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Biological validation of a novel process and product for quantitating western blots

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ABSTRACT

Protein normalization of western blots has relied upon housekeeping proteins which exhibit signal saturation and varied cellular expression level variations. These issues can produce spurious results leading 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 enabling Total Protein Normalization, and we demonstrate its superior protein normalization capabilities through analysis of target proteins in different cell backgrounds. These data illustrate how housekeeping proteins exhibit signal saturation, yield erroneous normalization data, and display sample-to-sample variations averaging 48.2 % overall. Signal intensities obtained using our new method show a linear relationship to protein sample load, thus providing accurate protein normalization with an overall average variation of 7.7 %.

1. Introduction

Western blotting, or immunoblotting, is a method for detecting a single protein of interest within a complex, multi-protein sample. While western blotting methods have been in practice for over three decades, it has only been in recent years that peer reviewers and journal editors have advised or required authors to more rigorously demonstrate the validity of their technique for quantifying western blot results. Such a demonstration would include the use of an internal loading control (ILC) for protein normalization, a technique to account for western blot method inaccuracies and other inherent experimental variability that is presumably unrelated to the system attributes being studied.

Protein normalization is a means by which the amount of a target protein can be determined relative to a reference ILC, often a housekeeping protein (HKP) like α-tubulin, β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or heat shock protein 90 (HSP90). An HKP is chosen and used as an ILC under the assumption that the level of the chosen HKP is directly proportional to the total amount of protein in the samples being studied. Changes in a sample's target protein levels that arise from designed experimental perturbations are then expressed

Abbreviations: 2-ME, 2-mercaptoethanol; AKT1, protein kinase B protein; BCA, bicinchoninic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CV, coefficient of variation; DMA, dimethyl acrylamide; DTT, dithiothreitol; *E. coli* BL21, *Escherichia coli* bacteria, B strain, used for recombinant protein expression, lacking the lon protease, and deficient in the outer membrane protease OmpT; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor protein; H+L, heavy plus light chains; Em, emission wavelength maximum; Ex, excitation wavelength maximum; FL, fluorescent; FQ, 3-(2-furoyl) quinoline-2-carboxyaldehyde; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HeLa, an immortal cell line derived from cervical cancer cells taken from Henrietta Lacks on February 8, 1951; hEGF, human epidermal growth factor protein; HKP, housekeeping protein; HSP90, heat shock protein 90; ILC, Internal Loading Control; IVT, *in vitro* translation; LDS, lithium dodecyl sulfate; M, moles per liter; MCF-7, Michigan Cancer Foundation-**7**, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers; MES, 2-(N-morpholino)ethanesulfonic acid; mM, millimoles per liter; nm, nanometer; PVDF, polyvinylidene fluoride; R^2, R-squared = the coefficient of determination = 1 – (sum of squares of residuals)/(total sum of squares); SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, tris buffered saline; Tris, tris(hydroxymethyl)aminomethane; TM, trademark; TPN, total protein normalization; USD, United States Dollars; UV, ultraviolet; W, watt; w/v, weight per volume.

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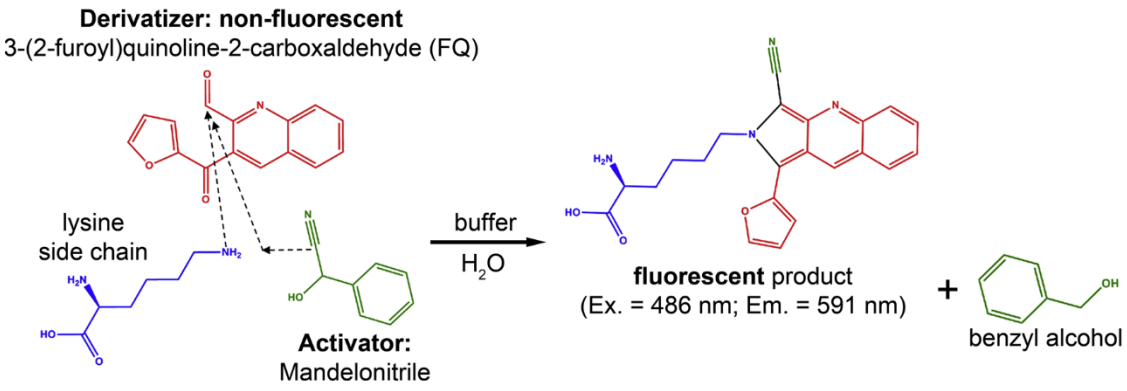

Fig. 1. The No-Stain reaction. The Activator and non-fluorescent Derivatizer molecules react with a primary amine like a lysine side chain to form covalently a highly fluorescent product and benzyl alcohol.

as ratios to the level of the measured HKP. Protein normalization using HKPs thereby also serves as an ILC to correct for minor sample-to-sample and gel lane-to-lane variations. While the assumptions about HKPs have enabled researchers to normalize changing levels of their studied targets to the presumed unchanging levels of HKPs, the assumptions often lack validity, and this potential invalidity leads to inaccuracies, inconsistencies, and incorrect conclusions (Ghosh et al., 2016).

Potential issues with HKPs when used for protein normalizations and as ILCs have been extensively reported and reviewed (Pillai-Kastoori et al., 2020):

- 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 (Dittmer and Dittmer, 2006) 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 saturation also makes HKPs poor ILCs (Aldridge et al., 2009)
- 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 (Barber et al., 2005; Eaton et al., 2013; Wilhelm et al., 2014) or other cellular states (Li and Shen, 2013)
- Expression of HKPs can vary with cellular treatment being studied (Bass et al., 2017)
- HKP expression levels may change with the density of cultured cells (Greer et al., 2010)
- The solubility and, therefore, apparent expression levels of HKPs are dependent on the cell lysis buffer and method used (Janes, 2015)
- Proteins of interest may be present in a culture medium that is devoid of HKPs
- HKPs can be of a size that approximates a protein of interest, thereby co-migrating
- Antibodies for HKPs can cross-react with proteins of interest

To address these cited and other potential issues, researches have needed to perform laborious, time-consuming optimizations and control experiments to validate their experimental methodologies to ensure that their results are a consequence of perturbations being studied as opposed to underlying variations (Taylor et al., 2013; Eaton et al., 2014; Pillai-Kastoori et al., 2020). To overcome these obvious issues and ease the validation burden required for using HKPs for protein normalization and as ILCs, a method affording greater ease and accuracy is needed. Such a new method for improving protein normalization and protein quantitation using total protein as the ILC should:

