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Stain Free Total Protein Staining is a Superior Loading Control to β -Actin for Western Blots

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Abstract

Semi-Quantification of proteins using Western blots typically involves normalization against housekeeping genes such as β -actin. More recently, ponceau S and Coomassie blue staining have both been shown to be suitable alternatives to housekeeping genes as loading controls. Stain free total protein staining offers the advantage of no staining or destaining steps. Evaluation of the use of Stain free staining as an alternative to β -actin or the protein stain ponceau S showed that Stain free staining was superior to β -actin and as good as or better than ponceau S staining as a loading control for Western blots.

Keywords

Stain Free; Ponceau S; β -actin; Western blotting; Nitrocellulose

Western blot analysis is one of the most widely used methods to semi-quantify concentrations of specific proteins in a tissue. To correct for possible protein loading inaccuracy, a “housekeeping protein” with relatively constant expression in the tissue being investigated is usually used as an internal loading control. The two most commonly used loading controls are β -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). However several recent reports suggest that the use of housekeeping proteins as a loading control has to be carefully evaluated [1].

β -actin is a 43 kDa protein that is found in nearly all eukaryotic cells at concentrations over 100 μ M. One advantage of β -actin is that it is a highly conserved protein and most antibodies to β -actin can be used for many different species. However, β -actin participates in many intracellular processes and can be affected by different cellular processes such as changes in cell configuration and during differentiation of neuronal cells [2; 3]. Other experimental results suggest that housekeeping genes such as β -actin fail to distinguish differences in actin protein levels at higher total protein loads that are typically used for the detection of low-abundance proteins [1; 4].

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GAPDH is a cytoplasmic protein that is expressed at high levels in most tissues. Recently reports have shown changes in GAPDH levels under specific conditions and variations with fiber type [5]. GAPDH was also found to inaccurately reflect differences in protein concentration at higher total protein loads [4]. Another report suggests that GAPDH was not a suitable loading control for rat skeletal muscles from animals that were younger than 14 days [6]. Because of these problems with housekeeping controls we have used ponceau staining as a loading control [7]. Several advantages of ponceau staining include the low cost, the fast staining time, and easy removal of the dye from the bound proteins. However, the easy removal of the dye bound to the proteins can sometimes be a problem. Once the membrane was stained with ponceau S the intensity of the stained bands decreased with time such that if the researcher is delayed by just 10 minutes or longer the staining intensity was significantly lower resulting in poor quality images and substandard quantification.

We started using Criterion Stain free pre-cast gels which cost \$1 more than the regular pre-cast gels (based on list price) because it provided a quick and easy way to detect protein problems in the gel before and after transfer. Ponceau S is not suitable as a gel stain. The convenience of not having to stain the blot combined with the high quality of the images obtained from the Stain free staining on nitrocellulose membranes prompted us to determine the suitability of this method as a loading control for Western blots using a tissue (rat liver) commonly used for Western blots.

Rat livers (obtained from Pel-freez, AR) were weighed and cut into small pieces with a razor blade and homogenized with a hand-held *Potter-Elvehjem* homogenizer in 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.5 mM DTT, pH 7.5. The homogenate was subsequently centrifuged at 12,000 xg for 30 min at 4°C to remove the tissue debris. The supernatant (liver lysate) was removed and quantified using a Nanodrop 2000C (Thermo Scientific, MA). The lysate was then mixed with 4x Laemmli loading buffer (250 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 20% (v/v) β -mercaptoethanol, and 0.01% (w/v) bromophenol blue) and heated at 96°C for 3 minutes.

Western blots were carried out using 4–20% Criterion Stain free gradient gels (Bio-Rad, CA) loaded with 10–45 μ g or 10–50 μ g of liver lysate and subsequently transferred to nitrocellulose membranes (Bio-Rad) using a Transblot Turbo apparatus (Bio-Rad). Gels were activated by UV exposure for 2 minutes using a Bio-Rad Chemidoc MP imager. After protein transfer wet and dry membranes were imaged for Stain free staining and total protein quantified using Imagelab 4.1 (Bio-Rad). After Stain free imaging of the gels, the gels were stained with ponceau S (Amresco, OH) for 1 minute and quickly destained in water to remove non-specific ponceau staining. The membranes were then imaged and total protein quantified using Imagelab 4.1. After destaining in TBST (Tris-buffered saline (TBS) with tween, 0.05 M Tris, pH 7.4, 0.1 M NaCl, 0.05% (v/v) Tween 20), membranes were blocked with 3% (w/v) non-fat milk in TBST for 1 hour and then incubated with anti- β -actin (13E5, 1:2000, Cell Signaling Technologies, MA) for 2 hours. Membranes were then washed three times for 5 minutes and then incubated with goat anti-rabbit horseradish peroxidase antibody (1:5000, Sigma, MO) for 1 hour. Membranes were again washed with TTBS three times for 5 minutes and incubated with Clarity chemiluminescence substrate (Bio-Rad), imaged on the Chemidoc MP, and the bands detected analyzed with Imagelab 4.1. Our results show that Stain free is superior to β -actin as a loading control for liver samples (Fig. 1). The results also suggest that ponceau S is a better loading control than β -actin for liver samples. The poor linearity of actin at higher protein concentrations was not surprising as this is consistent with recent publications [1]. While the correlation coefficient (R^2) for linear regression lines of Stain free and ponceau were both >0.99, the slope of the Stain free regression line was significantly greater than that of the ponceau. This is likely due to the higher sensitivity of

