

## Experion™ Automated Electrophoresis System: Enhancing Sensitivity in SDS-Protein Electrophoresis

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### Introduction

The development, manufacture, and quality control of protein therapeutics require state-of-the-art analytical techniques to elucidate protein structure and to assure product purity, homogeneity, and stability (Ma and Nashabeh 2001). SDS-PAGE has been the benchmark methodology for monitoring impurities and assessing consistency in the manufacture of biologics, with silver-staining techniques required to detect minor impurities. However, SDS-PAGE is time consuming and requires the use of toxic reagents. An automated and quantitative alternative to SDS-PAGE has long been desired. The advent of capillary electrophoresis (CE) provided a solution (Wehr et al. 1998). The entangled polymer sieving systems used in CE exhibit separation power similar to that of SDS-PAGE, and automated injection, on-tube detection, and peak integration permit unattended analysis of multiple samples. However, the UV absorbance detectors employed in most commercial CE systems have insufficient sensitivity for detection of low-level impurities. By prelabeling proteins with a fluorescent tag and using laser-induced fluorescence (LIF) detection, CE can achieve sensitivity comparable to silver stains, and this method is currently in use in the biopharmaceutical industry (Hunt and Nashabeh 1999).

A drawback of both CE-UV and CE-LIF methods for separation of SDS-proteins is the requirement for extensive capillary preparation before each injection, which increases analysis time and reduces throughput. Automated microscale electrophoresis systems provide a rapid and convenient alternative to conventional CE instruments for the separation of SDS-proteins. The Experion automated electrophoresis system, for example, is a chip-based separation system that can process ten samples in 30 min. The protein mass range covered by the system is 10–260 kD, and the resolution is similar to that of a 4–20% gradient gel. The system employs a dynamic staining-destaining chemistry with fluorescence detection that provides sensitivity comparable to colloidal Coomassie staining. To enhance the sensitivity of the Experion system for detection of low-level impurities in preparations of biotechnology products, a method for prelabeling proteins with a fluorescent dye was investigated.

The effect of the dye:protein molar ratio was also studied. The method also incorporates modifications in the Experion system sample preparation procedure to enhance injection efficiency and a modification in the Experion gel staining procedure to reduce background fluorescence of the Experion Pro260 stain. The method uses the Experion system with no hardware or software changes and employs the Experion Pro260 chip and components of the Experion Pro260 analysis kit.

### Methods

The workflow for protein labeling and sample preparation for analysis on the Experion system is shown in Figure 1.

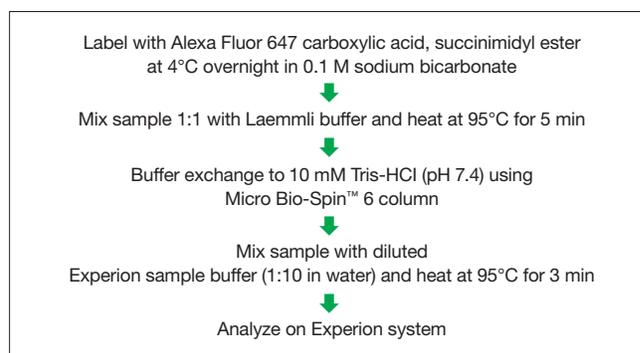


Fig. 1. Workflow for protein labeling and sample preparation.

### Protein Labeling

Prior to labeling, samples were buffer exchanged into labeling buffer (0.1 M sodium bicarbonate) using Micro Bio-Spin 6 columns equilibrated with 0.1 M sodium bicarbonate. Proteins were labeled with Alexa Fluor 647 carboxylic acid, succinimidyl ester dye (Invitrogen Corporation), which has an excitation wavelength that closely matches the Experion laser wavelength (635 nm excitation, 675 nm emission). A stock solution of dye was prepared in dimethyl sulfoxide (DMSO) at a concentration of 1.4 mg/ml, and a dye working solution was prepared by diluting the stock solution to 350 µg/ml in DMSO. BSA or porcine IgG (Sigma-Aldrich, Inc.) were used as protein standards, and the final protein content of antibody samples was estimated by interpolating absorbance at 280 nm against a standard curve prepared with BSA. These standards were prepared in labeling buffer. Proteins were mixed with aliquots of dye stock or working solution to provide a dye:protein

molar ratio of 1:1, 5:1, 10:1, or 100:1. Dye-protein solutions were incubated overnight at 4°C.

