Reports

Optimization of western blotting for the detection of proteins of different molecular weight

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ABSTRACT

Protein samples electroblotted onto nitrocellulose membranes and quenched with a mixture of blocking agents produced a strong signal for cystic fibrosis transmembrane-conductance regulator (CFTR), a high-molecular-weight protein, in western blotting. Optimized conditions for CFTR were then extended to medium- and low-molecular-weight proteins (LAMP1 and Rab11a, respectively) to determine the effects of methanol concentration (0–20%) in Towbin's transfer buffer (TTB). Methanol in TTB appears to have little to no effect on CFTR signal. However, for medium-sized (LAMP1) and small (Rab11a) proteins, a lower concentration of methanol (10%) was sufficient to produce a maximal signal. Therefore, methanol, a toxic solvent, can be removed from or reduced in TTB without compromising signal strength. Here, we show modifications that may be useful in detecting and/or improving the signal of low-abundance proteins.

METHOD SUMMARY

Cell lysates following SDS-PAGE were electroblotted onto nitrocellulose or polyvinylidene difluoride membranes and blocked with a variety of blocking agents (bovine serum albumin, gelatin, nonfat dry milk, fetal bovine serum or with a mixture of these components). Optimized variables (nitrocellulose membrane and a mixture of blocking agents) were then used to determine the methanol concentrations needed in Towbin's transfer buffer to obtain and/or retain maximal signals for markers representing high-, medium- and low-molecular-weight proteins.

Western blotting is a commonly used procedure for the detection and characterization of proteins. In this procedure, crude lysates are first separated based on their molecular weight by SDS-PAGE [1], transferred to a solid membrane surface and detected with the help of protein-specific antibodies. The term 'western', for protein transfer from gel to membrane, has been used [2] to maintain a geographical naming tradition after 'Southern' blotting, first described by EM Southern, for the transfer of DNA to membrane [3] and later 'northern' blotting for the transfer of RNA to membranes [4]. The techniques used in western blotting have evolved greatly [5–9] since its inception in 1979 by Towbin et al. [10]. However, obtaining maximal sensitivity for the detection of a specific protein remains a fundamental issue, leading for new ways of enhancing detection sensitivity by making changes in the protocol for specific proteins [11–15].

To establish the optimal detection conditions for specific protein in western blotting, immunotitration is generally performed. Primary and enzyme-conjugated secondary antibodies are titrated at various dilutions against a specific amount of protein sample that is then electrophoresed. The optimal combination of primary and secondary antibodies is then used to obtain the maximal signal for the protein of interest. In addition to the ratio between protein applied to the gel and the concentration of primary and secondary antibodies used, other variables also influence protein detection sensitivity in western blotting. Here, our focus is on three of the variables, including type of membrane, type of blocking agents and concentration of methanol in Towbin's transfer buffer (TTB), that we believe play a critical role in enhancing the detection sensitivity of proteins. This study was conducted with a set of three randomly chosen membrane proteins of high, medium and low molecular weight.

Plastic- and cellulose-based solid surfaces, in the form of thin, microporous sheets of about 100-μm thickness, are used for the transfer of biomolecules, such as nucleic acids [3,4] and proteins [2,10]. Nitrocellulose (NC) and polyvinylidene difluoride (PVDF) membranes with a pore size of 0.2 μm are the most common types of membranes used for the transfer of proteins [16]. Unlike PVDF membranes, NC membranes are fragile and, as a result, cannot be reused for multiple probing with different antibodies. However, more recently, NC membranes have become available with a supported base (e.g., with Hybond-C), giving them PVDF-like mechanical strength. As a result, researchers have a choice of using either type of membrane without any restriction. NC membranes are reported to be slightly less sensitive than PVDF membranes but enable a higher signal-to-noise ratio. However, no data are available on the sensitivity of supported NC membranes. NC membranes are the most useful of all types of membranes with respect to the transfer of proteins, glycoproteins

and nucleic acids, because of their high binding capacity and low background noise [3,4,17]. Additionally, NC membranes are recommended for the detection of low-molecular-weight proteins, whereas PVDF membranes are recommended for high-molecular-weight proteins [16].