Stain free (2–28ng, Bio-Rad website, <http://www.bio-rad.com/evportal/en/US/LSR/Solutions/LUSQ3K15/Total-Protein-Detection#4>) compared to ponceau (>100ng [8]).

We used pre-cast gels for these studies because it is easy to get large numbers of gels from the same production batch. This reduces possible error(s) associated with differences between different batches of hand cast gels, as the quality of SDS polyacrylamide gels is affected by many factors [9]. Bio-Rad technical notes recommend PVDF low fluorescent membranes for Western blots using Stain free staining. We found that nitrocellulose works quite well but a critical experimental note is that nitrocellulose membranes need to be wet when imaged for Stain free total protein staining, otherwise the image quality obtained was significantly worse (Fig. 2). Membranes can be imaged wet soon after transfer or can be dried and re-wet with TBS, TTBS or water and imaged (Fig. 2 shows a representative image for a nitrocellulose blot re-wet with TTBS). The wet nitrocellulose membrane had less background and greater protein intensity than the dry membrane. Besides the increased signal, linear plots of intensity versus liver lysate amount (10–50µg) showed that wet membranes had better linearity than dry blots (R^2 for wet membrane 0.99, for dry membrane 0.90, for re-wet membrane 0.99, Fig. 2).

The detection of proteins on Stain free gels are based upon trihalocompound modification of tryptophan residues in proteins run on Stain free gels which are exposed to UV. The modified tryptophans give a fluorescent signal that can be readily detected by a CCD camera. The sample preparation and gel electrophoresis protocol are the same as those used for standard SDS-PAGE. For researchers that prefer to make their own gels, the proteins in gels can be visualized by adding trichloroacetic acid or chloroform followed by illumination with UV light [10]. The modification of tryptophan residues raises concerns about possible complications with downstream applications. However, the Stain free gel detection approach has been shown to have certain advantages such as with MALDI-MS analysis [11]. A potential problem would be the use of antibodies raised towards a peptide region that contains a tryptophan. The stain free modification may disrupt the antibody-antigen interaction. However, we have used more than ten antibodies with normal and Stain free gels and were unable to detect any significant differences with antibody reactivity. This may be due to the activation time that we utilized (2 minutes). The Imagemag software which controls the Chemidoc has three activation settings, low (1 minute), medium (2.5 minute), and high (5 minute). The activation time in our experiments is set to medium and manually stopped after 2 minutes. While longer times gave higher intensities, the linearity of the total protein staining was not improved (data not shown). Since longer activation times gave higher intensities it suggests that more tryptophans were being modified, so at 2 minutes a proportion of the tryptophans would not be modified, allowing tryptophan-specific antibodies that interact with tryptophan residues to detect the protein.

Total protein staining methods (Coomassie, ponceau S, Stain free) have the advantage of not relying on a single protein as the loading control. The most common error associated with Western blotting quantification is due to overloading of the loading control. One of the major problems with Western blotting quantification is the relatively limited dynamic range of tissue and cell lysates. The dynamic range of lysates varies depending on the tissue or cell type and the protein determination method used, such as bicinchoninic acid assay, Bradford, or absorbance at 280nm. Coomassie staining of the blots was not compared as we are unaware of any Coomassie blue staining method which gives satisfactory staining on nitrocellulose membranes. This is due to the high background staining of the membrane because of the lower concentrations of methanol for destaining that must be used (when compared to PVDF membranes) to prevent the nitrocellulose membrane from dissolving.

