Review Article:
IMPROVING THE QUALITY OF WESTERN BLOT RESULT

Viskasari Pintoko Kalanjati
Department of Anatomy and Histology
Faculty of Medicine Airlangga University
Surabaya, Indonesia

ABSTRAK

Western blot adalah tekhnik untuk mendeteksi suatu protein spesifik dalam suatu sampel, seperti juga pada tekhnik imunohistokimia dan ELISA (enzyme-link immunosorbent assay) meskipun terdapat perbedaan mendasar dalam pelaksanaan dan analisisnya. Dengan membandingkan kadar protein spesifik yang dicari dengan kadar rekombinan protein target yang diketahui sebagai suatu standar pada western blot, kadar absolut protein spesifik tersebut dapat dianalisis secara densitometri. Sedangkan bila standar yang digunakan adalah gabungan dari semua protein sampel, atau adalah suatu sampel protein house-keeping yang telah diketahui kuantitasnya, maka nilai relatif kadar protein spesifik tersebut dapat dihitung. Banyak faktor dapat mengurangi kualitas hasil western blot (dan dapat mempengaruhi hasil densitometrinya). Dalam artikel ini, akan dibahas beberapa cara yang dapat dikerjakan untuk meningkatkan kualitas hasil western blot sehingga hasil perhitungan dari western blot menjadi lebih akurat.

ABSTRACT

The western blot is one of useful techniques in detecting specific protein in the given sample, other than the immunohistochemistry and ELISA (enzyme-linked immunosorbent assay). When probed against the recombinant target protein as the standard sample, it can be used to analyze the exact densitometry value of target protein in the sample, whilst probing against the protein-pool sample or house-keeping protein samples are relatively quantified the densitometry value of the target protein (Buckley et al. 2006). Many factors may affect the western blot results, and here we will discuss about how to trouble-shoot some of these difficulties to increase the quality the results.

Keywords: *immunoblotting, trouble-shooting*

Correspondence: Viskasari Pintoko Kalanjati, Department of Anatomy and Histology, Faculty of Medicine Airlangga University, Jl Prof dr Moestopo 47, Surabaya 60131, Indonesia. Email : viskasaripk@yahoo.com

INTRODUCTION

The western blot method was developed by the laboratory of George Stark at Stanford and the name was came from W. Neal Burnette, where it is a play on the name Southern blot (a technique for DNA detection developed earlier by Edwin Southern), northern blot (an RNA detection technique) and eastern blot (a technique for detecting post-translational modification of protein) (Harlow & Lane 1988).

The western blot is a technique used to detect specific proteins in the homogenated or extracted tissue sample (Laemmli 1970). In this technique, gel electrophoresis is used to separate the native or denatured proteins according to their polypeptide length (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). After the electrophoresis step, the proteins are then transferred to a membrane, usually nitrocellulose or PVDF (polyvinylidine fluoride) membranes where they are ready to be probed by specific antibodies against the target protein (Harlow & Lane 1988).

Nowadays, companies have been developing new apparatus to shortcut the long-winding steps in the traditionally done western blot. This equipment can cut down the duration of the membrane blocking, and also the probing of the membrane against the primary and secondary antibodies. However, optimalization must be done to get the best result for each antibody. The blocking solution is usually specified by the company, where the percentage is also much lower than the usual percentage when using the skimmed-milk solution. Some of these machines used the compression and vacuumed chamber-principles to force the antibody goes through the membrane, thus more efficiently ensure the contact between the membranes and the antibody, results in shorter blocking duration. Filter papers are used to sandwich the membrane, however, sometimes the blocking solution and the solutions contain the antibody can be re-used with the small well

located under the membrane. You may find further details on these apparatus at the company websites.

Other updated techniques come with the idea of improving the way to read the probed proteins at the membranes by escaping the use of X-ray film as the media to visualize the bands, instead of directly read the bands from the membrane or gel using the spectrophotometry or densitometry machines after exposing the membrane. Further paragraphs in this article will be focused on the trouble-shooting of the traditionally done western blot technique, where the blocking step and the step of probing the membrane with the primary and secondary antibodies are done by submerging the membrane in the solutions put in the container on the orbital shaker prior to the visualization process.

