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Review

Approaches to Prevent Saturation in Western Blot Analysis

Short Title: Prevention of Saturation in Western Blotting

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ABSTRACT

Western blotting is widely used to quantify protein levels in various samples. The procedure includes transferring proteins from a gel to a membrane and then blocking the membrane to avoid non-specific binding with particular antibodies. Identification is carried out with secondary antibodies connected to enzymes or fluorescent dyes, enabling the visualization of the target protein bands. Quantification is subsequently performed by measuring the intensity of the bands, so it is necessary to employ appropriate experimental techniques and approaches. Potential mistakes may be encountered when quantifying Western blotting results, but many can be easily avoided. Some images selected for Western blotting may exhibit over-saturation, which can result in inaccuracies in quantification. Over-saturated bands cannot be accurately quantified as they fall outside the linear detection range, causing their signals to look the same. Therefore, we will focus on essential approaches to prevent signal saturation and accurately quantify the signal intensity of the target protein in Western blotting.

Keywords: Protein quantification, Saturation, Western blotting

1. Introduction

The popular technique of Western blotting is widely used for evaluating protein expression levels in various samples[1]. The process involves transferring proteins from a gel to a membrane, followed by blocking the membrane to prevent non-specific binding with specific antibodies[2]. Detection is performed with secondary antibodies linked to enzymes or fluorescent dyes, allowing visualization of the target protein bands. Quantification is then carried out by measuring the intensity of the bands using an imaging software. Sample protein levels are then normalized to a reference protein such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin that is expected not to be affected by the treatment. Consequently, a comparative analysis of the relative protein levels is conducted[3,4].

Signal saturation is a common issue that can arise during the quantification process in Western blot analysis. This phenomenon is an error that can impede precise quantification and interpretation of results[5]. Hence, it is crucial to employ approaches that prevent signal saturation to ensure the reliability and precision of the results. This manuscript will focus on approaches to avoid saturation in Western blotting.

1.1. Definition of linear detection range

As shown in **(Figure 1)**, linear detection range refers to the range of signal intensities that exhibit a linear and proportional relationship between the amount of target protein and the strength of the signal captured by the detector. In the Western blotting, it is assumed that there is a linear relationship between the quantity of the loaded sample and the density of the protein bands. Therefore, it is critical to confirm that the protein bands are situated within the linear detection range[6]. When the signal is weak and falls out of the linear range, it gets affected by noise. Conversely, the imaging system cannot accurately increase the signal intensity if the signal is excessively strong and beyond the linear range[3].

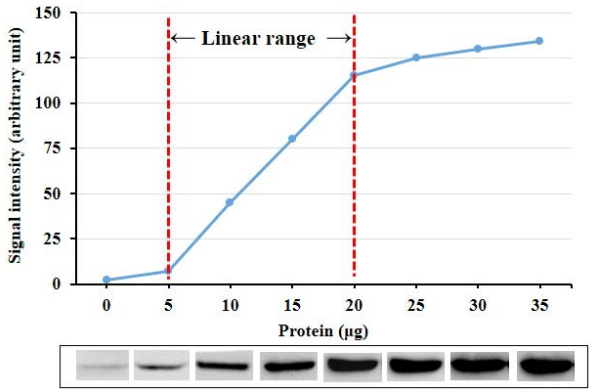

Figure 1: Linear range of detection between the amount of protein and signal intensity.

1.2. Saturation in western blot analysis

In the Western blot technique, saturation occurs when an increase in the amount of target protein does not result in the expected increase in band intensity. When saturation occurs, the sensor pixels reach their maximum capacity to convert incoming photons into electrons.

Two common types of saturation observed when quantifying the results of a Western blot are membrane saturation and signal saturation. Membrane saturation occurs when an excessive amount of proteins load onto a membrane. If proteins exceed the localized binding capacity of the membrane, no more targets can bind[7]. In cases where the intensity of a strong band exceeds the capability of the detection system, signal or detector saturation happens. Beyond this point, increasing the amount of protein fails to produce the expected increase in signal and underestimate differences between samples[8,7].

