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APPLICATION NOTE

BETTER HOUSEKEEPING: PROTEIN NORMALIZATION ON JESS

INTRODUCTION

Quantitative interpretation of Western blot data is often a challenge due to improper standardization. For researchers trying to perform comparative analyses of protein expression between biological samples that vary in complexity, disease state or applied external stimuli, an approach that reproducibly measures legitimate variance is especially important.

"Housekeeping" proteins (HKPs), also known as loading controls, are commonly used for protein normalization and to rule out technical variations in Western blot data. This approach compares the relative expression of a target protein to that of an unrelated, thought-to-be ubiquitously and constitutively expressed loading control—most often β-actin, glyceraldehyde-3-phosphate dehydrogenase, or β-tubulin, among others. However, more and more published work points to HKPs as an unreliable choice for normalization since their expression, too, can be influenced by various factors such as experimental treatment and growth conditions, stress, cell cycle phase, proliferation status, age or sex of biological source and pathological state[1-3]. Therefore, assuming a comparable expression of HKPs between your samples for normalization purposes may lead you to make inaccurate hypotheses and conclusions about the target(s) you are investigating.

Instead, normalizing target protein abundance to the overall amount of protein present in a sample is a more accurate means for eliminating technical errors and determining fold-change in protein expression. This method, for which total protein stains and stain-free products are commercially available, is antibody-independent and minimizes the impact of varying expression of a loading control. On Jess, it's a simple-to-perform added step in a Simple Western size assay. In this application note, we'll walk you through the protocol and show you that protein normalization on Jess is indeed superior to the HKP approach.

HOW DOES PROTEIN NORMALIZATION ON JESS WORK?

Jess gives you an easy way to see if your samples contain a consistent protein load—just load the proprietary in-capillary protein normalization reagent into the assay plate and she'll take care of the rest. The fluorescent reagent binds to proteins immobilized in the same capillary as your immunoassay via primary and secondary amine interactions. The result? You can quickly see if your samples contain a consistent protein load, identify experimental setup and user errors and effectively normalize expression of your target protein to get accurate and consistent data, giving you the confidence you need in your results.

MATERIALS

Products used in this application note are presented in TABLE 1.

PRODUCT	VENDOR	PRODUCT NUMBER
Protein Normalization Module	ProteinSimple	DM-TP02
Separation Module	ProteinSimple	SM-W004
β-Actin Mouse Monoclonal Antibody	R&D Systems	MAB8929
14-3-3 gamma Mouse Monoclonal Antibody	Novus Biologicals	NB100-406
Anti-Mouse Secondary NIR Antibody	Protein Simple	043-821
Jurkat Cell Lysate	Santa Cruz Biotechnology	sc-24788
HUMAN WHOLE TISSUE LYSATES		
Liver	Novus Biologicals	NB820-59232
Lung	Novus Biologicals	NB820-59237
Kidney	Novus Biologicals	NB820-59231
Colon	Novus Biologicals	NB820-59205
Breast	Novus Biologicals	NB820-59203
Tonsil	Novus Biologicals	NB820-59272
Brain	Novus Biologicals	NB820-59177

TABLE 1. Products used in this application note.

METHODS OF PREPARATION

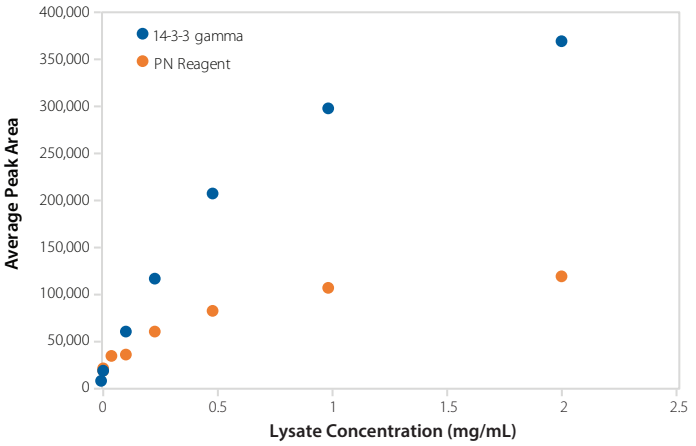
We diluted 2.5 mg/mL Jurkat cell lysate and 5 mg/mL human whole tissue lysates in 0.1X ProteinSimple Sample Buffer and supplemented the dilution with 40 mM DTT and fluorescent standards as described in the Jess Separation Module product insert. The final concentration for Jurkat cell lysate samples ranged from 0.5 mg/mL to 2.0 mg/mL, and we used 0.3 mg/mL for all human whole tissue lysates. All samples were denatured and reduced for 5 minutes at 95 °C.

