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Transfer of high molecular weight proteins using the iBlot 2 Gel Transfer Device

Successful transfer of high molecular weight proteins with the speed and convenience of a dry blotting system

Reliable transfer of high molecular weight (HMW) proteins from a gel to a membrane during western blotting is a common challenge among life science researchers. The Invitrogen™ iBlot™ 2 Gel Transfer Device offers a buffer-free system with a total preparation and run time of less than 15 minutes per blot. This application note presents information to optimally transfer HMW proteins (i.e., >150 kDa) from a gel to a membrane during western blotting with our user-friendly iBlot 2 Gel Transfer Device. Compared with conventional wet tank transfer techniques, transfer of high molecular weight proteins using the iBlot 2 device can save hours of time.

Optimizing conditions for dry transfer of >150 kDa proteins

Recommendation 1: Increase transfer time

The iBlot 2 Gel Transfer Device has enabled many users to achieve rapid protein transfer for a broad range of proteins. The preprogrammed default 7-minute transfer conditions of the iBlot 2 device typically work well for a mixed range of proteins. While these conditions work for many protein samples, parameters may need to be optimized for your proteins of interest and their respective molecular weights. Proteins >150 kDa migrate more slowly in a gel matrix relative to smaller molecular weight proteins, and as a result, require more time to transfer. For these HMW proteins, transfer times should be increased to 8–10 minutes regardless of the gel type selected. As demonstrated to the right, the most efficient transfer of the ~190 kDa epidermal growth factor (EGFR) protein is achieved after 8–10 minutes of transfer (Figure 1).

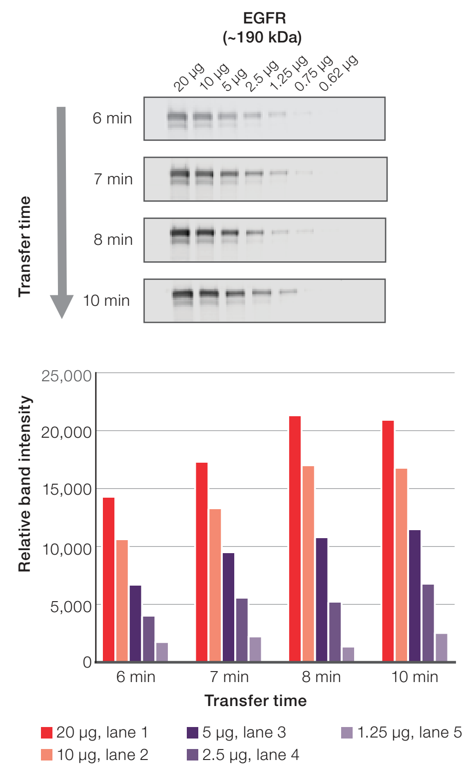

Figure 1. Dilution series of A431 cell lysate, separated on a 4–20% Tris-glycine gel, was transferred with the iBlot 2 device using transfer times of 6–10 minutes at 25 V.

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Table 1. Recommended running parameters for proteins with molecular weights >150 kDa.

Transfer stack	Method	Volts	Run time
Regular transfer stack, Nitrocellulose or polyvinylidene fluoride (PVDF) membrane (one midi gel or two mini gels)	P0, P3	20–25	8–10
Mini transfer stack, Nitrocellulose or PVDF membrane (one mini gel)	P0, P3	20–25	8–10

Using the right gel for high molecular weight protein separation

Recommendation 2: Use Tris-acetate gels

Choosing the right gel is a key factor in the successful transfer of HMW proteins. A popular general-use gel is a 4–20% Tris-glycine gel, which can effectively separate a mixed range of proteins. However, HMW proteins will be compressed into a narrow region at the top of these gels. A better option for HMW proteins is a Tris-acetate gel or a low-percentage, nongradient Tris-glycine or Bis-Tris gel. Optimal transfer can be achieved with a Tris-acetate gel. Tris-acetate gels maintain a neutral pH and separate HMW proteins with higher resolution than Bis-Tris or Tris-glycine gels (Figure 2). Comparison of HMW protein separation using different gel chemistries and gradients shows best separation and resolution of HMW proteins using 3–8% Tris-acetate gels. This increased resolution leads to increased transfer efficiencies and higher sensitivity. As seen in Figure 2B, better transfer is seen using the Tris-acetate gel over a 4–20% Tris-glycine gel—9 ng visualized on the Tris-acetate gel vs 620 ng visualized on the Tris-glycine gradient gel.

