Screening on Simple Western™

Introduction

When expressing an antibody or recombinant protein in a heterologous host, getting to the right clone quickly is critical. Obtaining enough material to verify clone positivity can require growth or expansion of the clones, so there is a need for a sensitive detection method to minimize the amount of sample that is required. While sensitive detection of expressed proteins can be achieved by ELISA, it lacks the ability to provide any information on the molecular weight of the protein being detected, making this method insufficient for most clone selection. On the other hand, while the traditional Western blot can provide molecular weight information, it is often not as sensitive as an ELISA and it is even more time consuming and labor intensive.

ProteinSimple's Simple Western offers a powerful solution by coupling protein separation and sensitive detection in high-throughput. This allows you to screen up to 96 samples per day in a single run, and obtain truly quantitative data with molecular weight for the expressed protein. Here we describe a workflow for both Simple Western Wes™ and Peggy Sue™/Sally Sue™ that will allow you to get the most out of your instruments. In addition, the ability to prepare and freeze your Simple Western samples a day ahead allows for continuous screening throughout the week, up to 480 samples per week per instrument. We've also got epitope-tag antibody recommendations so you can start screening with no optimization of the detection. Finally, we describe examples of how you can use this workflow for common tasks, including screening for His tag proteins, epitope tag antibody optimization, and screening for BSA contamination.

Recommended Tag Antibodies

We've optimized the most popular HRP-conjugated epitope-tag antibodies (HA, His, and FLAG) so you can quickly start screening your samples. Shown in **Table 1** are the details including dilution and incubation time for each antibody.

Using HRP-conjugated epitope-tag antibodies allows for a single-step assay to be run. Doing so speeds up the overall assay by eliminating the need for additional detection through a secondary antibody.

ANTIBODY	SUPPLIER	PART NUMBER	ANTIBODY DILUTION FOR SIMPLE WESTERN	INCUBATION TIME
Anti-HA-HRP	R&D Systems	HAM0601	1:5	30 minutes
Anti-His-HRP		MAB050H	1:10	
Anti-FLAG-HRP		HAM85291	1:25	15 minutes

TABLE 1. Optimized conditions for HRP-conjugated epitope tag antibodies.

Screening Workflow

MATERIALS

ProteinSimple

- Instruments: Peggy Sue, Sally Sue or Wes
- 12-230 kDa Wes Separation Module (SM-W004)
- 12-230 kDa Peggy Sue or Sally Sue Separation Module (SM-S001)
- No secondary Detection Module for Wes, Peggy Sue or Sally Sue (DM-003)

Other materials

- 96-well PCR plates (Bio-Rad, HSP9601)
- PCR plate seals (USA Scientific, 2923-0100)
- Multichannel pipet capable of pipetting 2.5 µL for Wes
- 96 to 384-well adjustable pipet (For example, E1-ClipTip Electronic Pipette, Thermo Scientific) for Sue
- Vortex mixer
- Centrifuge with plate adapter
- Thermal cycler

METHODS

Whether you plan on screening your samples on Wes, Peggy Sue or Sally Sue, we recommend that the samples first be placed into a 96-well plate in order to make sample preparation as simple and as straightforward as possible. Chances are, if you are screening early in your process, for example cell supernatants, you already start from a 96-well format. The general workflow will be similar between Wes and the Sue instruments, except for the sample prep plate layout, as the Wes and Sue assay plate layouts vary significantly.

Sample preparation

1. Prepare a 2X Master Mix (2X MM) by reconstituting a tube of Fluorescent Standards as follows (each tube makes enough 2X MM for approximately 40 samples) and place on ice until ready to use. Add and mix:

   a. 60 µL milliQ H_2O

   b. 20 µL 10X Sample Buffer

   c. 20 µL 0.4 M DTT

2. Add 2.5 µL of 2X MM to the corresponding wells of your 96-well sample prep PCR plate.

3. Using a multichannel pipet, transfer 2.5 µL of undiluted sample to the sample prep plate as noted on the plate layout.

4. Seal the sample prep plate with a PCR plate seal and centrifuge for 30 seconds at 1000 x *g* to ensure all the sample is at the bottom of the plate.

