A Novel Method for Total Protein Normalization in Western Blotting That Avoids Invalid Results Obtained When Using Housekeeping Proteins

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ABSTRACT

Protein normalization of western blots has relied upon housekeeping proteins which exhibit signal saturation and varied cellular expression levels across different cell types and conditions. Consequently, these issues can produce spurious results that lead to erroneous conclusions. A superior method to protein normalization using housekeeping proteins is Total Protein Normalization, a method now recognized as the gold standard for quantitative westerns. Total Protein Normalization requires that all proteins on a membrane be stained or labeled uniformly, imaged, and then analyzed for total protein. It is important that such a normalization process not interfere with typical immunodetection methods, fits within existing western workflows, and exhibits a linear relationship of signal intensity to protein load under all experimental conditions. Here we report that we developed a new reagent that enables Total Protein Normalization, and we demonstrate its superior protein normalization capabilities through our analysis of four target proteins (AKT1, cyclophilin b, EGFR, and phospho-EGFR) in four different cell backgrounds (HeLa, MCF-7, Jurkat, and A431 cells). These data illustrate how three housekeeping proteins exhibit signal saturation, yield erroneous normalization data, and display sample-to-sample variations averaging 48% overall. Signal intensities obtained using our new method, however, show a linear relationship to protein sample load, thereby providing accurate protein normalizations with an overall average variation of 8%. Utilization of housekeeping proteins for protein normalization can lead to errors in quantifying westerns and subsequently invalid conclusions from experimental studies; in contrast, this novel and improved Total Protein Normalization method provides an elegant alternative for achieving accurate quantitative western blots.

INTRODUCTION

Although western blotting methods have been in practice for over three decades, only recently have peer reviewers and journal editors advised authors to more rigorously demonstrate the validity of the means by which they quantify their western blot results. Such a demonstration would include protein normalization, a technique to account for western blot method inaccuracies and other inherent experimental variability that would presumably be unrelated to attributes being studied. Protein normalization is a means by which the amount of a target protein can be determined relative to a reference, often a housekeeping protein (HKP) like α-tubulin, β-actin, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). An HKP is chosen and used as a reference with the assumption that the level of the HKP would be directly proportional to the total amount of protein in samples being studied. Changes in a sample's target protein levels arising from designed experimental perturbations are then expressed as ratios to the level of the measured HKP. Unfortunately, the assumptions underlying the use of HKPs for protein normalization of western blot results frequently lack validity, and this can result in inaccuracies, inconsistencies, and invalid conclusions[1].

Cited issues with using HKPs for protein normalization of western blot results:

- HKPs, in most samples and as one consequence of their high abundance relative to the often limited abundance of a target protein of interest, exhibit signal saturation[2-4] in the typical range of protein loads being studied (i.e., the levels of HKPs do not display a linear response to increasing protein load), and this also makes HKPs poor internal loading controls[3]
- Not all cell lines and tissue types express the desired HKP or, if the chosen HKP is expressed, its expression level can vary with tissue type[4] or other cellular states[5]
- Expression of HKPs can vary with cellular treatment being studied[6]
- HKP expression levels may change with the density of cultured cells[7]

To overcome these issues with using HKPs for protein normalization, another method is needed, and that method is Total Protein Normalization (TPN). We have recently developed the Invitrogen™ No-Stain™ Protein Labeling Reagent that enables reliable TPN of western blots.

The No-Stain reagent consists of three components: No-Stain Labeling Buffer, No-Stain Activator, and No-Stain Derivatizer. The active ingredient of the Activator is mandelonitrile with its electrophilic cyano-carbon, while the active ingredient of the Derivatizer is the fluorogenic amine-derivatization molecule 3-(2-furoyl)quinoline-2-carboxaldehyde. In the presence of the Activator and as shown in Figure 1, this fluorogenic molecule reacts rapidly with primary amines in the presence of nitriles to form highly fluorescent conjugates with visible wavelength excitation and freedom from the background fluorescence typical of most other reactive fluorophores, while enabling ultrasensitive detection of primary amines (e.g., lysine sidechains and the amino termini of proteins)[8]. Protein normalization that utilizes the No-Stain reagent – according to the method depicted in Figure 2 – is ideally suited as an alternative to other existing, more cumbersome total protein visualization methods like Coomassie gel staining or Ponceau S membrane staining.

