Application Note

The Influence of Chemiluminescent Substrate on Protein Detection Using PVDF Membranes

Introduction

Western blotting is an analytical method for the immunodetection of proteins, particularly low-abundance proteins in a given sample. This process involves the transfer of protein patterns from sodium dodecyl sulfate-polyacrylamide electrophoresis gel (SDS-PAGE) to a microporous membrane under the influence of an electric current. Transfer of proteins from membrane by means of western blotting or electrophoretic elution can be performed by wet, semi-dry, or dry blotting method. The choice of blotting method depends on the type of required information from the blot as well as protein, time, and cost. The wet transfer that is the focus of this application note is the most common method of blotting and will be described in more details in following paragraph. The most common microporous membranes used for blotting are cellulose, nitrocellulose (NC), polyvinylidene difluoride (PVDF), cellulose acetate (CA), polyethersulfone (PES), and nylon (NY). PVDF membranes are mostly used for western blotting due to their mechanical properties, surface characteristics, and high binding capacity.

The process of wet western blotting can be divided into three steps: SDS-PAGE electrophoresis, electrotransfer (where sandwich consists of electrophoretic gel and membrane submerged in transfer buffer), and chemiluminescent detection. Each of these steps can influence the final results. The efficient transfer of proteins from a gel to a membrane depends significantly on the nature of the gel, the molecular weight of the proteins being transferred, and the membrane. For example, too light or missing bands could be due to too large pore size of the membrane or too long transfer time. Moreover, the sensitivity of the chemiluminescent substrate is also an important factor in performance of western blot. In the detection step, the local concentration of antibodies and substrate influences the rate of the chemiluminescent reaction. Thus, the reaction rate changes over time and across the surface of the blot. Low-intensity bands consume the available substrate slower than a strong band with a high local concentration of horseradish peroxidase (HRP) reporter which may rapidly deplete the available substrate.

Following the GVS general protocol for Western blot, this application note compares the performance of GVS transfer membranes with competitors for 11 kDa, 42 kDa, and 87 kDa molecular weight proteins. Different molecular weight proteins are applied to confront the membrane's pore size with the protein size. In the detection step, the blots were incubated in distinct chemiluminescent substrates to illustrate their sensitivity to low concentrations of the targeted protein. In case of 42 kDa proteins three different chemiluminescent substrates have been used in order to present the influence of substrate on detection limits.

Materials and Methods

Materials:

The HeLa Whole Cell Lysate was purchased from Boster Bio®. Three primary antibodies: Beta Actin Polyclonal Antibody, Anti-Cytochrome C and Anti-Furin were purchased from Bioss® and Abcam respectively. Secondary antibody, Goat Anti-Rabbit IgG Antibody (H+L), was purchased from Bioss®. Chemiluminescent substrates *LightWave™ (PN: LW001, LW002), LightWave™ Plus (PN: LW003, LW004), and LightWave™ Max (PN: LW005, LW006)* were provided by GVS. Transfer membranes are GVS®-PVDF membrane (0.45 µm 300×300 mm, PN: 1212639 and 0.22 µm 300×300 mm, PN: 1214429) and equivalent competitor's membrane. Skim milk as the blocking buffer was commercial retail trade.

Methods:

Two-fold serial dilution of Hela whole cell lysate (Boster Bio®) from 8 µg to 0.03 µg of total protein was prepared for the electrophoresis step. Lysate dilution was loaded into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and run at 120 V for 20-30 minutes (or until the sample reached the stacking gel) and then, 180 V for 30-45 minutes (separation of the proteins under constant voltage). After electrophoresis, transfer of proteins was performed at 120 V for one hour, followed by blockading of the membrane for one hour at room temperature (RT) in the blocking buffer (5% skim milk in PBST buffer); and incubation with primary antibody (dilution in accordance to manufacturer instruction) for one hour at RT. After each antibody incubation blots were washed with PBST three times. Incubation with secondary antibody (1:10000 dilution in blocking buffer) was performed for 45 minutes at RT. In the end, blots were incubated for 2-3 minutes in the chemiluminescent substrate and subjected to the detection system with an exposure time of 180 seconds.

Results

The important factors to achieve reliable results from a Western Blot are pore size, protein binding capacity, morphology, and surface properties of the membrane. The molecular weight of the testing protein, type, quality and affinity of the antibodies, buffers, blocking material and the performance of the electrophoresis SDS-PAGE gel is influencing the desired results as well. In addition, the detection step, or in other words chemiluminescent substrate can crucially affect the result at very end of analysis.

In this application note, the impact of membrane pore size and the sensitivity of chemiluminescent substrate on the blots is studied. The 0.22 µm and 0.45 µm GVS PVDF transfer membranes were tested and compared with competitor. Moreover, the influence of the protein size on the transfer and final results are evaluated as well. The results are presented in the following sections according to the pore size of the membranes.

