Rapid Validation of Purified Proteins using Criterion Stain Free™ Gels

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Introduction

Affinity chromatography is a widely used protein purification method. It has the advantages that it can be made selective for a target molecule of interest and can be readily scaled to meet the needs of an experiment. Chromatographic separations are usually monitored by spectrometry at 280 nm as the fractionated protein mixtures flow through a flow cell at the end of a column. Monitoring separations in this manner is useful for determining when a wash or elution is complete and for monitoring column separation efficiency, but offers little value for validating the final sample purity. Polyacrylamide gel electrophoresis (PAGE) is usually the method of choice for identifying which fractions have material sufficiently pure for use in downstream applications. PAGE has the advantages that it can test multiple fractions at a time, is highly sensitive, and is information rich. However, the process is time consuming, taking several hours before results are available. It can also suffer from loss of information when background stain is not washed away completely or when stained proteins within the gel are overly destained and intensity is lost.

Modern automated chromatography systems, such as the Profinia™ protein purification system (Bio-Rad Laboratories, Inc.), can complete an affinity purification protocol in as little as 30 min. With PAGE taking several hours, it becomes the rate-limiting step in the effort to determine if a protein purification experiment has been successful. There have been efforts to develop stains that accelerate the gel staining and destaining process such as Bio-Safe™ Coomassie stain (Bio-Rad) and InstantBlue stain (Expedeon Protein Solutions). While these stains have greatly reduced the time it takes to stain a gel, it still takes significant time to remove the background of these dyes to a level where reliable quantitative data may be obtained from the gels. Furthermore, residual background dye in these gels may obscure the visualization of impurities that are present. Here we present a new technology that dramatically reduces the time required to detect and quantify proteins in a gel, and improves the user's ability to reproducibly validate an affinity purification procedure.

A new PAGE chemistry, Criterion Stain Free gels (Bio-Rad), which allows for protein visualization in polyacrylamide gels within 5 min of completing the electrophoretic sample separation, has become commercially available.

Materials and Methods

Chromatography

Samples consisted of a His-tagged protein (Bio-Rad) that was purified using the Profinia protein purification system fitted with a 5 ml Profinity™ IMAC resin cartridge (Bio-Rad). Fractions were pooled and labeled as: flow-through, wash-1, wash-2, and eluent. Eluted samples were stored at –80°C until analysis.

Gel Electrophoresis

Aliquots of each IMAC elution fraction (flow-through, wash-1, wash-2, and eluent) were prepared for gel electrophoresis by adding Laemmli sample buffer. Samples were loaded onto Criterion Stain Free 4–20% Tris-HCl gels as follows: lysate 7.5 µl, flow-through 15 µl, wash-1 15 µl, wash-2 15 µl, and eluent 15 µl. All gels were run at 200 V constant for 60 min using a Criterion™ Dodeca™ cell (Bio-Rad) and Tris/Glycine/SDS running buffer (Bio-Rad).

Imaging

Following electrophoresis, gels were activated on a Gel Doc™ EZ imaging system (Bio-Rad) for 5 min, and then imaged with the Optimize for Intense Bands option selected.

Gel Staining

Following the stain-free imaging process, the gels were stained by one of three methods as described below:

- Bio-Safe Coomassie stain — Gels were rinsed three times for 5 min each with water and then stained with Bio-Safe Coomassie stain for 1 hr. The gels were destained in water for 30 min and imaged. The gels were further destained for an additional 24 hr with two changes of water and imaged again
- InstantBlue stain — Gels were stained with InstantBlue stain for 1 hr and imaged. The gels were then destained for 24 hr with two changes of water and imaged again
- Coomassie Brilliant Blue (CBB) R-250 stain — Gels were stained with CBB R-250 (Bio-Rad) for 1.5 hr followed by destaining four times for 30 min each with CBB R-250 destaining solution (Bio-Rad) and imaged. The gels were further destained for 24 hr with two changes of destaining solution and imaged again

Imaging of Coomassie-stained gels was performed on a Gel Doc EZ imaging system fitted with a white light conversion screen. The Gel Doc EZ system was controlled through Image Lab™ 2.0.1 software with the Optimize for Intense Bands and Criterion gel imaging size options selected.

Data Processing

All images were processed and analyzed using Image Lab 2.0.1 software. Images were cropped to the region of interest and then lanes were automatically detected. Bands were detected with a sensitivity setting of 75 for Criterion Stain Free gels and 50 for Coomassie-stained gels. For baseline detection the lane disk size was set to 99 (minimum baseline correction) for Criterion Stain Free gels and as needed for Coomassie-stained gels, usually 10 (default) or smaller.