1.3. Approaches to prevent saturation

Enhancing the knowledge of Western blot data quantification undoubtedly plays a crucial role in obtaining more valid and accurate data in future studies. The following recommendations are suggested to ensure the achievement of valid results and to avoid inaccurate data caused by oversaturated bands in Western blot analysis.

1.3.1. Use an optimal amount of target protein: Loading an excessive amount of protein onto a sodium dodecyl sulfate (SDS)-PAGE gel can lead to data saturation in Western blotting[5]. Ensure that the amount of protein loaded on the gel is within the linear detection range. Optimal dilution depends on the expression level of a target protein. It is necessary to perform a serial dilution to determine the optimal concentration of a target protein that should be loaded on a gel. Proteins that are highly expressed require a greater dilution to avoid signal saturation and to bring the signal intensity within the optimal range for detection[3].

1.3.2. Optimize the concentrations of antibodies: Spatial crowding of antibody epitopes, quenching of fluorescently labeled secondary antibodies or oxidation of enzyme-conjugated secondary antibodies can play a role in the saturation process[9,8]. It is necessary to use the proper concentration of primary and secondary antibodies. An excessive amount of antibody may cause the signal to become saturated. As a result, it is recommended to utilize serial dilution to optimize the antibody concentration[10].

1.3.3. Choose a suitable detection chemistry: Two main types of detection chemistry used to visualize signals in Western blot are enhanced chemiluminescence (ECL) and fluorescence detection methods. ECL is an enzymatic method utilizing secondary antibodies labeled with horseradish peroxidase (HRP) as an enzymatic reporter. The HRP enzyme produces light following exposure to a luminol-based substrate and generates a signal that fluctuates over time[11]. Because this type of detection depends on an enzyme kinetic, the signals may not accurately reflect protein abundance and amplification of signals by ECL increases the probability of saturation. On the other hand, fluorescence detection is a non-enzymatic method that uses secondary antibodies labeled with fluorophores. Unlike enzyme kinetics, fluorescence detection provides signals that correlate directly with the amount of protein, thus minimizing the likelihood of saturation[9,7].

1.3.4. Choose an optimal exposure time: Optimizing exposure time prevents signal saturation and makes it possible to detect even minor changes in the band density. Limiting the exposure time during chemiluminescent detection can help to prevent signal saturation. Experiment with various exposure times to determine the optimal duration for a clear signal with minimal background interference[11]. Simultaneous analysis of proteins with both low and high levels of expression in Western blotting leads to challenges in visualization. Prolonged exposure times required for detecting proteins with low-expression levels may result in saturated bands for proteins with high-expression levels. On the other hand, short exposure times that are optimal for highly expressed proteins may result in signal loss and inability to detect proteins with low expression levels[12].

1.3.5. Choose a suitable imaging system with quantification software: Charge-coupled device (CCD) camera, a vital technology in digital imaging, provides a greater sensitivity and a broader dynamic range compared to traditional x-ray film. Digital imaging system by measuring the full range of the target protein bands prevents detector saturation. Therefore, digital camera-based detectors recognize saturation bands better than film-based processors[13]. Imaging systems require appropriate

software for quantifying the density of protein bands. Even though software analysis programs use slightly different techniques, the maximum height or region is commonly utilized to determine the level of the target protein[14].

2. Conclusion

To prevent signal saturation and accurately quantify the signal intensity of the target protein in Western blotting, it is recommended to consider to the following approaches: use an optimal amount of target protein, optimize the concentrations of antibodies, select suitable detection method and exposure time and employ a suitable imaging system with analysis software.

3. Authors' Contributions

Z.Z.; Original draft writing. M.M.; Original draft review and editing.

4. Conflict of interest

The authors have no conflicting interests to declare.

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