Several protein- and nonprotein-based agents are used for blocking spaces on membranes unoccupied by proteins to prevent sticking of the antibodies used in subsequent steps. Such agents are called 'blocking' agents and are important in reducing background noise and improving the signal of proteins of interest. Most of the commonly used blocking agents are protein based, such as nonfat dry milk [18], soy milk [19], fetal bovine serum [20], gelatin [21], and individual proteins, such as casein [9], bovine serum albumin [10], hemoglobin [22] or any purified protein. Nonprotein blocking agents, such as polyvinylpyrrolidone [23] and Tween-20 [24], are also used, primarily for the detection of phosphorylated proteins to avoid interference of residual phosphoproteins present in protein-based blocking agents [25].

Methanol is added to TTB with the purpose of removing SDS from proteins electrophoresed onto an SDS-polyacrylamide gel and assists proteins in binding effectively to NC membranes [3,10]. It was originally used at 20% in transfer buffer by Towbin *et al.* [10] and reported to be less important for the binding of high-molecular-weight proteins [16,26]. Methanol is a solvent also known for its severe toxicity, which may lead to loss of vision as early as 12 h after exposure [27] and can cause blindness by damaging optic nerves with consumption of a volume as small as 10 ml [28]. Although methanol poisoning is uncommon, once consumed it may break down into harmful chemicals, such as formaldehyde or formic acid, and cause gastrointestinal and urinary disorders that may be lethal [29].

Materials & methods

Materials
Precast Mini-PROTEAN® TGX™ gels, Immun-Blot® PVDF and supported nitrocellulose (NC) membranes (both of pore size 0.2 μm), Tris/glycine buffer, gelatin type-A from pig skin and all other routine chemicals required for SDS-PAGE and western blotting were purchased from Bio-Rad Laboratories (CA, USA). Halt™ protease inhibitor cocktail was purchased from Thermo Fisher Scientific (MA, USA). Bovine serum albumin (BSA) free of immunoglobulins and goat-anti-rabbit-HRP secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (PA, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (GA, USA). Nonfat dry milk powder (blotto) was purchased from a local grocery store. Anti-LAMP1 antibody was purchased from Cell Signaling Technology (MA, USA). Anti-Rab11a antibody was purchased from Santa Cruz Biotechnology (TX, USA), whereas the anti-cystic fibrosis transmembrane-conductance regulator (CFTR) antibody R3194 was a gift from CR Marino (VA Medical Center, TN, USA). General laboratory chemicals and reagents for tissue culture were purchased either from Sigma-Aldrich Chemical Co. (MO, USA) or Invitrogen (CA, USA).

Cell lysates
CFBE41$_0$, a human lung epithelial cell line transfected with the wild-type CFTR gene (CFBE-wt), was generously provided by B Stanton (The Geisel School of Medicine at Dartmouth, Hanover, NH, USA). This transfected cell line was developed by JP Clancy (University of Cincinnati, OH, USA) [30]. Cells were grown in a humidified 37°C incubator in the presence of 5% CO_2 in Dulbecco's modified Eagle medium with L-glutamine (2 mM), sodium bicarbonate (35 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), plasmocin (5 μg/ml), puromycin (0.5 μg/ml) and 10% FBS. CFTR expression in these cell lines was stimulated as previously described [31]. The cells were then harvested and lysed by sonication in the presence of 1% SDS and Halt™ protease and phosphatase-inhibitor cocktail.

Protein assay
Total protein content in cell lysates was measured by an improved amidoschwarz protein assay [32].

SDS-PAGE
Known quantities of protein samples were electrophoresed using precast Mini-PROTEAN® TGX™ gels on a Mini-PRETEAN® Tetra cell (Bio-Rad) according to standard procedures [1] and the manufacturer's instructions. Samples were electrophoresed on 7.5% gels for the detection of CFTR, 10% gels for the detection of LAMP1 and 12% gels for the detection of Rab11a proteins.