- Be compatible with existing western workflows and typical immunodetection methods
- Exhibit a linear relationship between signal intensity and protein sample load under all experimental conditions
- Stain or label all proteins present on a membrane generated within the western workflow without bias
- Enable acquisition of a total-protein-stained membrane image that can be compared directly to the immunodetected signal from the same membrane

The need for such a new method can now be met with the Invitrogen™ No-Stain™ Protein Labeling Reagent (Thermo Fisher Scientific catalog numbers A44717 for ten membranes and A44449 for 40 membranes at a current cost of 50 USD and 150 USD, respectively) ("No-Stain Protein Labeling Reagent," 2019) that can rapidly and fluorescently label a western blot for total protein. In contrast to the labor-intensive methods required for using HKPs and validating their usage, this No-Stain Reagent which we developed now enables reliable performance of Total Protein Normalization (TPN) (i.e., using total protein instead of an HKP as ILC) with ease. The No-Stain Reagent kit, when stored properly, is stable for at least one year from the date of manufacture and consists of only three components: a No-Stain Labeling Buffer, the No-Stain Activator, and the 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--carboxyaldehyde (FQ). In the presence of the Activator, 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 (Pinto et al., 1995). This fluorogenic molecule provides ultrasensitive detection of primary amines (e.g., lysine sidechains and the amino termini of proteins) (Beale et al., 1990; Pinto et al., 1997) and, when used as a component of the No-Stain Protein Labeling Reagent, enables a detection sensitivity of 20 ng per protein. For these reasons, a method of protein normalization that utilizes the No-Stain Protein Labeling Reagent as an ILC is ideally suited as an alternative to other existing, more cumbersome total protein visualization methods like Coomassie gel staining or Ponceau S membrane staining. Note that the No-Stain Reagent can be used more safely as a total protein stain compared to other staining and de-staining methods that require the use of damaging UV light for imaging, hazardous organic solvents, acids, or carcinogens like the ruthenium used in some stains (Aldridge et al., 2009; Kirshner and Gibbs, 2018).

The reaction mechanism of the Invitrogen No-Stain Protein Labeling Reagent shown in Fig. 1 is a variation of the four-component Ugi reaction (Marcaccini and Torroba, 2007; Ugi et al., 2003), and results in a covalently linked, conjugated ring system at the nitrogen of lysine side chains. The No-Stain reagent labels lysine residues, and the amino acid lysine is one of the most abundant of the 20 amino acids commonly found in proteins: a 7.2 % observed frequency in vertebrate proteins with the frequencies of all amino acids ranging from 1.3 % for

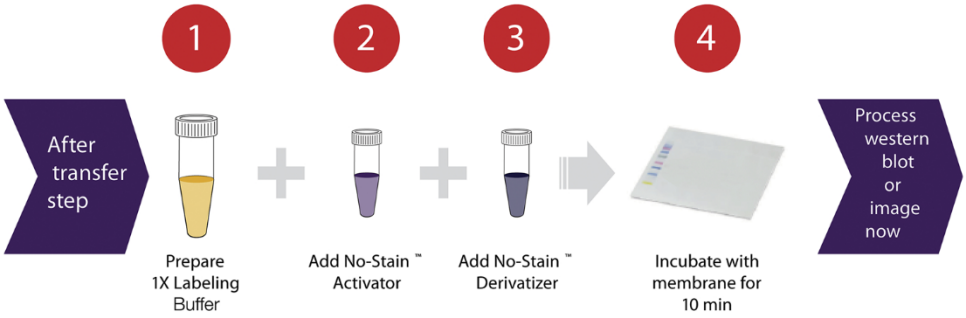

Fig. 2. The Invitrogen No-Stain Protein Labeling Reagent is easy to use. Shown is the rapid (less than 15 min) workflow for labeling proteins on a membrane after transferring from a gel, although a gel that has not been transferred or otherwise stained can also be labeled if desired. Note that the No-Stain Protein Labeling Reagent is for Research Use Only and not for use in diagnostic procedures.

tryptophan to 8.1 % for serine (King and Jukes, 1969). The prevalence of lysine in proteins gives the lysine-based No-Stain labeling method an advantage for total protein visualization: downstream signals from labeled lysine residues can yield greater sensitivity and more closely approximate total protein. Perhaps the greatest value of this No-Stain labeling method for total protein visualization is that it can be used to perform TPN of target proteins on a western blot with a single imaging step. As described previously, TPN requires that a western blot be stained or otherwise labeled for total protein and this total protein staining or labeling can then be used as an ILC for a target protein being studied.

The method of TPN has begun to gain traction. In fact, the *Journal of Biological Chemistry* – in its guidelines for presenting data – advises authors to normalize signal intensity in western blots to total protein loading and cautions against the use of HKPs for such normalizations ("Instructions for Authors," 2018).

Furthermore, a TPN system should work over a broad range of conditions and not suffer from the previously described issues inherent in the use of HKPs for protein normalization. In conjunction with an Invitrogen™ iBright™ Imaging System or other appropriate imager, the No-Stain Protein Labeling Reagent satisfies these criteria and can be easily used to perform TPN accurately and reliably.

2. Materials and methods

2.1. Samples

2.1.1. Bacterial cell lysate

An overnight culture of *E. coli* BL21 cells was harvested, pelleted, and frozen. Cell pellets were subsequently resuspended in Tris buffer containing EDTA and protease inhibitors. Suspensions were then sonicated followed by the addition of Triton X-100 detergent. After shaking and centrifugation, the supernatant was collected and prepared for SDS PAGE.

2.1.2. Spinach leaf extracts

A spinach leaf extract was prepared as previously described (Porubleva et al., 2001) with some modifications. Spinach leaves frozen in liquid nitrogen and pulverized with mortar and pestle were incubated at −20 °C after resuspending in cold acetone containing 10 % trichloroacetic acid and 0.07 % 2-mercaptoethanol (2-ME). Precipitated proteins were pelleted by centrifugation, washed several times in cold acetone with 0.07 % 2-ME, and dried under vacuum. Dried proteins were solubilized in 7 M urea, 2 M thiourea, 4 % CHAPS, and 20 mM DTT by incubation in a sonicating water bath. Insoluble debris was removed by centrifugation, and the remaining soluble proteins were alkylated with 50 mM DMA followed by quenching with DTT and storage at −80 °C. This soluble protein preparation was subsequently thawed and prepared for SDS PAGE.

