

Tissue Standards for Aging Research**Abstract:**

A good loading control is critical for accurate comparison of tissue protein levels in aged and young tissue. After reviewing the literature we were surprised to discover the housekeeping proteins commonly used in Western analysis had significant drawbacks. The purpose of this study was to evaluate the linearity and reproducibility of common housekeeping genes tubulin, actin, and GAPDH compared to measures of total protein staining using Direct Blue and Stain Free gels. We found that measurements of total protein staining were superior to housekeeping genes for use as a loading control in young and old mouse, liver, heart, and brain. Among the total protein staining methods, Stain Free gels were superior to Direct Blue.

Abbreviations

CoV, coefficient of variation; R^2 , coefficient of determination

Keywords

Protein standards, Stain Free, Aging

1. Introduction

Proteomics is rapidly changing the way we define gross biological changes; and we use gene expression profiling to tract expression of particular proteins in animals as they age. These experiments typically entail preparing tissue or cell lysates from mice of various ages, and analyzing them by Western blotting. The intensity of a Western signal is then compared in animals of various ages. Although this seems like a straightforward analysis, the key to getting sound data should be no surprise—is a good loading control.

What are the properties of a “good” loading control? It must show whether each lane has loaded the same amount of tissue or cell lysate; and, for our experiments, it must be unaffected by aging and experimental conditions. It also must be simple to use and not interfere with downstream applications. It should be expressed uniformly in various tissues; the signal intensity must rise linearly in the range of extract loaded (difficult if the loading control is a highly expressed housekeeping gene and the protein of interest is expressed at a low level); the molecular weight must differ enough from the target protein that the bands are distinct, and yet similar enough to correct for possible differences in transfer efficiency. Finally, the control and experimental proteins should share the same subcellular compartment.

During our literature search for the best loading control we found that the most oft used control for loading typically entails normalizing to a Western signal from a housekeeping protein. Since this method seemed to us to be subject to multiple drawbacks, we carefully compared the reliability of three popular single protein loading controls: GAPDH, tubulin, and actin, with two Western-based methods of “staining” gels or blots for total protein.

2. Materials and Methods

2.1 Tissue Lysates

Healthy mice determined by visual observation and necropsy used as controls in previous work (Glenn et al. , 2008, Mallinger et al. , 2011, Nelson et al. , 2007) and were anesthetized with Xylocaine/Ketamine, and perfused with cold PBS plus EDTA-free protease inhibitors (Complete, Roche, Alameda, CA). Organs were quick-frozen on dry ice, then 2 mL FLAG lysis buffer, 50 mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100 containing EDTA-free protease inhibitors plus 0.5% antifoam agent was added per mg of dry weight. The mixture was homogenized on ice, with twenty strokes of a Polytron homogenizer set on “20” power, and spun at 16,000 x G for 50 minutes, to separate the debris. Supernatant was removed and samples frozen in aliquots, at -80°C. The protein concentration of lysate was determined by the Bradford protein assay (Bio-Rad, Hercules, CA). For analysis, samples were thawed, diluted to 1 mg/ml in Laemmli buffer with 10% 2-mercaptoethanol, and resolved by 4-15% SDS-PAGE Criterion Stain-Free gels (Bio-Rad).

2.2 Western Blot Analysis

Gels were transferred to PVDF membranes with a Trans-Blot turbo transfer system (Bio-Rad). Blots were blocked 1 h with 5% nonfat dry milk; then, primary antibody was applied for 1 h at room temperature or overnight at 4 °C. Blots were washed four times with TBS containing 0.1% Triton X-100 and then incubated with peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse, 1:10,000; Jackson ImmunoResearch (West Grove, PA) for 1 h at

room temperature. Primary antibodies were α -Tubulin (monoclonal, 1:1,000; Sigma T5168), β -Actin (monoclonal, 1:1,000 Sigma A1978) or GAPDH (monoclonal, 1:500; Millipore Mab 3474). Bands were visualized using enhanced chemiluminescence (Perkin Elmer). Images were captured on an Epi-Chemi³ system (UVP, Upland CA). Bands were quantified by Image J software.

2.3 On-blot Total Protein Analysis

For Direct Blue staining, blots were equilibrated in 40% EtOH, 10% acetic acid, and stained with 0.8 mg/ml Direct Blue 71 (Sigma) in 40% EtOH, 10% acetic acid, as described by Hong et al (Hong et al. , 2002, Hong et al. , 2000). For analysis by the Stain Free system, gels and blots were analyzed by the Gel Doc EZ Imager system (Bio-Rad). Image Lab (Bio-Rad) software was used to quantify protein concentration in samples visualized by Direct Blue staining and Stain Free gels.

