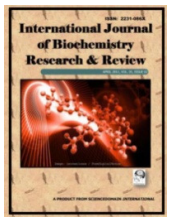

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Tissue Standards Using Stain Free Gels as a Superior Approach for Aging Research

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HMW and KAG designed the study, wrote the protocol and supervised the work. Authors HMW and GMD carried out all laboratories work and performed the statistical analysis. Author KAG managed the analyses of the study, wrote the first draft of the manuscript and managed the literature searches. Author HMW edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

A good loading control is critical for accurate comparison of tissue protein levels in aged and young tissue. After reviewing the literature we discovered that 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 proteins 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 proteins both in linearity and reproducibility in young and old mouse, liver, heart, and brain. Among the total protein staining

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methods, the Stain Free method was superior to Direct Blue, in terms of lower variability and higher accuracy in all tissues, at both ages.

Keywords: Aging research; data normalization; housekeeping proteins; loading control; protein electrophoresis; protein standards; stain-free technology; total protein blot stain; Western blotting.

ABBREVIATIONS

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

1. INTRODUCTION

Proteomics is rapidly changing the way we define gross biological changes; now allowing us to follow 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 one of three common housekeeping proteins. While some studies have suggested that, in principle, normalizing to any housekeeping protein has multiple drawbacks [1,2], most studies have examined the applicability of each of the housekeeping proteins individually.

Actin has often been used as a standard housekeeping protein and its linearity was comparable to reversible ponceau staining for total protein in colon, liver and kidney [3]. In some tissues such as the retina, spinal cord, and skeletal muscle actin has the smallest variation, while in other closely related tissues such as the brain, it has the highest variation among housekeeping proteins [4,5]. This was confirmed by another group where it was found that brain lysates using actin as a loading control revealed both high variation and poor linearity [6]. Others have argued that actin performs poorly when comparing samples between different animals or tissue culture conditions, because of the high variation based upon both animal sex, injury, and biochemical stimuli [2,7,8]. It also performed poorly in tissue culture as increasing amounts of human breast adenocarcinoma blotted for actin demonstrated no correlation between actin and protein levels [9].

GAPDH is another highly used housekeeping protein. However, caution should be used when studying protein degradation, as we do, since GAPDH is substrate for chaperone mediated autophagy and levels will be reduced when autophagy is stimulated [7,10-12]. Additionally, GAPDH is a glycolytic enzyme, hence levels may also be altered by conditions that increase glycolytic enzymes, i.e., the Warburg effect [13]. In studies of brain tissue GAPDH levels had virtually no correlation with protein levels [6]. In contrast, others have found GAPDH to have low levels of variation in brain tissue, but unacceptable variation in spinal cord and skeletal muscle [4]. GAPDH also performed poorly in tissue culture cells as the GAPDH signal saturates early in tissue culture. Compared to Coomassie staining for total protein, GAPDH signals saturate very early at 5μg of human squamous cell carcinoma cell lysate [14]. Furthermore, GAPDH is very dependent upon cell density, levels increased 3-fold with increasing cell confluence [15].

Tubulin completes the list of highly used housekeeping proteins. Caution should be used

when studying aging as tubulin levels are known to change with age and phosphorylation of tubulin increases with age which may affect both gel mobility and antibody binding [16,17]. Tubulin is an excellent loading control in some tissues, but has very high variation in other tissues [4]. Overall when comparing multiple tissues, tubulin demonstrated the highest variability [13]. Tubulin also performed poorly in tissue culture being highly dependent on cell confluence [15].

Finally, there are methods measuring total protein levels. These include the fluorescent-based systems such as Stain Free blotting and the densitometry bases systems such as Direct Blue, Coomassie, amido black and India ink.

To find the best loading control 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. To our knowledge this is the largest side-by-side comparison of housekeeping proteins to total protein staining. Moreover, we believe it is the first study to examine these different types of loading controls both in multiple tissues and at different ages.

