Common mistakes in Western blot

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Abstract

High specificity and sensitivity are the key enabling characteristics that made Western blot the gold standard approach for protein detection and quantification. However, poor understanding of the physical and chemical basis of the technique results in common mistakes that could seriously affect the reliability of blot readout. Specifically, issues concerning poor gel casting could result in non-homogeneous distribution of pores between gel lanes, that in allowing faster migration of proteins in some lanes compared to others, result in a wavy line of detected bands. This could impact on the identification of the target protein through the molecular weight marker. Secondly, although overloading of samples may not seriously impact on protein detection, it generates blots which could not be used for the quantification of relative abundance of proteins. Another problem is the non-specific binding of non-target proteins by primary antibodies which manifest as multiple bands on the same membrane strip. This came about due to the presence of multiple binding clefts on the antibody, which could bind to different proteins. Finally, incomplete washing of secondary antibody yields blots with large smear and dark patches that makes the quantification of relative abundances of proteins in different samples difficult. Hence, although Western blot is a standard technique in the life sciences for the specific detection and semi-quantitative analysis of protein abundance, multiple issues ranging from gel casting to sample loading and choice of antibody could confound blot readout. However, simple steps exist to ameliorate the aforementioned problems. For example, greater care in gel casting would enable proteins to migrate uniformly across different gel lanes. Use of the Coomassie Blue stain would provide an indication of the relative abundance of different proteins separated by electrophoresis and help select a suitable dilution factor for a full Western blot. Greater care in washing and use of an appropriate secondary antibody concentration would reduce high background on the membrane strip. But, the most difficult issue remains the choice of primary antibody as it is hard to predict possible non-specific binding partners of the antibody. Overall, Western blot remains a reliable method for the confirmation of target proteins probed by mass spectrometry methods.

Keywords: Western blot, protein analysis, secondary antibody, washing step, wavy line of detected bands, sample overloading, non-specific binding, primary antibody, non-target proteins, pore structure,

Subject areas: biotechnology, molecular biology, biochemistry, cell biology,

Introduction

Using antibodies specific for particular proteins, Western blot is the gold standard technique for detecting specific proteins and quantifying their relative abundances. The method

is highly sensitive and provides confirmatory evidence for the presence or absence of specific proteins. Thus, it is a commonly used assay in biological sciences and medicine. However, Western blot is a laborious and high expense assay with many steps. Hence, efforts should be expended in reducing common errors typically encountered in Western blots, which affects the accuracy and sensitivity of the assay.[1] Ranging from overloading of gel to poor homogeneity of the casted protein gel and wrong choice of primary antibodies,[2] simple errors in conducting Western blot could result in the erroneous non-detection of a protein or a mistake in protein quantification.

Mistakes

Overloading of protein sample

The most common error in Western blot is overloading of gel with protein samples; thereby, resulting in blots with very large intense bands, which in saturating optical charge coupled device (CCD) detector, hampers the automated semi-quantitative assay of protein relative abundance (Figure 1). Specifically, the fourth membrane strip of Figure 1 exhibited high intensity protein band characteristic of overloading of protein sample during the electrophoresis step of Western blot. The problem is an important one given that the objective of Western blot is to obtain good readout from all target proteins on a single blot. However, for a mixture of proteins with substantial differences in relative abundances, difficulties may exist in using sample dilution as an approach for reducing sample overloading as large dilution could render some proteins non-detectable.

One possible approach for addressing the problem would be to conduct a Coomassie Blue stain on an initial run of protein electrophoresis. By analysing the relative band intensities that emerges from a Coomassie Blue stain, specific dilution factor could be employed to reduce sample overloading of high relative abundance proteins, while enabling the facile detection of less abundant proteins. Nevertheless, cases exist where it is not possible to arrive at a dilution factor that could allow reliable detection of all target proteins for semi-quantitative Western blot. In such cases, only qualitative detection of target proteins could be achieved by the method.