2.4 Statistical Analysis

To compare young to old tissue, samples from three young male mice (6 months) and three old male mice (18 months) were tested separately. Each mouse sample was run in triplicate on the same 18-well gel, such that no sample was consistently run at the gel edge. At least three gels were run, yielding an $n = 27$ for both young and old samples.

For linearity analysis, tissue lysates from three young male mice were pooled. Samples containing 10, 20, and 40 μ g of lysate were run in triplicate on the same 12-well gel. Each gel was derived from one tissue type. At least three gels were run for each tissue yielding an $n = 9$ for the samples. Statistical analysis was performed using Excel software.

3. Results

The brain, heart, and liver of three young and old mice were our sources of tissue. These organs were chosen, in part, because they are large and so yield a good amount of lysate. Moreover, each organ responds to aging with distinct gene expression changes: the heart changes significantly, the brain changes moderately, and the liver, comparatively little (Zahn et al. , 2007). Since many studies focus on proteins expressed at relatively low levels, we loaded our gels with moderate amounts of lysate, containing 10, 20, or 40 μ g of protein. This amount allows low-abundance proteins to be transferred and detected in a linear manner (Mollica et al. , 2009). Western blots of tubulin, actin, and GAPDH--all housekeeping proteins stably expressed in the aging heart, liver and brain (Zahn, Poosala, 2007), were compared to total protein staining of blots using Direct Blue or Stain Free gels to find the most reliable loading control. α -tubulin was chosen rather than β -tubulin because it is more abundant in the heart and liver (Lewis et al. , 1985).

For each potential control, we assessed three parameters: 1) the coefficient of variation or CoV, which is the standard deviation of a set of values, divided by the mean--a dimensionless term expressed as percent; 2) the mean difference total signal intensity in young vs. old mice; and 3) the coefficient of determination (R^2), which reflects the goodness of fit of a regression analysis. Each parameter allows us to consider distinct aspects of each control. For example, a control should be reproducible; thus, on a Western, the intensity of duplicate samples should have a low coefficient of variation (CoV). Since we study aging, samples derived from young and old tissues containing the same amount of total protein, should have equal mean intensity of

the loading control. To assess this we arbitrarily set the mean value for young tissue lysate at 1 and calculated fractional change in the mean value for old tissue. Finally, we want our loading control to be useful as a semi-quantitative normalization factor, so we measured the R^2 value. To do this the mean intensities of Western bands loaded at 10, 20 and 40 ug protein/lane were plotted; and linear regression was used to determine the best fit the R^2 values. The closer the R^2 value to 1.00, the better the control. Using these parameters, we assessed how each control performed in each tissue.

To demonstrate the variation in housekeeping genes in different tissues we ran a demonstration blot containing 40 ug of protein from two young and old mice and stained by all of the techniques used in this study (Figure 1). Westerns probing for α -tubulin revealed that α -tubulin was abundant in samples from the brain but not the heart, and scarce in those from the liver (Figure 1), consistent with reports suggesting the amount of soluble tubulin differs by >50-fold in mouse liver and brain (Miller et al. , 1987). β -actin appeared to be abundant in the brain but less so in the liver, and absent in the heart, consistent with others' reports (Ferguson et al. , 2005). Only GAPDH was readily detected in all three tissues; however, the expression level was not uniform, consistent with reports that each tissue has a distinctive pattern of GAPDH expression (Zhu et al. , 2008a, Zhu et al. , 2008b).

In liver extracts, the mean tubulin intensity from old mice was almost three fold higher than from young mice; and for both young and old, the CoV of the signal was high. The mean actin signal intensity was also higher in older livers (36%), though the actin CoV was low in both young and old livers. In contrast the mean signal intensities for GAPD, Direct Blue, and Stain Free did not vary with age. The GAPDH signal, however, had a high CoV in old liver (30%), making it unacceptable as a loading control. In contrast, both methods of determining total protein rendered low CoV values (14 to 22%). Moreover, the banding patterns on the gels and blots were unaffected by age. Overall, in the liver, measurement of total protein by both Direct Blue and Stain Free gels appeared to be the best loading control for comparing young and old hepatic tissue (Table 1).

In liver extracts assessed over a range of protein concentrations, the change in signal intensity of tubulin, actin, GAPDH, Direct Blue, and Stain Free was quite reasonable, as shown by the R^2 values in Table 2. In contrast, the CoV for tubulin, actin, GAPDH, and Direct Blue were high. The high CoVs, were in part due to saturation of the housekeeping protein signal, or in the case of Direct Blue in saturation of some of the more abundant protein bands. Only the Stain Free method yielded a small coefficient of variation, over the linear range examined. Hence, when examined over a range of protein concentrations Stain Free staining provides the most linear and proportional loading control in the liver (Table 2). When taken together with the results of comparison of young and old tissue, the Stain Free method was the best loading control for the liver.

