Evaluation of the Criterion Stain Free™ Gel Imaging System for Use in Western Blotting Applications

Ahmed Elbaggari, Julie Choe, Kevin McDonald, and Anthony Alburo,
Bio-Rad Laboratories, Inc., Hercules, CA 94547.

Introduction

Coomassie Brilliant Blue (CBB) staining has been the standard technique for visualizing proteins on polyacrylamide gels after electrophoresis for decades. The procedure, which has changed little since its first description, is time consuming and the results are subject to variability. While this technique is useful for visualizing proteins on a gel, it is of limited use for protein quantitation and comparison across experiments.

The Criterion Stain Free gel imaging system described in this tech note represents a novel technique for fluorescent visualization of proteins in polyacrylamide gels. Based on methods similar to those reported by Kazmin et al. (2002) and Ladner et al. (2004), this system does not require separate staining protocols. It relies instead on a UV-induced chemical modification by trihalocompounds of naturally occurring tryptophan (Trp) residues within proteins. The Criterion Stain Free system comprises an imaging instrument with a transilluminator for UV activation and visualization of proteins that have been separated on Criterion Stain Free gels and Image Lab™ analysis software for automated size determination and quantitation of protein bands.

The function of the Criterion Stain Free gel imaging system is based on a chemical component present in the Criterion Stain Free polyacrylamide gels that undergoes a reaction with Trp residues when exposed to UV light. This generates fluorescent protein bands in 2.5 minutes, which can be easily detected both within gels and on blots using the Criterion Stain Free imager. This method provides detection of as little as 1 ng of typical globular proteins, which is slightly more sensitive than the standard CBB staining method. The advantage of this technology for PAGE applications is fast, efficient, and sensitive detection of proteins in gels without extensive postelectrophoretic manipulation (McDonald et al. 2008).

In this report, we describe the compatibility of the Criterion Stain Free gel imaging system with blotting applications. We show that the technique allows rapid and direct confirmation of appropriate protein patterns and efficient transfer before immunodetection is performed. Use of the Criterion Stain Free system eliminates the need for the time- and reagent-consuming traditional methods such as Ponceau S staining used to asses protein transfer prior to detection in western blotting applications. We also demonstrate that, while binding of some monoclonal antibodies may be affected by the Criterion Stain Free method, we have not observed an effect on antigen recognition by polyclonal antibodies with the Criterion Stain Free method.

Methods

Proteins used in this work were purchased from commercial sources: human serum (Bioreclamation Inc.) and hemoglobin A0 and human apo-transferrin (Sigma Chemicals). Detection reagents were rabbit anti-human transferrin antibody (Dako), goat anti-rabbit and goat anti-mouse HRP conjugated antibodies (Bio-Rad Laboratories, Inc.), and monoclonal mouse anti-human hemoglobin H1850-10 (US Biological). The mouse anti-human hemoglobin monoclonals 3E5 and 12H12 were gifts from Roger Walker (Bio-Rad). All samples were run on 4–20% Tris-HCl Criterion Stain Free gels using a Criterion™ electrophoresis cell (Bio-Rad). Samples were diluted in Laemmli sample buffer containing 5% β-mercaptoethanol and heated at 95°C for 5 min prior to loading on gels. Electrophoresis was performed at 200 V for 55 min. Gels were run in duplicates. Criterion Stain Free gels were visualized by using the Criterion Stain Free gel imaging system (Bio-Rad). Gels were blotted onto Pall Fluorotrans PVDF membranes using a Criterion blotter (Bio-Rad). Membranes were blocked for 1 hr at room temperature with agitation in TBST (Tris buffered saline, 0.05% Tween 20) with 3% nonfat dry milk. Membranes were then washed in TBST prior to incubation with agitation for 1 hr at room temperature in primary antibody. Three washes of 10 min with agitation in TBST were performed after each antibody incubation. Secondary antibody incubations were performed at room temperature for 1 hr with agitation. Chemiluminescence detection was performed using the Immun-Star™ WesternC™ chemiluminescent kit (Bio-Rad) and the Molecular Imager® VersaDoc™ MP 4000 system (Bio-Rad). All antibodies were diluted in TBST 3% nonfat dry milk. The secondary antibody was used at a dilution of 1:25,000. H1850-10, 3E5, 12H12, and anti-transferrin were used at 1:1,000, 1:500, 1:100, and 1:1,000 dilutions, respectively.