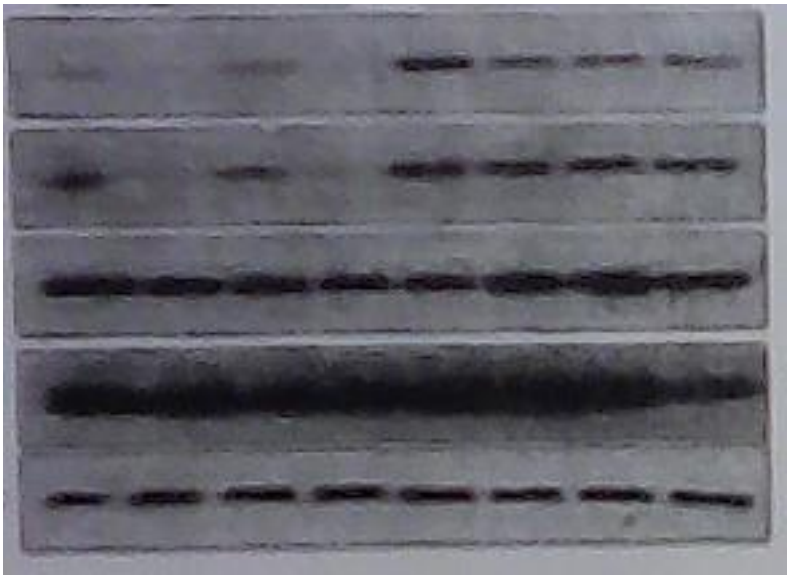

Figure 1: Overloading of protein gel. Dense indiscrete bands on the Western blot highlights the overabundance of protein sample for a particular molecular weight band. Given the difficulty of estimating the relative abundance of proteins in a complex protein mixture, use of the Coomassie Blue staining technique could provide a qualitative readout of the relative abundance of different proteins, that help suggest a dilution factor for simultaneous detection of high and low abundance proteins. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

Poor washing of secondary antibody

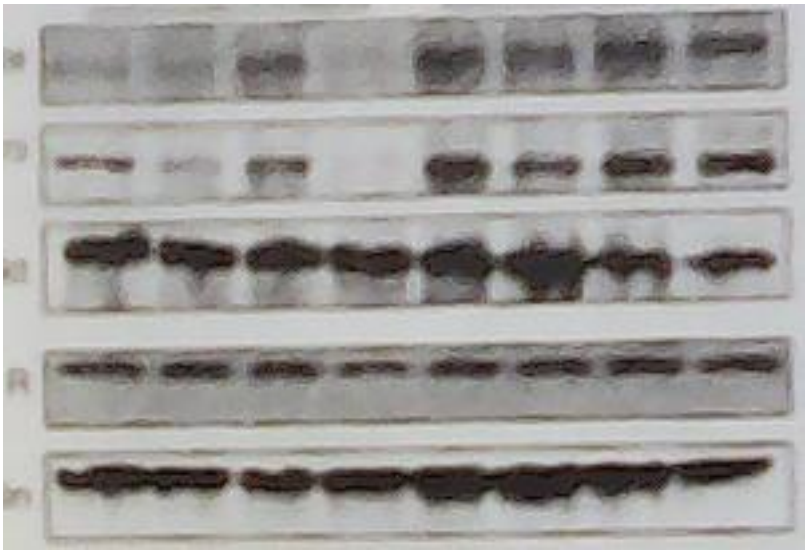

Figure 2: Poor washing of secondary antibody leaves behind a high background. Dark patches in membrane strip 4 (from the top) illustrates the binding of the secondary antibody on the membrane as it was incompletely washed off during the washing step. Membrane strip 3 (from the top), on the other hand, highlights that the electrophoresis step was not given enough time to run for all the proteins to concentrate within a narrow band. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis

configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

Poor washing of the membrane after secondary antibody staining is a common error in Western blot and leaves behind a dark smear on the membrane upon detection by chemiluminescence reagents applied to the membrane strip (Figure 2). Additionally, the problem is also related to the use of a high secondary antibody concentration, which, in turn, require more contact time during the washing step prior to application of the chemiluminescence reagents. Hence, conscientious attention to detail for the washing step is crucial to generating blots untainted by the binding of the secondary antibody to the membrane. Another example of poor washing of secondary antibody could be seen in the large dark patches and smear in Figure 3.

