Transfer of High Molecular Weight Proteins to Membranes: A Comparison of Transfer Efficiency Between Blotting Systems

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Introduction

Protein blotting has been a cornerstone technique in the field of biochemistry for over 30 years (Towbin et al. 1979). While the number of applications for this technique has grown (Bio-Rad bulletin 2895), the root technology has remained unchanged. For electrophoretic transfer, the gel containing the separated protein mixture is placed on a membrane and sandwiched between ion reservoirs. The blotting sandwich is then inserted between two electrodes and a current is applied, causing protein migration from the gel to the membrane. Efficiency of migration is based on a number of factors including the size and charge of the protein. Protocol optimization is often needed on a protein-specific basis, as the parameters needed for transferring large proteins will often cause small proteins to migrate through the membrane (membrane blowthrough). Once transferred, the proteins are accessible for downstream applications such as sequencing, immunodetection, or biochemical analysis.

There are two main gel blotting techniques: tank transfer (submarine) and semi-dry transfer (Bio-Rad bulletin 2895). In tank transfer the gel and the membrane, sandwiched between filter paper, are secured in a cassette and submerged in transfer buffer (the ion reservoir). This technique provides the largest flexibility in transfer conditions and is generally considered the gold standard when comparing efficiency of transfer techniques. Semi-dry blotting uses filter paper soaked in transfer buffer as the ion reservoir. The sandwich is placed in direct contact with plate electrodes and allows for fast transfers while using a limited amount of buffer. One benefit of this technique is that the user can apply a discontinuous buffer system, where different buffers are used for the reservoirs on the anode and cathode sides of the sandwich, which may help transfer efficiency. On the other hand, the small amount of buffer used in semi-dry blotting may cause ion depletion, which may limit transfer efficiency.

Recently, a new generation of transfer apparatuses has become available. These fast blotting systems aim to provide the efficiency of tank blotting with the simplicity of a semi-dry setup and do it in less time than either of the traditional techniques. The Bio-Rad® Trans-Blot® Turbo™ transfer system is a fast blotting system that provides protein transfer in 3–10 minutes, with prepackaged transfer packs that eliminate the need for buffer and membrane preparation. With two independently run cassettes, the Trans-Blot Turbo transfer system allows up to four mini-gels to be transferred at once.

In this study we compare the efficiency of different blotting techniques, including semi-dry, tank transfer, and fast blotting systems on proteins across a wide range of molecular weights with particular emphasis on large proteins (≥200 kD). We also conducted experiments transferring high molecular weight proteins that are difficult to transfect using conventional methods. The results demonstrate that the transfer efficiency of the Trans Blot Turbo transfer system meets or exceeds that of other comparable techniques.

Materials and Methods

General

Tank, semi-dry, and Trans-Blot Turbo transfers were performed using samples diluted in reducing Laemmli buffer and loaded onto 4–20% Mini-PROTEAN® TGX™ gels subsequently run at 200 V for 35 min. iBlot transfers were performed using samples diluted in reducing LDS sample buffer and loaded onto 4–12% NuPAGE gels run in MES buffer at 200 V for 35 min. SDS-PAGE broad range standards, Precision Plus Protein™ Unstained standards, SYPRO Ruby protein blot stain, Immun-Star™ goat anti-mouse, goat anti-rabbit HRP, and Immun-Star™ WesternC™ substrate were obtained from Bio-Rad. Antibodies were purchased from Santa Cruz Biotechnology, including KLH, U2AF65, myosin, and goat anti-mouse IgM-HRP. Thyroglobulin was purchased from Merck Chemicals.

Transfer Techniques

Trans-Blot Turbo Transfer System — after electrophoresis, gels were placed directly onto a Trans-Blot Turbo mini nitrocellulose transfer pack and transferred using the "HIGH MW" protocol for mini gels (1.3 A, 25 V, 10 min).

Trans-Blot SD Semi-Dry System — post-electrophoresis, gels were equilibrated in cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 min. The transfer sandwich was assembled using nitrocellulose membrane. Transfer occurred for 1 hr at a constant 25 V.