2. MATERIALS AND METHODS

2.1 Tissue Lysates

Mice were housed in controlled temperature rooms with 12-h light/dark cycles with food and water provided *ad libitum*. All protocols were approved by the University of Iowa Animal Care and Use Committee (ACURF #0609196). Healthy mice determined by visual observation and necropsy used as controls in previous work [18-20] and were anesthetized with Xylocaine/Ketamine, and perfused with 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 ºC. Blots were washed four times with TBS containing 0.1% Tween-20 and then incubated with peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse, 1:10,000; Jackson Immuno Research (West Grove, PA) for 1 h at room temperature. Primary antibodies were anti-Tubulin (monoclonal, 1:1,000; Sigma T5168), anti-Actin (monoclonal, 1:1,000 Sigma A1978) or anti-GAPDH (monoclonal, 1:500; Millipore Mab 3474). Bands were visualized using enhanced chemiluminescence (Perkin Elmer). Images were captured on an Epi-Chemi^3 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 [21,22]. 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. To determine, if protein loading control had changed with age, we determined P values of the sample means using an unpaired, two tailed, students T test.

For linearity analysis, tissue lysates from three young male mice were pooled. Samples containing 10, 20 and 40µg 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. Linear regression analysis to determine the best linear, non-proportional fit 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 [23]. 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 μg of protein. This amount allows low-abundance proteins to be transferred and detected in a linear manner without dangers of oversaturation [24]. We used the lowest reported concentration of antibody, and the shortest possible exposure time, as this is been shown to give most linear results[9].The linear curve of various proteins levels also served as our titration curve. Western blots of tubulin, actin, and GAPDH--all housekeeping proteins stably expressed in the aging heart, liver and brain [23], 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 [25].

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, P values from the unpaired, two tailed, students T test were used to demonstrate which methods perform the best in aging tissue [7,26]; and 3) the coefficient of determination (R^2), which reflects the goodness of fit of a regression analysis. Because Beer's law is a logarithmic relationship, a linear fit can only be assumed for discrete sections. Hence we chose to use a linear, non-proportional curve fit, which other researchers have shown to give more accurate results [24,27].

Each of these three parameters 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, we examined samples derived from young and old tissues. A good loading control would have equal mean intensity of in young and old tissue. 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. To determine whether the changes between young and old tissue were significant we calculated the P value, the larger the P value the less significant the change. 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 and 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.

All of the gels used in this study used a single tissue under varying conditions. To visually highlight the remarkable variability in housekeeping protein staining in multiple tissues that we and others described, we ran a single 12 well multi-tissue blot (Fig. 1). This multi-tissue blot contained 40 μg of protein from two young and old mice stained by all the techniques used in this study. The multi-tissue blot was for visual representation only, and no Image J analysis or statistical studies were done (Fig. 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 (Fig. 1), consistent with reports suggesting the amount of soluble tubulin differs by >50-fold in mouse liver and brain [28]. β-actin appeared to be abundant in the brain but less so in the liver, and absent in the heart, consistent with others' reports [13]. 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 [29,30].

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 GAPDH, 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. The P values were best for the Stain Free method. In contrast the P values were the worst for actin, where there was a statistically significant difference between young and old tissues. Overall, in the liver, measurement of total protein by both Direct Blue and Stain Free gels had the lowest CoV and highest P values in young and old hepatic tissue (Table 1, Fig. 2).

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 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 most linear and reproducible 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 P values obtained where the best for the Stain Free method. The worst performance was by tubulin with where the difference between young and old tissue almost reached statistical significance with a P value of 0.07.The signal from the Stain Free method was unchanged in young and old heart and had a small CoV, and the highest P value, demonstrating it again was the best loading control for comparing young and old cardiac tissue (Table 1, Fig. 2).

In 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, due to signal saturation. 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 CoV values of Direct Blue in young and old tissues limits its use a loading control in the heart. Hence, the Stain Free method was the superior loading control as assessed by CoV and P values 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, where expression in older tissue declined (13%), which may reflect loss of α–tubulin isoforms and increase in β–tubulin isoforms [17]. 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. The P values obtained where the best for the Stain Free method. The worst performer in the brain was GAPDH, the P value though poor at 0.32 was not statistically significant (Table 1, Fig. 2).