Table 1
Antibodies used in this work. Included are Thermo Fisher Scientific catalog numbers, host species, and antibody dilutions used.

Antibody	Host	Dilution	Catalog Number
alpha Tubulin Monoclonal Antibody	Mouse	1:2000	138000
beta Actin Monoclonal Antibody (AC-15)	Mouse	1:2000, 1:1000	AM4302
GAPDH Monoclonal Antibody (ZG003)	Mouse	1:2000	398600
GAPDH Loading Control Monoclonal Antibody (GA1R)	Mouse	1:1000	MA5-1573
HSP90 alpha Polyclonal Antibody	Rabbit	1:500	PA3-013
AKT1 Monoclonal Antibody (3A3)	Mouse	1:500	MA5-15591
Cyclophilin B Polyclonal Antibody	Rabbit	1:2000	PA1-027A
EGFR Monoclonal Antibody (F4)	Rabbit	1:1000	MA1-24226
Phospho-EGFR (Tyr1086) Polyclonal Antibody	Rabbit	1:5000	44-790G
Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680	Goat	1:5000	A21058
Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 790	Goat	1:5000	A11369
Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 680	Donkey	1:5000	A32802
Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 800	Donkey	1:5000	A32789

2.1.3. Rat liver tissue

Whole rat livers were finely chopped and added to conical tubes with chilled lysis buffer consisting of 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 20 mM DTT, and protease inhibitors. After sonicating this mixture, the lysate proteins were alkylated using DMA. After centrifugation of the lysate, the supernatant was collected and prepared for SDS PAGE.

2.1.4. Mammalian cell lysates and extracts

HeLa lysate was prepared for SDS PAGE from material included in 1-Step Human IVT (*in vitro* Translation) Kits from Thermo Fisher Scientific. Total extracts were prepared for SDS PAGE from MCF-7 and A431 cells that were serum starved overnight. A population of these A431 cells were challenged with 100 ng/mL hEGF for five minutes before harvesting. Total cell extracts from Jurkat cells, serum starved overnight and then treated with 50 µM of LY294002 for one hour, were prepared for SDS PAGE.

2.2. SDS PAGE and western blotting

Dilutions of the cell extracts were prepared for loading in

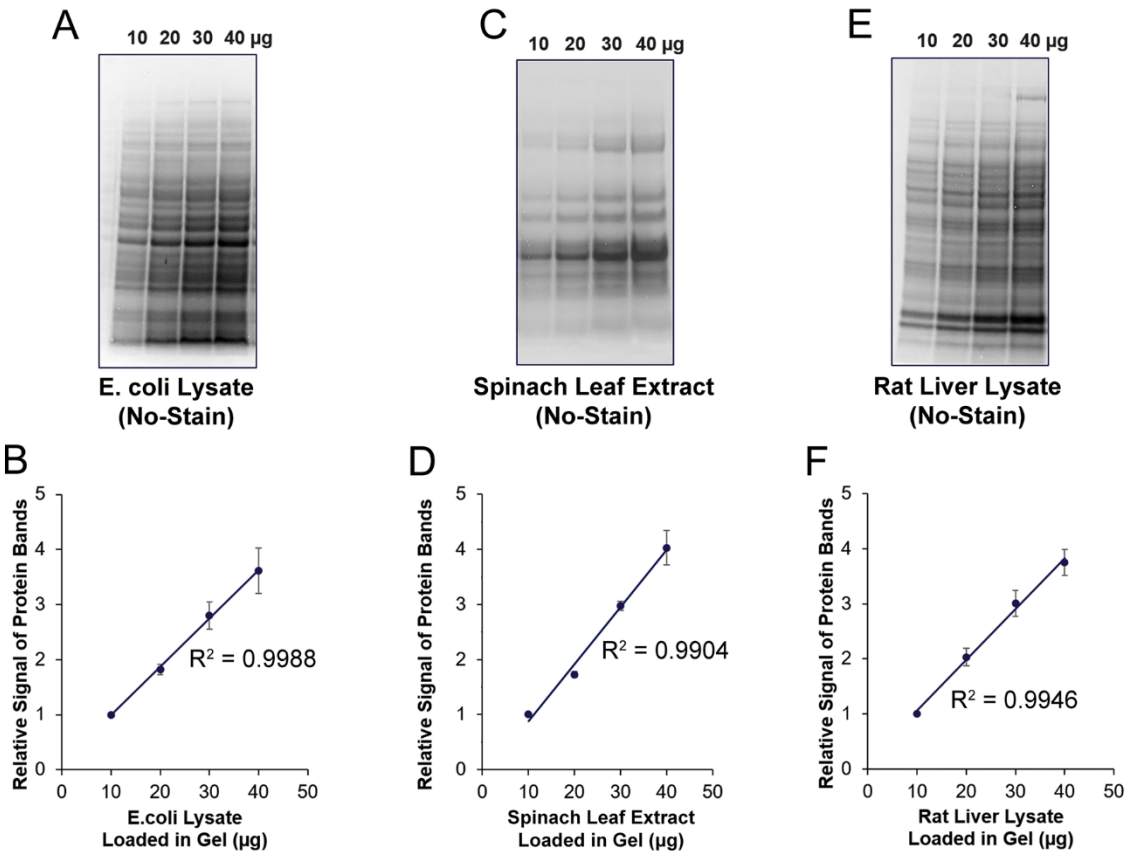

Fig. 3. The No-Stain Reagent provides a linear response of signal intensity to total protein load. Fluorescent images of membranes generated from three different cell types are shown in **Panels A**, **C**, and, **E**. The densitometric signal intensities for each respective cell type versus protein load are shown in **Panels B**, **D**, and **F**. R^2 values for each line through plotted data are also displayed.

