Using the Simple Western Total Protein Assay to Normalize Immunoassay Data in the Same Run

Introduction

Housekeeping proteins such as GAPDH, tubulin, and others are typically used to normalize the target protein signal on traditional Western blots. But, many of these proteins aren't expressed as consistently as previously thought, making single target normalization less than ideal. Finding an unbiased way to normalize all the protein immobilized on a membrane or capillary is critical.

Scientific journals, like the Journal of Biological Chemistry, have recently updated their Western blot submission guidelines[1] and are starting to recommend the use of total protein normalization for better data quality that reflects expression changes more accurately. To analyze total protein using traditional Western blots, they ask that authors stain the blot membrane with Coomassie or Ponceau S and generate a normalizing value per lane.

In this guide, we'll show you how to use the Total Protein Detection Module (DM-TP01) with any immunoassay detection module of your choosing to get total protein and immunoassay data in the same run. Going this route gives you a high throughput and more accurate way of getting that normalization factor you'll need when submitting articles to journals, without adding any experimental time per run.

How the Simple Western Total Protein Assay works

The total protein assay shown in **Figure 1** is an in-capillary labeling technique where proteins are separated by molecular weight, immobilized in the capillary, and then labeled with biotin before blocking. Next, the biotin-labeled proteins in the capillary are bound by HRP-conjugated streptavidin (SA-HRP) for detection in a chemiluminescent reaction.[2]

In Compass for Simple Western, you can select the Total Protein Assay from the assay menu. This assay has optimized incubation times for the Total Protein Detection Module reagents. Check out the product insert and the Total Protein Assay Plate Layout included with the Total Protein Detection Module (DM-TP01) for more information.

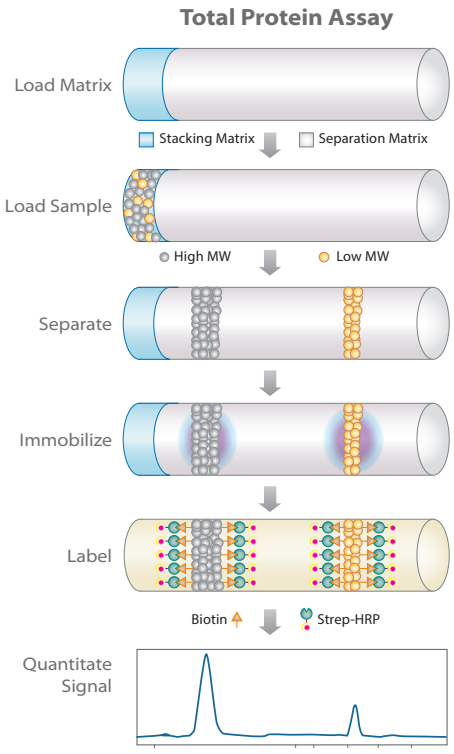

FIGURE 1. Proteins are separated and then UV-captured in the capillary the same way as the immunoassay. Next, captured proteins are exposed to the biotin labeling reagent, which allows for recognition by streptavidin-HRP.

Running the Total Protein assay and Immunoassay at the same time

To get total protein and immunoassay data on the same set of samples, the two assays would need to be run in separate capillaries. If they're done in separate runs on Wes™, that would be ~6 hours of total run time as each

assay takes about 3 hours. We've designed a way to cut this time in half by running both assays at the same time and on the same plate using the Total Protein Assay as the template for both.

To get started, let's first look at the default assay conditions for the total protein and immunoassay, shown side by side in **Figure 2**.

The two assays are slightly different, but have the same number of steps. The Total Protein Assay needs a 30-minute biotin labeling time (highlighted in blue in **Figure 2**) compared to the Immunoassay, which has a 5-minute blocking step at the same step in the assay. Of these two incubations, the biotin labeling time is more critical. So, when running both assays in the same Wes run, you'll want to use the default Total Protein Assay protocol conditions. You should run a sample dilution series first to identify the linear detection ranges for both the total protein and any immunodetected targets. Working within the linear range for both assays is important to get the most accurate and reproducible quantitation. **Figure 3** shows a plate layout of how to set both assays up on one plate.

And when you're ready to normalize the AKT1 immunoassay area based on the total protein area, first determine a total protein normalization factor in Microsoft® Excel®. The normalization factor is calculated as follows:

1. Choose a reference total protein area (in our case, from capillary 2). Next, divide by the area from each total protein capillary (capillary 2, 4, 6, 8 10, 12, 14, 16, 18 and 20) to obtain normalization factors for each sample.

2. Multiply the target protein areas (in our case AKT1) in capillaries 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21) by the corresponding normalization factor from Step 1 to obtain total protein normalized immunoassay data.

Immunoassay

	Value
Separation Matrix	
Stacking Matrix	
Sample	
Separation Time (min)	25.0
Separation Voltage (volts)	375
Matrix Removal	
Antibody Diluent Time (min)	5.0
Primary Antibody Time (min)	30.0
Secondary Antibody Time (min)	30.0
Detection	

Total Protein Assay

	Value
Separation Matrix	
Stacking Matrix	
Sample	
Separation Time (min)	25.0
Separation Voltage (volts)	375
Matrix Removal	
Biotin Labeling Time (min)	30.0
Primary Antibody Time (min)	30.0
Total Protein HRP Time (min)	30.0
Detection	

FIGURE 2. Default assay protocols for the Immunoassay and Total Protein Assay on Wes. The main difference in the protocols is a single incubation, highlighted in blue in each protocol.