Results and Discussion

Use of the Criterion Stain Free System in Evaluating Protein Transfer

Since the Criterion Stain Free system enables quick and easy protein visualization without staining, it constitutes a fast, effective, and easy method for assessing the quality of protein separation both prior to and subsequent to protein transfer onto the membrane. Prior to transfer, the quality of protein separation is traditionally evaluated either by running prestained protein standards in parallel to protein samples or by staining a replicate of the gel to be transferred. Similarly, the uniformity and overall effectiveness of transfer of protein from a gel to a membrane can be evaluated by looking at the transfer of prestained standards to the membrane, by reversible staining of the blot with Ponceau S reagent, or by assessing the amount of proteins remaining in the gel after transfer by CBB staining.

These methods are not very sensitive or precise and are time consuming. In contrast, the Criterion Stain Free system offers direct visualization of proteins both in the gel and on the membrane (Figure 1) without any intervening staining steps. After transfer, both the membrane and gel can be placed in the imager (independently) to see what remained in the gel and what transferred to the membrane (Figure 1).

To demonstrate the utility of the Criterion Stain Free system in assessing transfer efficiency, a serial dilution of human hemoglobin was loaded into the wells of a Criterion Stain Free gel. Before blotting, the gel was imaged using the Criterion Stain Free system to assess the quality of the electrophoretic run. After blotting, both the membrane and the gel were imaged in the Criterion Stain Free imager to assess the quality of protein transfer across all lanes.

The sequence of images presented in Figure 1 shows that the Criterion Stain Free system can be used to rapidly assess protein transfer on a membrane prior to performing immunodetection. Because of its high sensitivity, the Criterion Stain Free system can detect minute amounts of proteins that remain in the gel after transfer. Thus, transfers can be rapidly optimized by tracking their efficiency. While nonuniform protein transfer due to uneven current distribution or other factors may occur, the quantitation feature of the Criterion Stain Free system enables researchers to take into account uneven transfer for more precise quantitative immunodetection.

Evaluation of Compatibility of the Criterion Stain Free System with Immunodetection Using Monoclonal Antibodies

The Criterion Stain Free system uses a UV-induced modification of the Trp residues within the proteins to generate a fluorescent signal. These modifications may affect the protein recognition by antibodies, particularly when the affected Trp is in an epitope. To demonstrate the applicability of this system for western blotting, we examined the effects of the Trp modification on recognition by monoclonal and polyclonal antibodies.

Human hemoglobin β-chain and two monoclonal antibodies (mAb) raised against a 13-amino-acid peptide in hemoglobin were used to test antigen recognition. Epitope mapping of the binding region for the two antibodies showed that each recognizes an epitope containing a Trp residue. One of the epitopes (shown in bold type), recognized by mAb 3E5, has a Trp (W) at the carboxyl end (KSA**VTALW**GKVNV), and the other, 12H12, has the Trp residue in the middle (KSAVT**ALWGK**VNV). Competitive binding experiments using alanine-substituted peptides demonstrated that the Trp residue was of moderate consequence for recognition by 3E5 and that it was of critical consequence to binding by 12H12 (Roger Walker, personal communication). A mAb generated against whole human hemoglobin with an unmapped epitope was used as a control. There is a high probability that this mAb does not recognize an epitope containing Trp, since there are only two tryptophans in the 147 amino acids composing the β-globin protein.

In the first experiment, human hemoglobin samples were prepared in sample buffer containing BSA as a carrier, separated by electrophoresis on Criterion Stain Free gels, and blotted to Pall FluoroTrans membranes. Before blotting, one gel was imaged using the Criterion Stain Free system to induce fluorescence and assess the quality of the electrophoretic run. Both gels were blotted and the blots obtained from the activated and nonactivated gels were probed with the 3E5 and 12H12 mAbs.