### Sample Preparation

Prelabeled protein samples were mixed with an equal volume of Laemmli sample buffer with 5% β-mercaptoethanol and heated for 5–7 min at 95°C. Samples were then buffer exchanged into 10 mM Tris-HCl (pH 7.4) using Micro Bio-Spin 6 columns. Sample buffer was prepared by diluting Experion Pro260 sample buffer containing 0.03% β-mercaptoethanol 10-fold with distilled water. For analysis, 4.5 μl of diluted sample buffer was mixed with 9 μl of prelabeled sample, heated at 95°C for 5 min, and loaded directly onto the Experion Pro260 chip.

Unlabeled protein samples and the Experion Pro260 ladder were prepared according to the Experion Pro260 analysis kit instructions. Briefly, 4 μl of ladder or sample was mixed with 2 μl of Experion Pro260 sample buffer containing 0.03% β-mercaptoethanol, heated at 95°C for 5 min, and then diluted with 84 μl of deionized water and loaded onto a primed chip.

### Preparation of Diluted Gel Stain

For experiments using diluted gel stain, the reagent was prepared by adding 2 μl of Experion Pro260 stain and 18 μl of 6.75% SDS (w/v in DMSO) to one tube of Experion Pro260 gel. The diluted gel-stain mixture was vortexed and filtered according to the Experion Pro260 analysis kit protocol.

### Experion System Electrophoresis and Analysis

Priming of the Experion Pro260 chip with gel stain and loading of the chip with gel stain, samples, and ladder were performed according to the Experion system instructions. Electrophoresis and analysis were performed using the Experion Pro260 analysis kit protocol.

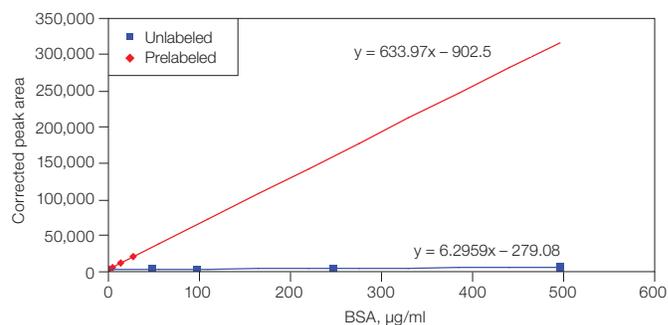
## Results and Discussion

### Comparison of Experion System Analysis of Unlabeled and Prelabeled Proteins

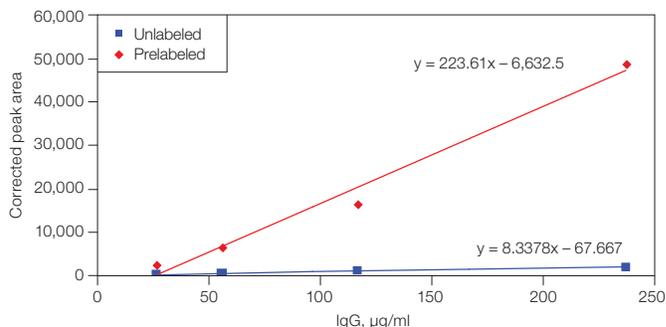
To determine the sensitivity enhancement obtained by prelabeling proteins with Alexa Fluor 647, unlabeled and prelabeled samples of BSA and porcine IgG were prepared over an approximately 1,000-fold concentration range using 2-fold serial dilutions. Both proteins were pre-labeled using a dye:protein molar ratio of 1:1. Sensitivity was measured as the slope of the corrected peak area vs. concentration plot. For BSA, sensitivity enhancement was 100-fold (Figure 2). For IgG, sensitivity was calculated using the heavy chain peak and was found to be enhanced 27-fold relative to unlabeled IgG heavy chain (Figure 3). An overlay of the electropherograms of the unlabeled and prelabeled IgG samples is shown in Figure 4. The higher sensitivity gain for BSA vs. IgG heavy chain may reflect differences in dye incorporation due to the higher lysine content in BSA.