In the heart the tubulin signal again increased significantly in the old tissue (2.4 fold), and had a high CoV, regardless of age. The actin signal also again increased moderately, by 40%, in older heart tissue, enough to exclude it as a loading control. Additionally, the CoV for the actin signal was very high in young tissue. Though GAPDH had a small CoV, the signal decreased by 5% in the old heart. The banding pattern revealed by the Direct Blue and Stain Free methods was the same in young and old heart tissue. However, the Direct Blue staining intensity decreased by 13% in older samples and had the highest coefficient of variation. The signal from the Stain Free method was unchanged in young and old heart and had a small CoV, demonstrating it again was the best loading control for comparing young and old cardiac tissue (Table 1).

In the heart extracts assessed over a range of protein concentrations the signals from actin, Direct Blue, and stain free all had reasonable R^2 values. In contrast, signals from tubulin and GAPDH had extremely low R^2 values, in part because high expression saturated the signal, so the change in signal intensity was not linear. Tubulin, actin, and GAPDH also had high CoVs. In contrast, both Direct Blue and stain free had low CoVs, making them reasonable loading controls for comparing heart extracts (Table 2). However the poor performance of Direct Blue in young and old tissues limits its use as a loading control in the heart. Overall the Stain Free method was the best loading control for heart tissue.

In the brain, assessments of young and old tissue yielded markedly different results. Although the mean tubulin signal again changed the most, this time the expression in older tissue declined (13%), which may reflect loss of α -tubulin isoforms and increase in β -tubulin isoforms (Hungen et al. , 1981). Additionally, the CoV for tubulin was 28% for both ages. The expression patterns of actin, GAPDH, Direct Blue, and Stain Free were uniform. Additionally, the CoV of all four controls was small, ranging from 6 to 17%, making these equally acceptable as loading controls for the brain.

In brain extracts, low R^2 values were only found for the concentration curves of tubulin, GAPDH, Direct Blue, and Stain Free. The CoV was only acceptable for the Stain Free method, making it the only acceptable method for controlling for brain extracts.

4. Discussion

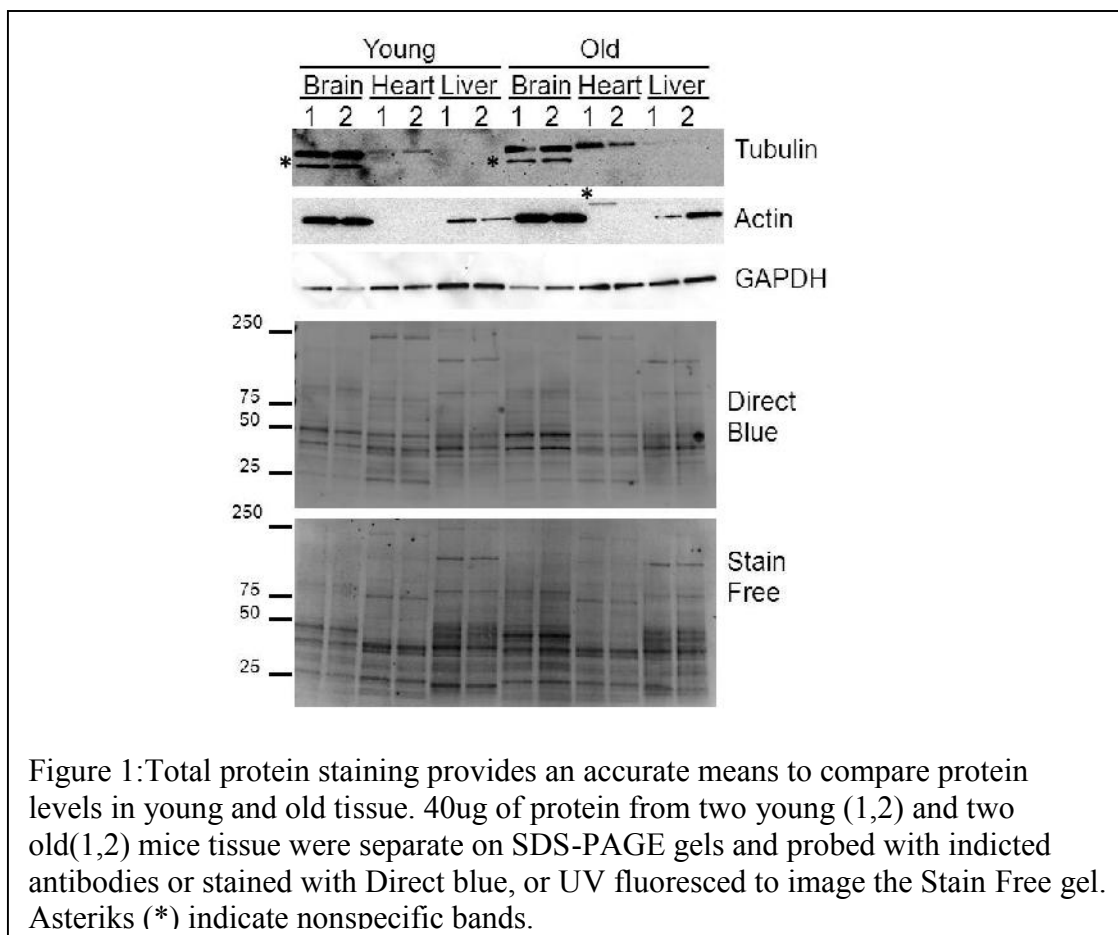
In summary, for our studies on aging in these tissues, staining for total protein was the best method for quantifying the amount of lysate loaded. Other groups have come to similar conclusions comparing Westerns of housekeeping genes to various methods for staining for total protein in cell lysates (Aldridge et al. , 2008, Colella et al. , 2012, Gürtler et al. , 2013, Romero-Calvo et al. , 2010). Staining for total protein has another advantage: the relatively nonspecific probing reveals the overall degradation and quality of a lysate. Among the two methods studied for measuring total proteins there is additional consideration that favors the Stain Free method. The Stain Free signal is fluorescent, generated by UV light; Direct Blue is a stain, the signal generated by absorbent light. The signal of absorbing stains is saturated at lower concentrations than those of fluorescing stains, such that absorbent light comparisons are generally accurate when signals differ by less than an order of magnitude (Hong, Yoo, 2000, Miller et al. , 2006, Rees and Laurence, 1955). Stain Free signals, on the other hand, depend on protein fluorescence and can compare differences of up to two orders of magnitude (Gürtler, Kunz, 2013, Ladner et al. , 2004).