5. Vortex the sealed sample prep plate with 3 second pulses several times (3 to 5) along the length of the plate to ensure proper mixing of sample and 2X MM.

6. Centrifuge for 30 seconds at 1000 x *g*.

7. Transfer the sealed sample prep plate to a PCR thermocycler and denature (5 minutes at 95 °C is our default condition).

8. Cool plate down on ice for 5 minutes.

9. Centrifuge for 2 minutes at 1000 x *g*.

10. Vortex sample prep plate with 3 second pulses several times (3 to 5) along the length of the plate.

11. Centrifuge for 30 seconds at 1000 x *g*.

Note: *At this point you have the option of snap-freezing your samples on dry ice and storing at -80 °C for screening the next day. Longer term storage of prepared samples is not recommended.*

   a. Thaw prepared sample plate on ice. (There may be cases where reheating is necessary.)

   b. Vortex sample prep plate with 3 second pulses several times (3 to 5) along the length of the plate.

   c. Centrifuge for 30 seconds at 1000 x g.

   d. Continue with normal protocol.

12. For Wes runs, use a standard 8-channel pipet to transfer 3 µL of sample from the sample prep plate to a Wes assay plate. For Sue runs, use an adjustable multichannel pipet to transfer 5 µL of sample to a Sue assay plate. Make sure to follow your plate layout precisely.

Note: *If you are running multiple Wes assay plates from the same PCR sample prep plate, make sure to only cut the seal off to access the samples for a given run. Keep the rest of the plate sealed and on ice until ready to screen the next set of samples to prevent evaporation.*

13. Add other Simple Western reagents to the assay plate.

14. Centrifuge the assay plate for 5 minutes at 1000 x *g* at room temperature and run on Wes or Sue using the recommended incubation times listed in **Table 1**.

Sample preparation on 96-well PCR plate for Wes assay

A single 96-well PCR plate can be used to prepare up to 96 samples for up to 4 Wes runs (**Figure 1**). If you are planning to run more than one Wes assay plate, use the same loading scheme in subsequent wells on the sample preparation plate (Run 2, Run 3, Run 4).

After preparing the sample preparation plate, you are ready to start setting up your Wes assay plate. Follow the instructions in **Figure 2** to get your Wes assay plate filled.

Sample preparation on 96-well PCR plate for Sue assay

The sample prep plate layout for Peggy Sue and Sally Sue is different than for Wes. Since the Sues can run up to 96 samples, the entire 96-well sample preparation plate can be filled with samples (**Figure 3**). It is recommended that the Biotinylated Ladder (L) be added to the sample prep plate and denatured with the samples as to make the transfer to the assay plate as straightforward as possible.

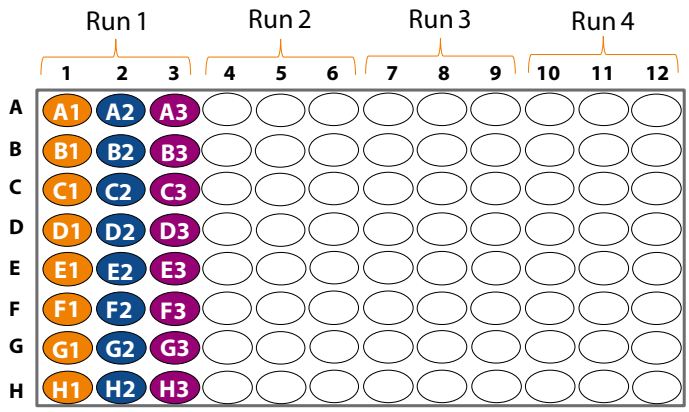

FIGURE 1. Example of a sample preparation plate layout for Wes.

