Simple Western Streamlines Serum Antibody Analysis

Introduction

Scientists measure serum antibody levels to confirm immune responses against a bacteria to diagnose infections[1], to test for antibody production after vaccination, and to detect the presence of autoantibodies in autoimmune diseases.[2] Traditional Western blots are often used to detect these antibodies, but testing with Western blots means a *lot* of hands-on time. After the antigen of interest is separated by SDS-PAGE and transferred to a membrane, each lane has to be cut into individual strips so patient serum samples can be individually tested for the presence of specific antibodies. Then you have to process and analyze them manually.

Simple Western assays happen in individual capillaries, and everything from sample separation to data analysis is completely automated. No more cutting individual strips, washing and incubating them, or lining them all up with a molecular weight marker before detection. Just pipette your sample into the wells of your assay plate, set up your run, and you're done! And all that manual data analysis is gone too — Compass for Simple Western does it all for you. Did we mention you only need 10 µL of diluted serum per data point?

In this application note, we used Simple Western to detect autoantibodies in lupus patient serum as a model system to generate proof-of-concept data for the assay. But you can use this method any time you need to detect and quantitate specific serum antibodies. In fact, check out how researchers are using this assay to detect *Salmonella* antibodies without having to cut blots into individual strips.[1]

Serum antibody assay

Because it's a completely open platform, you've got the flexibility you need to modify the Simple Western standard assay whenever you want to analyze serum antibodies (**Figure 1**). When you prep your assay plate, instead of using primary antibody, you simply pipette 10 µL serum sample diluted in ProteinSimple Antibody Diluent 2 into the designated primary antibody row instead. Serum dilutions will range anywhere from 1:10 to 1:20000 depending on the amount of antibody in your sample. Next, prepare the purified antigens with 5X Fluorescent Master Mix, heat denature, and pipette into the row you'd normally pipette your samples. The final concentration of recombinant antigen will be assay specific. We recommend start out around 0.05–1 mg/mL and adjusting as needed to keep the antigen saturating. Everything else stays the same, and you'll still get your analyzed data in just 3 hours with only 30 minutes of hands-on time.

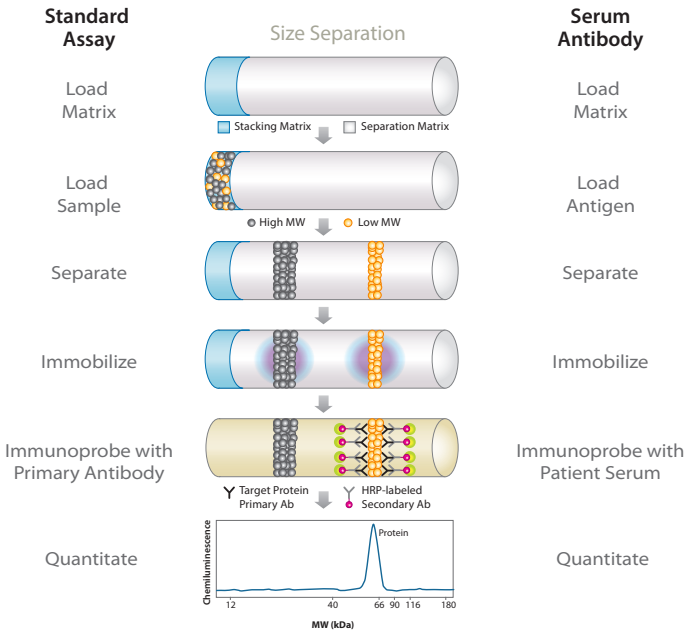

FIGURE 1. Comparison of standard Simple Western assay and Serum Antibody Assay.

When the assay starts, both Separation Matrix and Stacking Matrix are loaded into the capillary first followed by purified antigen. The antigen is separated by size and is immobilized to the capillary wall. Diluted serum then fills the capillary during the primary incubation step, so the antigen-specific antibody present in the serum binds to the antigen on the capillary wall. A species-specific HRP conjugated secondary antibody that recognizes the Fc region of the serum antibody is then introduced to the capillary during the secondary incubation step. Finally, the Luminol/peroxide substrate reacts with the HRP, and the chemiluminescence generated is detected and used for quantitative results.

