Peggy™: size- or charge-based western blotting at the push of a button

Western blotting continues to be the most popular method used for protein analysis, in spite of its drawbacks in quantitation and reproducibility and its laborious workflow. Protein Simple's platform of Simple Western™ products provides a new standard in instrumentation that fully automates the archaic western blotting process and provides highly reproducible and quantitative data. Now Peggy™, the newest addition to the Simple Western™ family, provides immunoassays with separation by size or charge in a single instrument.

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Introduction

The Simple Western™ products have completely integrated the western blotting process, which otherwise has remained essentially unchanged in the 34 years since it was first reported[1,2]. Peggy™ combines the power of Simple Western™ size and charge assays, allowing users to perform both assays on a single platform. Size-based assays can provide protein molecular weight and absolute quantitation of proteins in a complex sample. With the use of post-translational modification (PTM)-specific antibodies, these assays can provide information about relative levels of known phosphorylation sites and other PTMs. Charge-based assays (using isoelectric focusing, or IEF) can provide enhanced sensitivity and exquisite resolution for nearly all variant forms of a protein. These variant forms, once spatially resolved by electrophoresis, can be identified and measured with a single, pan- specific protein antibody[3]. The relative abundance of PTM forms in the charge assay can provide insight into the activity and processing of the protein of interest.

How Peggy™ works

Samples are collected using standard techniques and lysed in one of Protein Simple's lysis buffers according to procedures provided by the company. For size assays, samples are diluted into Simple Western™ sample buffer, reduced and denatured, and 5 μL of this sample will generate 8 data points per run. For charge assays, the sample is diluted into the appropriate ampholyte premix with isoelectric point (pI) standards, and 8 μL of sample is used to generate 8 data points per run. After less than 1 h of setup, the prepared samples, primary and secondary antibodies and chemiluminescent substrate are dispensed into designated wells in a low-volume, 384-well assay plate. Simple Western™ assay buffers, capillaries and the prepared assay plate are placed in Peggy™, which carries out all assay steps automatically. Proteins are separated into the capillaries as they migrate through the size or charge resolving matrix. The separated proteins are immobilized to the capillary wall via a proprietary, photoactivated capture chemistry. Target proteins are then identified using a primary antibody, and subsequent immunodetection is accomplished using a horseradish peroxidase–conjugated secondary antibody and chemiluminescent substrate. Molecular weight or pI and signal for immunodetected proteins are reported automatically. Simultaneous analysis of up to 96 samples can be performed in a single experiment, and results are available immediately once the assay is complete.

Representative data

Peggy™ size-based assays can provide critical information on overall protein expression and relative levels of specific, known protein variants. **Figure 1a** shows data from peripheral blood mononuclear cell (PBMC) samples that were probed with an ERp44 antibody. Relative protein expression can be determined by automated peak area analysis using the integrated Simple Western Compass™ software.

Peggy™ charge-based assays can provide additional information, resolving variant forms of a protein for characterization as shown in **Figure 1b**. By separating protein samples based on properties unrelated to size separation, a complementary profile is obtained that provides additional insights into protein function and mechanism of action.

In addition to control PBMC samples, afflicted PBMCs were analyzed and compared. In **Figure 2**, PBMCs from people with autoimmune disease were profiled with LMP2 and ERK antibodies. **Figure 2a** shows the size-based data from this run. Compass™ software automatically calculates the molecular weight and peak area, allowing for straightforward comparison of protein levels (**Table 1**).

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APPLICATION NOTE

Table 1 | Summary of LMP2 data on six PBMC panels as seen in Figure 2.

Sample	Size		Charge	
	Apparent molecular weight (kDa)	Peak area (relative luminescence units)	pI = 4.8 (% area)	pI = 5.2 (% area)
AI - 1	24	947	23.7	76.3
AI - 2	26	1,882	12.8	87.2
N - 1	24	1,008	99.7	0.3
N - 2	25	1,859	97.2	2.8
N - 3	24	1,235	99.8	0.2
N - 4	25	2,640	98.4	1.6