Tank Transfer — after electrophoresis, gels were equilibrated in cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 min. The transfer sandwich was assembled in a Mini Trans-Blot® cell using nitrocellulose membrane. The transfer occurred for 1 hr at 100 V constant or overnight (15 hr 40 min) at 30 V.

iBlot Transfer System — post-electrophoresis, gels were placed on a Mini Nitro iBlot stack and assembled on the iBlot system following manufacturer's instructions. Proteins were transferred using program P3 for 10 min.

SYPRO Ruby Staining

After transfer, membranes were placed in 10% acetic acid, 7% methanol and agitated for at least 15 min. Membranes were subsequently washed four times in deionized water for 5 min each, then placed in 15 ml of SYPRO Ruby protein blot stain for 15 min. Blots were washed in deionized water six times for approximately 1 min each before imaging. Blot images were taken on a VersaDoc™ MP 4000 system using green LED excitation and analyzed using Image Lab™ software.

Immunodetection

After transfer, blots were blocked using 3% BSA in Tris-buffered saline containing 0.05% Tween-2A (TTBS) for 1 hr with vigorous agitation. Blots were subsequently incubated in a primary antibody diluted in blocking solution for 1 hr, then washed in TTBS five times for 5 min each. The blot was incubated in an HRP-conjugated secondary antibody diluted in TTBS for 1 hr, then washed six times for 5 min each. Blots were developed using Immun-Star WesternC substrate, imaged on a ChemiDoc™ XRS+, and analyzed using Image Lab software.

Results and Discussion

Combined with the use of the total protein blot stain SYPRO Ruby, the nine natural proteins in Bio-Rad's SDS-PAGE broad range standards provide a convenient tool for determining the transfer efficiency of different types and sizes of proteins. These standards were used to compare the transfer efficiency of different systems: the Trans-Blot Turbo system, semi-dry transfer, the iBlot system, and tank transfer. Transfer efficiency was assessed by measuring the intensity of fluorescently stained protein bands on the membrane after transfer (post-transfer membrane). The images of the blots show that for most of the proteins, transfer efficiency of the Trans-Blot Turbo system is comparable to or better than that of other systems including overnight tank blotting (Figure 1A). A ten-minute transfer with the Trans-Blot Turbo system performs well across the range of proteins tested, from the 200 kD myosin to the 7 kD protein aprotinin (Figure 1B). Some proteins display notable differences in transfer efficiency between the techniques. Phosphorylase b (97 kD), for instance, shows similar transfer efficiency with a ten-minute Trans-Blot Turbo transfer and overnight tank blotting, yet it is barely visible on a blot generated using the iBlot transfer system. The 200 kD protein myosin transfers