Detection and analysis of autoantibodies in lupus patient samples

SLE PATIENT SAMPLES AND ASSOCIATED ANTIGENS

For this study, we used normal and systemic lupus erythematosus (SLE) patient serum samples from Bioreclamation IVT (**Table 1**). Blood was collected in SST tubes to speed up the clotting process for serum isolation and frozen immediately after to complement-preserve the samples.

A list of diagnosis and medications were provided for each patient as well as the SLEDAI Score that indicates the severity of SLE flare at the time the patient sample was collected with a score of >3 indicating a mild to moderate flare and a score of >12 indicating a severe flare. Samples were aliquoted upon arrival to avoid the sample degradation that can happen when you freeze-thaw serum multiple times.

We also purchased SLE-associated antigens from Surmodics (**Table 2**). All of the antigens were recombinant proteins. The prevalence of each antigen in SLE patients as well as other autoimmune diseases have also been reported.[2]

SIMPLE WESTERN SETUP

Purified antigens were mixed with 5X Fluorescent Standard, DTT and 1X Sample Buffer for a final concentration of 0.02 mg/mL according to the Wes Size Assay Product Insert. Samples were heat denatured at 95 °C for 5 minutes and 3 µL of sample per well was transferred to row A of the plate. Patient serum diluted 1:10 in Antibody Diluent 2 with 10X System Control Rabbit Antibody (PN 042-196) diluted to 1X was transferred to row C of the plate (**Figure 2**). 20X ProteinSimple Anti-Rabbit HRP Conjugate[3] was diluted into ProteinSimple ready-to-use Anti-Human IgG HRP Conjugate provided in the Anti-Human IgG Detection Module (PN DM-005) and

PATIENT	GENDER	AGE	MEDICATIONS	DIAGNOSIS	SLEDAI SCORE
Normal	Male	58	N/A	N/A	N/A
Systemic Lupus Erythematosus (SLE)	Male	30	Prednisone, Lisinpril, Vicodin HP, Clonidine HCL, Furosemide, Cyclobenzaprine	SLE, nephritis and nephropathy, HTN, lupus nephritis	10

TABLE 1. Patient gender, age, medication, diagnosis and SLEDAI score of normal and SLE patient serum purchased from Bioreclamation. Patient info was provided by Bioreclamation.

ANTIGEN	PREVALENCE IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)	ANTIGEN INDICATED IN OTHER AUTOIMMUNE DISEASE
PCNA	2-7%	N/A
Ro/SS-A	24-60%	Sjögren's syndrome, neonatal lupus syndrome
U1-snRNP 68/70	12-28%	Mixed connective tissue disease
U1-snRNP A	13-23%	Mixed connective tissue disease

TABLE 2. Antigens the normal and SLE patient samples were tested against. Recombinant antigens were purchased from Surmodics.

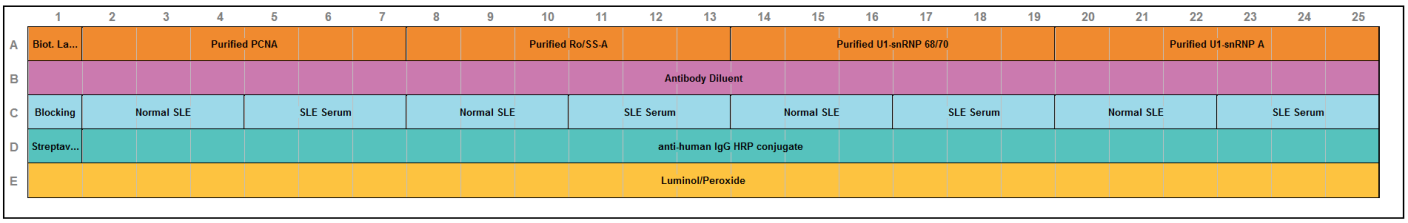

FIGURE 2. Compass for Simple Western plate map used to screen for SLE serum antibodies.