The Invitrogen™ NuPAGE™ Tris-Acetate protein gels provide excellent separation of large molecular weight proteins. NuPAGE Tris-Acetate gels are available in 2 different polyacrylamide concentrations: 7% and a 3–8% gradient. The NuPAGE Tris-Acetate gels can be run with Tris-acetate SDS running buffer to resolve proteins under denaturing conditions, or with Tris-glycine native running buffer to resolve proteins under nondenaturing (native) conditions.

Recommendation 3: Use a quick alcohol equilibration step of the gel if you can't utilize Tris-acetate gels

Adding a quick alcohol equilibration step before transfer can greatly enhance the transfer of HMW proteins when not using the ideal gel chemistry. Equilibrating the gel in alcohol removes contaminating electrophoresis buffer salts and prevents an increase in the conductivity of the transfer, which can increase the amount of heat generated. In addition, the alcohol equilibration step allows the gel to adjust to its final size before transfer. Heat generated during the electrophoresis step can cause certain gels to expand; alcohol can help shrink the gel to its final size. To improve transfer efficiency, submerge the gel in 20% ethanol (prepared in deionized water), and equilibrate for 5–10 minutes at room temperature on a shaker prior to transfer.

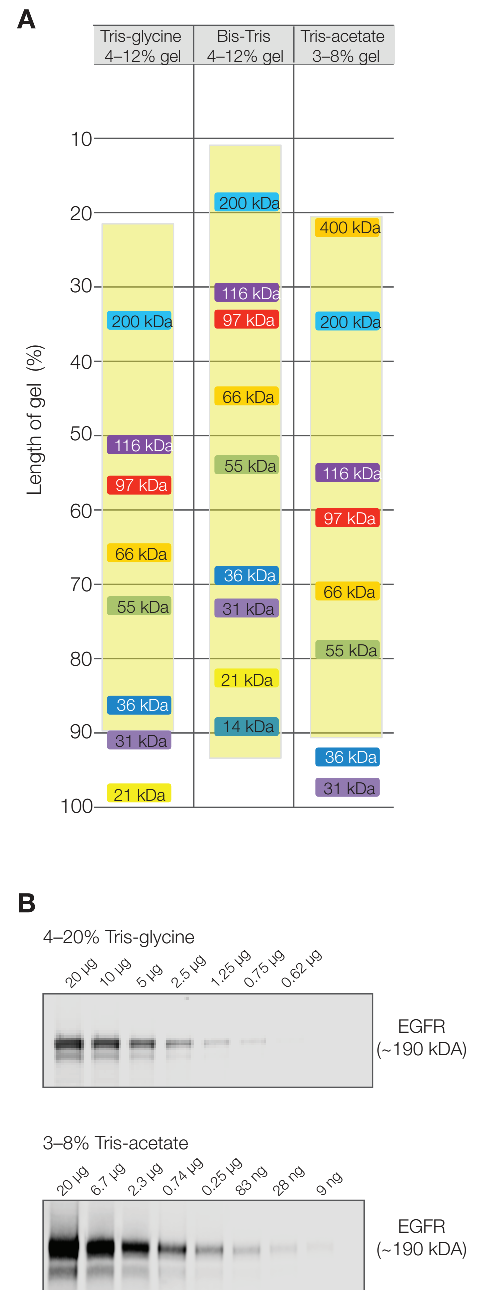

Figure 2. (A) Tris-acetate gels enable optimal separation and transfer of HMW proteins. Optimal results are obtained in the yellow shaded areas. (B) Western blotting analysis of EGFR from A431 lysates transferred from an Invitrogen™ Novex™ 4–20% Tris-Glycine gel and a NuPAGE™ 3–8% Tris-Acetate gel using the iBlot 2 Gel Transfer Device.

Figure 3 demonstrates the increased transfer efficiency of keyhole limpet hemocyanin (KLH), a ~360–400 kDa protein, when the gel was equilibrated with 20% ethanol prior to transfer. Our data suggests that an equilibration step may not be needed with the Tris-acetate gels since large proteins from these gels transfer more efficiently than from Bis-Tris gels.

