RESEARCH TIPS:
The Power of Calibrated Western Blotting for Accurate Protein Quantification

Q&A with Robyn M. Murphy
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Question: What are the most important aspects to understanding the theory and technique of quantitative Westerns?

Western blotting is a widely used technique in molecular biology and biochemistry for detecting specific proteins in a sample. Traditional western blotting methods often assume linearity and proportionality between the amount of protein loaded and the signal detected. We have shown this is often not the case (see Mollica et al 2009, Murphy and Lamb, 2013) and, as a consequence, inaccuracies can be introduced into the analyses. This is where calibrated or quantitative western blotting comes into play, which we have developed and refined, providing a more reliable and accurate method for protein quantification.

Question: What do you feel are the requirements for achieving accurate data?

Calibration curves are essential in quantitative biochemical techniques. Broadly speaking, to determine the absolute amount of a given analyte, a set of standards of known concentrations are used and the Beer-Lambert Law applied, where Absorbance (Abs) is proportional to concentration (C), $Abs \propto C$. As a biochemical approach, this can be applied to the amount of protein in each sample using Western blotting. In our lab, the sample is typically skeletal muscle, where a purified protein with a known concentration is necessary to determine the concentration of the target protein in the muscle. See Murphy et al, 2011; Murphy et al, 2009; Larkins et al, 2012.

In further context of western blotting, calibration curves consisting of multiple input amounts of the sample of interest are used to establish the relationship between the amount of protein loaded and the signal intensity detected. The linear portion of the relationship is then applied to experimental samples to obtain accurate quantification using the linear regression equation, $y = \mathrm{m}x + c$ and importantly, identifying the upper and lower limits of detection along a linear range. See MacInnis et al, 2017; Wyckelsma et al, 2017; Wyckelsma et al, 2016.

In our research, we have demonstrated that small differences in protein abundance, such as 10-20%, can be biologically relevant, but which, in the absence of calibration curves, may not be detected.

Question: What tips do you have for other researchers related to quantitative Westerns?

The main advantage of calibrated western blotting is its ability to provide accurate and reproducible relative abundance of proteins in samples of all types. This is particularly important when considering the physiological consequence of a higher or lower abundance of a given protein, where small changes can have significant biological implications. Understanding the abundance of dysferlin in muscle tissues is important to understanding its function. Traditional methods like immunohistochemistry lack the ability to be calibrated and so cannot be used to determine dysferlin quantification. Our calibrated quantitative western blotting approach can overcome this limitation and is currently being addressed in our research program.

To appreciate the need to always use calibrated western blotting, researchers are encouraged to think about why they are performing western blotting and what differences they are trying to see. Once applied, and one sees that the slope of the line is typically not equal to '1' which would indicate a proportional relationship between input and output values, one equally sees that performing western blotting without calibration curves will hinder the ability to obtain robust data.

Conclusions about the Power of Calibrated Westerns:

Calibrated western blotting is a powerful technique for accurate protein quantification. By using calibration curves, we can overcome the limitations of traditional western blotting methods and obtain reliable data on protein abundance. This is particularly valuable for studying dysferlin and other low-abundance proteins, providing insights into their role in muscle function and disease.

Our lab continues to refine and apply calibrated western blotting techniques to advance our understanding of muscle biology and develop potential therapeutic strategies for muscular dystrophies.

Published Resources to Share

Publications that (i) describe calibrated western blotting in the greatest detail and (ii) set the context on why they are necessary.

Mollica JP, Oakhill JS, Lamb GD and Murphy RM (2009). Are genuine changes in protein expression being overlooked? Reassessing Western blotting. **Anal Biochem***,* 386: 270-275.

Latchman HK, Wette SG, Ellul DJ, Murphy RM[#], Frankenberg NT[#] (2023) Fiber type identification of Human Skeletal Muscle. **J Visual Exp (JoVE)** DOI: 10.3791/65750. PMID: 37811931

Murphy RM and Lamb GD. (2013) Important considerations for protein analyses using antibody-based techniques: Down-sizing western blotting up-sizes outcomes. **J Physiol**, 591 (23): 5823-5831

Papers published using calibrated western blotting

MacInnis MJ, Zacharewicz E, Haikalis ME, Martin BJ, Skelly LE, Tarnopolsky MA, Murphy RM* Gibala MJ*. (2017) Superior mitochondrial adaptations in human skeletal muscle after interval compared to continuous single-leg cycling matched for total work. **J Physiol**, 595 (9): 2915-2930

Murphy RM, Mollica JP, Beard NA, Knollmann BC and Lamb GD (2011). Quantification of calsequestrin 2 (CSQ2) in sheep cardiac muscle and Ca^{2+}-binding protein changes in CSQ2-knockout mice. **Am J Physiol Heart***,* 300 (2): H595-604

Murphy RM, Larkins NT, Mollica JP, Beard NA & Lamb GD (2009). Calsequestrin content and SERCA determine normal and maximal Ca^{2+} storage levels in sarcoplasmic reticulum of fast- and slow-twitch fibers of rat. **J Physiol** 584: 443-460.

Larkins NT, Murphy RM and Lamb GD. (2012) Absolute amounts and diffusibility of HSP72, HSP25 and αB-crystallin in fast- and slow-twitch skeletal muscle fibers of rat. **Am J Physiol Cell Physiol***,* 302: C228-239

Wyckelsma VL, McKenna MJ, Levinger I, Petersen AC, Lamboley CR, Murphy RM. (2016) Cell specific changes in the abundance of GAPDH and Na^+, K^+-ATPase proteins in skeletal muscle from aged individuals, implications for protein measurements. **Exp Gerontol**, 75: 8-15

Wyckelsma VL, Levinger I, McKenna MJ, Formosa L, Ryan MT, Petersen AC, Murphy RM. (2017) Preservation of skeletal muscle mitochondrial content in older adults: a relationship between mitochondrial dynamics, fibre type and exercise training. **J Physiol**, 595 (11): 3345-3359