Western blotting

Optimizing western blot transfer for high and low molecular weight proteins

Introduction

Western blotting is a widely utilized technique in protein biology research, enabling the detection and analysis of specific proteins within complex biological samples. One of the critical steps in this process is the transfer of proteins from a gel onto a membrane, which can be challenging, particularly for proteins on each end of the molecular weight spectrum. Here we provide information on how to optimize the transfer of both high molecular weight (HMW) proteins (>150 kDa) and low molecular weight (LMW) proteins (<120 kDa) from a gel to a membrane using the Invitrogen™ iBlot™ 3 Western Blot Transfer System. This system is a buffer solution–free, dry blotting system that enables researchers to quickly and easily set up, run, and clean up after a transfer in less than 10 min. In comparison to traditional wet tank and semi-dry transfer methods, the iBlot 3 system offers a significant time-saving advantage while delivering equivalent or better transfer efficiency for both high and low molecular weight proteins. It is compatible with all commercially available gel chemistries, including Tris-glycine, Bis-Tris, Tris-acetate, and tricine.

Methods

Electrophoresis was carried out as described in the user manuals for each gel type. Wet transfer was performed according to the manufacturer's instructions using PVDF membranes. For transfers on the iBlot 3 system, Invitrogen™ iBlot™ 3 Transfer Stacks (mini size, PVDF) were used except where the figure legend indicates that nitrocellulose was used. Voltage and transfer times for each experiment are noted in the figure legends. Semi-dry transfers were performed using Bio-Rad™ Trans-Blot™ Turbo™ PVDF Transfer Packs. Immunodetection was completed using the Invitrogen™ Bandmate™ Automated Western Blot Processor. Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate was used for chemiluminescent detection. Imaging was performed using the Invitrogen™ iBright™ FL1500 Imaging System, and relative quantitation (band volume, which is band area multiplied by band intensity) was performed using iBright™ Analysis Software.

Optimizing protein separation for HMW and LMW protein transfer

To achieve efficient transfer of both HMW and LMW proteins, the selection of an appropriate gel type is a critical factor. Tailoring the gel chemistry to the protein molecular weight ensures optimal separation, which is the foundation for successful protein transfer.

Separation of HMW proteins

For optimal separation of HMW proteins, use of a Tris-acetate gel is recommended. Invitrogen™ NuPAGE™ Tris-acetate gels minimize protein modifications by maintaining a pH 8.1 environment that results in sharper bands. Additionally, a specialized gel buffer and lower polyacrylamide concentration near the top of the gel allow larger proteins to transfer more efficiently than from Bis-Tris or Tris-glycine gels, as demonstrated in Figure 1.

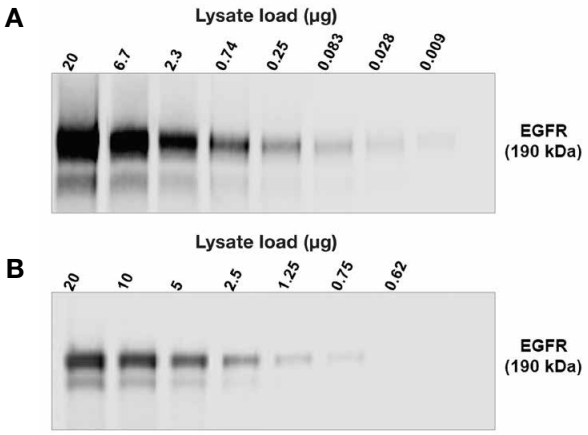

Figure 1. Tris-acetate gels offer superior transfer efficiency of HMW proteins compared to 4–20% Tris-glycine gels. A dilution series of A431 cell lysate was loaded onto **(A)** an Invitrogen™ NuPAGE™ 3–8% Tris-acetate gel and **(B)** an Invitrogen™ Novex™ 4–20% Tris-glycine gel, for gel electrophoresis. Proteins were subsequently transferred to a membrane and detected using anti-EGFR primary antibody, HRP-conjugated secondary antibody, and a chemiluminescent substrate. The blot that was made with the Tris-acetate gel had a detection limit of 9 ng, compared to the detection limit of 620 ng with the 4–20% Tris-glycine gel.