### Effect of Dye:Protein Molar Ratio on Sensitivity and Peak Width

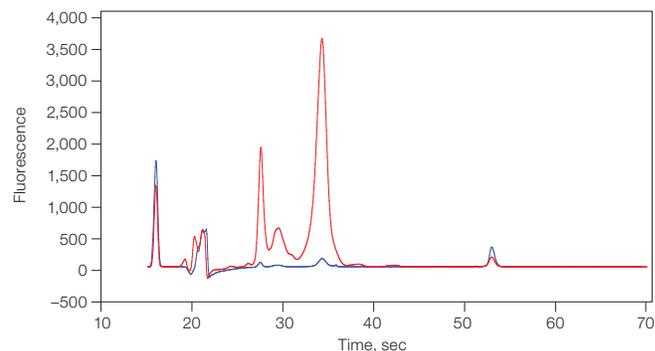
To determine the effect of the dye:protein molar ratio on sensitivity and peak width, BSA was pre-labeled with varying



**Fig. 2. Sensitivity comparison of unlabeled and prelabeled BSA.** Sensitivity is increased 100-fold for prelabeled BSA.



**Fig. 3. Sensitivity comparison of unlabeled and prelabeled porcine IgG.** Sensitivity, calculated using the IgG heavy chain peak, is increased 27-fold for prelabeled IgG.



**Fig. 4. Overlay of electropherograms of unlabeled (—) and prelabeled (—) porcine IgG (35 μg/ml).**

amounts of Alexa Fluor 647 and analyzed on the Experion system. Results demonstrate that a more than 500-fold increase in sensitivity can be achieved at a dye:protein molar ratio of 1:100 (Table 1). However, significant peak broadening

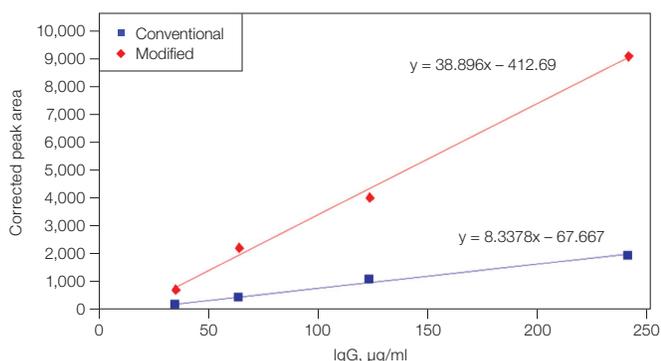
**Table 1. Effect of dye: protein molar ratio on peak width for prelabeled BSA.**

Dye: Protein Molar Ratio	Protein Concentration, μg/ml	Sensitivity Increase Relative to Unlabeled BSA	Peak Width at Half Height, sec
1:1	7.4	99x	1.30
1:10	7.0	344x	1.53
1:100	6.8	539x	2.09

is observed using ratios greater than 1:1. In applications where minor contaminants are poorly resolved from major components, the loss in resolution could offset the gain in sensitivity.

#### Contribution of Sample Preparation Procedure to Sensitivity Enhancement

The amount of protein sample introduced into the separation channels by electrokinetic injection depends on the concentration of the sample, as well as on the stacking effects due to differences in ionic strength between the sample and the gel. When using the Experion system, the conventional sample preparation procedure requires dilution of 4  $\mu$ l of the protein sample and 2  $\mu$ l of Experion Pro260 buffer in 84  $\mu$ l of water prior to loading onto the gel, which results in a 22.5-fold dilution of the protein sample and a 45-fold dilution of the buffer's ionic strength. With the sample preparation procedure modified for high sensitivity, the protein sample is first diluted 2-fold (prior to the buffer exchange step using a Micro Bio-Spin 6 column) and then 9  $\mu$ l of the diluted protein sample is mixed with 4.5  $\mu$ l of Experion Pro260 buffer that has been diluted 10-fold with water. This results in an overall 3-fold dilution of the protein sample and a 30-fold dilution of the buffer's ionic strength prior to loading. Consequently, with the high-sensitivity procedure, the protein sample is 7.5-fold more concentrated than with the conventional procedure. Because the ionic strength of the sample is 1.5-fold higher with the high-sensitivity procedure, its loading efficiency is reduced proportionally. Taking into account these two factors, the high-sensitivity procedure should allow loading of five times more protein into the Experion system than the conventional procedure. To investigate this, IgG was prepared using the two procedures without addition of the Alexa Fluor dye. The results indicate that the modified sample preparation procedure contributes a 4.7-fold increase in sensitivity (Figure 5) as expected.