The 14-3-3 gamma primary antibody (1 mg/mL) was diluted 1:100 in Antibody Diluent 2. The lyophilized β-actin primary antibody was reconstituted in 0.5 mL of sterile PBS, then diluted 1:50 in Antibody Diluent 2. Protein Normalization Reagents were diluted and prepared based on the dilution matrix found in the Protein Normalization Module product insert.

WORKFLOW OVERVIEW

Many labs will not measure the amount of total protein in their samples before running a Western blot, but this also often overloads highly expressed proteins and makes normalization—especially to HKPs—inaccurate. Because signal intensity has a clear correlation with antigen loading, reliable normalized Western blot data can only be generated if the proper amount of protein is known to be loaded. This is done by establishing the linear range of detection for your target and normalization standard to ensure your normalized assay conditions fall within this range.

To accurately normalize protein expression using the Protein Normalization (PN) Module on Jess, start by determining the amount of sample to load so both the PN Reagent and target protein can be detected within the same linear range. In other words, begin by running a titration experiment to plot the signal intensity of both your target protein detected by immunoassay and the PN reagent. From here, you can establish a combined target and PN Reagent linear range from which you'll determine the optimal concentration to use in your assay. ProteinSimple recommends a titration curve ranging from a lysate concentration of 2.0 mg/mL down to 0.05 mg/mL. In FIGURE 1, we demonstrate the signal detection results of this dilution series for both the PN Reagent and the target of interest, 14-3-3 gamma, a member of a family of proteins that affect a myriad of cell-signaling pathways.



FGIURE 1. Titration results showing the signal detection of the PN Reagent and 14-3-3 gamma (peak area, y-axis) over a series of lysate concentrations (x-axis).

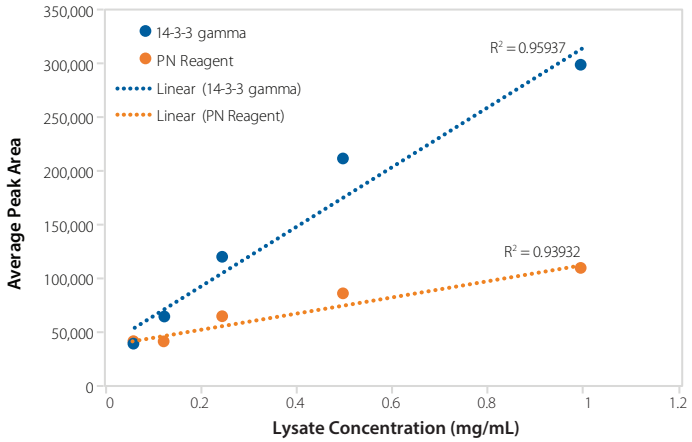


FIGURE 2. The concentration of lysate you load in your assay should fall within the linear range of the signal for both your target and the PN Reagent.

In **FIGURE 2**, we apply linear regression analysis to our titration data from **FIGURE 1** using Excel and demonstrate a strong relationship between the expression of our target protein and PN Reagent within the shown concentration range (14-3-3 gamma, R^2=0.9594; PN Reagent, R^2=0.9393). From here, the concentration of lysate that is within both linear ranges can be selected for loading in a Simple Western assay on Jess. For example, in this case, an amount from 0.06 mg/mL to 1 mg/mL can be selected, although a good place to start is in the middle of this range at 0.3 mg/mL to 0.5 mg/mL.

You're now ready to load and run the optimized assay with the correct concentration of lysate! Follow the "Pipette your plate" diagram located in the Protein Normalization Module product insert for further instructions on dispensing the correct volume of reagents and samples in their designated wells. Then follow the "Start Jess" steps to get your assay loaded and started in Compass for Simple Western Software.

Once your run is complete, you're ready to normalize your target protein expression to the overall amount of protein present in each capillary. Compass for Simple Western will automatically transform the raw peak area values for your target (**FIGURE 3**, *Area* column) into normalized peak areas (**FIGURE 3**, *Corr. Area* column) without requiring any intervention by you, saving you from the hassle of manually calculating normalized values.