Separation of LMW proteins

Resolving LMW proteins with small differences in size is a common challenge when using popular general-use protein gel chemistries such as 4–20% Tris-glycine. In contrast, tricine gel chemistry offers enhanced separation and improved resolution of LMW proteins. The Invitrogen™ Novex™ tricine gel system is a modification of the Tris-glycine system in which tricine replaces glycine in the running buffer. This system utilizes a discontinuous buffer system that is specifically designed for resolving LMW proteins. A comparison between the tricine and Tris-glycine gels is depicted in Figure 2, which shows that both α-tubulin and cleaved caspase are well resolved by a tricine gel but appear to be poorly separated by a 4–20% Tris-glycine gel. Furthermore, the tricine gel offers superior sensitivity and a greater limit of detection for both α-tubulin (50 kDa) and cleaved caspase-3 (17 and 19 kDa). Once protein separation is sufficiently optimized, the transfer procedure can be refined.

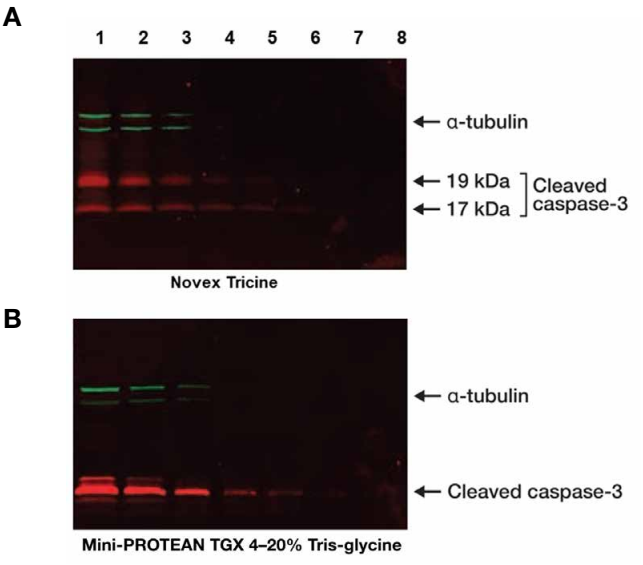

Figure 2. Optimizing separation and transfer of LMW proteins with gel chemistry. After separation on Invitrogen™ Novex™ 16% tricine and Bio-Rad™ 4–20% Mini-PROTEAN™ TGX™ gels, proteins were transferred to membranes and probed with primary antibodies against α-tubulin and caspase-3, followed by secondary antibodies labeled with Invitrogen™ Alexa Fluor™ Plus 800 and Alexa Fluor™ Plus 680 dyes, respectively. Lanes 1–8: A 1.5x dilution series of cytochrome c–treated Jurkat cell lysate was prepared, and 1 µL was loaded into each well. **(A)** Separation and transfer using the Novex tricine gel. **(B)** Separation and transfer using the Mini-PROTEAN TGX Tris-glycine gel.

Optimizing parameters for HMW and LMW protein transfer

Setting the right transfer conditions is of utmost importance, as it directly impacts the efficiency and reproducibility of the protein transfer step as well as the ability of a protein to bind to the membrane. Traditional wet and semi-dry transfer systems allow adjustment and optimization of three key transfer parameters: voltage, current, and run time. The iBlot 3 system enables users to adjust an additional variable: temperature. Typically, transfer of HMW proteins will benefit from slightly longer transfer times, higher voltage, and slightly warmer transfer conditions, while transfer of LMW proteins can benefit from shorter transfer times and cooler conditions. For convenience, the iBlot 3 system is preprogrammed with three transfer methods that are optimized to transfer proteins of HMW, LMW, and a broad molecular weight range. Although these methods are an excellent starting point, transfer parameters may occasionally need to be refined to achieve maximum transfer efficiency of the proteins being studied.

