⌘ *Author's Choice*

Western Blots *versus* Selected Reaction Monitoring Assays: Time to Turn the Tables?

Ruedi Aebersold, Alma L. Burlingame, and Ralph A. Bradshaw

As of January 1, 2013, the paper entitled "Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications," by Towbin and colleagues (1), had been cited 52,488 times (ISI Web of Knowledge v5.8), placing it among the elite group of papers that have truly transformed life science research. For more than 30 years, the nitrocellulose-based Western blotting technique introduced by this paper has been a principal method for the detection of specific proteins in complex biological samples. In the original paper, the authors already anticipated that refinements and variations of the basic technique could lead to the determination of properties of a protein other than its mere presence, and indeed such extensions have been exceedingly successful. The state of phosphorylation, the presence of other post-translational modifications, domain boundaries, estimation(s) of the molecular weight of the protein(s), and the approximate location(s) of antibody epitopes, among other important parameters, have been determined via Western blotting. However, these workers likely could not have anticipated that it would also become the *de facto* "gold standard" method for quantifying a protein in a complex sample. In fact, in the third sentence of their abstract (1) they wrote, "For sodium dodecyl sulfate gels, the original band pattern was obtained with no loss of resolution, but the transfer was not quantitative," suggesting that they considered accurate protein quantification by means of Western blotting to be a challenge.

Over the past two decades, advances in mass spectrometry (MS)[1] and bioinformatics have revolutionized the analysis of proteins and proteomes. Initially, the development of proteomic technology, and therefore most proteomic studies, focused on the reliable identification of an ever increasing number of proteins contained in biological samples. This was spectacularly successful, and today proteomics papers routinely report the identification of thousands of proteins and/or phosphorylation events (or other types of modifications). Accordingly, the analytical objectives of MS-based proteomics have diversified. The determination of the relative abundance of specific proteins across samples (relative quantification) and the determination of the concentration of a given protein in a sample (absolute quantification) have become recognized as among the biologically most important attributes of proteins. A rich literature on the quantitative MS of small molecules and seminal early studies that indicated that the main principles of quantitative MS, particularly isotope dilution (2) also applied to polypeptides (3), raised the expectation that MS could be used as the basis for quantitative proteomic experiments. Importantly, these expectations have been largely met. Numerous studies have indicated that protein quantification via MS can be robust, accurate, and reproducible and can achieve low limits of detection, provided that technical pitfalls, such as incomplete protein extraction, incomplete proteolysis, and artifactual protein modifications, are appropriately controlled and considered.

Over the past few years, the methods used to quantify proteins by MS—in particular, data generated by means of data-dependent analysis methods, used with or without stable isotope labeling—have steadily evolved and have been widely deployed. More recently, targeted proteomic methods—specifically, selected reaction monitoring (SRM), also referred to as multiple reaction monitoring—have become prevalent. SRM has been the quintessential quantitative MS method for small molecules, and its favorable performance characteristics also apply to peptide analytes. The method is conceptually similar to Western blotting. Both use assays that must be developed for each target protein to detect and quantify specific, predetermined (sets of) analytes in complex samples. However, the methods differ substantially in their implementation, the reliability of the resulting assays, and the quality of results they produce. A Western blotting assay essentially depends on the specificity of the antibody used. In contrast, an SRM assay depends on multiple parameters, such as the retention time, the mass-to-charge ratio of the precursor ion and selected fragment ions of the targeted peptide, and the relative signal intensities of the detected fragment (transition) signals. These values are then weighted and combined to derive a score that indicates the probability that the targeted peptide has been detected. This technology has been progressing rapidly. Highly multiplexed data acquisition techniques that support the quantification of hundreds of peptides in a single injection, software tools to set up targeting measurements and to statistically evaluate the acquired data, and methods for the rapid development of SRM

Received June 9, 2013, and in revised form, June 9, 2013
⌘ *Author's Choice*—Final version full access.
Published, MCP Papers in Press, June 10, 2013, DOI 10.1074/mcp.E113.031658

[1] The abbreviations used are: MS, mass spectrometry; SRM, selected reaction monitoring.

assays and resources for their public accessibility have made this technology simpler to use and more accessible (4). Targeted proteomics has also been recognized by the journal *Nature Methods* as the method of the year 2012 (5).