To demonstrate the impact of gel type and transfer conditions when analyzing larger protein transfer, KLH was loaded onto a NuPAGE 4–12% Bis-Tris gel and a NuPAGE 3–8% Tris-Acetate gel and transferred to a nitrocellulose membrane. Optimal performance is observed using the 3–8% Tris-acetate gel transferred with the iBlot 2 device. This combination shows greater sensitivity in comparison to the 3–8% Tris-acetate gel transferred with a traditional wet system, and greater sensitivity when compared to the Bis-Tris gel with or without equilibration using the iBlot 2 device. This indicates that for HMW proteins, using the iBlot 2 device and Tris-acetate gels together may be more efficient than using Bis-Tris gels with either the iBlot 2 transfer device or wet transfer techniques, or using Tris-acetate gels with wet transfer techniques (Figure 4).

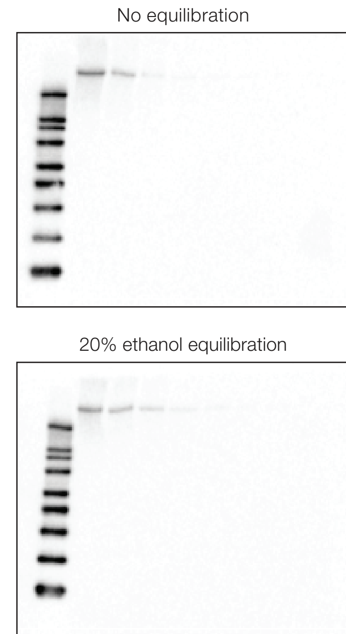

Figure 3. Increased transfer efficiency of KLH with pretreatment of a NuPAGE 4–12% Bis-Tris gel with 20% ethanol before transfer using the iBlot 2 transfer device.

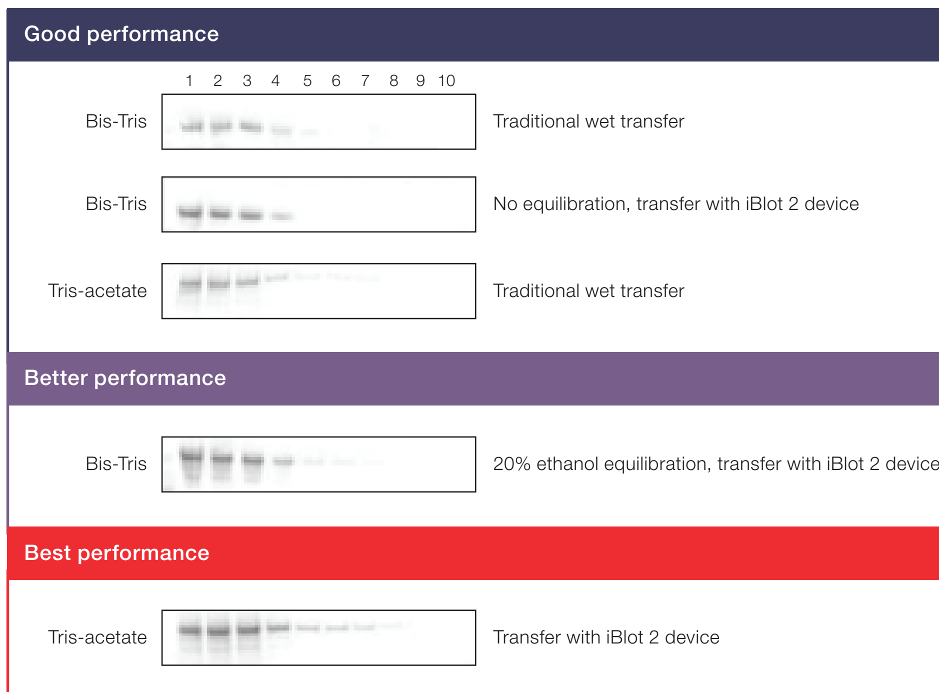

Figure 4. Protein blots after transfer times of 8 min at 20–25 volts with the iBlot 2 device, and wet transfer (60 minutes at 30 volts) for KLH large protein separated with NuPAGE 4–12% Bis-Tris and 3–8% Tris-Acetate Protein Gels. Lanes 1–10: 10 ng, 7.5 ng, 5 ng, 2.5 ng, 1 ng, 750 pg, 500 pg, 250 pg, 100 pg, and 50 pg purified KLH protein.