Transferring proteins across a broad molecular weight range

When detecting high and low molecular weight proteins in the same blot simultaneously (multiplex western blotting) or sequentially (after stripping and reprobing), it is important to consider potential transfer differences between proteins of differing size. In protein transfer, HMW proteins generally require higher voltage or longer run times compared to LMW proteins. While these conditions may be optimal for HMW proteins, they can cause LMW proteins to pass through the membrane, resulting in reduced signal.

Conversely, using low voltage or short run times may yield excellent results for LMW proteins, but may not effectively transfer HMW proteins from the gel to the membrane. If the preprogrammed broad range method does not efficiently transfer the specific HMW and LMW proteins of interest, optimization may be necessary. Begin by identifying the range that exhibits lower-than-expected transfer efficiency. If this is the case for LMW proteins, reduce the transfer time in subsequent optimization experiments by 1-minute increments until sufficient signals are obtained from both high and low molecular weight proteins. For further optimization, voltage can be gradually increased in increments of 2–5 V until maximum signals are obtained. If LMW proteins are exhibiting optimal signals while HMW proteins show low transfer efficiency, complete this process in reverse, slowly increasing time or voltage until good transfer is achieved.

This strategy enables maximum customization of the transfer process. If satisfactory results are still not obtained, it may be preferable to split the experiment into two parts, optimizing transfer parameters for HMW proteins and for LMW proteins separately.

Transferring HMW proteins

As mentioned above, transfer time is a crucial parameter in protein transfer. Unlike wet transfer methods that typically require 1–2 hr, the iBlot 3 system offers the advantage of optimizing transfer time within the range of 3–10 min without compromising signal quality. To demonstrate this, an experiment was conducted involving the fluorescent detection of a 190 kDa protein, the epidermal growth factor receptor (EGFR), in an A431 cell lysate. A lysate dilution series was loaded onto a gel, subjected to electrophoresis, and transferred at times ranging from 1 to 10 min (Figure 3). Imaging was performed on the iBright FL1500 Imaging System, and the iBright Analysis Software was used to measure the relative band volume of EGFR. The results demonstrated that even after only 3 min of transfer time, EGFR was easily detected, and maximum transfer efficiency was achieved at 6 min. While the optimal transfer time for other HMW proteins may vary, typically no more than 8 min are necessary.

Transferring LMW proteins

Optimization and benefits of cooling

The iBlot 3 system incorporates an automated cooling mechanism that swiftly returns the system to its initial temperature between transfers. Accurate temperature control results in more consistent electrical current and transfer efficiency over repeated runs. This is evident in the results presented in Figure 4, in which the transfer temperature of the iBlot 3 system was consistently no higher than 30°C (Figure 4A). In contrast, the temperature of the Bio-Rad™ Trans-Blot™ Turbo™ system reached 40–42°C after a 5 min transfer. The precise temperature control in the iBlot 3 system contributes to the consistent electrical current profiles during consecutive transfers, enhancing the reliability and reproducibility of protein transfer (Figure 4B).

Reducing the transfer temperature can help reduce blow-through and improve the transfer of low to medium molecular weight proteins. As shown in Figure 5, transfer of the LMW protein p23 is improved with increased cooling. When transferring LMW proteins, it is best to start with the preprogrammed LMW protein method that has a default cooling setting of “med cooling”.

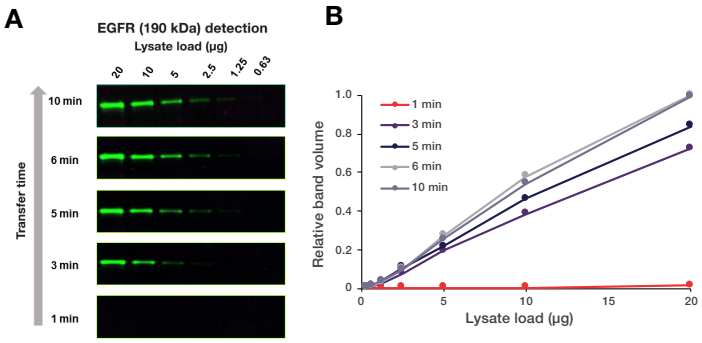

Figure 3. Detection of EGFR in a dilution series of A431 cell lysate separated on an Invitrogen™ Novex™ Tris-glycine gel, 4–20%, and transferred to 0.2 µm nitrocellulose with the iBlot 3 system using transfer times of 1–10 min at 25 V. (A) Detection of EGFR using an Alexa Fluor Plus 800 secondary antibody and imaged on the iBright FL1500 imaging system. **(B)** Normalized band volumes with transfer and detection of EGFR in A431 cell lysate at transfer times of 1–10 min. Maximum transfer efficiency was obtained at 6 min.