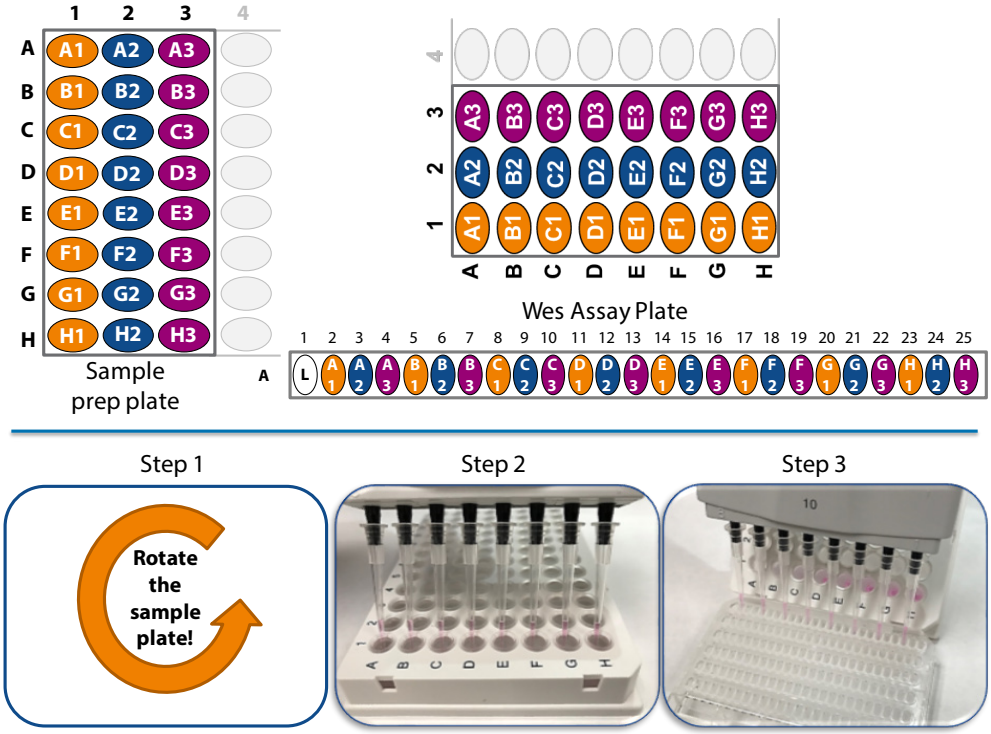

FIGURE 2. To load a Wes assay plate from the sample preparation plate, first rotate the sample prep plate 90° counter-clockwise (Step 1). Then transfer the prepared samples using an 8-channel pipet (Step 2), which will pipet into every third Wes sample well (Step 3). Therefore, column 1 samples from the 96-well preparation plate will go into every 3rd well starting in well A2, with well A1 reserved for the ladder (L).