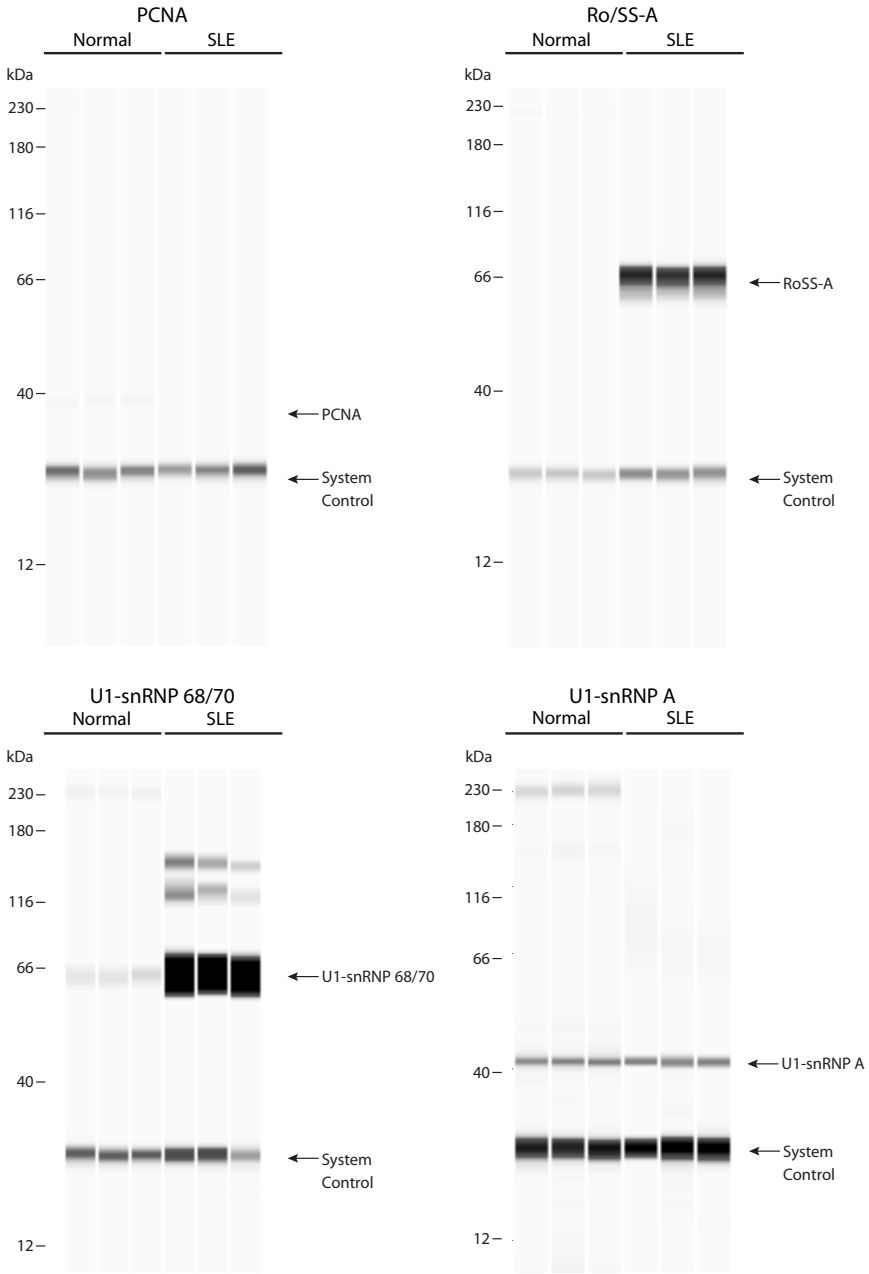

FIGURE 3. Normal and SLE patient serum samples were diluted 1:10 in Antibody Diluent 2 and introduced in the primary incubation step to capillaries already immobilized with known lupus antigens. Neither patient had autoantibodies for PCNA (top left) while both had equivalent amounts of U1-snRNP A (bottom right). Only the SLE patient had Ro/SS-A autoantibodies (top right), and the SLE patient also had 40X more U1-snRNP 68/70 compared to the normal patient (bottom left).

transferred to row D. Wes Luminol/peroxide was prepared as usual and transferred to row E.