**Fig. 5. Contribution of sample preparation procedure to sensitivity enhancement.** Sensitivity, calculated using the IgG heavy chain peak, is increased 4.7-fold when using the modified procedure.

#### Effect of Gel Stain Dilution on Sensitivity

The fluorescent dye in the Experion Pro260 gel-stain mixture serves two purposes. At the beginning of an Experion system run, the signal from the dye is used to autofocus the Experion instrument optics. During analysis, the dye is part of a dynamic protein detection system. The dye (stain) partitions into SDS micelles, which complex with proteins in the sample, and dye fluorescence intensity is enhanced in the hydrophobic interior of the micelle. Reduction of background fluorescence from the stain is accomplished by introducing stain-free medium by electromigration into the region between the separation segment and the detection segment of the channel. This forms an SDS-free region adjacent to the migrating sample bands. Diffusion of free SDS into this region reduces the detergent concentration below the critical micelle concentration, releasing dye from unbound micelles. This reduces background fluorescence (the destaining process). However, some residual fluorescence from the free dye remains.

With Experion system analysis of pre-labeled proteins, the stain is no longer needed for sample detection, but is required for the focusing step, detection of the Experion ladder, and detection of the upper alignment marker. The drawback is that its presence in the gel contributes to background fluorescence. To reduce this background, a reformulated gel stain was prepared in which the stain concentration was reduced 10-fold, yet still allowed for instrument autofocusing. The noise reduction of the diluted gel stain caused an additional 3-fold increase in sensitivity (data not shown).

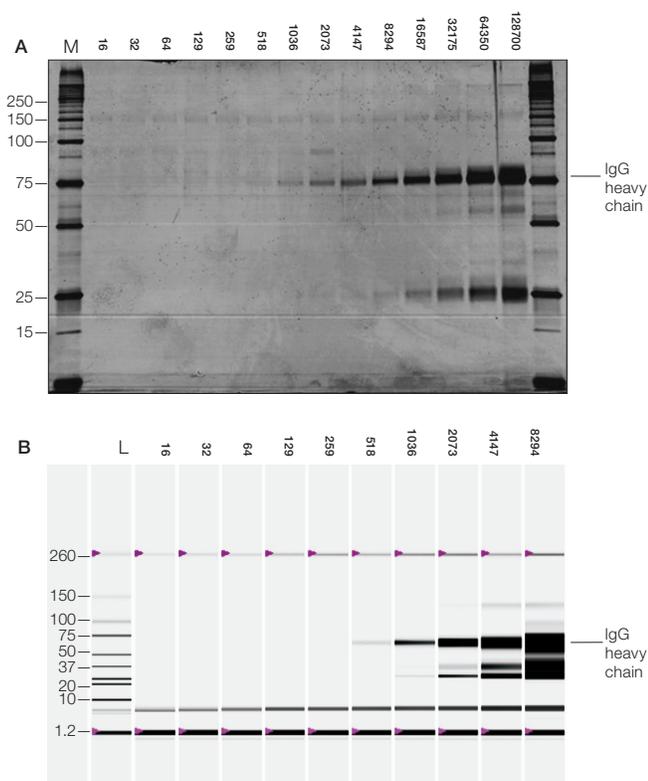
The contributions of prelabeling, sample preparation procedure, and stain dilution are summarized in Table 2.

**Table 2. Effect of procedure modifications on IgG detection sensitivity.**

Experion Sample Preparation Procedure	Sensitivity Increase
Conventional (without prelabeling)	1x
Modified	
Without prelabeling	4.7x
With prelabeling	27x
With prelabeling and Experion Pro260 stain dilution	80x

#### Comparison of Detection Limits of SDS-PAGE and Silver Staining to the Experion System

The detection limits of silver staining proteins on a polyacrylamide gel were compared with those of dye-labeled proteins that have been run on the Experion system. Two-fold serial dilutions of IgG were analyzed using both techniques, using the IgG heavy chain as a reference. For analysis on the Experion system, an aliquot of a 1 mg/ml stock solution of IgG was labeled overnight with Alexa Fluor 647 as described in Methods using a 10-fold molar excess of dye. The labeled IgG was heated in Laemmli buffer under reducing conditions and



**Fig. 6. Comparison of detection limits achieved with SDS-PAGE/silver staining and the Experion system.** **A**, silver-stained SDS-PAGE gel image of serial dilutions of IgG proteins; **B**, Experion virtual gel of diluted IgG samples that were pre-labeled with Alexa Fluor 647 and analyzed on the Experion system. Note that the IgG heavy chain band in the virtual gel from the Experion system can be easily detected at a concentration of 518 ng/ $\mu$ l, whereas it is barely visible on the silver-stained polyacrylamide gel. Lane M, molecular marker; lane L, Experion Pro260 ladder. Numbers indicate IgG concentration ( $\mu$ g/mL) in samples at loading of 6  $\mu$ l into the chip well.