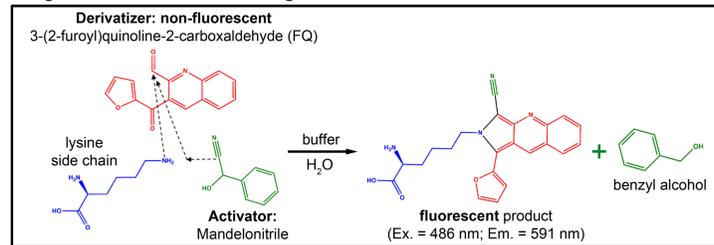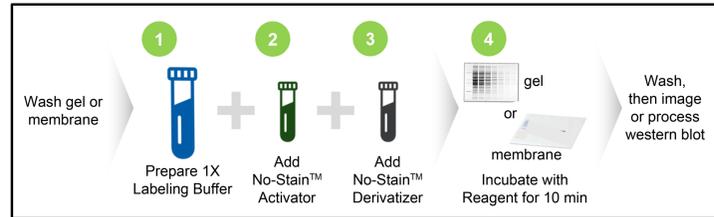### Figure 1. The No-Stain Labeling Reaction

Derivatizer: non-fluorescent
3-(2-furoyl)quinoline-2-carboxaldehyde (FQ)

lysine side chain

buffer
H$_2$O

Activator: Mandelonitrile

fluorescent product
(Ex. = 486 nm; Em. = 591 nm)

+ benzyl alcohol

Figure 2. The No-Stain Labeling Method

Wash gel or membrane

1 Prepare 1X Labeling Buffer
2 Add No-Stain™ Activator
3 Add No-Stain™ Derivatizer
4 gel or membrane Incubate with Reagent for 10 min

Wash, then image or process western blot

RESULTS

The capacity of the No-Stain Protein Labeling Reagent to serve as a means for performing TPN was assessed in various cell lines and compared to quantification results obtained using HKPs. For a method to be viable for protein normalization, the method must at least demonstrate a linear relationship between protein load and measured signal intensity.

Different, yet typical protein loads of HeLa cell lysate were electrophoresed, transferred to PVDF, No-Stain labeled, and then immunodetected for three HKPs (i.e., α-tubulin, β-actin, and GAPDH). Membrane images shown in Figure 3 were acquired using an iBright imager. The iBright software was used to quantify the fluorescent signals. The dotted Ideal Response line shown in the plot of Figure 3 represents the predicted quantitative response for each protein load. Results displayed in Figure 3 demonstrate that the No-Stain reagent provides a linear signal response to protein load over a range of HeLa protein loads that researchers typically use (10-40 µg per gel lane), and this response matches closely the predicted, Ideal Response. Densitometric signal intensities of HKPs, however, show a non-linear relationship to protein load within this typical protein load range, thereby underestimating the predicted response and being indicative of signal saturation (e.g., densitometric signal intensity does not increase proportionately with protein load). This signal saturation behavior of HKPs arises from the high cellular expression levels of HKPs, and this behavior limits the ability of HKPs to be used accurately as loading controls or to perform valid protein normalizations of target proteins under typical experimental conditions.
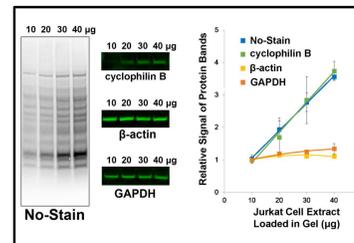
Figures 4 and 5 show results – similar to those shown in Figure 3 – that were obtained using extracts from cell lines other than HeLa.

Figure 3. HeLa Cell Lysate
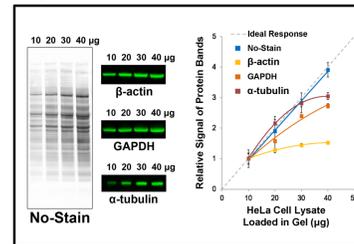
Densitometric signal linearity versus protein load was compared for three HKPs and No-Stain labeled HeLa lysate proteins. The No-Stain labeling reagent shows improved accuracy for normalization compared to HKPs in HeLa lysate.