Western blotting
After electrophoresis, gels were electroblotted either onto a PVDF or a supported NC membrane with TTB (25 mM Tris, 192 mM glycine, 0.01% SDS, pH 8.3) containing various concentrations of methanol (0–20%). Protein transfer was carried out using a Bio-Rad Mini *Trans-*Blot® electrophoretic transfer cell. After transfer, the membranes were incubated separately with 5% BSA, 5% FBS, 1% gelatin, 5% blotto or with a mixture of these components maintaining the same percentage, prepared in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20). All of these blocking agents are readily soluble in TBS-T buffer at room temperature, except for 1% gelatin, which requires heating to 37°C for 15 min. The western blotting procedure for the detection of CFTR protein was carried out according to previously established procedures using the anti-CFTR antibody R3194 [31,33], whereas LAMP1 and Rab11a proteins were detected according to the manufacturer's instructions.

Table 1. Effects of various blocking agents on the expression of CFTR.

Blocking agent[†]	Intensity/mm^2 (×1000)[‡]		Increase (%)[§]
	PVDF	NC	
BSA	11.5	11.9	3.5%
FBS	11.4	13.8	21%
Gelatin	12.1	14	16%
Blotto	13	15.7	21%
Mixture	12.9	16.8	30%

[†]See 'Materials & methods' section for concentrations and composition.
[‡]Data representing CFTR band C (see Figure 1).
[§]Percentage increase in CFTR band C signal from PVDF to nitrocellulose membrane.
BSA: Bovine serum albumin; FBS: Fetal bovine serum; NC: Nitrocellulose; PVDF: Polyvinylidene difluoride.

Data analysis

Band intensities were quantified by Quantity One® 1D analysis software (Bio-Rad) as previously described [34]. The density volume within each protein band was measured as intensity/mm^2. The average density volume for at least three independently run gels were used to calculate the standard error of the mean using GraphPad Prism version 8.0.0 (GraphPad Software, CA, USA).

Results & discussion

Several factors determine the maximal signal for a specific protein in western blotting. Some of these factors are protein specific, such as the expression levels in a given sample and the quality of the primary antibody. Other factors that apply to a wide range of proteins are the efficiency of transfer, the type of membrane, the blocking agents, and the concentration of methanol in TTB. In this study, we focused on factors that may influence many proteins. Membrane type, type of blocking agents and methanol in TTB were tested using three proteins of different molecular weights (CFTR, LAMP1 and Rab11a). A mutated CFTR (MW 170 kDa) impairs chloride ion channel function in epithelial cells of many organs and causes the genetic disease cystic fibrosis [35]. LAMP1 (MW 110 kDa) is a heavily glycosylated lysosomal membrane protein that is involved in lysosomal motility [36]. Rab11a (MW 25 kDa) belongs to a family of small GTP-binding proteins with roles in endocytosis and transport of newly synthesized proteins [37]. In our laboratory, we routinely employ proteins, such as LAMP1 and Rab11a, as markers of cellular trafficking in our ongoing studies on the degradation of CFTR protein [38]. All selected proteins in this study are membrane proteins that are difficult to solubilize and resolve owing to their complexity compared with cytosolic proteins [39].

Effects of membranes & blocking agents on western blotting

Because NC and PVDF are the two most commonly used membranes in western blotting, these membranes were used for determining the blocking agent that can provide optimum blocking of their free surfaces and increase the signal-to-noise ratio. Some of the most commonly used blocking agents, such as 'blotto' (nonfat dry milk), BSA, gelatin, FBS and their mixture were used with CFTR protein as a marker. CFTR can be visualized in the form of two electrophoretic bands: C (MW 170 kDa), a mature fully glycosylated form of the protein, and B (MW 130 kDa), an immature core glycosylated form [31]. Our blocking agents included single proteins (BSA and gelatin) and naturally occurring mixtures of proteins (blotto and FBS) from different mammalian sources. To expand the range of surfaces that could be efficiently blocked, a mixture of these agents was used as an alternative blocking agent.