We then loaded default assay parameters in Compass for Simple Western before starting the run. Antigen was run in six capillaries using the 12-230 kDa Separation Matrix. Three capillaries were used for normal patient serum and the remaining capillaries were used for SLE patient serum for triplicate data (**Figure 3**). We also multiplexed for the 29 kDa system control that Compass for Simple Western software uses to automatically normalize data from experiment to experiment.

DETECTING SLE AUTOANTIBODIES

Autoantibodies against PCNA weren't detected in either the normal or SLE patient sample, and only the SLE patient sample had Ro/SS-A autoantibodies. The SLE patient sample also had 40X more U1-snRNP 68/70 compared to the normal patient, but both had equivalent levels of U1-snRNP A (**Table 3**). Data was run in triplicate and was very reproducible with all CVs less than 10.8%.

To confirm our PCNA result and that the normal patient Ro/SS-A data wasn't a false negative, we analyzed the amount of antigen captured on the capillary wall in triplicate using the Simple Western Total Protein Assay (**Figure 4**).[4] For this assay, 0.02 mg/mL of immobilized antigens were labeled with biotin and detected with streptavidin-HRP in the secondary incubation step, so you'll get a measurable chemiluminescent signal. The amount of antigen in each capilliary was equivalent with CVs less than 5.7% (**Table 4**), confirming there were no autoantibodies against PCNA in the normal and PCNA serum samples. The additional band seen at 230 kDa is the 230 kDa fluorescent standard , which gets biotinylated and detected within the assay's LOD.

ANTIGEN	RO/SS-A		U1-snRNP 68/70		U1-snRNP A	
Serum	Normal	SLE	Normal	SLE	Normal	SLE
Average Peak Area	N/A	60524.1	9065.4	364559.6	5048.0	3437.0
Std. Dev	N/A	6528.3	983.3	35255.5	334.4	153.6
% CV	N/A	10.8	10.8	10.8	6.6	4.5

TABLE 3. Quantitation and statistical summary for the peak area of autoantibodies detected in normal and SLE patient serum. Compass for Simple Western software normalized peak areas to the 29 kDa System Control and calculated intra-assay CVs which were all under 10.8%.