DISCUSSION

Common problems that can be found in the western blot technique are:

No signal

This may be caused by the incompatibility of the primary antibody and the secondary antibody. You may use secondary antibody that was raised against the species in which the primary was raised (e.g. primary is raised in rabbit, use anti-rabbit secondary) (Harlow & Lane 1988). Other possible cause is there was not enough primary or secondary antibody bound to the target protein. Thus the use of more concentrated antibody and/or longer incubation period (e.g. overnight) at 4ºC may overcome this problem. Sometimes cross-reaction between blocking agent and primary or secondary antibody occurs, where the use of a mild detergent such as Tween20 or switch blocking reagent (i.e. commonly used blocking reagents are milk, BSA, serum or gelatin). This problem may arise when the primary antibody does not recognize the protein in the species being tested; polypeptide chain comparison between species should be checked in the datasheet or by performing a ClustalW alignment to ensure that your antibody should react with the target protein. You may also run the recommended positive control as parts of the optimalization step. Next thing that may cause this problem is the lack of antigen level. At least 20-30 ug protein per lane should be loaded, use of protease inhibitors may improve the results with running the recommended positive control. When the target protein is not abundantly present in the tissue, you may add several steps to maximize the signal (e.g. prepare nuclear lysates for a nuclear protein, etc.). Poor transfer of protein to membrane may result from the poor contact between the gel and the membrane due to the equipment failure, failure of the electric charges or simply because of the inappropriate contact between the lid and the transfer chamber that may cause the insufficient voltage transferred. You should then check the transfer with a reversible stain such as Ponceau S; check that the transfer was not performed the wrong way; if using PVDF membrane make sure you pre-soak the membrane in MeOH then in transfer buffer due to the hydrophobic membrane character. An excessive washing of the membrane should be avoided, whilst too much and/or too long in blocking step may inhibit you from visualizing the target protein. Some antibodies do not work in the milk-blocked membranes, but would work when changed into BSA (bovine serum albumine) with shorter period of blocking. Over-use of the primary antibody or use of re-used blocking and/or antibody solutions may decrease the effectiveness of these solutions, sodium azide may inhibit some type of the secondary antibodies and the horseradish peroxidase-conjugated antibodies. Carefully check the ECL (enhanced chemiluminescence) solution that can be damaged due to light over-exposing and/or have expired. Use of fresh substrate is the easiest way to solve this problem (Harlow & Lane 1988, Sambrook et al. 1989).

High background

This problem may arise from several causes including the blocking of non-specific binding might be absent or insufficient. Increased blocking incubation period and consider to change blocking agent percentage may solve this (e.g. 5% skimmed-milk, 3% BSA, or normal serum for 30 minutes). These solutions can be used in the antibody buffers as well. Other cause is primary antibody concentration that may be too high; in this matter the antibody titration to find the optimal concentration should be done and/or incubate with more dilute and longer period. Temperature during the incubation may cause this problem, usually it should be done at 4°C. Some secondary antibodies may be binding non-specifically or reacting with the blocking reagent, here you should run a secondary control without primary antibody to ascertain the specificity of your primary antibody. A cross-reaction between blocking agent and primary or secondary that may also cause this problem can be avoided by adding a mild detergent such as Tween20 to the incubation and washing buffer. A phospho-specific protein or milk contains casein which is a phosphoprotein; can cause high background because the phospho-specific antibody detects the casein present in the milk. Here, the use of BSA as a blocking reagent instead of milk. High background also can be caused by the unbound antibodies that should be washed properly by increasing the number of washes. Nitrocellulose

membrane is considered to give less background than PVDF. The membrane has dried out in between the blocking and the antibody probing steps. Carefully prevent this and make sure that the membrane is always in wet condition (Sambrook et al. 1989).