Results

Gel electrophoresis using Criterion Stain Free gels was used to assess the purity of a His-tagged protein following Profinity IMAC chromatography on a Profinia protein purification system. Following stain free imaging using the Gel Doc EZ imager, the gels were stained with Bio-Safe, instantBlue or CBB R-250 Coomassie stains according to manufacturer's directions. Destaining of the Bio-Safe Commassie and CBB R-250 stained gels was performed according to the manufacturer's fast destaining method. Following destaining, the gels were imaged and compared to the stain free gel results initially obtained using the Gel Doc EZ system.

Buffer Use and Time to Results

Use of the Gel Doc EZ system resulted in significant time and materials savings relative to staining with Coomassie stains. The Criterion Stain Free gels were removed from their cassettes, wetted, and imaged. There were no time consuming washing or staining steps performed to prepare the Criterion gels for imaging, resulting in significant time savings. Furthermore, no chemicals were required to stain or destain the gels thereby eliminating the cost associated with purchase and disposal of these chemicals. On the other hand, all three of the Coomassie stains required at least an hour to stain in order to obtain quantitative results, and further time to destain. The CBB R-250 had the added disadvantage of generating hazardous waste that required disposal. Coomassie-stained gels required multiple staining and destaining steps, which took between 1 and 3.5 hr (Table 1), depending on the staining procedure. Furthermore, the staining procedures required extended rocking in buffer and gel transfers, which in a few cases caused gels to crack or tear.

Table 1. Breakdown of time required to image Criterion Stain Free, Bio-Safe, InstantBlue, and Coomassie R-250 stained gels.

	Step	Time, min			
1	Chromatography	30			
2	PAGE preparation	15			
3	PAGE (200 V)	60			
	Subtotal	1 hr 50 min			
		Criterion Stain Free	**Bio-Safe Stain**	**InstantBlue**	**Coomassie R-250**
4	Rinse	0	15	0	0
5	Staining	5	60	60	90
6	Destaining	0	30*	0*	120*
	Subtotal	5 min	1 hr 45 min	1 hr	3 hr 30 min
	Total	1 hr 55 min	3 hr 45 min	2 hr 45 min	5 hr 20 min

* Fast-destaining method

Background Signal in Stained Gels

Destaining gels using quick destaining methods resulted in incomplete removal of stain and therefore, a high background signal in the Coomassie-stained gels. This high background made it difficult to detect weak bands in the gels. Additionally, the background stain levels were not uniform across the gels, which made it difficult to differentiate between the baseline and bands. This was due partially to the expected differential diffusion rates within the gradient gels resulting in a greater retention of the stain as the acrylamide percentage increased. While Image Lab software has the ability to automatically apply lane background corrections to improve data quality, it cannot restore data that is obscured or lost due to high background signal. For Coomassie-stained gels there is often not enough information to determine where the boundary between baseline and protein bands should be, due to the high background (Figure 1). The location of this boundary determines the accuracy of band quantitation.

The Criterion Stain Free gels had virtually no background signal and the background that was present was uniform across the gel. Because the agent used to stain the Criterion Stain Free gels is embedded in the gel matrix and is uniform throughout the gel, there are no diffusion effects, therefore the staining process was observed to occur uniformly across the gels. This resulted in bands that were clearly visible in the gels with little ambiguity between what constituted a band and what did not (Figure 1).

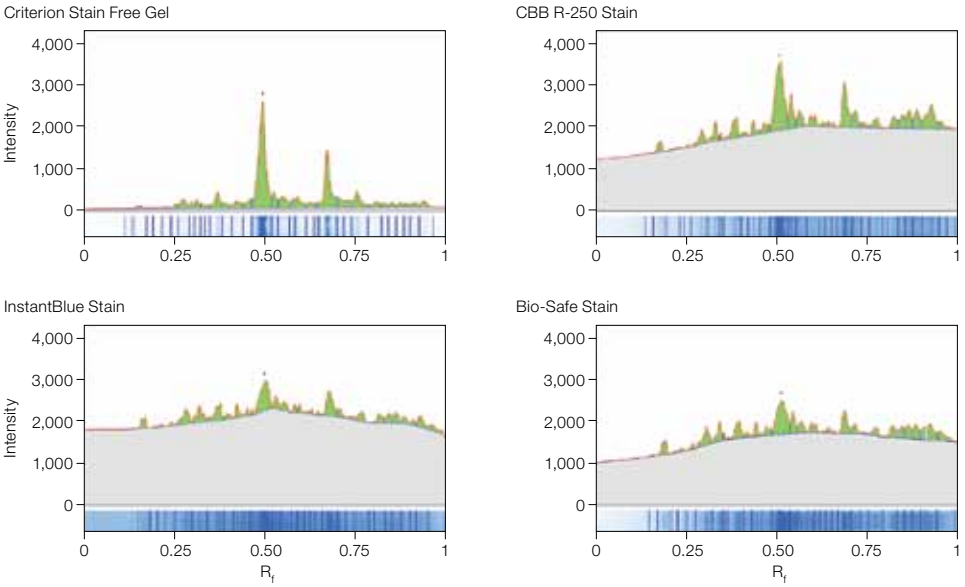
Quantitation

Four gel staining methods were examined for use in determining the relative abundance of the target protein in samples. All four staining methods tested gave reproducible results for the target protein (Table 2). However, the estimated amount of target protein relative to total protein (band %) varied depending upon the staining method. This was due to errors from residual background stain in the gel as well as the differential staining affinity of the dyes for proteins in the gel.