A pair of precast gels were electrophoresed with multiple samples of the same lysate and transferred simultaneously to minimize experimental variations. One gel was transferred to a supported NC membrane and the other to a PVDF membrane. The membranes were sliced and incubated with the various blocking agents as described in Materials & methods. CFTR produced a relatively stronger signal for both of its forms (bands C and B), on supported NC membranes than on PVDF membranes for each of the blocking agents. Mono-protein blocking agents, such as BSA or gelatin, appeared to be less effective than complex protein agents, such as blotto or FBS, as determined by the intensity of mature CFTR band C (Table 1). Furthermore, a mixture consisting of BSA, FBS, gelatin and blotto produced signals that were stronger than those produced by any one of the individual blocking agents (Figure 1 & Table 1). PVDF membranes are generally recommended for resolving high-molecular-weight proteins. However, in our current and previous studies [34], supported NC membranes produced stronger signals for CFTR, a relatively high-molecular-weight protein.

Effects of methanol concentration in TTB on western blotting

Since supported NC membranes produced a stronger signal with lower background when using the mixture of blocking agents, we chose these optimal conditions to conduct our next set of experiments. The goal of these experiments was to determine the effects of methanol on the transfer of proteins of various molecular weights and on their signal. Because blotto is a widely used blocking agent in many laboratories worldwide, we compared it with the mixture of blocking agents.

Methanol plays an important role in stripping SDS molecules from charged proteins during PAGE and in binding of proteins to membranes during electrotransfer [2,22,40,41]. However, for high-molecular-weight proteins, methanol has been reported to be unneces-
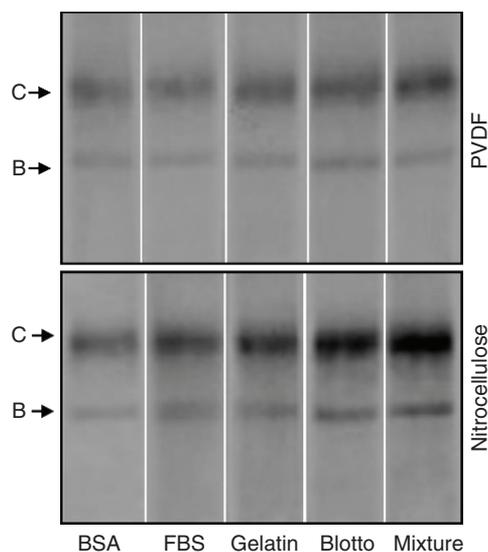
Figure 1. Effects of membranes and various blocking agents on the detection of CFTR signal by western blotting. Samples (25 μg per lane) of total cell lysates from CFBE (wt) cells were electrophoresed by 7.5% SDS-PAGE. Gels were transferred to either a PVDF or nitrocellulose (NC) membrane using Towbin's transfer buffer containing 20% methanol. After transfer, membranes containing individual lanes were carefully excised and blocked with various blocking agents (see 'Materials & methods' section) and immunoblotted with anti-CFTR antibody. CFTR-specific signal was detected using a c400 image analyzer (Azure Biosystems, CA, USA). The NC membrane produced stronger CFTR signals than the PVDF membrane, regardless of blocking agent used. A mixture of blocking agents (BSA, FBS, gelatin, and nonfat milk powder, also known as blotto) produced a synergistic effect. Arrows on the left indicate the fully glycosylated, mature CFTR (band C, MW ~170 kDa) and core glycosylated CFTR (band B, MW ~130 kDa).
BSA: Bovine serum albumin; FBS: Fetal bovine serum; PVDF: Polyvinylidene difluoride.
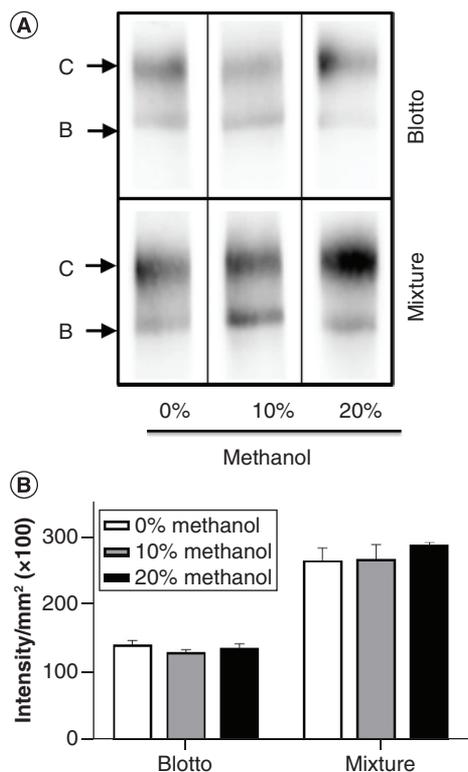
Figure 2. Comparative analysis of the effect of a traditional blocking agent (blotto) with that of a mixture of blocking agents under different concentrations of methanol in TTB on the strength of the CFTR signal. (A) Cell lysates (25 μg/lane) from wild-type CFTR-expressing CFBE cells were electrophoresed as described in Figure 1. Proteins were transferred to a supported NC membrane in the presence of 0, 10 or 20% methanol in TTB and blocked with either blotto (upper panel) or a mixture of blocking agents (lower panel). The mixture of blocking agents produced a stronger signal compared to blotto with no effect of methanol in TTB. **(B)** Fully glycosylated CFTR band C from western blot images from three independently conducted experiments **(A)** were scanned and plotted as described in the 'Materials & methods' section.