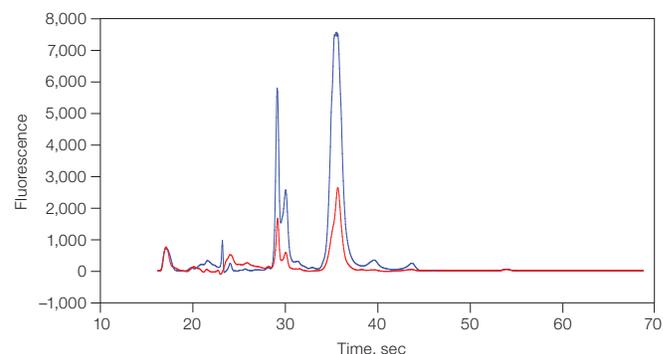
excess dye was removed using a Micro Bio-Spin 6 column. Serial dilutions of the dye-labeled IgG were prepared in water and analyzed with the modified sample preparation procedure using diluted Experion Pro260 gel-stain as described in Methods. For analysis with SDS-PAGE, an aliquot of the 1 mg/ml IgG stock solution was treated similarly, except that DMSO was used instead of Alexa Fluor dye, and the serial dilutions were prepared in Laemmli buffer. A 10  $\mu$ l aliquot of each dilution was electrophoresed on a 12.5% Criterion™ Tris-HCl gel using Precision Plus Protein™ standards as molecular mass markers. The gel was stained using the Dodeca™ silver stain kit according to the instructions provided with the kit. The gel image was acquired on the Molecular Imager® GS-800™ calibrated densitometer.

A comparison of the detection limits of the Experion system and SDS-PAGE is shown in Figure 6. Examination of the IgG heavy chain bands indicates that the highest dilution that produced visible bands with both techniques contained

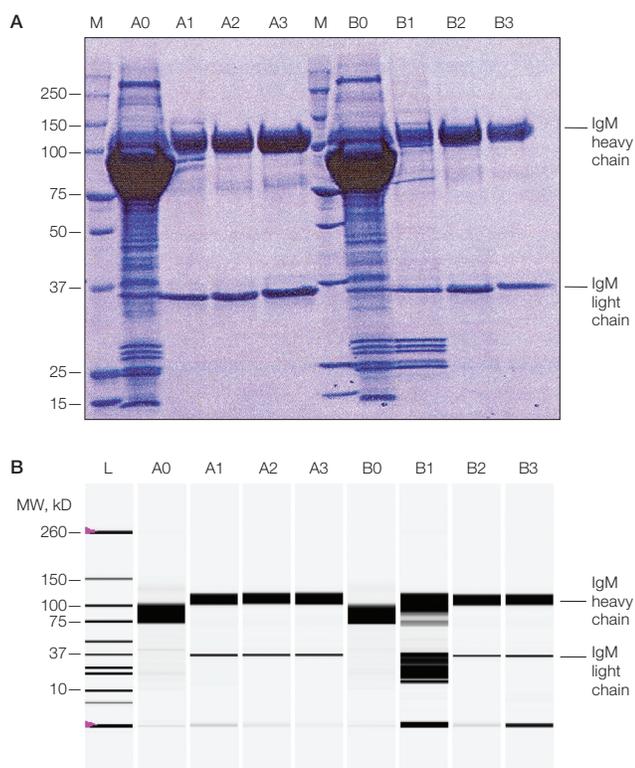
518 ng/ml of protein. However, the Experion system was able to easily detect the IgG heavy chain band at a concentration of 518 ng/ml, whereas the band was barely visible on the silver-stained polyacrylamide gel. Note that the detection limit for a gel-based electrophoresis assay is usually expressed in terms of mass. In contrast, the detection limit for a chip-based automated electrophoresis system, such as the Experion system, is expressed in terms of concentration because it is difficult to precisely calculate the mass injected into the separation chamber of the system. In this study, the detection limits for both SDS-PAGE and the Experion system are expressed in terms of the concentration of the sample solution. For example, a 10  $\mu$ l aliquot of the 518 ng/ml dilution contains 5.18 ng of IgG heavy chain.