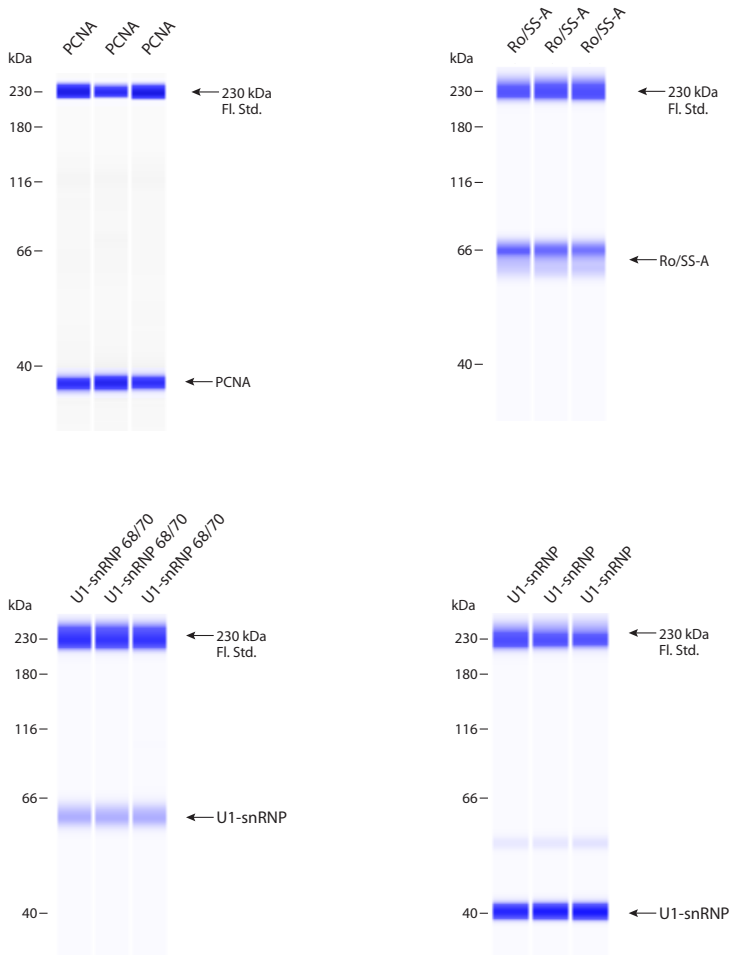

FIGURE 4. The Simple Western Total Protein assay was used as a positive control to confirm recombinant antigen was immobilized to the capillary wall, even when autoantibodies weren't detected in the serum. PCNA: top left, Ro/SS-A: top right, U1-snRNP 68/70: bottom left, U1-snRNP A: bottom right.

	PCNA	RO/SS-A	U1-snRNP 68/70	U1-snRNP A
Average Peak Area	18502.7	18047.0	8599.5	18752.7
Std. Dev.	530.5	342.8	144.6	1075.8
% CV	2.9	1.9	1.7	5.7

TABLE 4. Quantitation and statistical summary for the peak area of antigen bound to the capillary wall. Compass for Simple Western software averaged the peak area for the three data points and calculated CVs, which were all below 5.7%.

Spot-on Precision

We all know that immunoassays aren't always as reproducible as we'd like them to be. Wes' automation cuts out a lot of the user variability associated with traditional Western blots, so we also tested the serum antibody application robustness to see how it stacked up. The same samples were run on Wes on three different days, and peak areas were normalized to the 29 kDa System Control to remove day-to-day variability (**Figure 5**). The results showed inter-assay CVs were under 14% across all 3 days. With CVs like these you can be sure you'll get consistent data day in and day out (**Table 5**).