Multiple bands

If you find multiple bands visualized from your blot, may be your sample comes from the cell lines that have been frequently passaged gradually accumulate differences in their protein expression profiles. Fresh, non-passaged cell line should be run as a control sample. Post-transalational processes may modify the tissue including acetylation, methylation, myristylation, phosphorylation and glycosylation. It is worth it to check the literatures and use an agent to dephosphorylate, de-glycosylate, etc. the protein to bring it to the correct size. The target in your protein sample may have been digested (more likely if the bands are of lower molecular weight), where the use of sufficient protease inhibitors in your sample buffer can solve the problem. Unreported novel proteins or different splice variants that share similar epitopes and could possibly be from the same protein family are being detected in this matter, checking the literature for other reports and also perform a BLAST search with the use of the cell line or tissue reported on the datasheet may help. Too high concentration of the primary antibody can also cause this problem; decreasing the antibody concentration and/or the incubation period should answer this problem. Too high concentration of the secondary antibody that may be bound to the nonspecific proteins may cause this problem. Hence, decreased secondary antibody level with the secondary antibody control only (without the primary) may help. The antibody that has not been purified may give multiple bands too, problem dismissed by the use of the purified antibody. The multiple bands seen may be non-specific, some companies include the blocking peptides to differentiate between specific and non-specific bands to differentiate the specific from the non-specific bands. The multimeric protein target may be formed, and boiling in SDS-PAGE for 10 minutes rather than 5 minutes to disrupt multimers may help. The uneven white spots on the blot may be the air bubbles trapped against the membrane during transfer or the antibody is not evenly spread on the membrane (Figure 1). These can be avoided by removing bubbles when preparing the gel for transfer with the plastic plate and using the orbital shaker to gently agitate the membrane during incubation (Harlow & Lane 1988, Sambrook et al. 1989).

The black dots on the blot may cause by the antibodies' binding to the blocking agent, thus try to filter the blocking agent. On the other hand, the white bands on a black blot (negative of expected blot) may be due to too much primary and/or too much secondary antibody. Diluting the antibodies further may solve this problem. Sometimes the molecular weight marker lane is black when the antibody reacts to it. Adding a blank lane between the molecular marker and the first sample lane can be done (Figure 1) (Harlow & Lane 1988, Sambrook et al. 1989).

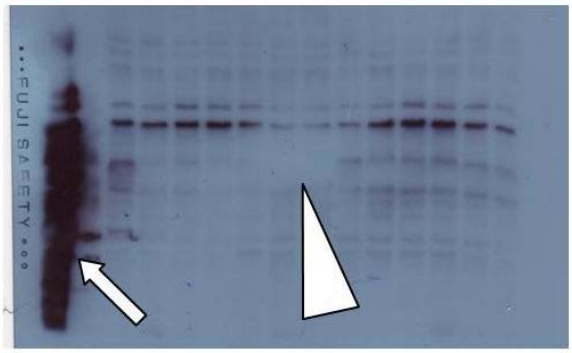

Figure 1. Representative western blot film with molecular weight marker reacted to the secondary antibody results in black dots (white arrow) that may compromise the visual and densitometry value of the bands right at the next lane. White dot (white arrow-head) results from air bubble.

When the band of interest is very low/ high on the blot, it can be due to the inefficient way of separation. Changed gel percentage: a higher percentage for small protein, lower percentage for large proteins may help separating the protein bands better. Smile effect of the bands that looks like the convex bands may be resulted from too fast migration process, or the temperature during the migration was too hot (changing the pH and altering the migration). Thus, slowering the migration and/or run the gel in the cold room or on ice should be done.

Uneven band size in lanes probed for the same protein can be because of g too quickly set-gel while casting and the acrylamide percentage is not even along the lanes. You might want to review the recipe of the gel and the addition of TEMED to the gels and/or add a little 0.1% SDS in water to the top of the migrating gel while it sets may stop it from drying thus more evenly set.

The uneven staining of the gel may be due to bacterial contamination and/or not enough antibody. Here you may want to try keeping antibodies at 4°C and use fresh buffer covers the gel. Making sure that the membrane is

always covered with the antibody/ incubate under agitation can help get rid of this problem (Harlow & Lane 1988, Sambrook et al. 1989).

CONCLUSION

The western blot analysis depends heavily on the quality of the target protein band resulted. Because the quantification is done by quantifying either the densitometry or spectrophotometry of the bands; clearness and specificity of these bands should be achieved. Certain techniques can trouble-shoot these problems and may help improving the result quality.

REFERENCES

1. Buckley ST, Foley PF, Innes DJ, et al.: GABAA receptor b isoform protein expression in human alcoholic brain: Interaction with genotype. Neurochem International 2006;49:557-567.
2. Harlow E, Lane D: Immunoblotting--Immunohistochemistry, ed 1st. Cold Spring Harbour, Cold Spring Harbour Laboratory, 1988.
3. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.
4. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning. A laboratory manual, ed 2nd. New York, Cold Spring Harbor New York, 1989.