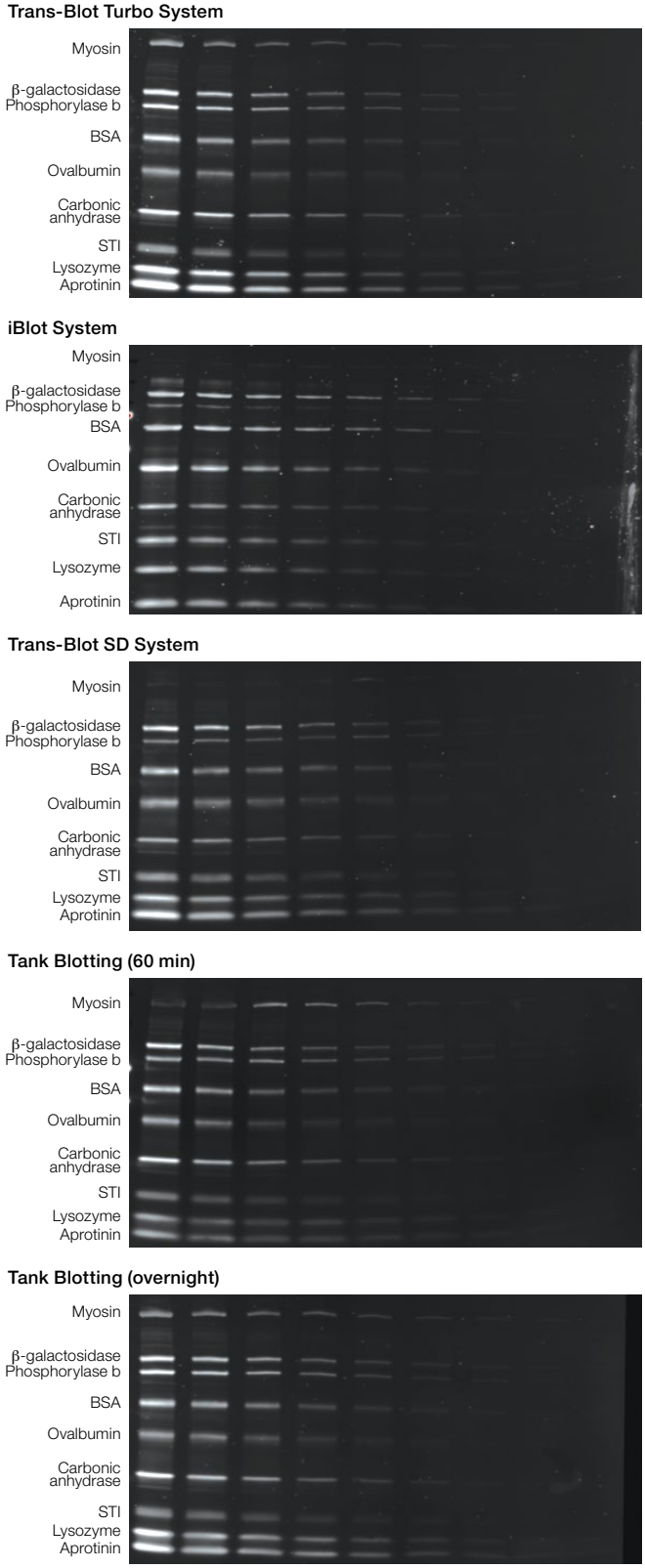

Fig. 1A. Transfer efficiency of broad range standards using different methods. A twofold dilution series of broad range standards, starting at 0.5 µg load (each protein), was transferred from a 4–20% TGX or a 4–12% NuPAGE gel (iBlot) onto nitrocellulose membranes. Total protein was detected using SYPRO Ruby protein blot stain and imaged on a VersaDoc 4000 MP imager using green LED excitation. Integration time and transform settings are the same for each image.

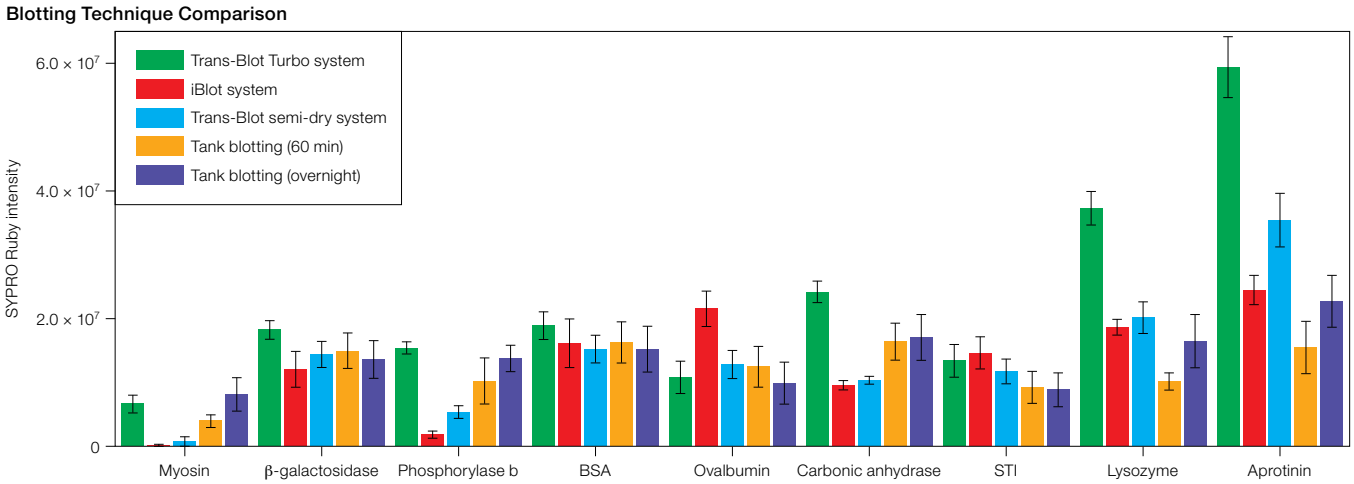

Fig. 1B. Intensity quantitation of broad-range protein standards. Band intensity of the nine proteins in the second dilution of each image was quantitated using Image Lab software and plotted. Error bars represent the standard deviation from the average of four blots.