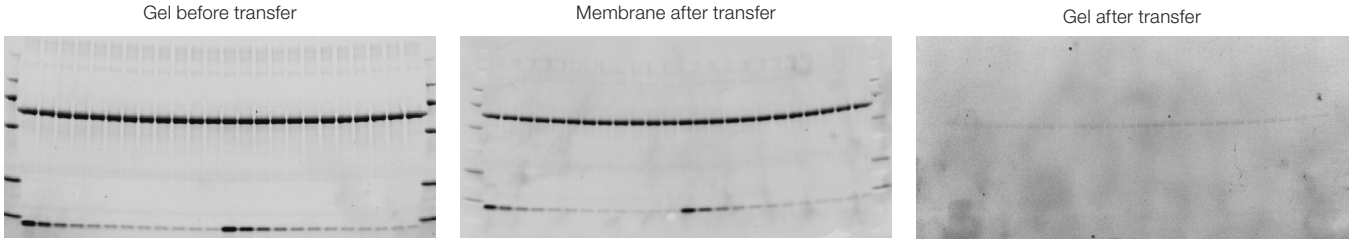

Fig. 1. Assessment of protein transfer using the Criterion Stain Free gel imaging system. Images of the gel before and after transfer and of the membrane after transfer were taken using the Criterion Stain Free imager. Serial 1:2 dilutions of hemoglobin (starting quantity, 80 ng), with 1.8 µg of BSA/lane as a carrier (top band), were electrophoretically separated on a 4–20% 26-well Criterion Stain Free gel.