Navigating to the lane view in Compass for Simple Western will display the total protein in each capillary, or "lane," a visual that you'll find relatable to traditional total protein membrane stains (**FIGURE 4**, left). ProteinSimple lets you go a step further with a dot overlay feature, allowing you to get an immediate and quantitative visual summary of your normalized data (**FIGURE 4**, middle and right). Please refer to the Compass for Simple Western Quick Reference Guide for further guidance on the analysis of Simple Western assay results.

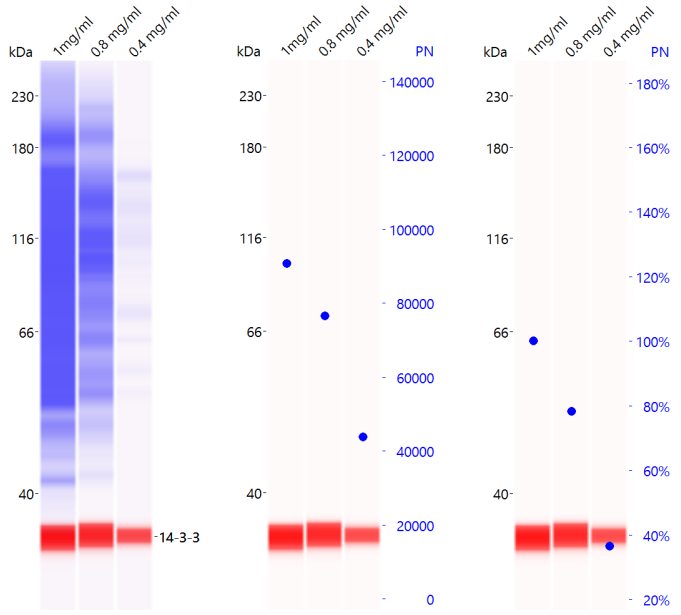


FIGURE 4. Lane view of protein normalization on Jess in Compass for Simple Western Software. Shown are three options for visualization: the traditional total protein "membrane stain" (left); dot overlay of the raw total protein area measured in each "lane" (middle); dot overlay of the normalized percent total protein area measured relative to the chosen reference "lane" or capillary (right), in this case, the 1-mg/mL sample.

Peaks | Capillaries

Sample	Primary	Secondary	Cap	Peak	Name	Position	MW (kDa)	Height	Area	% Area	Corr. Area	Width	S/N	Baseline	Channel
0.8 mg/ml	14-3-3	Anti-Mo...	15	1	14-3-3	330	35	21797.2	231044	100.0	236682.0	10.0	832.8	295.1	NIR
0.6 mg/ml	14-3-3	Anti-Mo...	16	1	14-3-3	331	36	19807.8	195776	100.0	238724.3	9.3	958.6	275.0	NIR
0.4 mg/ml	14-3-3	Anti-Mo...	17	1	14-3-3	332	35	15598.2	142643	100.0	255462.8	8.6	633.1	280.9	NIR
1mg/ml	14-3-3	Anti-Mo...	18	1	14-3-3	331	35	24625.7	257740	100.0	242451.3	9.8	1368.7	292.7	NIR
0.8 mg/ml	14-3-3	Anti-Mo...	19	1	14-3-3	332	35	23805.1	242293	100.0	254687.8	9.6	891.0	298.7	NIR
0.6 mg/ml	14-3-3	Anti-Mo...	20	1	14-3-3	333	36	21008.5	194730	100.0	263617.6	8.7	1011.5	332.4	NIR

FIGURE 3. Peaks Table in Compass for Simple Western Software showing raw peak area values in the Area column (circled left) and normalized peak area values in the Corr. Area column (circled right).

The Peaks Table shown in **FIGURE 3** can then be exported for further analysis. As an example, in **FIGURE 5**, we chose to represent the exported data in a bar graph using Excel. Both raw (blue bars) and normalized (orange bars) 14-3-3 gamma protein expressions are shown as a measure of peak area at various concentrations of Jurkat cell lysate. Six replicates of each sample concentration were run, which produced intra-assay CV values well below the Simple Western intra-assay specification of 20%, attesting to the effectiveness of protein normalization on Jess.