Invitrogen™ Bolt™ 4–12 % Bis-Tris Plus 10-well gels at constant sample volumes with varied total protein masses (10, 20, 30, and 40 µg). The concentrations of the bacterial cell, rat liver, and HeLa lysates were determined by BCA assay; the concentrations of the other extracts and lysates were determined from densitometric comparisons to known HeLa lysate loads in Coomassie-stained gel images. Samples in Bolt LDS Sample Buffer with 50 mM DTT were heated for ten minutes at 70 °C, loaded in gel wells in duplicate, and electrophoresed at 200 V (constant) using MES Running Buffer in Invitrogen™ Mini Gel Tanks with Invitrogen™ PowerEase™ 300 W Power Supplies. Proteins in electrophoresed gels were then transferred to PVDF membranes using Invitrogen™ iBlot™ 2 Gel Transfer Devices with Method P0 for 7 min. Proteins on membranes were next labeled with ease in following the protocol for the No-Stain Protein Labeling Reagent shown in Fig. 2.

2.3. Immunodetection

Antigen detection was accomplished after No-Stain labeling by first using Blocker™ FL Fluorescent Blocking Buffer to block for 30 min. Incubations with primary antibodies shown in Table 1 were performed for one hour at room temperature. Membrane washes were performed using TBS with 0.05 % Tween-20. Secondary antibody incubations were done for one hour at room temperature.

2.4. Membrane imaging

No-Stain imaging was performed using an iBright Imaging System with 455–485 nm excitation and 565–615 nm emission. Each membrane was simultaneously imaged for No-Stain, Alexa Fluor 680, and Alexa Fluor 790 fluorescence depending on the secondary antibodies used for each membrane.

2.5. Image analysis

Densitometric quantitation of protein bands on fluorescent images of membranes was conducted using the iBright system's onboard software. For No-Stain fluorescence, signal intensities were determined by summing the densitometric volumes of all bands in a lane. For the 680 and 790 fluorescence from secondary antibodies, signal intensities were generated from single bands corresponding to either an HKP or a target protein.

Next, relative densitometric signal intensities were calculated by first assigning the fluorescence for a 10 µg loada value of 1. Then the densitometric fluorescence at each of the other protein loads was normalized to the 10 µg fluorescence for that HKP or No-Stain within the same membrane image. Since we assumed that a plot of the predicted response of relative signal intensities versus protein load would pass through the origin, an ideal or predicted response line could be generated for each data set. The calculated densitometric values for each protein load on the ideal response line were then used as the reference values from which percent errors could be computed for every densitometric measurement.

Computations for TPN using the No-Stain reagent and an iBright imager are based on the iBright software's densitometric analysis. The iBright software performs densitometry on the No-Satin fluorescence of each lane's total protein and assigns a normalization factor of 1.0 to the lane with the largest summed densitometric value. The densitometric sum of every other lane is then compared, and each other lane is given its own normalization factor (i.e., some value less than 1). These factors are then multiplied by the densitometric fluorescence of target proteins to obtain total protein normalized values. This is how Total Protein Normalization is achieved using the No-Stain Protein Labeling Reagent in conjunction with the iBright Imaging System.

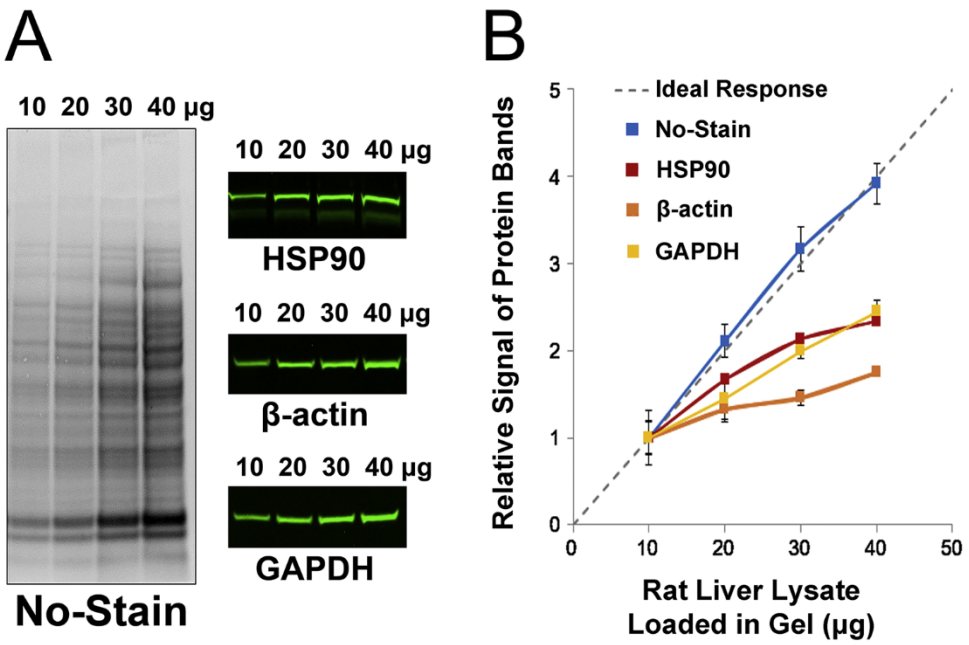

Fig. 4. The No-Stain Reagent yields improved accuracy for normalization compared to HKPs in rat liver lysate. Densitometric signal linearity versus protein load was compared for HKPs and No-Stain labeled rat liver lysate proteins. Results obtained using the No-Stain Protein Labeling Reagent were compared to those obtained using three different HKPs. **Panel A:** Fluorescent images of the No-Stain labeled and immunodetected western blot were acquired using an iBright Imaging System with the No-Stain and 680 nm channels, respectively. **Panel B:** Densitometric values (i.e., protein band volumes) – relative to that of the 10 µg load– from images were plotted versus total protein loaded in a gel well for the No-Stain and each of the three HKPs. An idealized linear, predicted response is shown as a dotted line.

3. Results and discussion

The capacity of the No-Stain Protein Labeling Reagent to serve as a means for performing TPN and as an ILC was assessed with various lysate and extract sources (i.e., bacteria, plant, and animal) and compared to quantification results obtained alternately using HKPs. For a method to be viable for protein normalization as an ILC, the method must demonstrate a linear relationship between protein load and measured signal intensity, presumably in the range of protein load that researchers desire to use in studying their protein of interest.