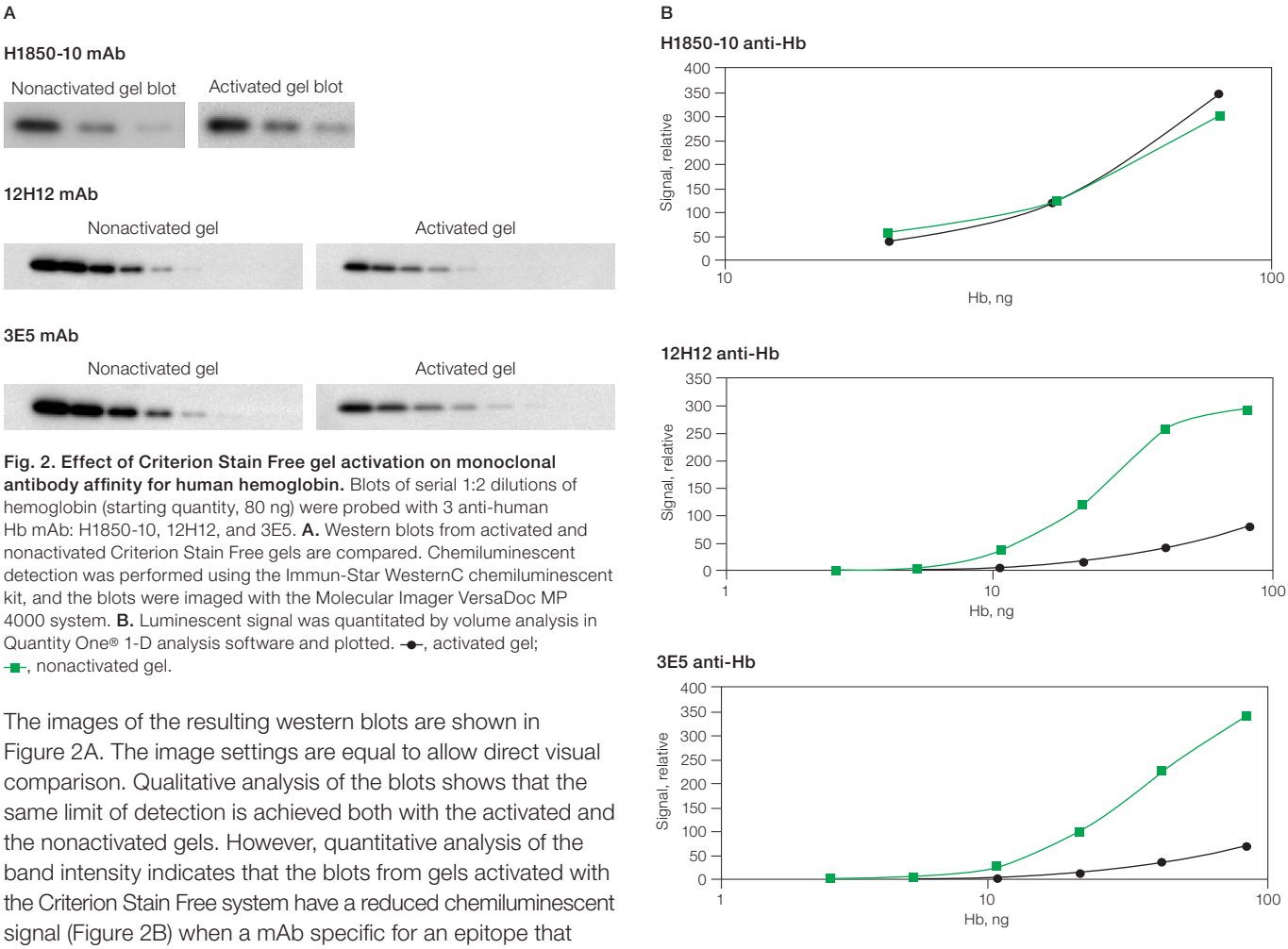

Fig. 2. Effect of Criterion Stain Free gel activation on monoclonal antibody affinity for human hemoglobin. Blots of serial 1:2 dilutions of hemoglobin (starting quantity, 80 ng) were probed with 3 anti-human Hb mAb: H1850-10, 12H12, and 3E5. **A.** Western blots from activated and nonactivated Criterion Stain Free gels are compared. Chemiluminescent detection was performed using the Immun-Star WesternC chemiluminescent kit, and the blots were imaged with the Molecular Imager VersaDoc MP 4000 system. **B.** Luminescent signal was quantitated by volume analysis in Quantity One® 1-D analysis software and plotted. –●–, activated gel; –■–, nonactivated gel.

The images of the resulting western blots are shown in Figure 2A. The image settings are equal to allow direct visual comparison. Qualitative analysis of the blots shows that the same limit of detection is achieved both with the activated and the nonactivated gels. However, quantitative analysis of the band intensity indicates that the blots from gels activated with the Criterion Stain Free system have a reduced chemiluminescent signal (Figure 2B) when a mAb specific for an epitope that contains a Trp residue is used. Mean reduction of intensities of 79% and 78% were seen for all dilutions of the 12H12 mAb and 3E5 mAb, respectively. This suggests that chemical modification of Trp induced by UV exposure during the activation step of the Criterion Stain Free imaging process interferes with recognition by antibodies when the epitope contains Trp. However gel activation does not inhibit binding of the H1850-10, a mAb that was raised against the whole β-globin protein, and that is unlikely to have Trp in its epitope. This control mAb had a mean signal reduction of only 7% when averaging data over 16 replicate antigen dilutions across two individual blots. This value is well within typical random deviation between blots. The reduction of signal intensity for mAbs that have tryptophan in their epitopes has two possible explanations. One explanation is that the modified tryptophan prevents antibodies from binding completely, and only a subset of the tryptophans are actually modified. The second possibility is that the binding is not completely blocked but that affinity for the site is reduced, with a commensurate reduction in signal intensity. Regardless of the mechanism of interference, the limit of detection was still 10 ng with or without activation of the gels.

Evaluating Compatibility with Immunodetection Using Polyclonal Antibodies

Because of their specific recognition of a single epitope, binding of monoclonal antibodies is more likely to be affected by the gel activation process than is the binding of polyclonal antibodies. To verify this, recognition of transferrin by polyclonal anti-transferrin antibodies was monitored. Serial dilutions of human transferrin were separated by electrophoresis on two duplicate Criterion Stain Free gels. One gel was activated prior to transfer and imaged using the Criterion Stain Free system, while the other one was transferred without preactivation. Transferrin was detected using rabbit anti-human transferrin antibodies (1:1,000).