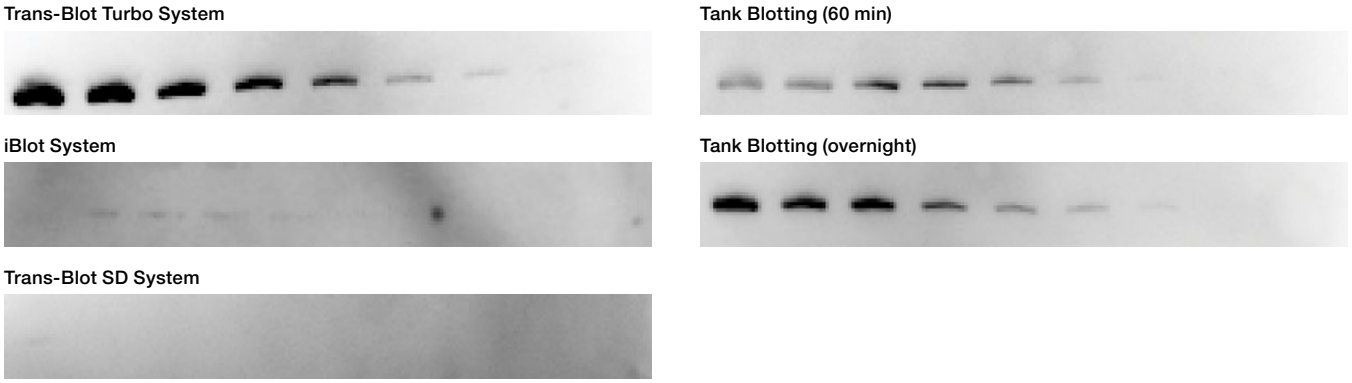

Fig. 2. Transfer efficiency of myosin (200 kD). Blots shown in Figure 1 were probed with a 1:200 dilution of myosin antibody. All blots were developed simultaneously and imaged on a ChemiDoc XRS+ imaging system using identical integration times. The images were transformed to show the highest limit of detection for each transfer technique and are representative of two experiments.

to a similar degree with the Trans-Blot Turbo system and overnight tank blotting, yet this protein transfers poorly with other techniques. To determine if the lack of transfer is an artifact of SYPRO Ruby staining, the blots were subsequently probed with a myosin antibody. The results (Figure 2) show that immunodetection reflects the trends seen in total protein staining. Signal intensity is strongest on the blots transferred using the Trans-Blot Turbo system as well as with overnight tank blotting, leading to higher sensitivity for protein detection on these blots compared to those transferred using other techniques.

A HeLa cell nuclear lysate dilution series was used to assess transfer efficiency of a complex sample onto nitrocellulose membrane (Figure 3). The results are similar to those observed for the broad range standards, where the efficiency of an overnight tank transfer and a ten-minute Trans-Blot Turbo transfer are comparable across a wide range of protein molecular weights. A ten-minute transfer using the iBlot system gives strong signal intensity for proteins between 20 and 100 kD, but very weak transfer for proteins falling outside of this size range. Following blot staining, the membranes were probed for U2AF65, an RNA splicing factor present in nuclear lysate (Pastuszak et al. 2011) (Figure 4). Immunodetection reveals higher U2AF65 signal intensity for transfer performed with the Trans-Blot Turbo system compared to other techniques.