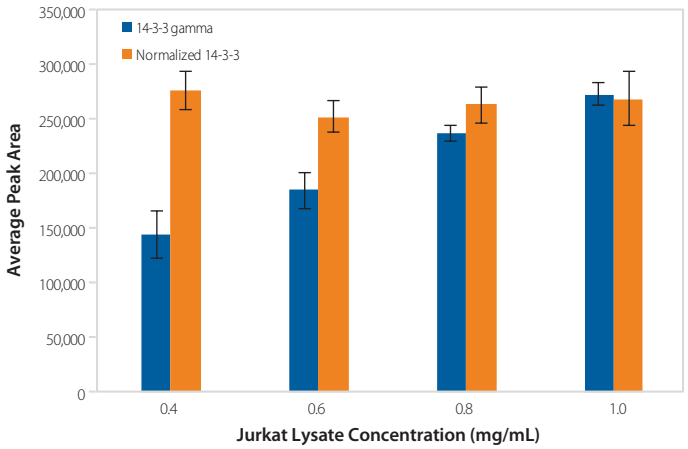


FIGURE 5. Comparative data showing 14-3-3 gamma protein expression (blue bars) and the normalized expression (orange bars) in various concentrations of Jurkat cell lysate. Targets were detected by the NIR channel on Jess.

PROTEIN NORMALIZATION ON JESS VERSUS β-ACTIN HKP

Quantitating target protein expression is essential for the identification of novel disease biomarkers, development of various targeted therapies and the comparison of large-scale protein expression profiles, among other applications. Without a stable reference for monitoring change in abundance, you cannot draw accurate conclusions from Western blot data. β-actin is one such popularly chosen reference, but differential β-actin expression has been observed and documented in various tissues from available disease models, as well as in their healthy counterparts.

To further illustrate this point, we evaluated the expression profile of β-actin in six different human whole tissues using a lysate concentration of 0.3 mg/mL (n=4) for all samples and compared the results with ProteinSimple PN Reagent staining on Jess (**FIGURE 6**). Indeed, a large variance in β-actin expression was observed when comparing across tissue types, whereas the overall protein expression measured by the PN Reagent remained comparable. These data support the conclusion that PN on Jess is a far more stable and reliable reference for normalizing and quantitating protein expression changes than the HKP, β-actin. Normalizing target protein expression data to the overall amount of protein present in your sample is also sure to boost your confidence with regard to the quality of your data submitted during peer-review and publication of your next Simple Western dataset.

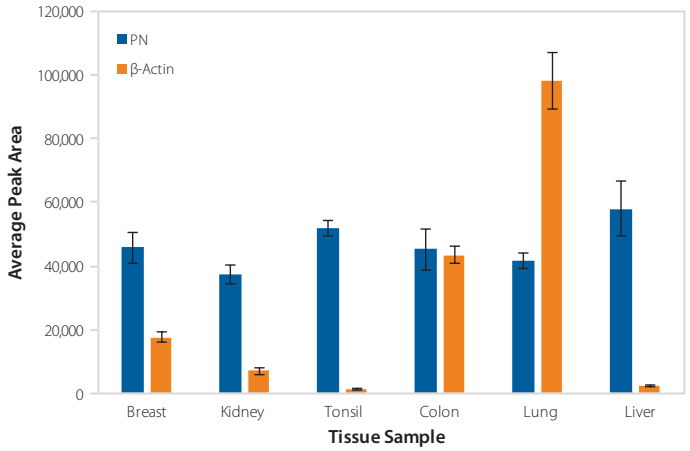


FIGURE 6. Comparative total protein data (blue bars) and the expression of β-actin (orange bars) in six human whole tissue lysates (0.3 mg/mL) tested using Jess.

FIGURE 7. Workflow to develop and optimize a Simple Western assay for total protein analysis and target normalization.

CONCLUSION

In this application note, we've shown you how you can perform accurate protein normalization using a simple workflow on Jess and why frequently used HKPs are not the preferred reference method. The recommended procedure can be easily summarized and is shown in FIGURE 7.

REFERENCES

1. Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies, RE Ferguson, HP Carroll, A Harris, ER Maher, Selby PJ and RE Banks, Proteomics, 2005; 5:566-71.

2. Beta-actin is not a reliable loading control in Western blot analysis, A Dittmer and J Dittmer, Electrophoresis, 2006; 14:2844-55.

3. Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting, SL Eaton, SL Roche, M Llavero Hurtado, KJ Oldknow, C Farquharson, TH Gillingwater and TM Wishart, PLoS One, 2013; 8:e72457.

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