It is likely that α -actin would be suitable as a loading control in most tissues when the total protein loaded on the gel is low to moderate. It is also likely that α -actin may be appropriate as a loading control for some samples such as blood samples [4]. However, in liver samples α -actin semi-quantification was not linear above 25 μ g of total protein, limiting its use as a housekeeping protein for this tissue as most Western blots are done using >25 μ g of total protein. This result is consistent with recent results which suggest that α -actin was an unreliable loading control [1]. Two other total protein stains, SYPRO Ruby and Amido Black, were found to be satisfactory alternatives to the single-protein controls GAPDH and α -actin [4]. However, SYPRO Ruby is costly and contains heavy metals which require special disposal methods in some states, while Amido Black has a lower limit of detection than Stain free staining. Both stains, like ponceau, have to be incubated with the membranes and destained. The compatibility of SYPRO Ruby or Amido Black staining of gels, before blotting to detect initial problems with protein loading, with subsequent western blotting is not known. In conclusion, Stain free staining was found to be a superior loading control to the commonly used loading control α -actin and was as good as or better than ponceau S for liver samples.

Acknowledgments

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References

1. Dittmer A, Dittmer J. Beta-actin is not a reliable loading control in Western blot analysis. *Electrophoresis*. 2006; 27:2844–5. [PubMed: 16688701]
2. Farmer SR, Wan KM, Ben-Ze'ev A, Penman S. Regulation of actin mRNA levels and translation responds to changes in cell configuration. *Mol Cell Biol*. 1983; 3:182–9. [PubMed: 6835208]
3. Castaño Z, Kypta RM. Housekeeping Proteins: Limitations as References During Neuronal Differentiation. *The Open Neuroscience J*. 2008; 2:36–40.
4. Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. *J Neurosci Methods*. 2008; 172:250–4. [PubMed: 18571732]
5. Galpin AJ, Raue U, Jemiolo B, Trappe TA, Harber MP, Minchev K, Trappe S. Human skeletal muscle fiber type specific protein content. *Anal Biochem*. 2012; 425:175–82. [PubMed: 22469996]
6. Mutungi G, Bell C. Commonly used loading controls in Western Blot studies are not suitable for use in post-natal rat skeletal muscles. *Proc Physiol Soc*. 2008; 11:93.
7. Gomes AV, Waddell DS, Siu R, Stein M, Dewey S, Furlow JD, Bodine SC. Upregulation of proteasome activity in muscle RING finger 1-null mice following denervation. *FASEB J*. 2012; 26:2986–99. [PubMed: 22508689]
8. Sasse J, Gallagher SR. Detection of proteins on blot transfer membranes. *Curr Protoc Immunol*. 2008; 8(Unit 8):10B. [PubMed: 19016449]
9. Gomes AV, Barnes JA. Cost- and time-efficient gel electrophoresis for mini-gel systems. *Anal Biochem*. 1998; 260:106–8. [PubMed: 9648661]
10. Kazmin D, Edwards RA, Turner RJ, Larson E, Starkey J. Visualization of proteins in acrylamide gels using ultraviolet illumination. *Anal Biochem*. 2002; 301:91–6. [PubMed: 11811971]
11. Susnea I, Bernevic B, Wicke M, Ma L, Liu S, Schellander K, Przybylski M. Application of MALDI-TOF-Mass Spectrometry to Proteome Analysis Using Stain-Free Gel Electrophoresis. *Top Curr Chem*. 2013; 331:37–54. [PubMed: 22547356]

Figure 1.

Semi-Quantification of Liver Lysate using different loading controls.

A. From left to right, Stain free gel showing liver lysate (10–45 μg) after activation with UV light for 2 minutes; Stain free blot; and ponceau S stained blot. B. Western blot of liver lysate (10–45 μg) probed with α -actin (1:2000). Each figure is representative of three independent blots. C. Graph showing the relative intensity of the α -actin on the membrane versus the amount of protein loaded on the gel (n=3 for each plot). Data is presented as mean $\pm$ SEM. D. Graph showing the relative intensity of the total protein on the membrane versus the amount of protein loaded on the gel (n=3 for each plot). Data is presented as mean $\pm$ SEM.

Figure 2.

Comparison of Stain free imaging when the nitrocellulose membrane is wet or dry.

A. From left to right, blot showing liver lysate (10–50 μg) Stain free image when the nitrocellulose membrane is wet; blot showing Stain free image when the nitrocellulose membrane is dry; and blot showing Stain free image when the nitrocellulose membrane is re-wet after drying. Each figure is representative of three independent blots. B. Graph showing the relative intensity of the proteins on the wet, dry, and re-wet membranes versus the amount of protein loaded on the gel (n=3 for each plot). Data is presented as mean $\pm$ SEM.