To examine the transfer efficiency of proteins larger than 200 kD, the transfer of two 330 kD proteins was assessed using the various systems in this study. A dilution series of purified human thyroid thyroglobulin (a protein that comprises two 330 kD subunits) was transferred and the membranes were stained with SYPRO Ruby stain. Signal intensity and the limit of detection of this protein are comparable between Trans-Blot Turbo and overnight tank transfers. Similar results are obtained for immunodetection of a dilution series of a purified keyhole limpet hemocyanin, a mollusk tyrosinase comprised of multiple subunits, the largest of which is 400 kD (Swerdlow et al. 1996) — turbo transfers yield a performance comparable to that of overnight tank transfers.

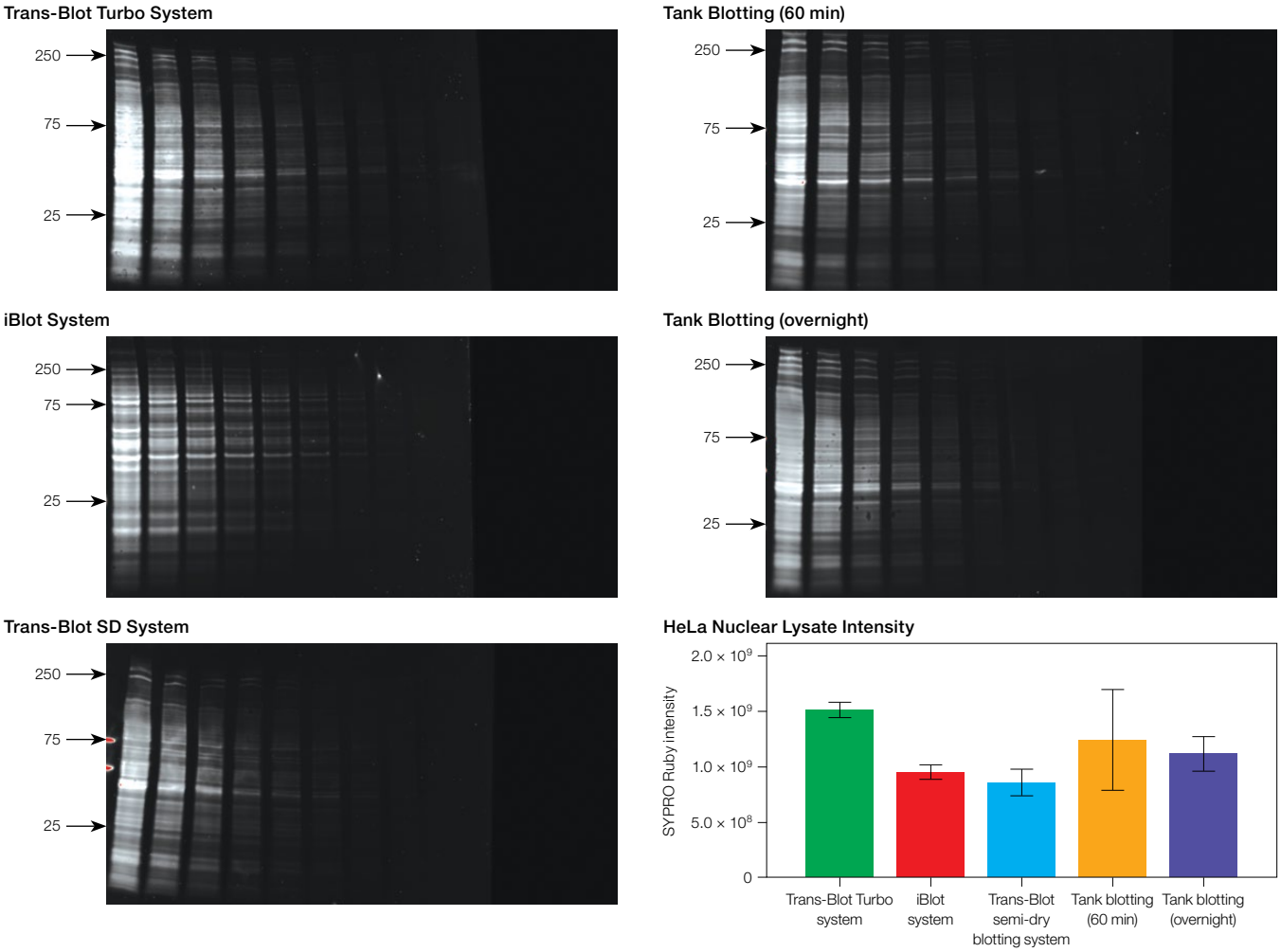