To examine and demonstrate the capability of the No-Stain Reagent to provide a linear signal response to total protein loaded in an SDS PAGE gel well, three diverse sources were chosen: bacteria cells, spinach leaves, and rat livers. The range of total protein assayed and loaded in gel wells was chosen to be 10–40 µg because this range would encompass the amount of total protein load typically required for researchers to be able to visualize their low abundance proteins of interest. Dilution series of the *E. coli* lysate, spinach leaf extract, and rat liver tissue extract were prepared, electrophoresed, and transferred to PVDF membranes. The membranes were labeled for total protein using the No-Stain Reagent and then imaged. Fluorescent signal intensities were acquired and plotted versus total protein loaded in gel wells. Images of stained membranes and plots are shown in Fig. 3. Lines were drawn through each set of data points and each line's R^2 value – the canonical measure of the degree of linearity of data – were determined using Excel. Since all lines drawn from our plotted data exhibit R^2 values of 0.99, it can be concluded that the No-Stain Reagent produces a signal that is directly proportional to the total protein load within the tested range and samples examined.

Having demonstrated that the No-Stain Reagent has the required

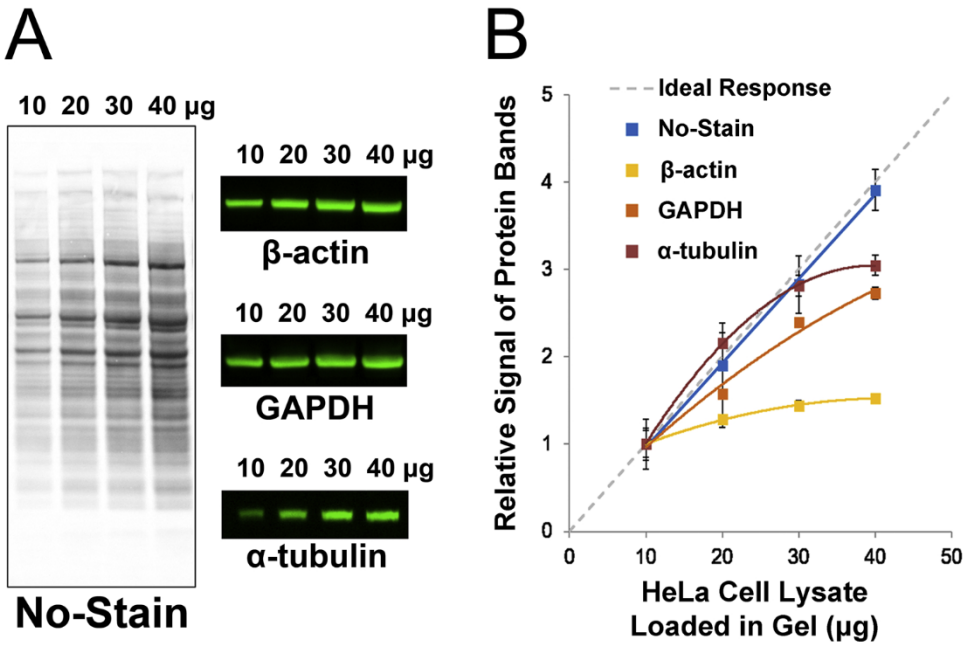

Fig. 5. The No-Stain Reagent yields improved accuracy for normalization compared to HKPs in HeLa lysate. Densitometric signal linearity versus protein load was compared for HKPs and No-Stain labeled HeLa lysate proteins. Results obtained using the No-Stain Protein Labeling Reagent were compared to those obtained using three different HKPs. **Panel A:** Fluorescent images of the No-Stain labeled and immunodetected western blot were acquired using an iBright Imaging System with the No-Stain and 680 nm channels, respectively. **Panel B:** Densitometric values (i.e., protein band volumes) – relative to that of the 10 µg load– from images were plotted versus total protein loaded in a gel well for the No-Stain and each of the three HKPs. An idealized linear, predicted response is shown as a dotted line.

Table 2
Percent error to predicted response of normalization methods using rat liver lysate.

Normalization Method	Percent Error of Method from Predicted According to Rat Liver Lysate Protein Loaded in Gel				
	10 µg	20 µg	30 µg	40 µg	Average (20–40 µg)
No-Stain	0.0 %	5.6 %	5.5 %	2.0 %	4.4 %
HSP90	0.0 %	16.4 %	28.6 %	41.4 %	28.8 %
β-actin	0.0 %	33.5 %	51.4 %	56.2 %	47.0 %
GAPDH	0.0 %	27.6 %	33.5 %	39.0 %	33.4 %

Table 3
Percent error to predicted response of normalization methods using HeLa lysate.

Normalization Method	Percent Error of Method from Predicted According to HeLa Lysate Protein Loaded in Gel				
	10 µg	20 µg	30 µg	40 µg	Average (20–40 µg)
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 %	12.7 %

attributes of an ILC when used with rat liver tissue extracts within a total protein range typically used by researchers, we assessed the No-Stain Reagent's capability as an ILC versus that of three commonly used HKPs. Membranes prepared and labeled using the No-Stain Reagent were immunodetected for HSP90, β-actin, and GAPDH. Fluorescent images were obtained and quantified. Acquired data plotted against total protein loaded in gel wells clearly shows that a linear response approximating the desired ideal response is only obtained when using the No-Stain Reagent as an ILC (Fig. 4). Each of the three HKPs tested deviate from the ideal response and linearity. These deviations occurring with the HKPs are a consequence of signal saturation and a failure of the HKPs to track proportionally with increasing total protein. In other words, these HKPs are not suitable as ILCs while the No-Stain Reagent is suitable as an accurate and reliable ILC for protein normalization in the range of total protein being studied by researchers.

Biological and medical researchers, however, often focus in their studies on higher level organisms than bacteria, plants, and rodents. These higher-level organisms (i.e., mammals and humans in particular) typically possess much more complex cellular components, protein structures, and responses. To validate the No-Stain Reagent with a more complex cellular environment, an investigation like that undertaken with the rat liver lysate was pursued using HeLa cell lysates. Again, the capability of the No-Stain Reagent as an ILC was compared to that of three HKPs (i.e., β-actin, GAPDH, and α-tubulin}.