sary [16]. In this study, we also observed no effects of methanol on the signal of CFTR, a relatively high-molecular-weight protein (Figure 2). However, for medium (LAMP1, MW 110 kDa) and small (Rab11a, MW 25 kDa) proteins, 10–20% methanol was required to achieve maximal signal (Figures 3 & 4). For all proteins, regardless of their molecular weight, a mixture of blocking agents enabled a stronger signal than did blotto. Interestingly, for Rab11a, a mixture of blocking agents produced a signal even in the absence of methanol, and this signal was several fold stronger than that produced with blotto in the presence of 20% methanol in TTB (Figure 4). Therefore, mixture of blocking agents allows us either to reduce and/or eliminate methanol in TTB.

In our laboratory, we have replaced blotto with the mixture of blocking agents and use supported NC membranes for all type of proteins, regardless of their molecular weight. As a result, we observed enhanced protein signals and, in some instances, signals that were previously undetectable. A good example is detection of a low-molecular-weight protein, Rab11a, in the absence of methanol when a mixture of blocking agents was used. The Rab11a signal produced under these conditions was much stronger than the signal obtained with a traditional method consisting of 20% methanol in TTB and blotto as the blocking agent (Figure 4). A strong signal with or without
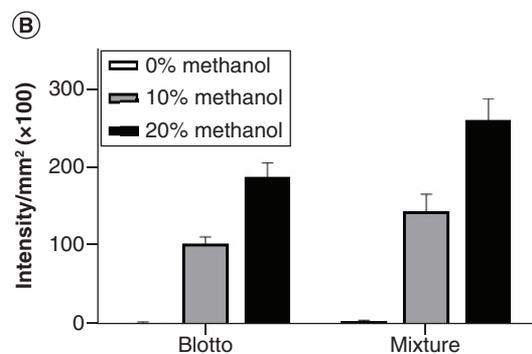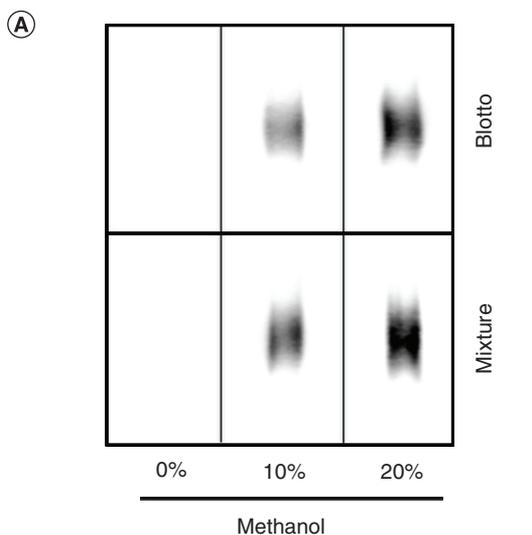
Figure 3. Comparative analysis of the effect of a traditional blocking agent (blotto) with that of a mixture of blocking agents with different concentrations of methanol in TTB on the strength of LAMP1 signal. (A) Cell lysates (20 μg/lane) from CFBE (wt) cells were electrophoresed by 10% SDS-PAGE, processed according to the conditions described in Figure 2, and immunoblotted with anti-LAMP1 antibody. LAMP1 (MW 110 kDa) signal appears to be dependent on the concentration of methanol, with maximum signal obtained when a mixture of blocking agents was used in the presence of 20% methanol in TTB. The results in this figure represent one of the three such experiments performed. **(B)** The LAMP1-specific protein band from western blot images in three independently conducted experiments **(A)** was scanned and plotted as described in the 'Materials & methods' section.
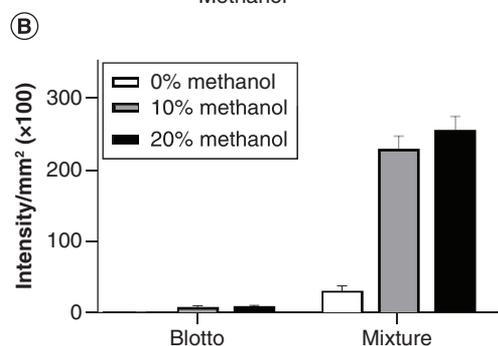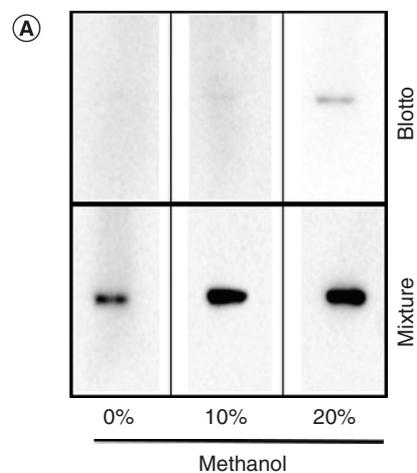