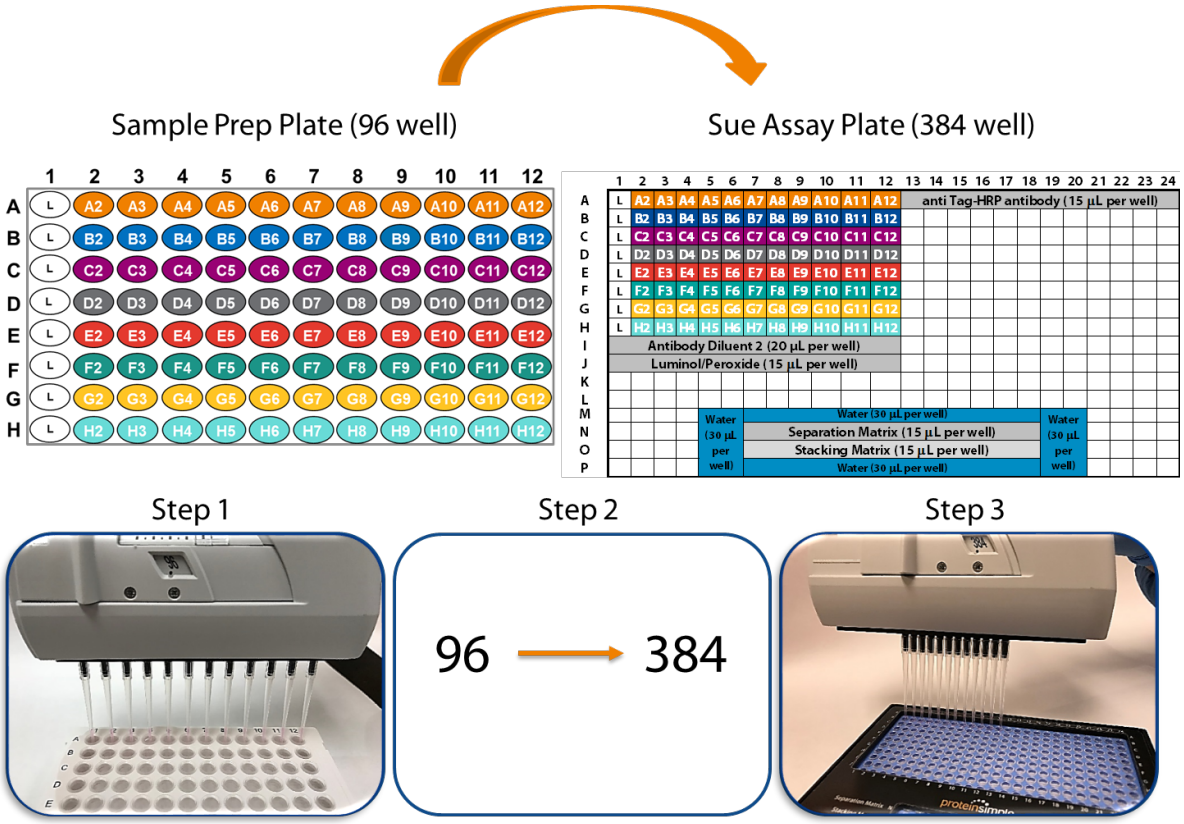

FIGURE 3. Sue Assay plate layout. After sample preparation in a 96-well plate, transfer samples to the Sue plate following the loading scheme shown. After sample transfer, other required reagents can be added to the plate.

To demonstrate the utility of using Simple Western for screening, we've included some examples for both Wes and Sally Sue/Peggy Sue.

Results

Example 1: His-tagged protein screen on Wes

14 HEK293 clones expressing secreted His-tagged protein constructs and two negative control clones were screened on Wes in four consecutive runs on the same day (**Figure 4**). Each run contained one technical replicate of a given sample and standard curve. Samples for all four runs were prepared in a single 96-well PCR plate the day before the screen (see optional procedure for preparing samples the day before screening). Other reagents (diluted primary antibody and Luminol/Peroxide) where made in bulk, for all 4 Wes runs, and kept on ice for the day protected from light until use.

The His-tagged samples screened on Wes resulted in data of high confidence. From Run 1 to Run 4, the average CV was 11.5% for the samples and 10.0% for the standard curve.

Example 2: Multiple epitope tag antibody optimization using a Multi Tag protein

The recombinant *E. coli* Multi Tag protein from Abcam (ab36791), which contains 12 epitope tags fused in tandem, allows for the optimization of a variety of anti-Tag antibodies with one tool. All it takes is two runs and you'll have optimized conditions for your anti-Tag antibody of interest. First, titrate both antigen and antibody to get an idea of the affinity of the antibody to the antigen. Then, perform a narrower titration of the antibody with a fixed concentration of the Multi Tag protein (determined in the first experiment). In addition, if you are using a Sue for optimization, you can also conduct a time course to determine the primary antibody incubation time required to reach saturation. This basic approach may also be used for screening multiple antibodies against a given lysate and other samples.

As an example, optimization of the anti-FLAG-HRP (R&D Systems, HAM85291) is shown in **Figure 5**. The anti-FLAG antibody was titrated against 0.1 mg/mL recombinant Multi Tag protein and shows signal saturation at 1:25 dilution.