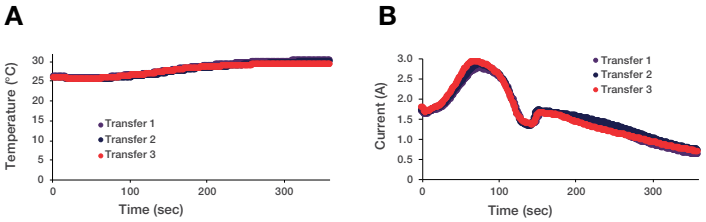

Figure 4. Temperature control provides consistent temperature and current in back-to-back transfers. (A) Cathode plate temperature and **(B)** current data from the iBlot 3 system’s data log were downloaded for three back-to-back transfers of A431 cell lysate that was separated on Invitrogen™ NuPAGE™ 4–12% Bis-Tris gels and transferred with iBlot 3 nitrocellulose transfer stacks at 25 V for 6 min.

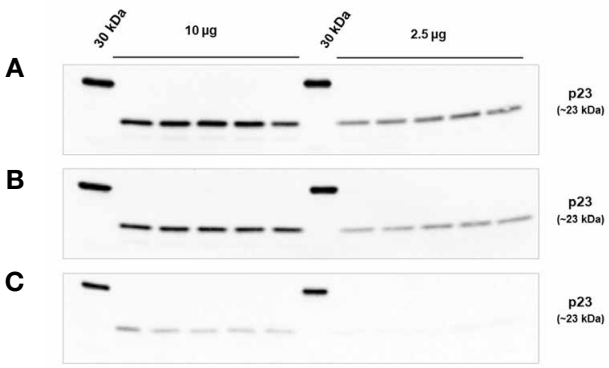

Figure 5. Detection of p23 and the impact of cooling during transfer. An A431 cell lysate was separated on a Novex 4–20% Tris-glycine gel and transferred to 0.2 µm nitrocellulose; p23 was detected with anti-p23 primary antibody, HRP-conjugated secondary antibody, and a chemiluminescent substrate. **(A)** Transfer using the iBlot 3 system with maximum cooling. **(B)** Transfer using the iBlot 3 system with no cooling. **(C)** Transfer with the Invitrogen™ iBlot™ 2 Gel Transfer Device with no cooling. It is important to note that the iBlot 3 system performs protein transfer at a lower temperature than the iBlot 2 system, even when the former is set to “no cooling”.

If improved transfer efficiency is needed, the transfer temperature can be further reduced by selecting “max cooling”. In general, lowering the transfer temperature improves transfer efficiency of LMW proteins; however, there may be protein-specific cases in which the opposite is true and selecting “low cooling” or “no cooling” has a positive effect on transfer efficiency.

Choosing the optimal system for HMW and LMW protein transfer

Dry transfer

Dry blotting utilizes a solid gel matrix, contained within the transfer stack, that serves as a unique ion source for protein transfer. This patented technology is utilized only in the iBlot devices and offers several benefits to the user. The iBlot 3 Western Blot Transfer System significantly decreases protein transfer duration from over 1 hr (wet transfer) to a mere 3–8 min, depending on the molecular weight of the protein in question. The iBlot 3 transfer chemistry can also result in tighter binding of proteins to the membrane compared to other transfer methods. Additionally, the iBlot 3 system offers faster setup and cleanup—the preassembled, self-contained iBlot 3 transfer stacks eliminate the need for membrane activation, transfer buffer preparation, and hazardous material disposal. The stack is self-contained within its own tray, eliminating any possible contamination such as from previously used equipment or tools as well as simplifying the cleanup process. Single-use electrodes are also contained within the transfer stack and are in direct contact with the gel matrix to further help generate a consistent and uniform electric field with no risk of degradation.