Fig. 3. Transfer efficiency of HeLa nuclear extract. A twofold dilution series of HeLa nuclear extract, starting at 20 µg, was transferred from a 4–20% TGX or a 4–12% NuPAGE gel (iBlot) onto nitrocellulose membranes. Total protein was detected using SYPRO Ruby protein blot stain and imaged on a VersaDoc 4000 MP imager using green LED excitation. Integration time and transform settings are the same for each image. The lane volume of the second dilution on each blot was calculated and plotted. Error bars represent the standard deviation of two experiments.

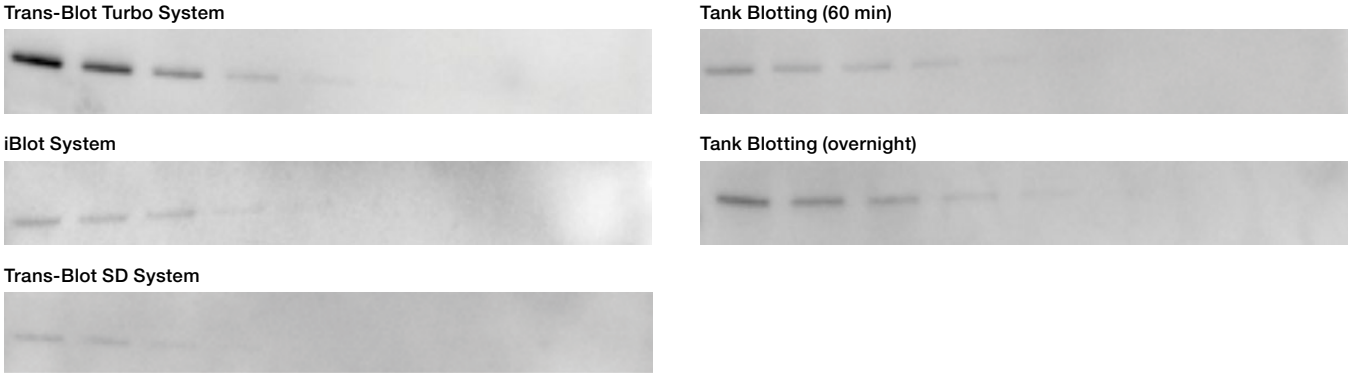

Fig. 4. Transfer efficiency of transcription factor U2AF65. Blots shown in Figure 3 were probed with a 1:250 dilution of U2AF65 antibody. All blots were developed simultaneously and imaged on a ChemiDoc XRS+ using identical integration times. The images were transformed to show the highest limit of detection for each transfer technique. The images are representative of two experiments.