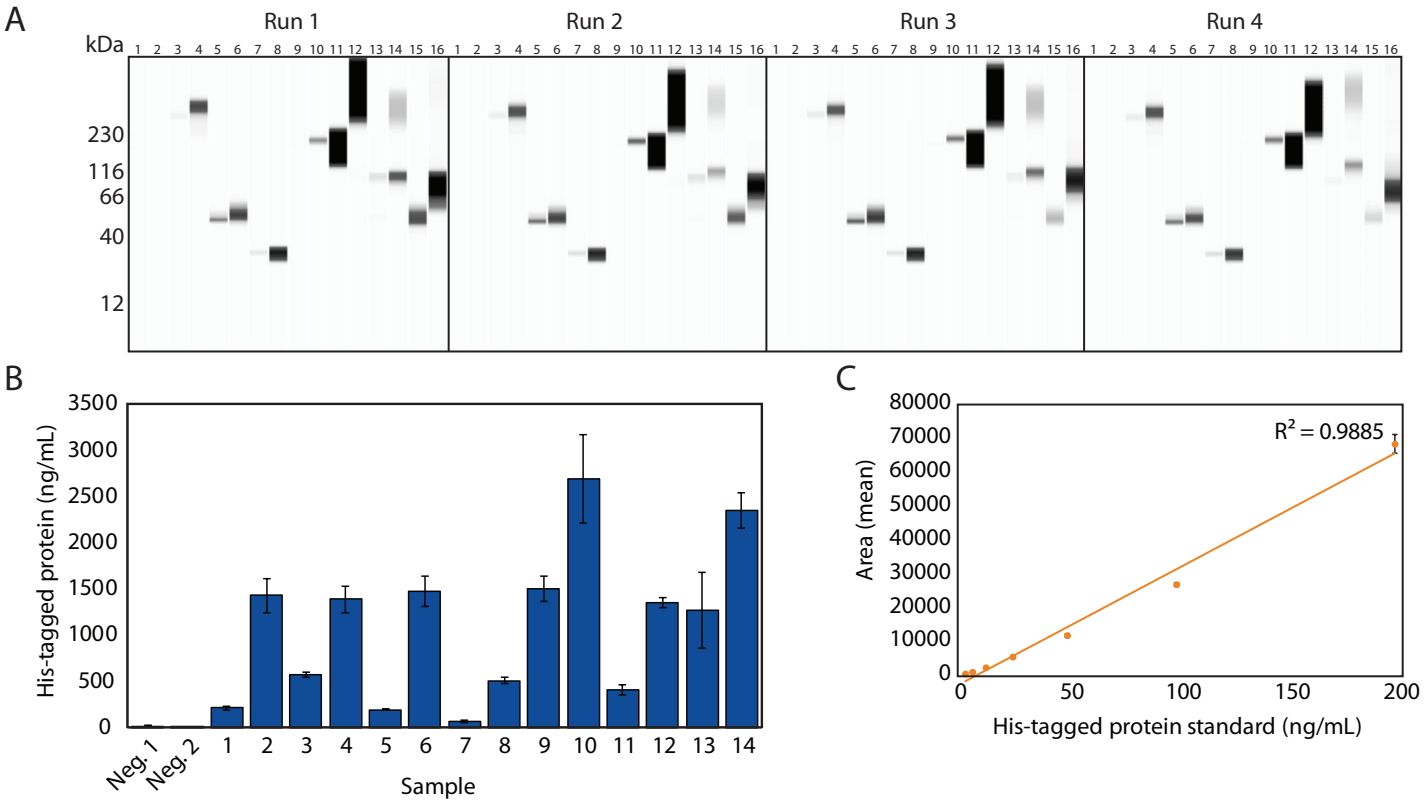

FIGURE 4. (**A**) Compass lane view of, from left to right, 2 negative controls, Neg. 1 and Neg. 2, and 14 His-tagged samples screened in four consecutive Wes runs, each run containing one technical replicate. (**B**) Average concentration of screened samples in panel A. (**C**) Standard curve over four Wes runs.