#### Application to Monitoring Monoclonal Antibody Purification

SDS-PAGE, CE-UV, and CE-LIF are all currently used as analytical tools in the development and manufacture of monoclonal antibodies. These techniques are used in product and process development and in determination of product purity and lot-to-lot consistency (Hunt and Nashabeh 1999, Ma and Nashabeh 2001). Of particular importance is the use of silver staining in SDS-PAGE and CE-LIF for the detection of low-level impurities that may be product related (mass variants, proteolytic fragments) or nonproduct related (host cell impurities, environmental contaminants). Therefore, it was of interest to investigate the use of the Experion system with prelabeling for characterization of monoclonal antibodies. The electropherogram shown in Figure 7 compares the level of monoclonal antibody in a crude cell culture filtrate and following purification. The relative intensities of light and heavy chain peaks demonstrate a 3.5-fold enrichment of antibody after purification, and the use of the prelabeling technique allowed visualization of contaminants such as those migrating at 40 and 44 sec. Figures 8A and 8B compare SDS-PAGE to the Experion system with prelabeling for monitoring an IgM purification process. In this study, the efficacy of two different purification schemes was evaluated. In the first scheme (samples A0–A3 in both images), IgM in a cell culture



**Fig. 7. Overlay of electropherograms of monoclonal cell culture filtrate (—) and purified monoclonal antibody fraction (—).**



**Fig. 8. Comparison of SDS-PAGE and the Experion system with prelabeling for monitoring an IgM purification process.** Coomassie-stained SDS-PAGE gel image (A) and Experion virtual gel (B) of IgM samples. Lane M, molecular marker; lane L, Experion Pro260 ladder; lanes A0 and B0, cell culture filtrate; lanes A1–A3, peak fractions from cation exchange, anion exchange, and hydroxyapatite purification steps, respectively; B1–B3, peak fractions from hydroxyapatite, cation exchange, and anion exchange purification steps, respectively. For Experion system analysis, samples were pre-labeled with Alexa Fluor 647.

filtrate was purified by three sequential chromatographic steps in the following order: cation exchange, anion exchange, and hydroxyapatite. In the second scheme (samples B0–B3 in both images), IgM was purified using the same chromatographic modes but in the sequence of hydroxyapatite, cation exchange, and anion exchange. A Coomassie-stained SDS-PAGE gel of the cell culture filtrate and the peak fractions of the three sequential purification steps for each scheme is shown in Figure 8A. An Experion virtual gel of the same samples pre-labeled with Alexa Fluor 647 and run on the Experion system is shown in Figure 8B. The cell culture filtrate exhibits overloaded bands of antibody heavy and light chains and many impurity bands. Impurities are progressively removed in the three purification steps, with most impurities removed following the second step in both schemes. The Experion virtual gel of the hydroxyapatite fraction in the second scheme (B1 in Figure 8B) exhibited a higher level of impurities than the Coomassie-stained SDS-

PAGE gel of the same material. This may indicate a greater response of these components with the pre-labeled Experion procedure than with Coomassie stain.

## Conclusions

Prelabeling proteins with the fluorescent tag Alexa Fluor 647 prior to analysis with the Experion system can provide an 80- to 500-fold enhancement in sensitivity compared to the conventional Experion method. Sensitivity enhancement arises from the increased fluorescence of the tagged proteins combined with a modified sample preparation procedure. Total sensitivity gain depends on the dye:protein molar ratio, and is a trade-off between increased signal and peak broadening. The prelabeling method requires no changes to the Experion instrument hardware or software, and uses the reagents from the Experion Pro260 analysis kit with the addition of a simple dilution of the sample preparation buffer and stain. A comparison of band intensities of IgG heavy chain using the Experion system and SDS-PAGE demonstrates that the Experion system can achieve detection limits comparable to those of silver staining of proteins resolved on a polyacrylamide gel. Since the protein band intensity achieved with both techniques depends on the number of basic groups, the detection limits for both silver staining and Experion analysis varies for different proteins. This procedure extends the detection capability of the Experion system to trace components in biotechnology products such as contaminants in monoclonal antibody preparations.

## Acknowledgement

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