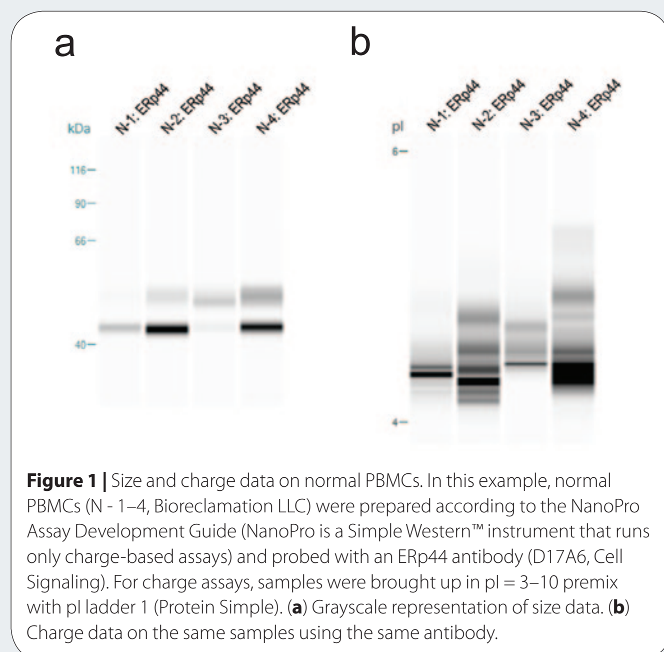

Figure 1 | Size and charge data on normal PBMCs. In this example, normal PBMCs (N - 1–4, Bioreclamation LLC) were prepared according to the NanoPro Assay Development Guide (NanoPro is a Simple Western™ instrument that runs only charge-based assays) and probed with an ERp44 antibody (D17A6, Cell Signaling). For charge assays, samples were brought up in pI = 3–10 premix with pI ladder 1 (Protein Simple). (**a**) Grayscale representation of size data. (**b**) Charge data on the same samples using the same antibody.

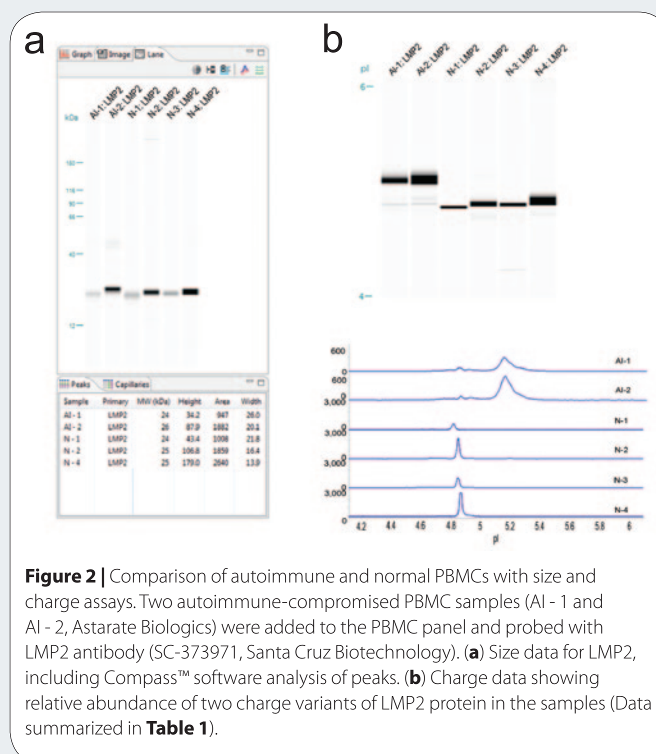

Figure 2 | Comparison of autoimmune and normal PBMCs with size and charge assays. Two autoimmune-compromised PBMC samples (AI - 1 and AI - 2, Astarate Biologics) were added to the PBMC panel and probed with LMP2 antibody (SC-373971, Santa Cruz Biotechnology). (**a**) Size data for LMP2, including Compass™ software analysis of peaks. (**b**) Charge data showing relative abundance of two charge variants of LMP2 protein in the samples (Data summarized in **Table 1**).

Charge-based assay data can provide additional insight into protein state, as PTMs such as phosphorylation, glycosylation and so forth will equate to shifts in the IEF matrix. In **Figure 2b** we show differences in the peak profile of LMP2 in afflicted versus nonafflicted PBMCs. Whereas the size data in **Figure 2a** show protein levels, the charge data in **Figure 2b** show differences in protein activity or processing, which may be a response to disease. By combining the size and charge assays, the researcher is provided with additional avenues for investigation or potential markers for a particular disease state.

Quantitation of results from Simple Western™ assays

Peggy™ data is digital, facilitating automated quantitation. The Compass™ software identifies the size or charge standards present in every capillary, assigns the chemiluminescent peaks to their appropriate molecular weight or pI and integrates peak area. Comparing data from two or more runs is as easy as opening the runs in Compass™ and selecting the data. Compass™ then analyzes peaks and graphs the data. Alternatively, the user can easily export tables of peak values or paste data into a spreadsheet application to compare peaks.

Summary

Peggy™ enables researchers to follow up a size-based immunoassay with a charge-based assay on one platform using the same sample. The charge-based analysis provides an information-rich, complementary data set, elucidating ratios of protein variants and providing leads for biomarker development. Like other Simple Western™ products, Peggy™ provides a fully automated solution, from loading samples all the way to peak analysis. For more information, please visit http://www.proteinsimple.com/.

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2. Renart, J., Reiser, J. & Stark, G.R. *et al.* Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with anti-sera: a method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. USA* **76**, 3116–3120 (1979).
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