Results of this investigation, displayed in Fig. 5, demonstrate that the No-Stain Protein Labeling Reagent provides a) 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 b) a response that matches closely to the predicted, Ideal Response. Conversely, the signal intensities of the three HKPs show a non-linear relationship to protein load within this typical range of protein, thereby underestimating the predicted response and being indicative of signal saturation as the signal intensity does not increase proportionately with protein load. This signal saturation behavior of HKPs that often arises from their high expression levels limits the ability of the HKPs to accurately serve as ILCs or to perform valid protein normalizations of target proteins under these typical conditions.

While the results shown in Figs. 4 and 5 graphically illustrate the signal saturation tendencies of HKPs, a more numerically quantitative analysis of the densitometric data was performed. Essentially, the percent errors for all methods (No-Stain and three HKPs) from the predicted response were computed and compared as shown in Tables 2 and 3. Since the relative intensities were normalized to the 10 µg loads, the relative densitometric signal measured would equate to the predicted densitometric signal, leading to the percent errors for the 10 µg loads being zero. The larger the percent error, the farther the densitometric signal is from the predicted response and the less accurate would be that method at that protein load. This analysis shows, as evidenced by the average percent errors for each method, that the No-Stain Reagent as an ILC for TPN provides significantly greater reproducibility and better concordance with protein load than protein normalization using HKPs.

While Figs. 4 and 5 illustrate the linearity of the No-Stain signal and

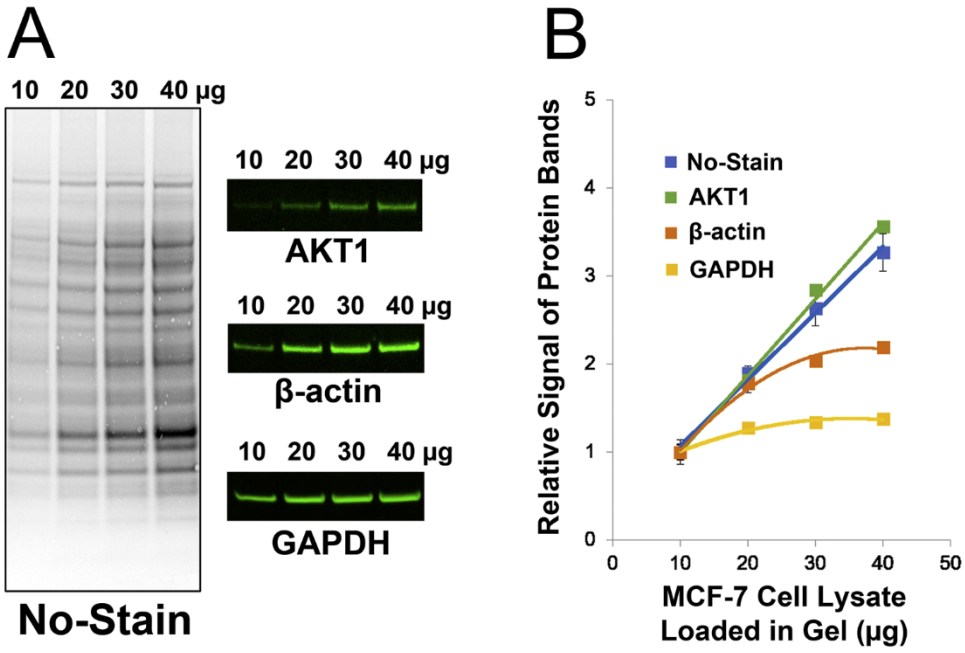

Fig. 6. The No-Stain Reagent yields improved accuracy for normalization compared to HKPs in MCF-7 cell lysate. Densitometric signal linearity versus protein load for HKPs and No-Stain labeled MCF-7 lysate proteins is illustrated. Results obtained using the No-Stain Protein Labeling Reagent and an AKT1 antibody were compared to results obtained using two HKPs. **Panel A:** Fluorescent images of the No-Stain labeled and immunodetected western blot were acquired using an iBright Imaging System with the No-Stain and 680 nm channels, respectively. **Panel B:** Densitometric values (i.e., protein band volumes) – relative to that of the 10 µg load– from images were plotted versus total protein loaded in a gel well for the No-Stain, target protein AKT1, and each of the two HKPs.

Table 4
Percent error to predicted response and statistical assessment of methods for protein normalization of AKT1 in MCF-7 cell extract.

Normalization Method	Percent Error of Method from Predicted According to MCF-7 Extract Protein Loaded in Gel					Normalized AKT1 Band Volume	
	10 µg	20 µg	30 µg	40 µg	Average (20–40 µg)	Range (x 10^6)	CV
No-Stain	0.0 %	5.2 %	12.3 %	18.3 %	11.9 %	0.40	5.8 %
β-actin	0.0 %	36.4 %	55.4 %	65.6 %	52.5 %	2.05	38.3%
GAPDH	0.0 %	11.2 %	32.1 %	45.3 %	29.5 %	1.32	23.1%