The low background signal in Criterion Stain Free gels made it much easier to distinguish between low level impurities in the samples and image baseline noise. High background in the Coomassie-stained gels made it difficult to identify weak bands. This was especially true for the CBB R-250 stained gels where many of the weak bands were not observed at all. Extended destaining (24 hr) did not improve detection of the weak bands even though the background was reduced (Figure 2). All staining methods appeared to give similar reproducibility when quantitating proteins, as long as the destaining times remained consistent.

CBB R-250, Bio-Safe, and InstantBlue stains were observed to give reproducible quantitative results with similar quality to the Criterion Stain Free gels (Table 2). However, the Coomassie-stained gels, with the fast destaining method, differed from the Criterion Stain Free gels in that they gave lower estimates for the quantity of His-tagged protein in the

A. Cell Lysate



B. Eluent

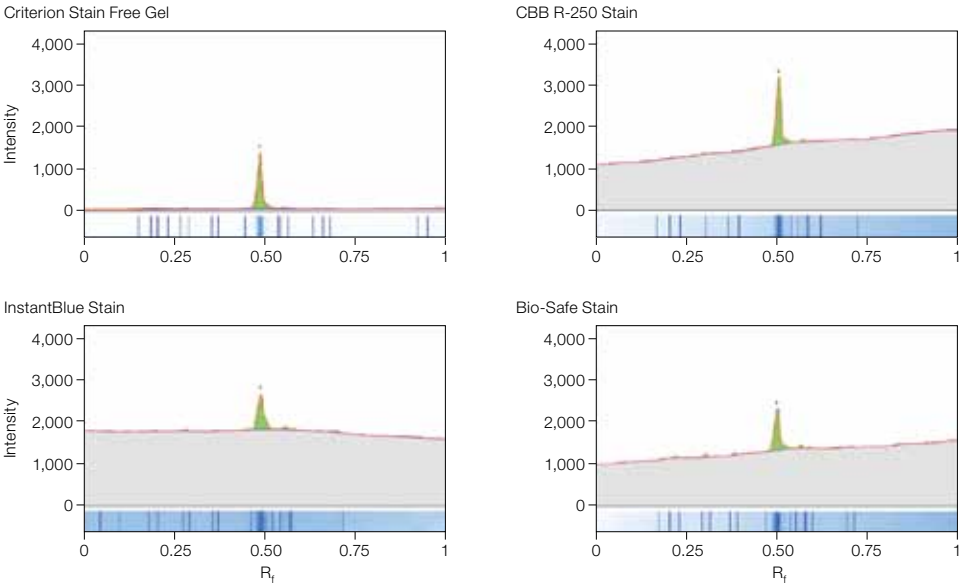


Fig. 1. Baseline comparison between Criterion Stain Free and Coomassie-stained gels. Protein migration profiles were analyzed using Image Lab software. **A**, analysis of cell lysate. **B**, analysis of eluent fraction containing the purified protein. Stained gel image is shown below the migration profile. Vertical blue lines indicate upper and lower limit of protein peaks. Criterion Stain Free gels consistently show low background compared to the other staining methods used.

Table 2. In-gel protein quantitation* comparison between Criterion Stain Free and Coomassie-stained gels.

Set	Stain, Destain Time	Lysate, %	Flow-through, %	Wash-1, %	Wash-2, %	Eluent, %
1	Stain Free, 0 hr	34.7 ± 1.2	4.2 ± 0.2	6.8 ± 0.2	87.4 ± 2.3	88.4 ± 3.1
1	Bio-Safe stain, 0.5 hr	19.8 ± 1.5	3.5 ± 0.3	5.3 ± 0.4	73.7 ± 4.5	78.9 ± 5.1
1	Bio-Safe stain, 24 hr	21.6 ± 1.7	3.9 ± 0.8	5.8 ± 0.8	84.9 ± 8.5	88.5 ± 2.6
2	Stain Free, 0 hr	32.6 ± 1.0	4.0 ± 0.5	6.9 ± 0.2	84.7 ± 3.7	89.3 ± 0.8
2	CBB R-250, 2 hr	27.5 ± 1.6	4.6 ± 1.0	6.9 ± 0.2	100 ± 0.0	90.4 ± 2.7
2	CBB R-250, 24 hr	33.3 ± 2.3	3.7 ± 0.5	7.0 ± 0.6	100 ± 0.0	96.7 ± 1.4
3	Stain Free, 0 hr	33.5 ± 2.0	4.1 ± 0.3	6.8 ± 0.8	83.9 ± 3.2	89.9 ± 0.9
3	InstantBlue, 0 hr	20.0 ± 2.5	3.1 ± 0.6	5.5 ± 1.3	91.1 ± 8.7	82.1 ± 3.7
3	InstantBlue, 24 hr	22.3 ± 2.9	4.4 ± 0.7	5.7 ± 1.0	98.2 ± 2.7	88.8 ± 3.5