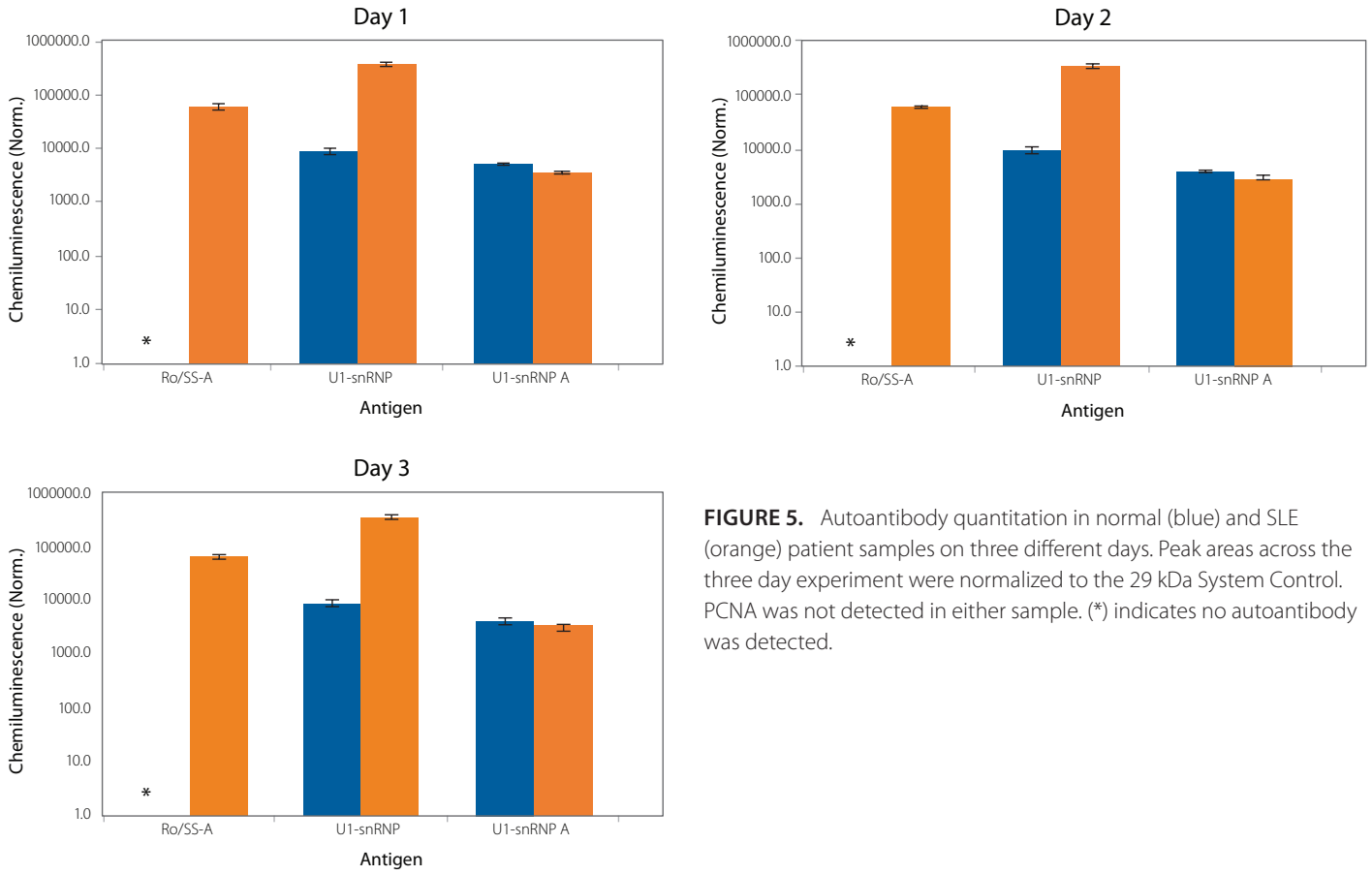

FIGURE 5. Autoantibody quantitation in normal (blue) and SLE (orange) patient samples on three different days. Peak areas across the three day experiment were normalized to the 29 kDa System Control. PCNA was not detected in either sample. (*) indicates no autoantibody was detected.

	RO/SS-A		U1-snRNP 68/70		U1-snRNP A	
	Normal	SLE	Normal	SLE	Normal	SLE
Average Peak Area	N/A	65116.9	9139.8	368115.0	4319.1	3644.1
Std. Dev.	N/A	6698.3	1041.2	32647.0	605.3	440.9
% CV	N/A	10.3	11.4	8.9	14.0	12.1

TABLE 5. Quantitation and statistical summary for the peak area of autoantibodies detected in normal and SLE patient serum across three days. Compass for Simple Western software normalized peak areas to the 29 kDa System Control and calculated inter-assay CVs, which were all less than 14%.

Conclusion

Now you can use Simple Western assays on Wes to run all your serum antibodies quickly and easily. Everything's completely automated and hassle-free, so you don't have to cut a single strip! Simple Western assays give you comparable data to traditional Western blot[1] but you'll get it faster, cut your hands-on time by at least 8-10X and use a lot less sample per data point. And because everything is automated, the data you get is much more precise.

To demonstrate this, we measured the levels of autoantibodies against known lupus antigens in normal and lupus patient samples. The Simple Western Total Protein assay was used to confirm the presence of antigen in the capillaries to rule out false negatives, and a Simple Western immunoassay was modified to measure levels of serum antibody. On average, it only took 30 minutes of hands-on time to prep each plate, and results were ready in three hours. Of the four antigens tested, PCNA wasn't detected in either sample which isn't surprising since it's only prevalent in 2-7% of all SLE patients. Ro/SS-A was only detected in the lupus patient sample, 40X more U1-snRNP was measured in the lupus samples compared to the normal samples, and comparable levels of U1-snRNP A were detected in both samples. We also got great precision with intra-assay CVs all under 10.8% and inter-assay CVs under 14% when we ran the same experiment on three different days.

References

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3. Improved Multiplexing with Simple Western Using a 20X Rabbit HRP Conjugate, ProteinSimple Technical Note.

4. Total Protein Analysis the Simple Western Way, ProteinSimple Application Note.

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