Figure 3A shows the image of the blots obtained from the activated and nonactivated gels. Quantitation of the signal intensity using the Molecular Imager VersaDoc MP 4000 system (Figure 3B) shows that the recognition of transferrin by the human anti-transferrin polyclonal antibodies is not affected by gel activation and that the UV activation does not affect specificity.

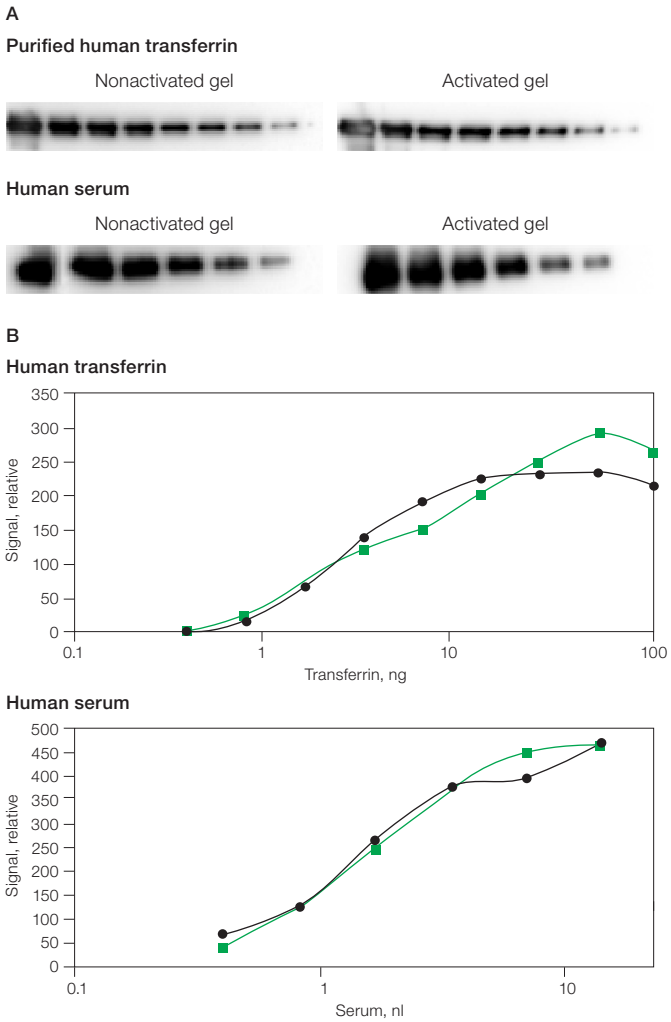

Fig. 3. Effect of Criterion Stain Free gel activation on polyclonal antibody affinity for proteins. A. Western blots from activated and nonactivated Criterion Stain Free gels are compared. Blots of serial 1:2 dilutions of transferrin protein (starting quantity, 100 ng) and human serum (starting volume, 12.5 nl) were probed with anti-human transferrin polyclonal antibodies. Chemiluminescent detection was performed using the Immun-Star WesternC chemiluminescent kit, and the blots were imaged with a Molecular Imager VersaDoc MP 4000 system. **B.** Chemiluminescent signal was quantified with Quantity One 1-D analysis software and plotted. –●–, activated gel; –■–, nonactivated gel.

Conclusions

The Criterion Stain Free gel imaging system is a fast, reliable, and sensitive tool to visualize proteins on polyacrylamide gels and on membranes. It offers a convenient alternative to the time-consuming Coomassie staining method. With the Criterion Stain Free system, it is possible to quickly visualize and quantify proteins after electrophoretic separation prior to transferring them to a membrane. Before immunodetection, the efficiency of the transfer can be easily monitored by imaging the membrane and the gel after transfer. Because the Criterion Stain Free system involves chemical modification of Trp residues, the binding of mAbs specific for epitopes containing a Trp residue may be affected. However, since Trp is typically between 1% and 2% of the amino acid residues in most proteins, most antibodies will not be affected by Trp modification. Examples of no change in western blotting chemiluminscent response were seen for both monoclonal and polyclonal antibodies to proteins transferred from an activated Criterion Stain Free gel, demonstrating the general compatiblity of Criterion Stain Free gels with western blotting applications.

References

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