Other considerations: choosing the right membrane

When it comes to western blotting, choosing the right membrane is also critical to the overall success of the transfer. When selecting the right membrane, a protein's properties (i.e., charge, hydrophobicity) and the downstream application will help determine which membrane to use. Finding the optimal membrane may require experimenting with your specific protein on different membranes. Knowing the properties and characteristics of each membrane will help determine the best format for your application.

The two membranes available for use with the iBlot 2 device are PVDF and nitrocellulose. Each offers key attributes that suit particular experimental conditions. PVDF has a higher protein binding capacity of ~240 μg/cm^2, while nitrocellulose has a protein binding capacity of ~209 μg/cm^2. The higher binding capacity of PVDF affords a high sensitivity to low-abundance proteins but can exhibit higher background noise. On the other hand, nitrocellulose membranes tend to exhibit lower background noise.

Proteins bind to nitrocellulose membranes through hydrophobic interactions while proteins bind to PVDF membranes through hydrophobic and dipole interactions. Due to the hydrophobicity of PVDF membranes, these are the preferred choice for hydrophobic proteins (i.e. membrane proteins).

If stripping and reprobing, PVDF membranes should be considered. Due to PVDF's greater hydrophobic properties, membranes can be stripped and reprobed easily, whereas nitrocellulose membranes can lose signal during processing. In addition, PVDF membranes demonstrate higher physical durability than nitrocellulose membranes.

Summary

HMW proteins can be successfully transferred using the iBlot 2 Gel Transfer Device. This dry blotting system provides overall visual immunodetection results that are either comparable to or more sensitive than the results obtained using a standard wet blotting system, but in a fraction of the time (Figure 5). When transferring large proteins, for best performance it is important to use Tris-acetate gels and longer transfer times of 8–10 minutes.

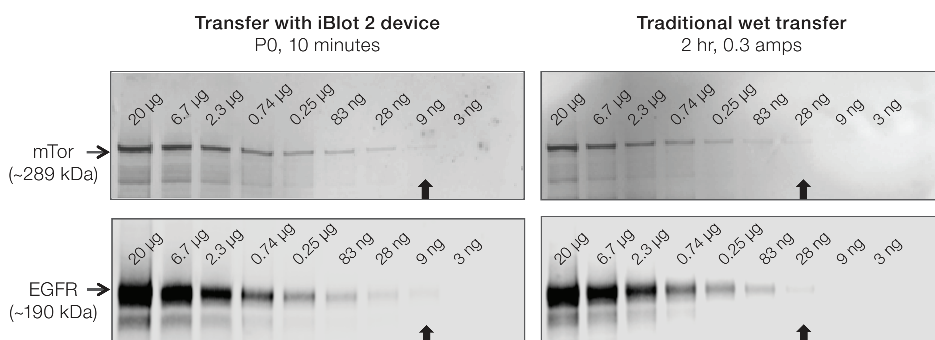

Figure 5. Western blotting analysis of mTOR and EGFR from A431 lysates transferred from a NuPAGE 3–8% Tris-Acetate gel using the iBlot 2 Gel Transfer Device and a traditional wet transfer system.

Methods

Transfer of EGFR time course

Western blot analysis of EGFR was performed by loading serially diluted A431 cell lysate from 20 µg to 312 ng per well onto a Novex™ 4–20% Tris-Glycine gel, WedgeWell™ format (Cat. No. XP04202BOX). Proteins were transferred using Invitrogen™ iBlot™ 2 Transfer Stacks, nitrocellulose, mini (Cat. No. IB23002) at 25 V for 6 min, 7 min, 8 min, or 10 min, and using the P0 program on the iBlot 2 device. Membranes were blocked for 30 minutes at room temperature and then probed overnight at 4°C with Invitrogen™ EGFR polyclonal antibody (Cat. No. PA1-1110) at a dilution of 1:500. After overnight incubation, the membranes were washed in TBST, probed with Invitrogen™ Goat anti-Rabbit (H+L) Highly Cross-Adsorbed Secondary Antibody, conjugated to Alexa Fluor™ Plus 800 (Cat. No. A32735) at a dilution of 1:5,000 for one hour at room temperature.