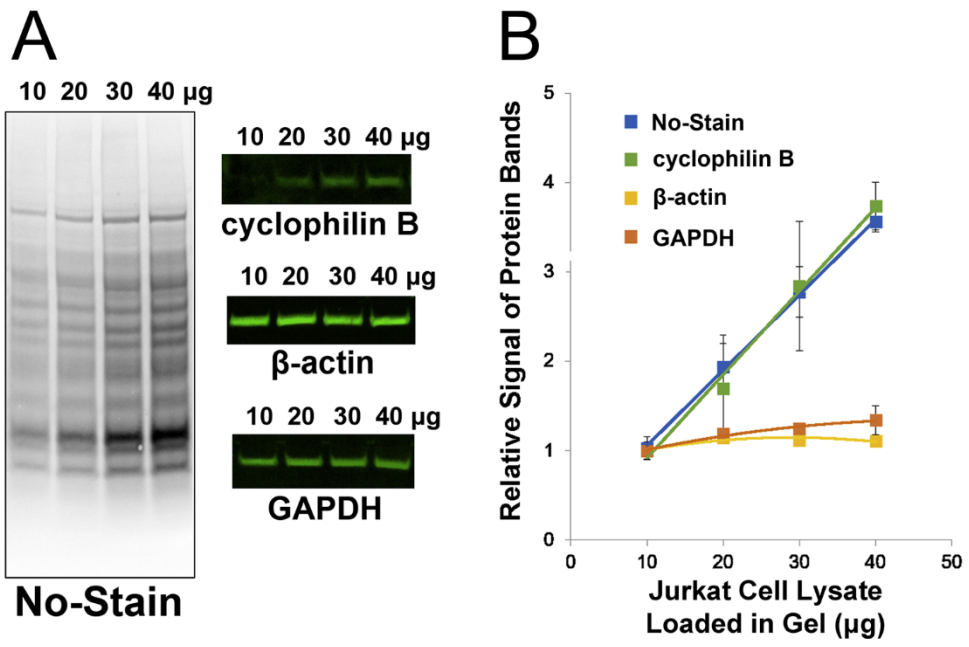

Fig. 7. The No-Stain Reagent yields improved accuracy for normalization compared to HKPs in Jurkat cell lysate. Densitometric signal linearity versus protein load for HKPs and No-Stain labeled Jurkat lysate proteins is shown. Results obtained using the No-Stain Protein Labeling Reagent and a cyclophilin B antibody were compared to results obtained using two HKPs. **Panel A:** Fluorescent images of the No-Stain labeled and immunodetected western blot were acquired using an iBright Imaging System on the No-Stain and 680 nm channels, respectively. **Panel B:** Densitometric values (i.e., protein band volumes) – relative to that of the 10 µg load– from images were plotted versus total protein loaded in a gel well for the No-Stain, target protein cyclophilin B, and each of the two HKPs.

the nonlinearity of HKP signal intensities derived from western blots of rat liver tissue extracts and HeLa cell lysates, target protein normalization was not directly assessed. Consequently, experiments were undertaken using target proteins in other extracts from mammalian cells. Quantifications using MCF-7 and Jurkat cell extracts were assessed alongside target proteins AKT1 and cyclophilin B, respectively. Additionally, both EGFR and phospho-EGFR levels in A431 cell extracts were studied by comparing measured signal responses from different mass loads of treated and untreated (control) extracts to expected responses. Protein normalizations using HKPs were performed and compared to TPN results obtained with the No-Stain reagent as a means for assessing their capabilities for serving as ILCs.

Results of experiments using MCF-7 cell extracts are shown in Fig. 6. The linear response of densitometric signal intensity from No-Stain labeled protein observed from rat liver and HeLa lysate loads was replicated with MCF-7 cell extracts. As the total protein load was increased, the densitometric value or signal intensity for total protein rose proportionately. This behavior was also expected of the target protein present in the extract and the signal intensities generated by both the No-Stain reagent and the fluorescent immunodetection of AKT1 were observed (Fig. 6). The non-linear response character of HKP densitometric signal intensities observed with both rat liver and HeLa lysate loads was replicated with MCF-7 cell extracts. This nonlinear behavior of HKP signal intensities, due to their signal saturation, means that accurate protein normalization cannot be achieved with these two HKPs with MCF-7 cell extracts at these total protein loads. As illustrated in Panel A and B of Fig. 6, HKPs do not accurately reflect the total protein load concentrations, and these non-linear signals generated by HKPs masks the level of target protein (i.e., AKT1 in MCF-7 cell extracts) when used to normalize target protein masses: the HKPs make poor ILCs under these conditions.

Data from the plot in Panel B of Fig. 6 was used to compute the percent errors from the predicted response as described previously for rat liver and HeLa lysates. Results for MCF-7 cell extracts are shown in Table 4, and this table includes the percent error from predicted for a target protein. Signal intensity data – used to plot the graph in Panel B of Fig. 6 – were also used to normalize target protein AKT1 levels (i.e., band volumes determined through densitometry). Statistical assessments of normalization data are also shown in Table 4. What is clear is how small the errors and variations of using No-Stain for TPN are compared to the significant variations and errors inherent when using the HKPs as ILCs for protein normalization.

Results of similar experiments with Jurkat cell extracts are shown in Fig. 7. The linear response of densitometric signal intensity from No-Stain labeled protein that was seen with rat liver, HeLa, and MCF-7 lysate loads is replicated with Jurkat cell extracts. As the total protein load is increased, the signal intensity for total protein is expected to rise proportionately, and this behavior of densitometric signal would also be expected of a target protein present in the extract. These expected behaviors of densitometric signal intensities generated by both the No-Stain reagent and the fluorescent immunodetection of cyclophilin B as the target protein in this case are in fact observed and shown in Panel B of Fig. 7. This behavior of the No-Stain signal intensities demonstrates the capability of the No-Stain Reagent to serve as an ILC. On the other hand, the non-linear response character of HKP signals that were seen previously with both HeLa and MCF-7 lysate loads is replicated with Jurkat cell extracts, and this nonlinear behavior of the HKP densitometric signal values due to signal saturation further confirms that accurate protein normalization cannot be achieved with either of these two HKPs using Jurkat cell extracts in this range of total protein loads. In other words, these HKPs do not accurately account for total protein load variations and they cannot be used reliably as ILCs under these

Table 5
Percent error to predicted response and statistical assessment of methods for protein normalization of cyclophilin B in Jurkat extract.

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 10^6)	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 %