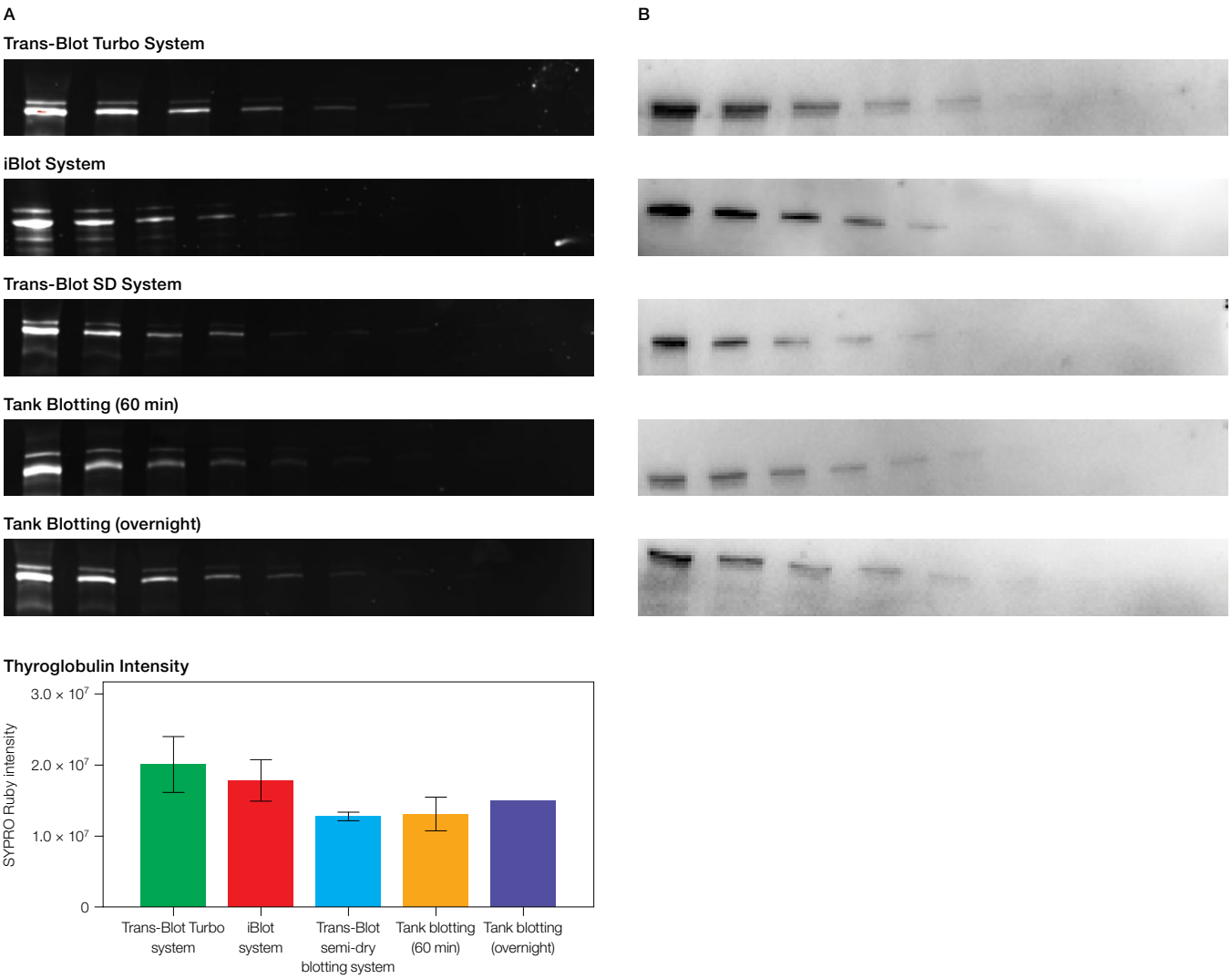

Fig. 5. Transfer efficiency of the high molecular weight proteins thyroglobulin and KLH. Twofold dilution series of purified human thyroglobulin (330 kD) and KLH (400 kD), starting at 500 ng load, were transferred from a 4–20% TGX gel onto nitrocellulose membranes. **A**, thyroglobulin was detected by SYPRO Ruby staining. The displayed images used identical integration and transform settings. Signal intensity analysis was performed on the second dilution in the series and plotted. Error bars represent the standard deviation between two replicates. **B**, KLH was detected on the membrane using a 1:1000 dilution of KLH antibody. Displayed images used identical integration time but were individually transformed to display the best limit of detection for each technique.

Conclusions

The variables that need to be considered when optimizing protein transfer are numerous. In this work we examined the impact of molecular weight on the efficiency of transfer. We compared the protein transfer efficiency of five different blotting systems by using fluorescent total protein staining and immunodetection of nitrocellulose membrane-bound proteins. Sets of discrete proteins, complex sample, and purified proteins were used for this work.

We have shown that the Trans-Blot Turbo and Mini Trans-Blot tank systems are equivalent to or outperform the iBlot system for all protein sizes tested in terms of transfer efficiency. Among the different transfer systems tested, the Trans-Blot Turbo system compares favorably to the others and matches or exceeds the transfer efficiency of the other techniques under the conditions tested. A key advantage of the Bio-Rad Trans-Blot Turbo system is the short setup and transfer time, compared to traditional tank blotting, for efficient transfer of complex samples and proteins of varying molecular weights.

References

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