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The use of total protein stains as loading controls: An alternative to high-abundance single-protein controls in semi-quantitative immunoblotting

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ABSTRACT

Western blots are used to estimate the relative concentrations of proteins of interest based on staining by specific antibodies. Quantitative measurements are often subject to error due to overloading of the loading control and over-reliance on normalization. We have found that at the protein concentrations normally used to quantify most low-abundance proteins of interest, frequently used single-protein loading controls, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin, do not accurately reflect differences in protein concentration. Two total protein stains, SYPRO® Ruby and Amido Black, were compared and found to be acceptable alternatives to single-protein controls. Although we cannot prove that high-abundance loading controls are inaccurate under all possible conditions, we conclude that the burden of proof should lie with the researcher to demonstrate that their loading control is reflective of quantitative differences in protein concentration.

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1. Introduction

The Western or immunoblot is widely used for determining the presence or absence of a protein within a cellular homogenate, limited only by the availability of a specific antibody. Demonstrating absence requires proof that protein is in each lane of the gel. A control antibody, or loading control (LC) often serves this purpose. Antibodies against β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), along with other high-abundance housekeeping proteins, are used most often because they bind to proteins in nearly any sample.

Increasingly, investigators are utilizing measurements of antibody binding such as fluorescence intensity to *quantify* differences between samples of interest. In these cases each sample must contain the same amount of total protein. Protein levels are first measured with colorimetric assays, such as the Bicinchoninic Acid (BCA) assay. However, gels relying on these tests may still be subject to differential protein transfer or human loading error, and thus journal reviewers usually require a second control. After measuring the protein of interest (POI) with a specific antibody (marked by a chemiluminescent reaction), a second set of antibodies is used to quantify the protein defined as the LC. The ratio of the POI to the LC is used by many laboratories to compare different samples, under the assumption that both measures vary to the same degree with concentration, and thus dividing or "normalizing" by the LC will correct for any loading errors or differential blot transfer (e.g., Asaka et al., 2006; Vasudevan et al., 2004; Wagner et al., 2006). For qualitative studies, the loading control is often just compared visually or displayed in the figure to provide evidence of even loading.

Two issues arise from the use of normalization. First, using a single-protein LC changes the fundamental hypothesis being addressed. A difference between two samples could be the result of an actual difference in the POI, or a difference in the abundance of the LC. Instead of quantifying protein relative to cell number, tissue volume, or total protein, one has reformulated the hypothesis to ask how much protein there is relative to, for example, β-actin concentration. For this reason, most loading controls are high-abundance housekeeping proteins whose levels are thought not to change under most circumstances. This assumption, however, appears imprudent. In the field of RT-PCR (a technique used to measure levels of mRNA), the use of these loading controls is also being questioned (Huggett et al., 2005; Yperman et al., 2004). In the case of each traditionally used loading control, circumstances have been described where the levels of the protein (or mRNA, e.g., Nahlik et al., 2003), differ between experimental groups. For example, it was observed that when cells of the rat spinal cord

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were exposed to traumatic injury, levels of β-actin were significantly altered (Liu and Xu, 2006). GAPDH and Tubulin levels have been found to change over the course of development (Alexander et al., 1985; Moskowitz and Oblinger, 1995), and it seems dubious to assume that no other experimental manipulation would affect the expression of other commonly used housekeeping proteins.

The second issue, and the focus of this study, stems directly from the use of *high-abundance* loading controls. Many proteins of interest, such as PSD-95 and pERK, are low-abundance compared with ubiquitous housekeeping or structural proteins. Unfortunately, this discrepancy in protein abundance between POI and the LC means that homogenate concentrations that allow the POI to be in the linear range of detection on a polyacrylamide gel, necessarily put the LC outside the linear range of detection. Recently it was shown that β-actin is a poor control for many Western blot analyses because at the protein concentrations most often used, optical density values are not only outside the linear range, but they become essentially uncorrelated with protein concentration (Dittmer and Dittmer, 2006). This second issue is pertinent even in the case of qualitative studies, where loading controls are just compared visually, because such studies often assume that if the protein bands *appear* to be equal, they must be very nearly so.

Given these issues, we decided to determine the feasibility of total protein alternatives to single-protein loading controls. We tested two total protein stains. SYPRO® Ruby is a commercially available protein stain that is used prior to antibody staining, thus circumventing a potential problem of extraneous antibody adhering to the blot and falsely elevating the measured protein concentration. Amido Black is a commonly used permanent post-antibody stain, and low-cost in comparison to SYPRO® Ruby. We compared linearity of these stains to the loading control GAPDH and in some cases β-actin. We used a serial dilution of cell homogenate spanning concentrations commonly used for post-synaptic density protein-95 (PSD-95) and pERK, two proteins that are of broad interest in neuroscience. PSD-95 is a synaptic scaffolding molecule and ERK is a ubiquitous signaling protein that is regulated by phosphorylation. In this study we show that total protein stains are acceptable alternatives to single-protein loading controls, and include additional cautionary notes about the use of semi-quantitative Western blotting.

