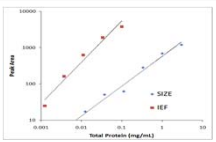
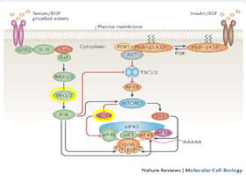


Figure 3: Sensitivity comparison between a Simple Western assay on Simon and a NanoPro assay on the NanoPro 100. K562 cells were lysed with ProteinSimple Bicine/CHAPS Lysis Buffer. Samples for assays were prepared at 3 mg/mL (size) and 0.3 mg/mL (charge) concentrations, and diluted as indicated. Samples run via Simple Western assay were separated 40 min at 250 V and samples run using the NanoPro assay were separated for 40 min at 20,000 µW. Detection for both assays used α-ERK1/2 antibody (ProteinSimple 040-474).

EGF Signaling

ERK1/2 as well as 4E-BP1 are central elements in the signal transduction cascade downstream from EGF and other growth factors.



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Monitoring Biological Activity

ERK

Figures 5 and 6 address the correlation between Simple Western (size) and NanoPro IEF (charge) assays. As expected, similar peak area ratios were observed between ERK1 and ERK2 in both assays when using untreated HeLa cells (5A), while in K562 cells, ERK2 expression is dominant in both assays (5B). Upon EGF stimulation of HeLa cells, results from the Simple Western using α-ERK1/2 antibody show no change in total ERK1/2 but an increase in phosphorylation is observed when using α-phospho ERK1/2 antibody (5A). Additionally, NanoPro IEF assay data show clear increases for distinct phosphorylated ERK1/2 isoforms in HeLa cells after EGF treatment using the phospho ERK as well as the total ERK antibody (5A).

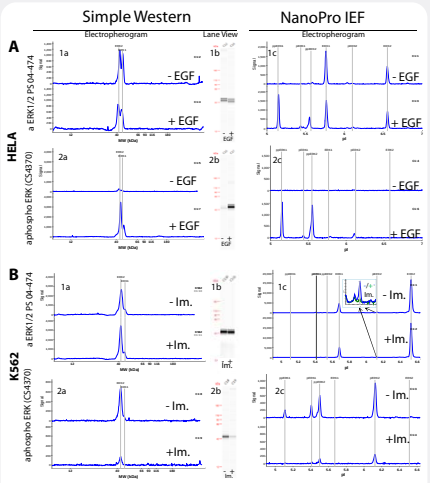

Figure 5: Correlation between Simple Western and NanoPro IEF assay separation and detection for ERK1/2. HeLa cells were treated for 15 min with and without 50 ng/mL EGF. K562 cells were treated for 4 hrs with and without 10 µM Imatinib (Im.). All samples were lysed with ProteinSimple Bicine/CHAPS Lysis Buffer. Samples analyzed via Simple Western assay were prepared at a concentration of 1 mg/mL and separated for 40 min at 250 V. Samples analyzed via NanoPro IEF assay were prepared at a concentration of 0.05 mg/mL and separated for 40 min at 21,000 µW.

In K562 cells, Imatinib treatment leads to a decrease of phosphorylation, as indicated by Simple Western and NanoPro IEF assays (Figure 5B). Using Simple Western assays, a clear reduction of ERK1/2 phosphorylation is observed with the use of α-phospho ERK1/2. However, more detailed information is present only when using an α-ERK1/2 antibody in NanoPro IEF, as it becomes evident how small the proportion of each phosphorylated isoform is in relation to the total amount of ERK. The insert exemplifies the Imatinib effect on mono phospho ERK2.

4E-BP1

4E-BP1 inhibits cap-dependent translation by binding to the translation initiation factor eIF4E. Hyperphosphorylation of 4E-BP1 disrupts this interaction and results in activation of cap-dependent translation. In K562 cells, 4E-BP1 phosphorylation is reduced upon Imatinib treatment.

As expected, both systems showed a decrease in 4E-BP1 phosphorylation upon Imatinib treatment when using either an α-4E-BP1 or α-phospho 4E-BP1 antibody. In addition, both data sets support Gingras findings (Gingras et al Genes Dev. 2001 15:2852-286), which reported that the phosphorylation of Ser65 is the last step of the sequential phosphorylation of 4E-BP1.

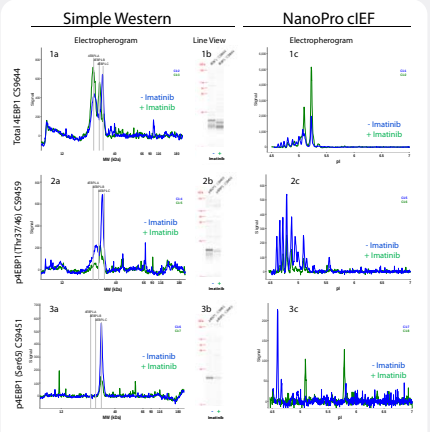

Figure 6: Correlation between Simple Western and NanoPro IEF assay separation and detection for 4E-BP1. HeLa cells were treated for 15 min with and without 50 ng/mL EGF. K562 cells were treated for 4 hrs with and without 10 µM Imatinib (Im.). All samples were lysed with ProteinSimple Bicine/CHAPS Lysis Buffer. Samples analyzed via Simple Western assay were prepared at a concentration of 1 mg/mL and separated for 40 min at 250 V. Samples analyzed via NanoPro IEF assay were prepared at a concentration of 0.05 mg/mL and separated for 40 min at 21,000 µW.

Conclusion

- Simple Western and NanoPro assays provide detailed analysis of both size- and charged-based characterization of proteins and data correlates well as demonstrated for ERK1/2 and 4E-BP1.
- Simple Western assays provide a fully automated alternative to Western blotting for immunodetection and quantitation of proteins.
- NanoPro assays are able to identify and characterize discrete and subtle modifications to target proteins and their isoforms.