Authors who submit papers containing quantitative protein data generated via MS are frequently asked by reviewers to validate some of the values with Western blotting. We believe that with the advances that have occurred, this request is now outdated, causing the unnecessary use of scarce resources and not achieving the main intent: objective cross-validation of results. Of course, confirmation of results with an orthogonal technique of *comparable quality* is sound scientific practice and should be encouraged. Unfortunately, the quality of quantitative data obtained via Western blotting is not comparable to that obtained with SRM. The quality of MS data is vastly superior for several reasons. (i) *Quality of the assay*: Quantification by Western blotting is based on a single reagent (antibody) that may be poorly characterized. Not uncommonly, neither its affinity for the antigen nor the epitope is known or disclosed. Further, frequently no *bona fide* reference sample is available to test the performance of the assay in the context of a particular experiment. In contrast, SRM assays depend on isotopically labeled reference peptides, the quality of which can be easily verified by a fragment ion spectrum. In addition, typically several independent peptides of the same protein are targeted to quantify a protein, although in specific cases (*e.g.* in studies targeting a post-translationally modified peptide) single peptide measurements have to suffice. The importance of each peptide assay can thus be independently verified in each laboratory or study and, in fact, for each sample type. (ii) *Quality of the results*: Protein quantification via Western blotting depends on a single signal: the intensity of a band on the blot. This signal can be specific (*i.e.* represent the targeted protein) or unspecific. A signal is generally declared specific if the electrophoretic mobility of a protein approximately corresponds to that expected based on the calculated molecular weight of the targeted protein. Generally, the quality of the result is therefore not known. However, the frequently observed (but generally not displayed) observation that a lane in a Western blot shows several positive bands suggests that the confidence in the identification of the target protein often might be rather low. SRM-based quantification, in contrast, uses multiple signals (multiple transitions per peptide, multiple peptides per protein, and multiple measurements of each signal) that are integrated into a composite score indicating the protein quantity. Even in cases when conclusions are drawn from a single peptide, such as for post-translationally modified peptides, multiple data points (transitions, repeat measurements) of that peptide are obtained. The availability of multiple independent data points for a particular analyte makes it possible to use statistical methods such as outlier detection, expectation maximization, or target-decoy strategies to differentiate between true and false results and to determine the statistical significance of the final value. The quality of the findings is therefore generally known. (iii) *Performance characteristics*: Each method is characterized by a number of performance characteristics such as limit of detection, linear dynamic range, ability to multiplex, and reproducibility. For most of these characteristics, MS-based methods now outperform Western blotting.

Although the case against using Western blotting as an orthogonal check on the validity of MS data is clear, this evaluation should not be construed as meaning Western blots have no value and that the technique should be dropped from the arsenal of useful biological methods. A considerable amount of important information has been obtained through the technique (consider the number of citations of the original report), and undoubtedly this will continue. Indeed, despite the limitations of immune-based reagents, they are, and will likely remain for a long time, an invaluable part of biological/biomedical research. But their usefulness as a means, or even as the "gold standard," for quantifying proteins in complex samples has to be seriously questioned now that SRM assays for proteins can be developed and used with comparative ease.

Therefore, we posit that the request to validate quantitative MS data by Western blotting is no longer justified. In fact, considering that the vast majority of protein identifications claimed from biological samples are still derived from Western blotting, it may be time to "turn the tables" and request that Western blotting results, or at least the assays that support these results, be validated by MS. There are factors such as the lower cost, lower complexity, and easier access of gel-based methods relative to MS that might be raised as arguments against this proposition. However, this is unsustainable, because data quality/validity is paramount for the scientific process, and if a demonstrably superior method is available, it must become the gold standard and be used if at all possible. Further, because validated SRM assays are now easily developed for essentially any protein, and because targeting methods continue to evolve (*e.g.* through their application to datasets acquired by data independent acquisition) the increased use of targeted MS will also make all those proteins for which no affinity reagent has been developed accessible for routine quantification, thus vastly expanding the scope of experimental biology and, importantly, its translational applications.

REFERENCES

1. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350–4354
2. Nocke, L., Siekmann, L. Z., and Breuer, H. (1970) New methods for the determination of steroid hormones. *Klin. Chem. Klin. Biochem.* **8,** 329–338
3. Desiderio, D. M., and Kai, M. (1983) Preparation of stable isotope-incorporated peptide internal standards for field desorption mass-spectrometry quantification of peptides in biologic tissue. *Biomed. Mass Spectrom.* **10,** 471–479
4. Picotti, P., and Aebersold, R. (2012) Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nat. Methods* **9,** 555–566
5. Anonymous (2013) Method of the year 2012. *Nat. Methods* **10,** 1