0.22 µm PVDF membranes

Figure 1 compares the binding capability of a 0.22 µm GVS PVDF membrane with a competitor's membrane in the transfer phase and the sensitivity of different chemiluminescent substrates in the detection of very low concentrations of proteins (Figure 1-a., b., c.).

The same serial dilution of HeLa Whole Cell Lysate (8 µg -0.03 µg) was introduced to the gels in the electrophoresis step for both membranes. Then, after the transfer step, the blots were incubated in the chemiluminescent substrate LightWave™. According to Figure 1-a, four lanes of Beta Actin (42 kDa) were detectable on both membranes (GVS and competitor) but the resolution of the lanes on the GVS membrane is stronger. This shows that GVS 0.22 µm PVDF membrane captures the proteins more efficiently in the transfer step. Using LightWave™ it was possible to detect the Beta Actin with concentrations up to 1 µg.

Applying the same procedure but performing the detection step with a stronger and more sensitive substrate, LightWave™ Plus, the number of visible bands increased and Beta Actin is detectable up to 0.12 µg for the GVS membrane and 0.25 µg for the competitor's membrane (Figure 1-b).

Finally, the Incubation of the blots in LightWave™ Max (Figure 1-c) enhances the number of visible bands compared to the LightWave™ Plus. Applying this substrate, even 0.06 µg of Beta Actin in the Lysate is detectable on 0.22 µm GVS transfer membrane. At the same condition, up to 0.12 µg of protein is visible on the competitor's membrane.

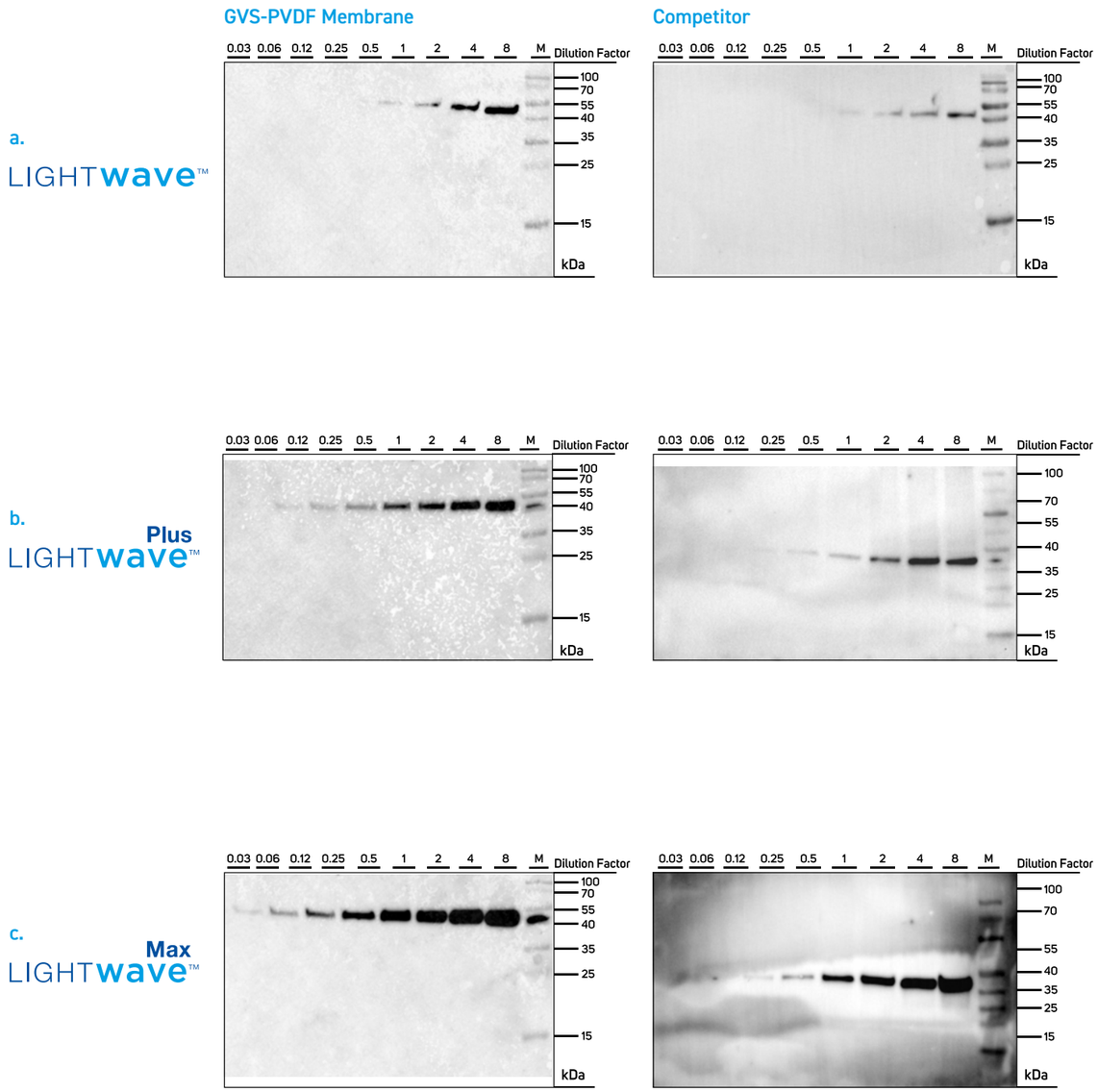
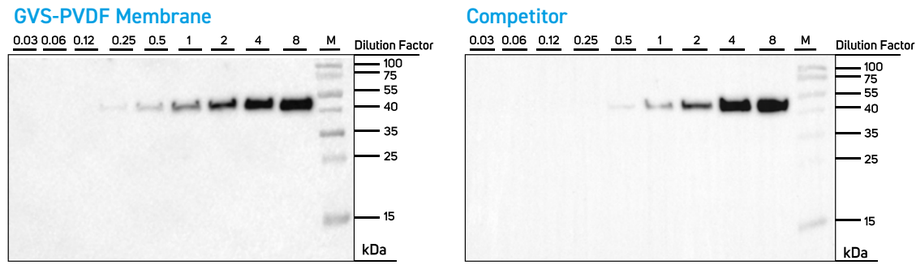