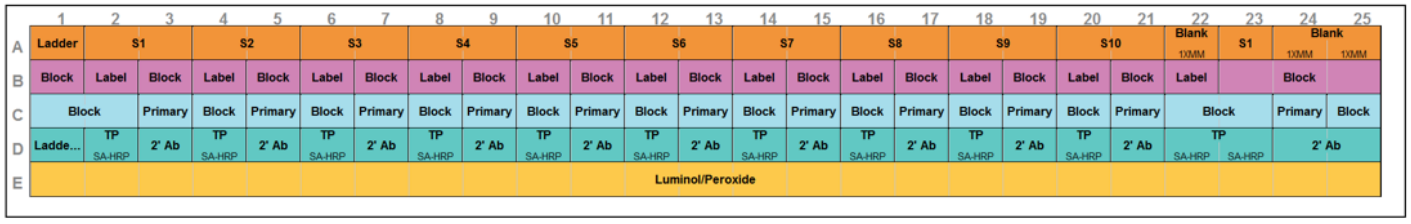

FIGURE 3. Suggested plate layout when running both the Total Protein and Immunoassay on the same Wes plate. For each sample (S1-S10), either a biotinylation reagent (Label) or antibody diluent (Block) is used in Row B. In Row C, either antibody diluent (Block) or primary antibody are used. In Row D, the total protein SA-HRP or anti-species secondary antibody (2' Ab) is used for detection. Controls are shown in wells 22-25.

To show the feasibility of running this dual assay, we used the 12-230 kDa Separation Module (SM-W004), HeLa lysate (0.2 mg/mL), an anti-AKT1 antibody and the Anti-Rabbit Detection Module (DM-001) for the immunoassay, and the Total Protein Module to detect the total protein in the sample (DM-TP01). Resulting data showed high reproducibility for both assays when run side-by-side (**Figure 4**). CVs for total protein came in at 4.23%, and 7.95% for the immunodetected AKT1. CVs for the immunodetected AKT1 decreased to 7.66% after total protein normalization.

The total area detected using the Total Protein Assay includes proteins biotinylated through the reaction as well as endogenously biotinylated proteins. Endogenous biotinylated proteins are identified by running a no-label control (**Figure 4**, lane 23). These proteins can be included in your analysis when quantifying your data.

The Total Protein and Immunoassay can also be combined and run together on Peggy Sue™ or Sally Sue™. For these instruments, the two assays can be run in separate cycles using the same wells loaded with sample.

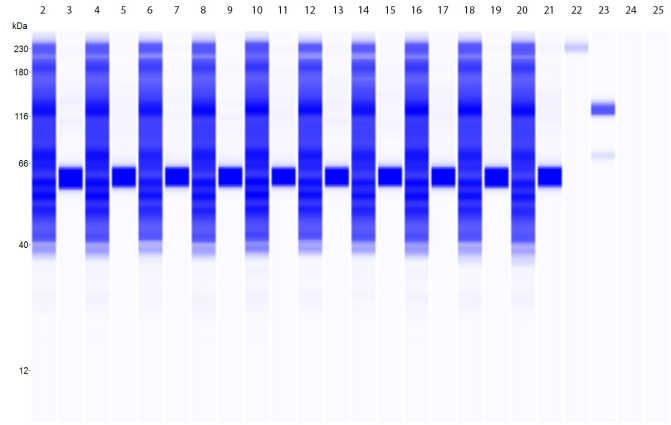

FIGURE 4. Example data set of a combined Total Protein and Immunoassay Wes run. HeLa lysate (0.2 mg/mL) was either examined with the Total Protein Assay (even-numbered lanes) or with an anti-AKT1 antibody (odd-numbered lanes). Controls (right) for the Total Protein Assay (lanes 22 and 23) show total protein labeling of the internal 230 kDa standard and detection of endogenous biotinylated proteins, respectively. Controls for primary antibody or secondary antibody-derived background are shown in lanes 24 and 25.

Conclusion

Given the need for better normalization of traditional Western blot data, you can use the Total Protein Assay to get the total protein data scientific journals need. It's easy, highly reproducible and can be truly quantitative with optimized assay conditions. As an added bonus, if you've already got an optimized Immunoassay, you can run the Total Protein and Immunoassay on the same plate and get data for both at the same time! Simply use the default Total Protein Assay in Compass for Simple Western and combine both detection modules onto one plate. Be sure to also include the appropriate controls to so your quantitation is as accurate as it can be.

References

1. Transparency is the key to quality, AJ Fosang and RJ Colbran, *J Biol Chem*, 2015; 290(50):29692-4.

2. Total Protein Analysis the ProteinSimple Way, ProteinSimple Application Note, 2015.

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