Abstract: 153P

What to do when Westerns go wild

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Western blotting is one of the most performed experimental protocols for semi quantitative analysis of protein expression. Although the procedure appears simple, difficulties often arise throughout the minimum two-day procedure. These complications can lead to issues such as the inability to detect the protein of interest, non-reproducible work, and false statistical data. Our laboratory has collectively run over 3000 gels and tested hundreds of antibodies over the last 15 years. We specialize in detecting proteins of low abundance in single skeletal muscle cells (fibres), or whole tissue samples with a total protein amount ranging from ~1 to 4 µg. Our experience with wild Westerns has led us to develop an improved Western protocol that includes key optimization steps for protein detection. Previously we showed that loading a low amount of protein sample (~2 µg) resulted in more proteins entering the gel during SDS-PAGE, allowing efficient protein migration and enhanced protein detection [1, 2]. We identified that even brief sample centrifugation led to loss of protein which subsequently interfered with the ability to obtain quantitative data [3]. We routinely do not heat protein samples, because it is either not necessary (e.g., AMPK isoforms) or results in loss of protein signal intensity (e.g., dihydropyridine receptor, DHPR) [4]. The adoption of stain free gel technology has eliminated the need to identify a suitable, unchanging, housekeeping protein [3, 5]. We have also stipulated that testing and validating commercially available primary antibodies is required to ensure which protein band is the protein of interest [2, 4, 6, 7]. Finally, incorporation of a calibration curve provides knowledge of upper and lower limits of detection and allows samples of interest to be calibrated to the given gel. Our comprehensive Western workflow is briefly summarized using specific examples in Table 1. Continuing from our previously published steps, this study shows additional steps that can be used to resolve detection problems. These include reapplication of sensitive chemiluminescence reagents, re-incubation of the secondary antibody or repeating the steps from incubation of the primary antibody.

Table 1. Dos and Don'ts of Western blotting.

Step	Do	Don't	Supporting Data
Sample preparation	Dilute sample to working concentration ~3 µg/µl wet weight	Centrifuge samples	~30% of the total sarcoplasmic reticulum protein Calsequestrin 2 would be present if centrifugation is used [8]. ~20-30% of the total mitochondrial protein pool would be lost if centrifugation is used [9].
	Load ~10 µg wet weight (equivalent to 2.5 µg total protein)	Overload protein	Loading more than 20 µg of wet weight resulted in the loss of proportionality and linearity required for accurate quantification of Calsequestrin 1 [8].
SDS-PAGE	Equilibrate loading volume, use calibration curve [8]	Use control proteins for quantification	2-fold more GAPDH in type II compared to type I fibres and is age-dependent [10].

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