In brain extracts, high R^2 values were 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

Studying protein expression in aging tissue can be particularly difficult. Transcriptional profiling of aging tissue indicates significant heterogeneity, with some tissues have little or no changes, while others have dramatic changes [23]. Unfortunately there has not been a similar study examining the protein levels. However the results from the transcriptional profiling suggest that there may be a significant heterogeneity among different issues with age. This is the first study to compare loading controls in aging tissues.

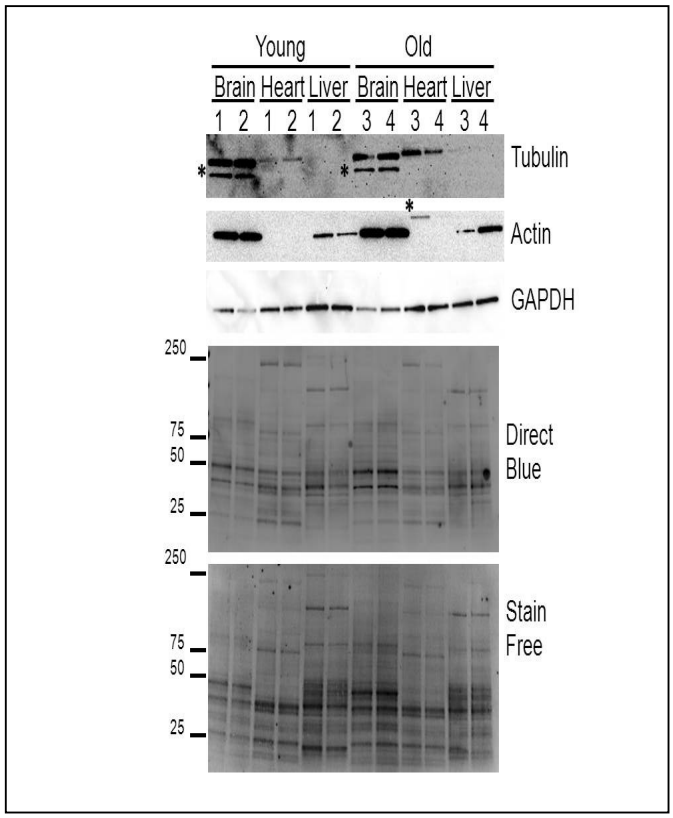

Fig. 1. Total protein staining provides an accurate means to compare protein levels in young and old tissue. 40ug of protein from two young (1,2) and two old (3,4) mice tissue were separate on SDS-PAGE gels and probed with indicted antibodies or stained with Direct blue, or UV fluoresced to image the Stain Free gel. Asteriks (*) indicate nonspecific bands