* His-tagged protein quantitation is reported as % of total protein ± standard deviation, N=5.

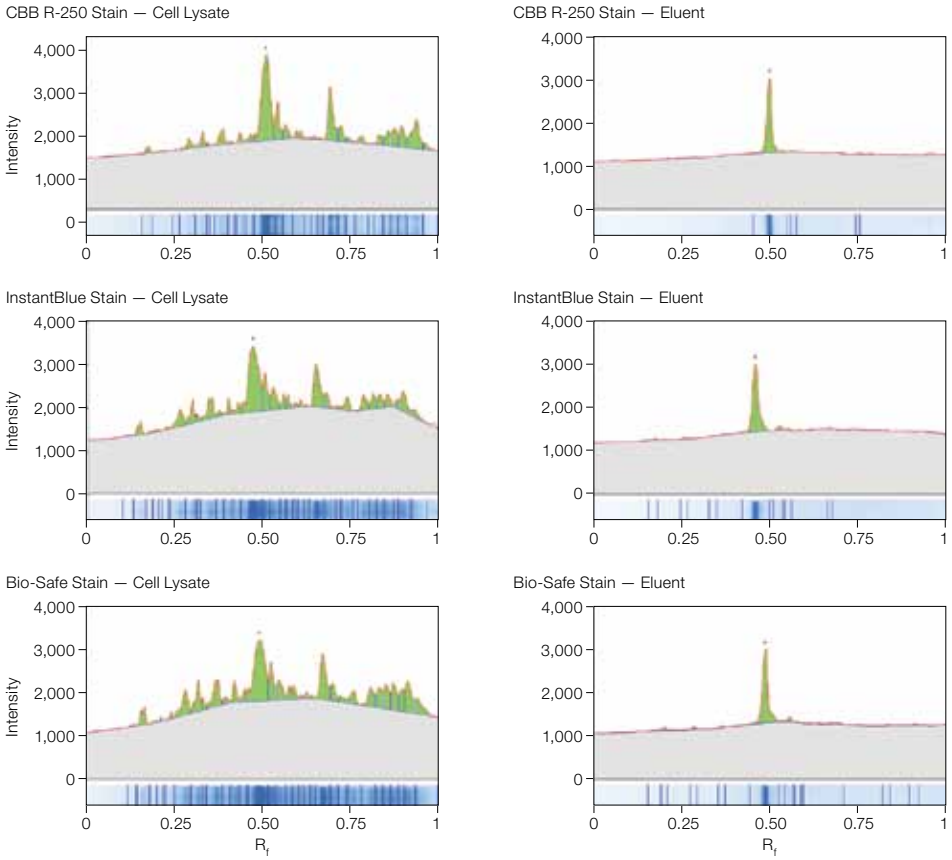


Fig. 2. Coomassie-stained gels after 24 hours of destaining. Analysis of eluent faction containing the purified protein. Protein migration profiles were analyzed using Image Lab software after 24 hours of gel destaining.

lysate and in the purified protein. This is most likely due to the differential staining abilities between the Coomassie dye and the stain-free method.

After 24 hours of destaining, the background staining levels were observed to significantly decrease for the Coomassie-stained gels. Unfortunately, this also resulted in the loss of a few weak bands in the gel. As shown in Table 2 the resultant eluent purity estimate generally increased with longer destaining times. Therefore, although long destaining times reduce gel background signal, they are counterproductive since they also result in the loss of weak bands. Stain-free gel imaging has the advantage that it has both a low background and allows weak bands to be observed.

Conclusion

The Gel Doc EZ imaging system, used in combination with stain-free gels, increases productivity, reduces costs, and improves results when performing gel electrophoresis and imaging. The low background images produced by Criterion Stain Free gels give superior results for non-quantitative and quantitative gel analysis. Weak bands are easier to observe and quantitate using the Criterion Stain Free system than with Coomassie stains since they are not obscured by high background signals. This makes data processing and analysis easier and less time consuming while producing consistent results. Furthermore, because Criterion Stain Free gels do not require post-electrophoresis staining or destaining they reduce the costs of materials and waste disposal. The Criterion Stain Free system also saves time with 5 min visualization and straightforward analysis.

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