Figure 1: Comparison of 0.22 µm GVS-PVDF transfer membrane with a competitor membrane using GVS chemiluminescent substrate: a. LightWave™, b. LightWave™ Plus, c. LightWave™ Max.

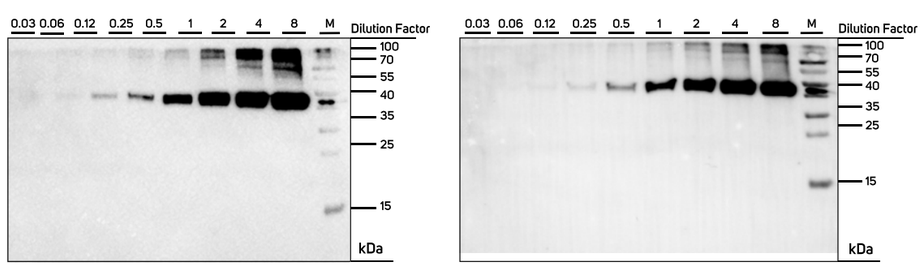
0.45 µm PVDF membranes

Similarly, testing of 0.45 µm PVDF membranes with different chemiluminescent substrates showed that by incubating the blots in LightWave™ it is possible to see Beta Actin (42 kDa) up to 0.25 µg of Lysate on 0.45 µm GVS membrane compared to 0.5 µg for the competitor (Figure 2-a).
Figure 2-b illustrates that using LightWave™ Plus where the intensity of the lanes for the 0.45 µm PVDF transfer membrane is up to 0.25 µg for both membranes. According to Figure 2-c, the number of detected lanes proved that LightWave™ Max can be applied for blotting very low concentrations. It is clear that with LightWave™ Max detection range increases to 0.03 µg of Lysate.