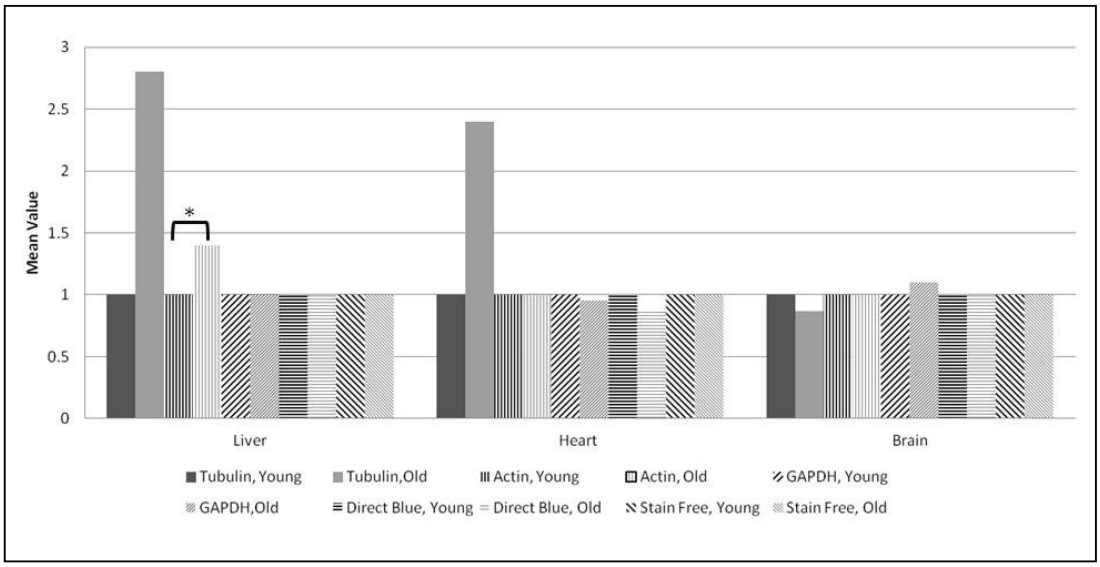

Fig. 2. Mean values for housekeeping and total proteins stains were only significantly different for actin in young and old liver. Mean values of fluorescent or densitometry measurements in young and old tissue from table1. * indicates p<.05

Table 1. Total protein as measured by stain free gels has the lowest CoV, the most consistent mean total values, as assessed by P values, between young and old tissue. CoV, the coefficient of variation, is a measure of the relative magnitude of standard deviation. It is the standard deviation divided by the mean. We used the unpaired, two tailed students t test to compare the means of the young and old samples. The P value is the probability, expressed as a decimal, that the mean values of the young and old tissue are the same

	Tissue	Liver		Heart		Brain	
	Age	Young	Old	Young	Old	Young	Old
Tubulin	Mean	1.0	2.0	1.0	2.4	1.0	.87
	CoV	.31	.39	.48	.36	.28	.28
	P	.053		.070		.57	
Actin	Mean	1.0	1.4	1.0	1.0	1.0	1.0
	CoV	.18	.23	.42	.25	.13	.17
	P	.027		.27		1.0	
GAPDH	Mean	1.0	1.0	1.0	.95	1.0	1.1
	CoV	.19	.30	.20	.14	.14	.06
	P	1.00		.74		.32	
Direct Blue	Mean	1.0	1.0	1.0	.87	1.0	.99
	CoV	.14	.20	.22	.36	.09	.17
	P	1.00		.59		.93	
Stain	Mean	1.0	1.0	1.0	1.0	1.0	1.0
Free	CoV	.20	.22	.18	.15	.15	.17
	P	1.00		1.00		1.00	

Table 2. Total protein as measured by Stain Free gels is the most linear standard as assessed by low CoV and high R2. CoV, the coefficient of variation, is a measure of the relative magnitude of standard deviation. It is the standard deviation divided by the mean

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

In summary, staining for total protein was the best method for quantifying the amount of lysate loaded. Total protein staining has another advantage, for aging researchers, as will it also measure extracellular protein such as that found in the extracellular matrix. This allows one to examine changes in secreted matrix proteins, a potentially significant source variation in aging [31,32].

Other groups have come to similar conclusions comparing Westerns of housekeeping proteins to various methods for staining for total protein in cell lysates. Some of the variations in housekeeping protein levels may be due to 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 [21,33,34]. However methods of stripping, or time of exposure to antibody [5]. Some studies recommended optimizing each the conditions for the use of each housekeeping protein to determine which housekeeping protein is the appropriate control [2].

Total protein stains do not require such an optimization. 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 florescent, generating and measuring a fluorescence signal requires additional equipment, a disadvantage for the Stain Free method. Stain Free signals, on the other hand, depend on protein fluorescence and can compare differences of up to two orders of magnitude [26,35].

We believe this is the most comprehensive comparison of Stain Free technology to housekeeping proteins. Some of the published literature on Stain Free gels is only descriptive and does not compare Stain Free gels to other methods [36]. Another study found Stain Free gels to be superior to the single housekeeping protein studied, GAPDH, but only examined a single tissue culture cell line [26]. Another study found Stain Free gels to be give the best results but only compared it to one alternative form of total protein staining, Sybro Ruby, and one housekeeping protein, actin, in a single tissue, the retina [5]. Finally, one study compared Stain Free gels to two methods of total protein staining and to two housekeeping proteins but only examined a single tissue, the brain [6].

5. CONCLUSION

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. 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 [4,6,9,13]. Still, it would be a poor control for our studies because the GAPDH expression levels change in aging tissue [37]. 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 [2,8,15,26]. The loading control with the lowest CoV's, highest P and R^2 values in all tissues, at both ages, was the Stain Free method.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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