Overall, the combination of the unique chemistry of the iBlot 3 transfer stacks and the consistent electrical current and temperature of the iBlot 3 transfer system generates excellent reproducibility with minimal time requirements. Figure 6 demonstrates the consistency in transfer and high levels of western blot sensitivity for both LMW and HMW proteins over repeated runs. The iBlot 3 system even exhibits improved transfer efficiency compared to its iBlot 2 predecessor, as shown in Figure 7.

Semi-dry transfer

Semi-dry transfer still relies on the use of a liquid buffer solution in the transfer stack, requiring tedious buffer preparation and device cleanup steps. Furthermore, in semi-dry transfer, the electrodes are not contained within the transfer stack, and this can sometimes lead to poor contact, reduced transfer efficiency, and uneven transfer of both LMW and HMW proteins. Both the LMW protein 4E-BP1, which runs as a 15 and 20 kDa doublet in SDS-PAGE, and the HMW protein mTOR are transferred more evenly and efficiently using the iBlot 3 dry transfer system than with a semi-dry transfer system (Figures 8, 9).

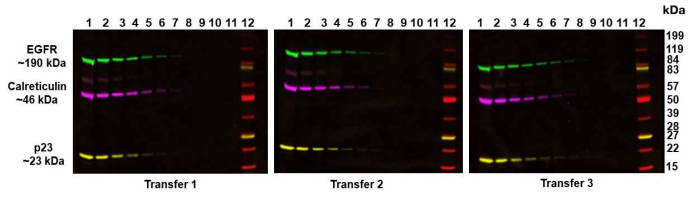

Figure 6. Consistency of multiplex immunodetection of three replicate transfers. Lanes 1–11: HEK293 lysate dilution series, 10–0.01 µg. Lane 12: Invitrogen™ iBright™ Prestained Protein Ladder. Invitrogen™ Bolt™ Bis-Tris Plus 4–12% gels were used, and transfer was performed using mini nitrocellulose iBlot 3 Transfer Stacks at 25 V for 6 min. Fluorescent detection was performed with Invitrogen™ Alexa Fluor™ 488, 546, and 800 labeled secondary antibodies.

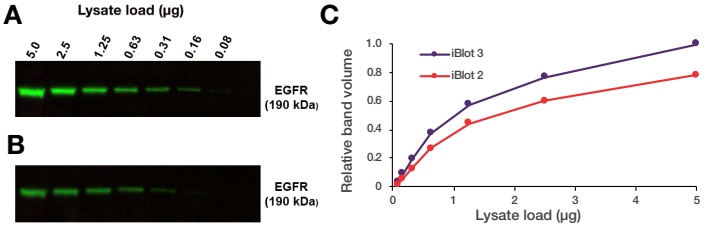

Figure 7. Comparison of EGFR transfer efficiency of iBlot 3 and iBlot 2 systems with a dilution series of HEK293 cell lysate. Bolt 4–12% Bis-Tris Plus gels were used for separation, and transfer was performed using mini nitrocellulose iBlot 2 or iBlot 3 Transfer Stacks. EGFR was detected using an Alexa Fluor Plus 800 secondary antibody, and the blot imaged on the iBright FL1500 imaging system. **(A)** Western blot transfer was performed on the iBlot 3 system at 25 V for 6 min with low cooling. **(B)** Western blot transfer was performed on the iBlot 2 system using the P0 program (stepped voltage at 20 V, 23 V, 25 V, over 7 min). **(C)** Normalized band volumes with transfer and detection of EGFR using the iBlot 3 system compared to the iBlot 2 system.