Table 1. Percent Errors from HeLa Lysate

Norm. Method	Percent Error of Method from Predicted According to HeLa Lysate Protein Loaded in Gel				
	10 µg	20 µg	30 µg	40 µg	Avg
No-Stain	0.0	5.1	5.8	2.3	4.4
β-actin	0.0	35.9	51.9	61.9	49.9
GAPDH	0.0	21.4	20.1	31.8	24.4
α-tubulin	0.0	7.9	6.3%	23.9	38.1

Percent errors from predicted responses for all methods were computed from data used to generated Figure 3 plots. The larger the percent error, the farther the densitometric signal is from the predicted, ideal response and the less accurate the method at that protein load. The No-Stain reagent for TPN provides better concordance with protein load compared to HKPs.

Figure 4. Jurkat Cell Extracts

The No-Stain reagent shows improved accuracy for normalization compared to HKPs.

Figure 5. A431 Cell Extracts
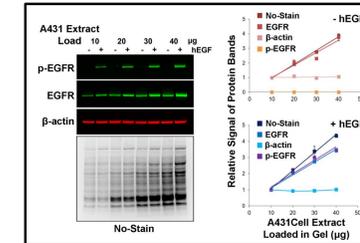
The No-Stain reagent shows accurate normalization while β-actin does not.

Data from the plots shown in Figures 4 and 5 were used to compute the percent errors from the predicted response as described previously for HeLa lysates. Results for Jurkat and A431 cell extracts are shown in Tables 2 and 3. These tables include the percent error from predicted for cyclophilin b as a target protein as well as EGFR and phospho-EGFR as target proteins. Data used to plot the graphs of Figure 4 and 5 were also used to normalize target protein levels (i.e., band volumes determined through densitometry), and results of statistical assessments of the normalization data are shown in Tables 2 and 4.

Table 2. Percent Errors from Jurkat Extract Data

Normalization Method	Percent Error of Method from Predicted According to Jurkat Extract Protein Loaded in Gel					Normalized Cyclophilin B Band Volume	
	10 µg	20 µg	30 µg	40 µg	Average (20-40 µg)	Range (x 10⁶)	CV
No-Stain	0.0	5.5	9.8	13.2	9.5	3.22	13.5%
β-actin	0.0	42.7	62.8	72.3	59.3	10.17	55.7%
GAPDH	0.0	40.5	58.5	66.5	55.2	10.23	49.8%

Table 3. Percent Errors from A431 Extract Data

Normalization Method for EGFR and Phospho-EGFR	Percent Error of Method from Predicted According to A431 Extract Protein Loaded in Gel									
	10 µg		20 µg		30 µg		40 µg		Average (20-40 µg)	
hEGF:	-	+	-	+	-	+	-	+	-	+
No-Stain	0.0	0.0	3.8	11.7	8.0	12.9	3.7	8.5	5.2	11.0
β-actin	0.0	0.0	45.8	53.6	67.5	68.7	73.3	74.7	62.2	65.7

Table 4. Normalization Accuracies from A431 Extract Data

Normalization Method for EGFR and Phospho-EGFR	Normalized EGFR Band Volume				Normalized Phospho-EGFR Band Volume			
	Range (x 10⁷)		CV		Range (x 10⁷)		CV	
hEGF:	-	+	-	+	-	+	-	+
No-Stain	0.34	1.09	4.4%	10.7%	---	2.32	---	5.8%
β-actin	2.58	3.40	47.9%	43.7%	---	11.77	---	46.2%

CONCLUSIONS

We have described and demonstrated issues inherent in using HKPs for protein normalization of western blots. Specifically, HKPs – at protein loads typically used for studying cellular proteins of low abundance – exhibit signal saturation that renders the HKPs unable to serve as loading controls. This saturation is seen as both a nonlinear signal response to and a significant deviation from values expected for increasing protein load. We have solved these issues with protein normalization using HKPs by utilizing for TPN the new No-Stain reagent, thereby affording much greater accuracy and reproducibility with results much closer to expected. Unlike HKPs, the No-Stain reagent provides a linear relationship of signal intensity to protein load over all cell lines tested. Variations – as given by average percent errors from predicted – using HKPs average 48% overall, while the variations using the No-Stain reagent average 8% overall.

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