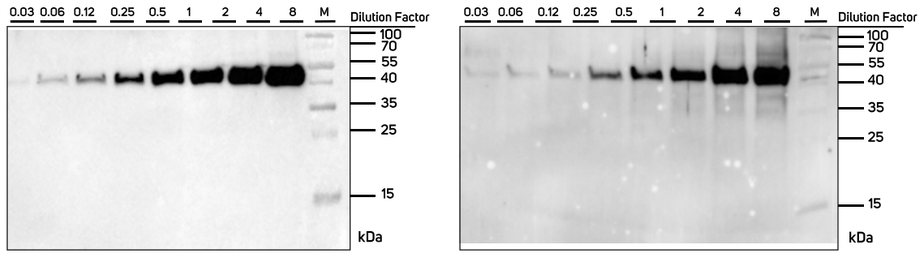
a.
LIGHTwave™



b.
LIGHTwave™ Plus



c.
LIGHTwave™ Max

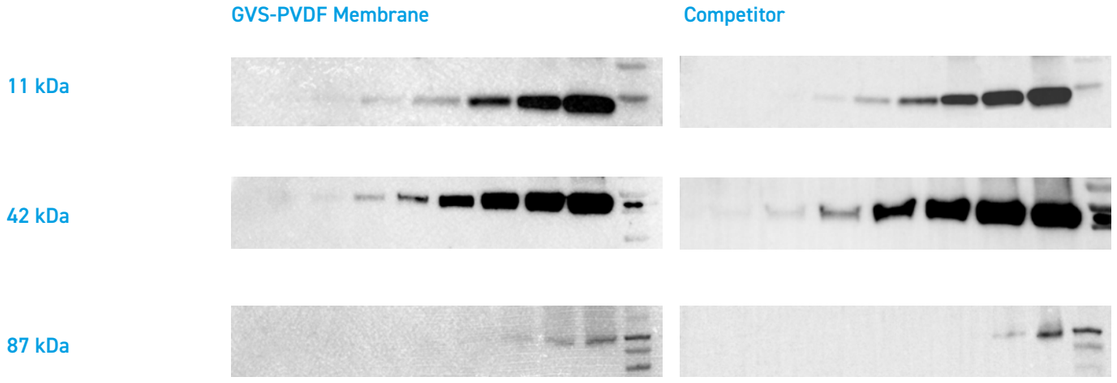


The effect of different molecular weights of the protein

In this section, the efficiency of the 0.45 µm and 0.22 µm membranes for transferring different molecular weight proteins (11 kDa, 42 kDa, and 87 kDa) detected with LightWave™ Plus is presented.

0.45 µm PVDF membranes

As can be seen in Figure 3, the performance of the membranes is the best for the 42 kDa proteins. For proteins with bigger size (87 kDa), the number of transferred lanes reduces to two for the competitor and three for the GVS membrane. For 11 kDa proteins, the transfer lanes are similar to those of 42 kDa, with slightly lower detection. According to these results, the protein size affects the transfer's efficiency. Thus, there should be a compromise between the protein and membrane pore sizes to have the best response from western blotting. It is recommended to use higher pore size membranes for bigger proteins or adjust protocol accordingly to obtain maximum performance.



0.22 µm PVDF membranes

The results showed that the number of 87 kDa lanes on the 0.22 µm membranes is less than those on the 0.45 um under the same condition. It is concluded that due to the smaller pores of 0.22 membranes, the transfer is more complicated or the electrotransfer step should be longer. Thus, in dealing with various protein sizes the western blot steps must be optimized and this variation must be considered also for membrane pore size selection.

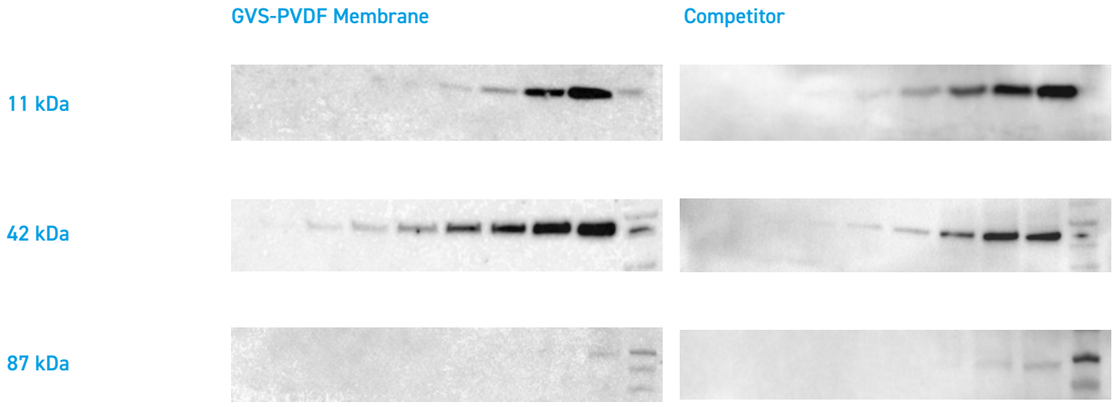


Figure 4: Comparison of the performance of 0.22 µm PVDF membranes for transferring proteins with different molecular weights (11 kDa, 42 kDa, and 87 kDa) using LightWave Plus.

Conclusion

The performance of 0.22 µm and 0.45 µm GVS PVDF membranes were compared to a competitor. The results showed that in all experiments the performance of GVS membranes was better or equal to the competitor membranes. Incubating the membranes in GVS chemiluminescent substrate LightWave™ Max helps detect very few proteins in each blot. Thus, according to the required accuracy different grades of the chemiluminescent substrate could be used. For higher concentrations of proteins, LightWave™ would be a reasonable option. Both pore sizes are able to transfer the proteins with different sizes. Under the same condition, membranes with larger pore sizes are required to transfer higher molecular weight proteins (87 kDa). It is possible to optimize the performance of the membrane for small protein sizes as well as large by adjusting the protocol in terms of transfer conditions.

References

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GVS S.p.A.
Via Roma 50 - 40069 Zola Predosa (BO) - Italy
tel. +39 051 6176311 | email: lifesciences.it@gvs.com