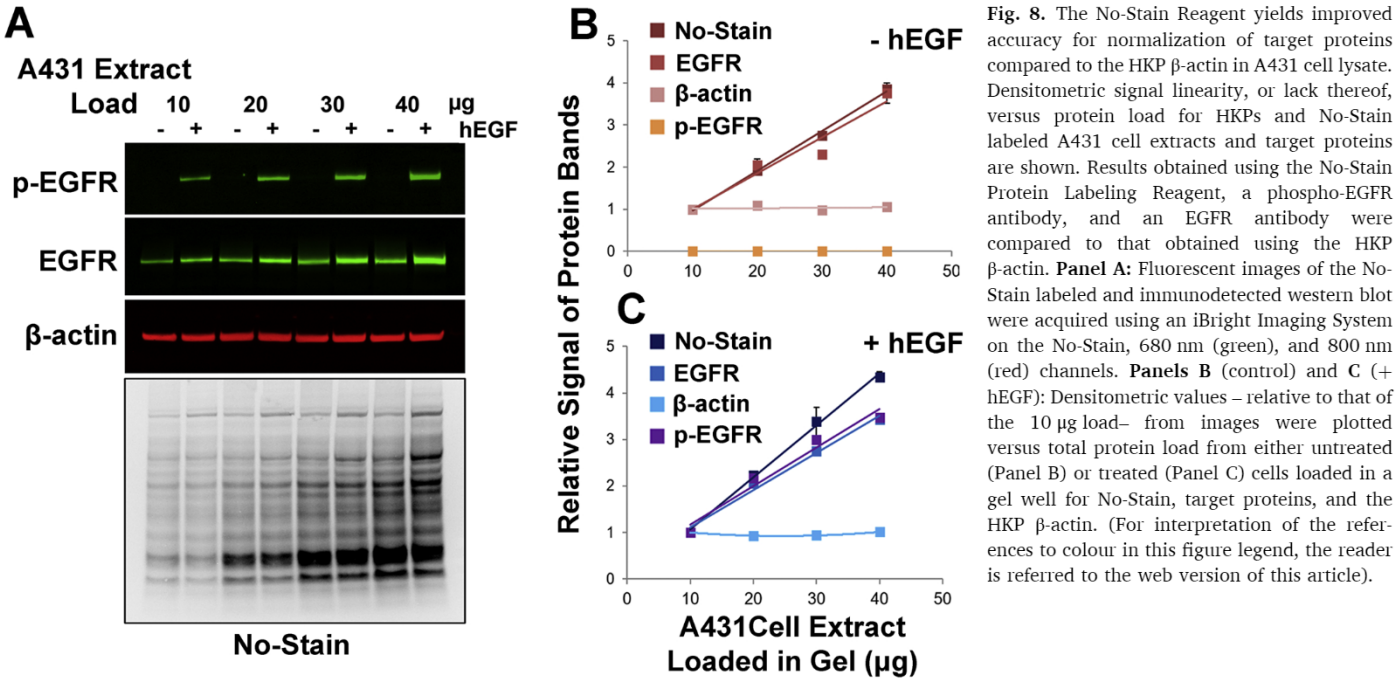

Fig. 8. The No-Stain Reagent yields improved accuracy for normalization of target proteins compared to the HKP β-actin in A431 cell lysate. Densitometric signal linearity, or lack thereof, versus protein load for HKPs and No-Stain labeled A431 cell extracts and target proteins are shown. Results obtained using the No-Stain Protein Labeling Reagent, a phospho-EGFR antibody, and an EGFR antibody were compared to that obtained using the HKP β-actin. **Panel A:** Fluorescent images of the No-Stain labeled and immunodetected western blot were acquired using an iBright Imaging System on the No-Stain, 680 nm (green), and 800 nm (red) channels. **Panels B** (control) and **C** (+ hEGF): Densitometric values – relative to that of the 10 µg load– from images were plotted versus total protein load from either untreated (Panel B) or treated (Panel C) cells loaded in a gel well for No-Stain, target proteins, and the HKP β-actin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

conditions.

Densitometric signal intensity data used to plot the graph in Panel B of Fig. 7 were used to normalize target protein cyclophilin B levels (i.e., band volumes) similarly to AKT1 in MCF-7 extracts. Statistical assessments of these normalization data are shown in Table 5. As observed with other lysates and extracts, the error and variation of No-Stain TPN with Jurkat cells is small compared to the significant variations and errors inherent with using HKPs for protein normalization under the same conditions.

To assess data that might be more representative of the kind of biologically significant results sought by a researcher studying signaling pathways involved in cancer, we compared quantitation of target proteins from A431 cell extracts that had been treated with hEGF to untreated A431 cell extracts. Our findings are illustrated in Fig. 8. As already demonstrated with other cell lines, the No-Stain Reagent as an ILC exhibits superior capability for protein normalization as compared to the HKP β-actin. In the case of the A431 cells, the HKP β-actin shows virtually no densitometric signal response to changing protein levels and would, therefore, not enable any protein normalization in this biological system. The No-Stain densitometric signal intensities, however, show a linear increase with protein load and track closely with target protein levels in both treated and untreated A431 cell extracts. The nonlinear behavior of the HKP signal intensities, or signal saturation, further confirms with these A431 extracts that accurate protein normalization cannot be achieved with the HKP β-actin under tested conditions. The failure of this HKP as an ILC to accurately normalize can be seen visually in Panel A and graphically in Panel B of Fig. 8, and since β-actin is unable to account for total protein load variations, the HKP β-actin cannot be used as an ILC in this system either.

Densitometric signal intensity data used to plot the graphs in Panels

Table 6
Percent error to predicted response and statistical assessment of methods for protein normalization of EGFR and phospho-EGFR in A431 cell extract.

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 %

Normalization Method for EGFR and Phospho-EGFR	Normalized EGFR Band Volume				Normalized Phospho-EGFR Band Volume			
	Range (x 10^7)		CV		Range (x 10^7)		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 %

B and C of Fig. 8 were used to normalize target protein levels (i.e., band volumes of EGFR and phospho-EGFR) in a fashion like that used with AKT1 in MCF-7 extract and cyclophilin B in Jurkat extract. Statistical assessments of these normalization data are shown in Table 6. These assessments show small errors with minor variation of No-Stain TPN compared to significant variations and major errors inherent with using HKPs for protein normalization.

4. Conclusions

We have listed, referenced, and demonstrated the many-fold issues inherent in using HKPs as ILCs for protein normalizations within the western workflow, particularly within biologically relevant systems. More specifically, HKPs – at protein loads typically used for studying low abundant cellular proteins - exhibit signal saturation that renders the HKPs unable to serve as ILCs. This saturation is seen as both a nonlinear signal response to, and a significant deviation from, values expected for proportionally increasing protein loads.

We have solved these issues with protein normalization using HKPs by utilizing for TPN the new No-Stain reagent as an ILC, thereby affording significantly 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, extracts, and lysates 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. Utilization of housekeeping proteins as ILCs 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 with the No-Stain Reagent as an ILC provides an elegant alternative for achieving accurate quantitative western blots.

CRediT authorship contribution statement

Thomas Diller: Conceptualization, Methodology, Visualization, Validation, Project administration, Resources, Writing - original draft, Writing - review & editing. **Jordan Thompson:** Investigation, Formal analysis, Validation, Visualization, Writing - review & editing. **Brian Steer:** Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

While it is true that the authors are employed by Thermo Fisher Scientific (the organization producing and selling the products used in this work) and such employment could appear to some to influence the work, such a relationship of the authors in no way affected the authors' objectivity or validity of the results that were attained objectively.

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