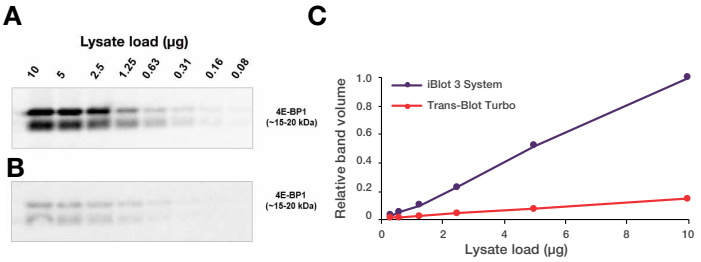

Figure 8. Comparison of chemiluminescent detection of 4E-BP1 with dry and semi-dry transfer of a dilution series of A431 cell lysate. Samples were separated with Invitrogen™ Novex™ Tricine Mini Protein Gels, 16%. **(A)** Dry transfer with the iBlot 3 system (5 min, 25 V, medium cooling). **(B)** Semi-dry transfer with the Trans-Blot Turbo semi-dry transfer device (5 min, 25 V). **(C)** Normalized band volumes with transfer and detection using dry and semi-dry transfer systems.

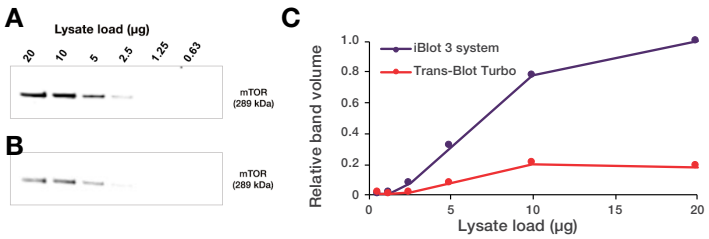

Figure 9. Comparison of chemiluminescent detection of mTOR with dry and semi-dry transfer of a dilution series of HEK293 cell lysate. Samples were separated on Invitrogen™ NuPAGE™ Tris-Acetate Mini Protein Gels, 3–8%, and transferred to nitrocellulose. **(A)** Dry transfer with the iBlot 3 system (8 min, 30 V, no cooling). **(B)** Semi-dry transfer with the Trans-Blot Turbo semi-dry transfer device (10 min, 25 V). **(C)** Normalized band volumes for transfer and detection using dry and semi-dry transfer systems.

Wet tank transfer

Wet tank transfer is performed on a transfer stack that is submerged in a transfer buffer that usually contains methanol. In addition to the laborious setup and cleanup, wet tank transfer has the drawback of requiring a long transfer time (1 hr to overnight). Despite these disadvantages, wet tank transfer remains a common transfer method due to the misconception that it can achieve better transfer efficiency than other methods. In reality, dry transfer consistently matches or outperforms wet transfer across the molecular weight spectrum; as shown in Figures 10 and 11, better transfer efficiency is observed with dry transfer of the LMW protein 4E-BP1 and the 57 kDa protein disulfide-isomerase (PDI).

There is a prevailing misconception among researchers that wet transfer methods yield the highest transfer efficiency for HMW proteins. However, our findings challenge this notion by demonstrating that the iBlot 3 system achieves superior transfer efficiency of the 290 kDa protein mTOR and the 380 kDa protein BRCA2, compared with wet transfer. Furthermore, protein transfer with the iBlot 3 can be completed in only 8 min instead of the 30 min to 1 hr necessary for the wet protocol. These results highlight the enhanced transfer efficiency and detection capabilities of the iBlot 3 system for HMW proteins.

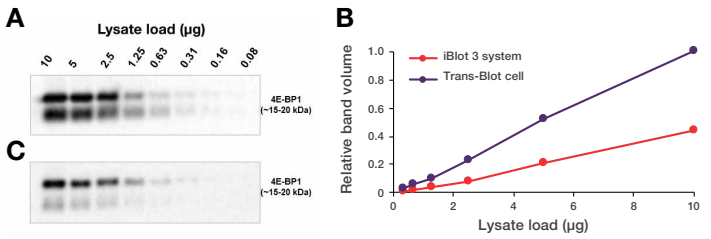

Figure 10. Comparison of chemiluminescent detection of 4E-BP1 with dry and wet transfer of a dilution series of A431 cell lysate. Samples were separated on Novex 16% tricine mini gels. **(A)** Dry transfer with the iBlot 3 system (5 min, 25 V, medium cooling). **(B)** Wet transfer with the Trans-Blot Mini Cell (1 hr, 100 V). **(C)** Normalized band volumes for transfer and detection using dry and wet transfer systems.