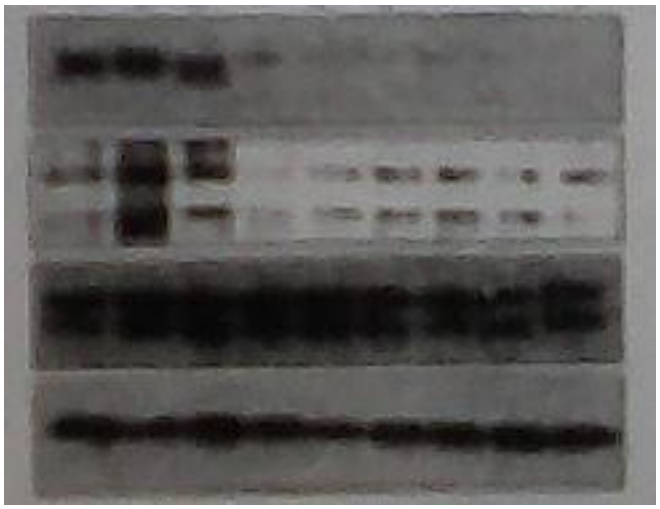

Figure 3: Large smear and dark patches on membrane resulting from poor washing of secondary antibody. Dark patches in membrane strips 1, 3 and 4 (from the top) highlights the poor washing of secondary antibody after the binding step. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

Poor gel casting and insufficient electrophoresis time

Another problem comes from poorly formed bands in membrane strip (e.g., membrane strip 3 from the top in Figure 2). This likely arise due to two factors. Firstly, insufficient time was allowed for the electrophoresis step, which resulted in the inability of the proteins to migrate as a distinct well-formed band across the gel, which after blotting to the membrane, resulted in the formation of poorly-formed bands across all lanes of the original gel. Secondly, poor pore characteristics that resulted from a poor gel casting technique could also hinder the migration and diffusion of proteins of various molecular weights through the pore structure of the polyacrylamide gel; thereby, resulting in poorly-formed bands across the gel.

Non-specific binding of primary antibody to a non-target protein

Specificity is the key enabling characteristic of Western blot, where antibodies capable of high specificity recognition of target proteins are chosen for specific binding to particular proteins of interest. However, given the myriad proteins present in a complex protein sample from lysed cells, as well as the possibility of non-specific binding between chosen primary antibody and other non-target proteins at various sites on the antibody, possibility exists for the concurrent binding between primary antibody to both target and non-target proteins (Figure 4 and 5). Specifically, while the primary antibody binds to the target protein at the main binding cleft, non-target proteins could also bind to the primary antibody at other binding sites.

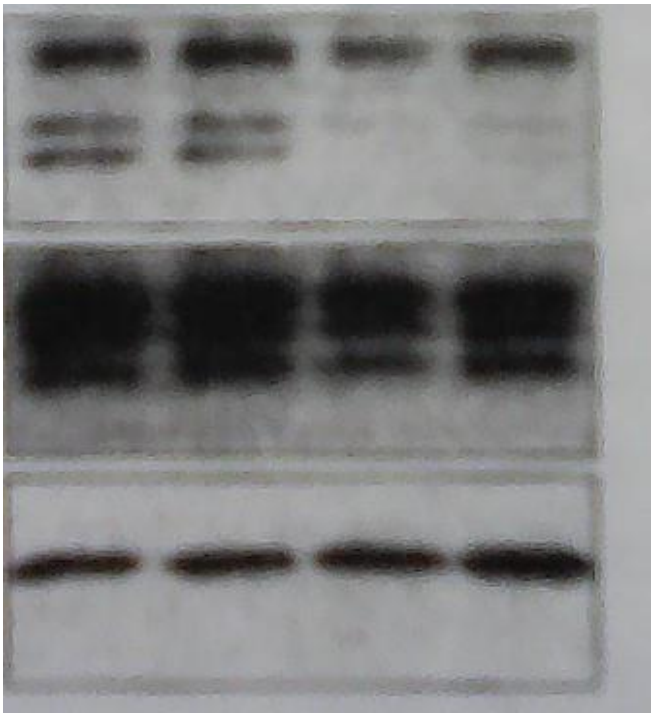

Figure 4: Non-specific binding of primary antibody to non-target proteins. Membrane strips 1 and 2 (from the top) highlights multiple dark intense bands from the same strip, which could be due to the non-specific binding of non-target proteins by the primary antibody. Hence, choice of primary antibody is crucial, but it is difficult to anticipate possible binding between chosen primary antibody with non-target proteins in a complex protein mixture from cells. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