Figure 4. Comparative analysis of the effect of a traditional blocking agent (blotto) with that of a mixture of blocking agents at different concentrations of methanol in TTB on the strength of the Rab11a signal. (A) Cell lysates (50 μg/lane) from CFBE (wt) cells were electrophoresed on a 12% SDS-PAGE, processed according to the conditions described in Figure 2, and immunoblotted with anti-Rab11a antibody. A mixture of blocking agents produced a stronger signal than with blotto, with little or no effect from the presence of methanol in TTB. The presence of 20% methanol appears to be essential when blotto is used as a blocking agent. However, with a mixture of blocking agents, a much stronger Rab11a signal (MW 25 kDa) can be seen even in the absence (0%) or at a lower concentration (10%) of methanol in TTB. The results in this figure represent one of three such experiments performed. **(B)** The Rab11a-specific protein band from western blot images in three independently conducted experiments **(A)** was scanned and plotted as described in the 'Materials & methods' section.

a reduced level of methanol may be owing to the synergistic effects of blocking agents in the mixture. In view of our findings, we are now using methanol, a toxic solvent, only when needed and at a lower concentration. This new laboratory policy helps not only in reducing the cost but also in avoiding the many medical risks posed by the use of methanol [27–29].

Maximal protein-specific signals in western blotting can be obtained when nitrocellulose membrane is used and blocked with a mixture of blocking agents. The presence of methanol in TTB appears to have little-to-no effect on improving signals of high-molecular-weight proteins. However, in the case of medium and small proteins, a lower concentration of methanol (10%) was sufficient to produce a nearly maximal signal. Methanol, a toxic solvent, can therefore be removed or reduced from TTB without compromising maximal protein-specific signals.

Future perspective

Researchers currently using blotto or any other individual blocking agents can use the mixture of blocking agents reported in this study to enhance the signal of their proteins of interest. This enhancement, coupled with other specific measures to improve the signal, can be useful in the detection of low-abundance proteins. Even if the detection of low-abundance proteins is not an issue, the modifications reported here can help researchers in cutting the cost of antibodies by allowing for their further dilution as well as reducing and/or avoiding the use of methanol, a potentially toxic agent, in transfer buffer.

Author contributions

Financial & competing interests disclosure

Open access

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