Increased transfer efficiency of KLH with pretreatment of gel with 20% ethanol

A dilution series of KLH was prepared for SDS-PAGE and loaded onto two Invitrogen™ Bolt™ 4–12% Bis-Tris Plus gels (NW04120BOX). Lane 1 was loaded with 5 µL of Invitrogen™ MagicMark™ XP Western Protein Standard (Cat. No. LC5602), lanes 2–10 were loaded with a 2-fold dilution series of KLH, 1,000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 pg, respectively, on each gel. After electrophoresis, one of the duplicate gels was incubated on a shaker in 20% ethanol for 10 minutes. The gels were then transferred using the iBlot 2 Gel Transfer Device to an iBlot 2 Transfer Stack, PVDF, mini (P0, 8 minutes). Immunodetection was performed on the two blots using the Invitrogen™ iBind™ Western Device (Cat. No. SLF1000) and associated Invitrogen™ iBind™ solutions (Cat. No. SLF1020), and Invitrogen™ iBind™ Cards (Cat. No. SLF1010). The primary antibody, anti-KLH was diluted 1:2,000 in iBind solution. The secondary antibody, HRP-conjugated goat anti-rabbit antibody was diluted 1:1,000 in iBind solution. Upon completion of the 2.5-hour iBind reaction, blots were removed from the devices, briefly rinsed with deionized water, and then incubated in Invitrogen™ ECL Chemiluminescent Substrate (Cat. No. WP20005) for 5 minutes, and imaged.

Transfer of KLH—a large molecular weight protein

A dilution series of KLH was prepared for SDS-PAGE and loaded on either a NuPAGE 4–12% Bis-Tris (Cat. No. NP0321BOX) or 3–8% Tris-Acetate Protein Gel (Cat. No. EA03752BOX). Lanes were loaded with 10 ng, 7.5 ng, 5 ng, 2.5 ng, 1 ng, 750 pg, 500 pg, 250 pg, 100 pg, and 50 pg purified KLH protein. After electrophoresis, one of the duplicate gels was incubated on a shaker in 20% ethanol for 10 minutes. Proteins were transferred to nitrocellulose membranes by conventional wet tank transfer or dry transfer using the iBlot 2 transfer device. For traditional wet tank transfer, 1 liter of Invitrogen™ NuPAGE™ Transfer Buffer (Cat. No. NP0006) was prepared with 20% ethanol and chilled to 4°C. Filter paper and membrane were equilibrated in cold transfer buffer for 15 minutes. Gel was rinsed in deionized water and equilibrated in cold transfer buffer for 15 minutes. Transfer was performed at 4°C for 60 minutes at 30 volts.

For dry transfer, gels were transferred using the iBlot 2 Gel Transfer Device to Invitrogen™ iBlot™ 2 Transfer Stack, nitrocellulose, mini (Cat. No. IB23002) (P0, 8 minutes). Immunodetection of proteins on the resulting membranes was performed using an Invitrogen™ WesternBreeze™ Chromogenic Immunodetection Kit (Cat. No. WB7105) for detection of rabbit primary antibodies. A rabbit anti-KLH primary antibody was used (1:10,000) with a chromogenic development time of 1 hour.

Rapid dry blotting vs. traditional wet transfer

Western blot analysis of 2 protein targets, mTOR and EGFR, was performed by loading serially diluted A431 cell lysate from 20 µg to 3 ng per well onto NuPAGE 3–8% Tris-Acetate gels (Cat. No. EA03752BOX). One gel was transferred with iBlot2 Transfer Stacks, nitrocellulose, mini (Cat. No. IB23002) using the P0 program (10 min) and the second gel was transferred using the Towbin wet tank transfer method (2 hours, 0.3 A). Membranes were blocked for 30 minutes at room temperature and then probed overnight at 4°C with an EGFR polyclonal antibody (Cat. No. PA1-1110) at a dilution of 1:500 and an mTOR monoclonal antibody at a dilution of 1:700. After overnight incubation, the membranes were washed in TBST, probed with Goat anti-Rabbit (H+L) Highly Cross-Adsorbed Secondary Antibody, conjugated to Alexa Fluor Plus 800 (Cat. No. A32735) at a dilution of 1:5,000 and with Invitrogen™ Goat anti-Mouse (H+L) Highly Cross-Adsorbed Secondary Antibody, conjugated to Alexa Fluor Plus 680 (Cat. No. A32729) at a dilution of 1:2,500 for one hour at room temperature.

Find out more at **thermofisher.com/iblot2**

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