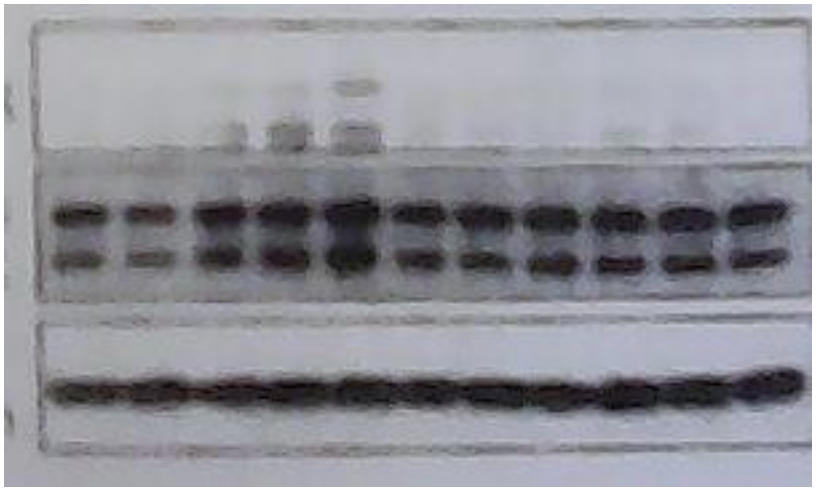

Figure 5: Binding of primary antibody to non-target and target proteins. Membrane strip 2 reveals two distinct bands belonging to two proteins of different molecular weights detected by binding to the primary antibody. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

Non-homogeneous distribution of small and big pores between gel lanes

Wavy lines of distinct protein bands are sometimes visible on Western blot readout, and highlighted poor gel casting. Specifically, non-homogeneous distribution of small pores between gel lanes could result in slight retardation of protein migration and diffusion between lanes, that results in a wavy line of detected protein bands at the lowest molecular weight band detected (Figure 6). For example, presence of a greater fraction of small pores on one side of the gel would enable the faster migration and diffusion of small, low molecular weight proteins on that side compared to the slightly retarded movement of the same protein in samples loaded on the other side of the gel.

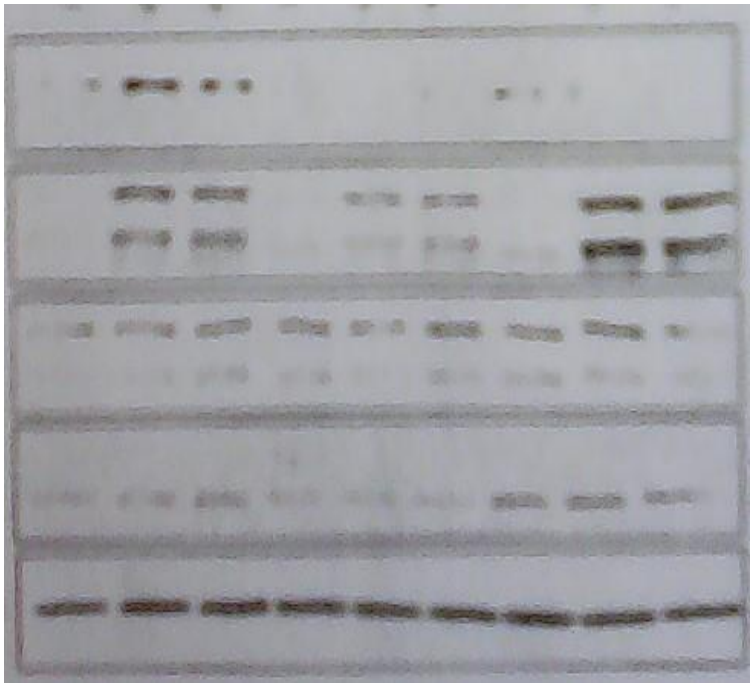

Figure 6: Non-homogeneous distribution of small pores between gel lanes. Specifically, presence of more small pores on the right side of the gel results in faster migration and diffusion of small proteins; thus, they were detected as a band at a lower position compared to the same protein in samples loaded on the left of the gel, where there was less fraction of small pores that facilitated the movement of small, low molecular weight proteins. Note that high molecular weight proteins separation did not manifest a wavy line in this blot, which indicated that there was equal distribution of large pores between gel lanes. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