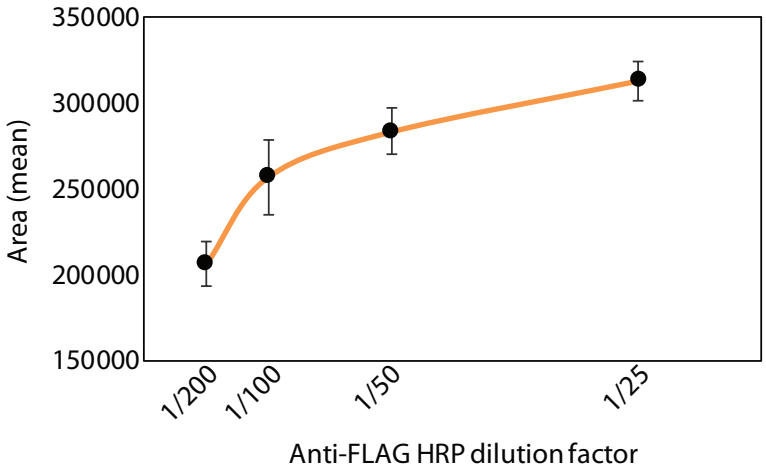

FIGURE 5. Optimization of the anti-FLAG-HRP antibody (R&D Systems, HAM85291). The antibody was titrated from 1:25 to 1:200 against 0.1 mg/mL recombinant Multi Tag protein.

Example 3: Screening for contamination

Screening on Simple Western is not just limited to finding the right clone; it has also shown great potential in detecting protein contamination in product development that rivals the traditional ELISA-based method of detection. For example, bovine serum albumin (BSA) is a common contaminant in the preparation of vaccines because it is a major component of the culture medium that is used during vaccine production[1]. The World Health Organization guidance requires that no more than 50 ng of BSA be present per human dose of vaccine[2]. Thus, there is a need for rapidly screening protein contaminants with sufficient sensitivity and reproducibility. Traditional ELISA-based methods are labor-intensive and do not provide size-based separation. Simple Western overcomes these limitations by providing fully automated operation and size-based differentiation, while maintaining the ability to screen large sample sizes (88 samples per run). This means Simple Western not only detects BSA with the necessary sensitivity and reproducibility, but it also distinguishes monomers from oligomers and higher order aggregates.

We used the same Peggy Sue workflow as described above to detect BSA in purification fractions by using a rabbit anti-BSA primary antibody available from Bethyl Labs.

Note: *Because BSA is present as a blocking agent in Antibody Diluent 2, you should use the Peggy Sue Charge Antibody Diluent (ProteinSimple, 040-309) in place of Antibody Diluent 2 for making antibody dilutions and for blocking.*

Briefly, the primary antibody was diluted 1:100 in Charge Antibody Diluent and incubated for 120 minutes, and then Goat-Anti-Rabbit HUX HRP Secondary Antibody (ProteinSimple, 041-081) was diluted to 1X in Charge Antibody Diluent and incubated for 60 minutes. This resulted in a broad range of precise BSA detection (**Figure 6**).

Conclusion

Simple Western lets you screen large sample sizes quickly while eliminating the manual washing and incubation

4821	235204	219	13831	880908	3998	40402	1427921
300	611	283	247	2797	189	32	9479
1571	64759	309	3542	295876	409	8006	838848
178	1822	210	305	7263	768	907	25574
14422	595522	1399	49630	1148400	3648	125229	2753867
158	431	885	176	1039	668	232	1636
78353	1375248	8371	319365	2912905	0	680851	5946550
277	4105	143	754	23294	329	1374	146950
298	471	476	79	1413	547	88	2545
666	19296	350	1517	111293	118	2460	380133
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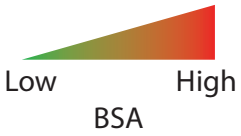

FIGURE 6. Detection of BSA on Peggy Sue using an anti-BSA antibody. The numbers represent the BSA signal or peak area in counts per second.

steps of traditional ELISAs and Western blots. To get you started on your Simple Western with little to no optimization, this present work describes a toolkit for screening some of the most commonly used epitope tags and contaminating proteins. Of course, the Simple Western platform is not limited to the antibodies listed here; any of these antibodies may be swapped out with your antibody of interest to meet your needs. No matter the antibody and target antigen you choose, the straightforward workflow described here will remain essentially the same.

References

1. Residual bovine serum albumin (BSA) quantitation in vaccines using automated capillary western technology, J Loughney, C Lancaster, S Ha and R Rustandi, *Analytical Biochemistry*, 2014; 461:49-58.
2. WHO expert committee on biological standardization, *WHO Technical Report Series 941*, 2007.

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