Overall, we found that Stain Free gels yielded the lowest CoV, highest R^2 , smallest mean signal variation, making them the best choice for our studies on aging. Others reported similar results (Colella, Chegenii, 2012, Gürtler, Kunz, 2013). Moreover, our careful analysis revealed that the oft used practice of normalizing to Western signals of housekeeping proteins is not recommended. If one insists on this method, however, normalizing to a GAPDH signal is the best choice because it varies relatively little (Aldridge, Podrebarac, 2008, Calvo et al. , 2008, Dittmer and Dittmer, 2006, Ferguson, Carroll, 2005). Still, it would be a poor control for our studies because the GAPDH expression levels change in aging tissue (Hachinohe et al. , 2013). Additionally, compared to using Western analysis of a housekeeping protein, using measurements of total protein are less affected by experimental conditions, cell cycle, or cell density (Greer et al. , 2010, Gürtler, Kunz, 2013, Liu and Xu, 2006, Ruan and Lai, 2007).

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Tissue	Age	Tubulin		Actin		GAPDH		Direct Blue		Stain Free	
		Mean	CoV	Mean	CoV	Mean	CoV	Mean	CoV	Mean	CoV
Liver	Young	1	.31	1	.18	1	.19	1	.14	1	.20
	Old	2.8	.39	1.36	.23	1	.30	1.0	.20	1.0	.22
Heart	Young	1	.48	1	.42	1	.20	1	.22	1	.18
	Old	2.4	.36	1.4	.25	.95	.14	.87	.36	1.0	.15
Brain	Young	1	.28	1	.13	1	.14	1	.09	1	.15
	Old	.87	.28	1.0	.17	1.1	.06	.99	.17	1.0	.17

Table 1: Total protein as measured by Stain Free gels has the lowest CoV and the most consistent mean total values between young and old tissue.

Tissue	Tubulin		Actin		GAPDH		Direct Blue		Stain Free	
	R ²	CoV	R ²	CoV	R ²	CoV	R ²	CoV	R ²	CoV
Liver	.99-1.00	.21-.46	.94-.99	.12-.27	.96-.99	.18-.54	.95-1.0	.16-.65	.96-1.00	.12-.18
Heart	.01-.99	.20-.65	.98-.99	.12-.36	.82-1.0	.31-.39	.88-.99	.13-.20	.97-.99	.11-.15
Brain	.94-.99	.08-.41	.01-.05	.24-.79	.92-1.0	.11-.38	.97-1.0	.25-.38	.98-.99	.11-.14

Table 2: Total protein as measured by Stain Free gels is the most linear standard as assessed y low CoV and high R².