The same situation also occurs for high molecular weight proteins (Figure 7). Specifically, wavy line of distinct bands of high molecular weight proteins could be seen near the top of the gel (i.e., membrane strips near the top) that results from preferential migration of large proteins through a higher fraction of large pores on some lanes of the gel compared to others.

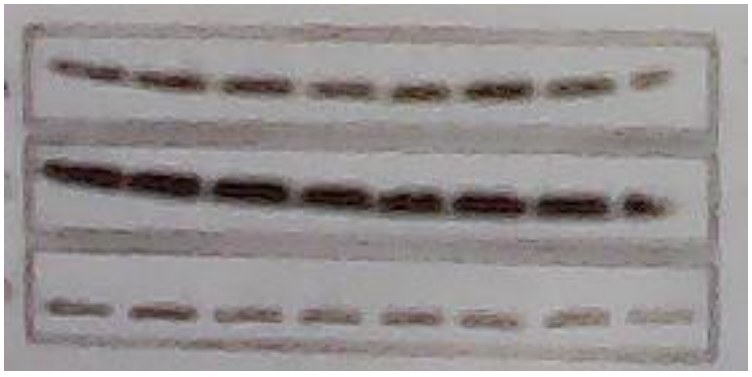

Figure 7: Non-homogeneous distribution of large pores between gel lanes. Specifically, wavy line of distinct bands of detected proteins revealed that large molecular weight proteins migrate faster near the center of the gel compared to the sides, due possibly to the presence of higher percentage of large pores in the middle of the gel than at the sides. Thus, high molecular weight proteins would be better able to migrate and diffuse within a pore structure which has a higher percentage of large pores compared to small ones. Note that the membrane strip at the bottom shows a straight line of low molecular weight detected proteins that highlighted the roughly equal distribution of small pores across gel lanes. Source: Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF (2016), Fatty acid synthesis configures the plasma membrane for inflammation in diabetes, *Nature*, Vol. 539, Issue, 7628, pp. 294-298.

Error in gel casting is the cause of non-homogeneous distribution of large and small pores across gel lanes. Specifically, if the various components of the protein gel are not added homogeneously across the face of the gel during gel casting, non-homogeneous distribution of pores could likely result.

Conclusions

Western blot is a gold standard method for confirming the detection of specific target proteins. But various errors in gel preparation, sample loading, choice of primary antibody, and washing would seriously affect the quality of blot readout. Care must be exercised in gel casting to reduce the non-homogeneous distribution of large and small pores that could, in impeding the migration of proteins, result in a wavy line of detected proteins that hamper the correct identification of protein through the molecular weight marker. On the other hand, good washing after application of the secondary antibody would reduce a significant source of detected background, which in allowing ease of adjusting contrast during image capture of blot, afford a more reliable method for quantifying the relative abundance of proteins across samples. Thirdly, careful selection of primary antibody would reduce non-specific binding of non-target proteins; thereby, enabling more specific and reliable detection and quantification of target proteins, even though it is very difficult to predict the binding partners of a primary antibody. Sufficient time should also be provided for electrophoresis to help in the formation of well-formed protein bands. Finally, overloading of protein sample is a common mistake which can be avoided by first performing a Coomassie Blue stain of the blot prior to proceeding to a full Western blot. Specifically, readout from the Coomassie Blue stain would provide a means for adjusting the dilution factor for all samples prior to sample loading on gel. Overall, simple steps exist to remedy some of the common errors in Western blot.

References

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Conflicts of interest

The author declares no conflicts of interest.

Author's contribution

The author wrote the manuscript.

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