2. Methods

2.1. Samples and blotting

Nuclear and cytoplasmic fractions extracted from pooled mouse cortex (adapted from Kitchener et al. (2004)) were recombined and aliquoted to create a standard protein homogenate that we have found remains stable over time. Protein concentration was determined using a Bicinchoninic Acid Solution (BCA) protein assay test (Sigma). 10% serial dilutions ranging from 21 to 41 μg per well were loaded in duplicate based on our experience that the optimal range for most low-abundance proteins of interest is 30 μg. Different dilutions served as intentional "loading errors", by which we could test the ability of a particular loading control to normalize differences due to loaded amount. Samples were denatured at 90 °C for 7 min in Laemmli's sample buffer plus 0.5 M DTT (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris, pH 6.8, 0.008% bromophenol blue) and run at randomly assigned positions or in order on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were stained with SYPRO® Ruby (Invitrogen) according to the manufacturer's instructions (before *any* other stain or blocker, as milk was observed to block SYPRO staining). A FluorChem™ 8900 Alpha Imager (Alpha Innotech) was used to capture digitally the emitted light of SYPRO® Ruby staining excited by 302 nm UV light, as well as chemiluminescence from the reaction of HRP-linked secondary antibody and SuperSignal West Pico (Pierce) solution. Signals were recorded as pixel intensity, and exposure times were adjusted such that there were no saturated pixels.

2.2. Antibody procedure

Blots were blocked in 5% milk in TBS + 0.1% Tween (1 h, room temperature). Primary antibodies were incubated at 4 °C overnight, and were as follows: mouse polyclonal anti-PSD-95 (1:2000, Affinity BioReagents), mouse anti-pERK (1:1000, Cell Signaling), rabbit anti-GAPDH (1:4000, Santa Cruz), mouse anti-β-actin (1:10,000, Sigma). Secondary antibodies were HRP-linked polyclonal anti-mouse and anti-rabbit (1:1000, Cell Signaling), incubated for 2 h at room temperature in 5% milk. All incubation steps and washes were completed on a Rocker Platform (Bellco), positioned perpendicular to the pivot axis such that each lane was evenly exposed to all reagents. Between antibodies, blots were reblocked, but no chemical stripping was used. Each blot was stained using submersion in Amido Black (0.03% napthol blue black in 3% acetic acid, Sigma.) for 3 min and allowed to air-dry overnight on plastic wrap before scanning at 300 dpi using a HP Scanjet 7400C. For the primary antibody GAPDH (Santa Cruz), concentrations ranging from 1:2000 to 1:10,000 of primary antibody and different incubation times (15 min, 1 h, overnight) were tested, but did not improve linearity. A second antibody to GAPDH (Sigma, G9545) was also tested using concentrations of 1:1000 and 1:10,000, with blots either stained solely with this antibody or after preliminary staining with PSD-95.

2.3. Densitometry

Images were analyzed using AlphaEaseFC software (Alpha Innotech). For relative quantification, the integrated optical density value (defined as Σ(each pixel value − background)) was determined for equal-sized boxes (for each antibody) drawn around bands, with background values taken below each band of interest to account for non-specific antibody staining in the lane. For total protein stains, different box sizes were tested, either around most of the lane, a small portion of the lane, or a thin strip through the center of the lane running from top to bottom. Background values were taken between lanes to remove background due to non-specific staining from neighboring lanes (see Fig. 1). However, in both cases background values did not significantly alter the data, and high values should be considered a warning sign. A preliminary test showed that the placement and size of the boxes shown in Fig. 1 (either a small rectangle, or a thin strip) is optimal for the total protein stains. A small rectangle allows multiple single proteins to be quantified, and can exclude the protein of interest, while a thin strip allows a majority of proteins to be included, while minimizing errors due to lane bending. The correlation coefficient data reported (Fig. 2B) are for small rectangles.

For calculations of coefficients of variation, blots were standardized so that data from replicate blots could be combined. When all experimental samples can be represented on a single gel, each band can be *standardized* (divided by) the mean of bands of that gel in order to average between technical replicates. (When several gels are needed to represent all groups, a standard protein or standard dilution series serves this purpose instead. Similarly, if the experimental groups vary enough that multiple digital exposures are necessary to avoid saturation, relative concentration should be extrapolated from a dilution series.)

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