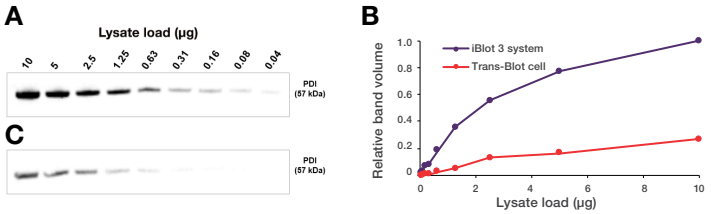

Figure 11. Comparison of chemiluminescent detection of PDI with dry and wet transfer of a dilution series of HepG2 cell lysate. Samples were separated on Invitrogen™ NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%, and transferred to nitrocellulose. **(A)** Dry transfer with the iBlot 3 system (6 min, 25 V, low cooling). **(B)** Wet transfer with the Trans-Blot Mini Cell (1 hr, 100 V). **(C)** Normalized band volume for transfer and detection comparing dry and wet transfer systems.

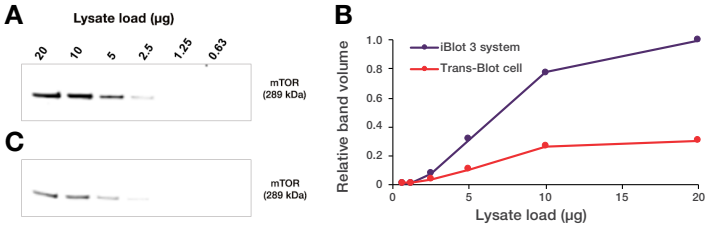

Figure 12. Comparison of chemiluminescent detection of mTOR with dry and wet transfer of a dilution series of HEK293 cell lysate. Samples were separated on 3–8% NuPAGE Tris-acetate mini gels and transferred to nitrocellulose. **(A)** Dry transfer with the iBlot 3 system (8 min, 30 V, no cooling). **(B)** Wet transfer with the Trans-Blot Mini Cell (1 hr, 100 V). **(C)** Plot showing normalized band volume for transfer and detection.

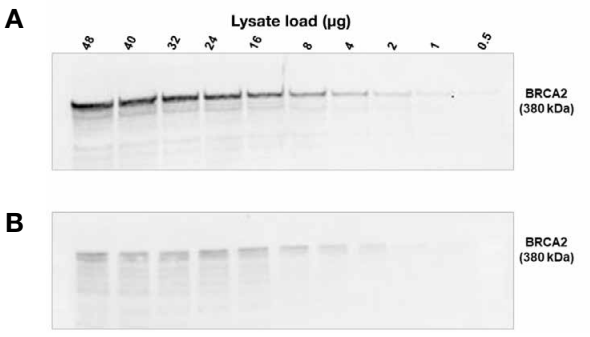

Figure 13. Detection of BRCA2 in a dilution series of HEK293 cell lysate comparing gel chemistries and dry and wet transfer systems. **(A)** Separation on a 3–8% Nu-PAGE Tris-acetate gel and transfer with the iBlot 3 system (8 min, 30 V, no cooling). **(B)** Separation on a 7.5% TGX gel and transfer with the Criterion Blotter (30 min, 100 V).

Summary

The optimization of key parameters is crucial for achieving efficient protein transfer in western blotting, particularly for high and low molecular weight proteins. The appropriate selection of gel type, careful adjustment of transfer time, voltage, and cooling, and consideration of protein-specific requirements are all vital elements in this process. The need for flexibility and customization in the transfer process is also highlighted, as optimal conditions can vary significantly depending on the specific proteins of interest. The iBlot 3 Western Blot Transfer System, with its preprogrammed methods for HMW and LMW transfer, offers a valuable tool for researchers, enabling efficient and reliable protein transfer in a significantly reduced time frame. When further optimization is needed, the system enables fully customizable programming to optimize protein transfer across the molecular weight spectrum as described in this application note.

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