# Biologics Analysis Workflow™ Model Comparability Study for Changes to Validated cGMP SDS-PAGE Assays with the GS-900™ Calibrated Densitometer

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Tech Note

**Imaging**

Bulletin 6387

## Introduction

In this report, we present a model comparability study for the evaluation of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) assays using calibrated densitometry. SDS-PAGE and calibrated densitometry are standard techniques used to resolve and quantitate proteins for purity/impurity analysis. This workflow involves the separation of the proteins on a gel, staining, image acquisition, and quantitative analysis of the gel image. There are multiple steps within this workflow and each step must be optimized to deliver the best performance for the assay. It is critical that this process be reproducible, accurate, and sensitive to a variety of proteins.

Bio-Rad has developed the Biologics Analysis Workflow specifically for GMP labs evaluating the composition of biologics with SDS-PAGE and calibrated densitometry. The workflow and each of the steps it comprises deliver ease of use, reproducibility, and accuracy. The Biologics Analysis Workflow includes all products necessary to evaluate the composition of biologics: Criterion™ TGX™ Precast Gels, Precision Plus Protein™ Standards, QC Colloidal Coomassie Stain, the GS-900 Calibrated Densitometer, an instrument qualification/operation qualification (IQ/OQ) kit, and Code of Federal Regulation Title 21 (21 CFR) Part 11 compliant Image Lab™ Analysis Software. Each product in the workflow was designed and optimized with the others to meet the needs of biologics analysis better than other available methods.

Bio-Rad has an extensive history in this area with the GS-800™ Calibrated Densitometer, which has enjoyed long-standing acceptance for use in regulated current good manufacturing practices (cGMP) environments. Bio-Rad is now introducing the Biologics Analysis Workflow, which expands beyond the imaging and analysis for this application and brings the biologics community a complete suite of products for their analyses. The workflow begins with the Criterion TGX Precast Gels, which increase assay throughput due to short run times and the ability to run numerous samples at once. The new QC Colloidal Coomassie Stain is an end-point stain with exceptional sensitivity and low background, making it ideal for the quantitation of impurities. Furthermore, it is ready to use and formulated without methanol, which requires hazardous waste disposal. The new GS-900 Calibrated Densitometer with 21 CFR Part 11 compliant Image Lab Software is a replacement for the GS-800 Calibrated Densitometer and Quantity One® Software. The GS-900 maintains the level of performance, sensitivity, and features of the GS-800 System while offering improvements to its design and reproducibility. The GS-900 uses LED lights for improved uniformity and stability and conducts a calibration step prior to each scan. Additionally, the IQ/OQ kit enables rapid calibration and validation of instrument performance.

As cGMP-regulated labs transition to the Biologics Analysis Workflow, they must obtain approval from the regulatory agencies for changes to existing protocols. The guidelines for changes to a protocol are outlined in the FDA's *Guidance for Industry: Comparability Protocols — Protein Drug Products and Biological Products — Chemistry, Manufacturing, and Controls Information* (fda.gov) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines, *Validation of Analytical Procedures: Text and Methodology Q2 (R1)* (ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf). To address these requirements, the GS-900 System was designed to support a seamless and compliant operational transition.

When changing a protocol, it is most efficient to make multiple changes (for example, to the gels, stain, and imager) within a single comparability study. The introduction of the GS-900 Densitometer provides an opportunity for labs to also optimize other parts of their current protocols. Adoption of Bio-Rad's new Biologics Analysis Workflow can improve several areas of the workflow by increasing speed, improving efficiency, and lowering costs while maintaining accurate and highly reproducible results.

To provide a guide for the approval process, this report presents an example of a comparability (bridging) study to aid a cGMP-regulated lab with the experimental design of its own comparability study. We focus on new protocols (outlined in Figure 1) that test for impurities using SDS-PAGE with an end-point Coomassie Stain and imaging on the GS-900 Densitometer.

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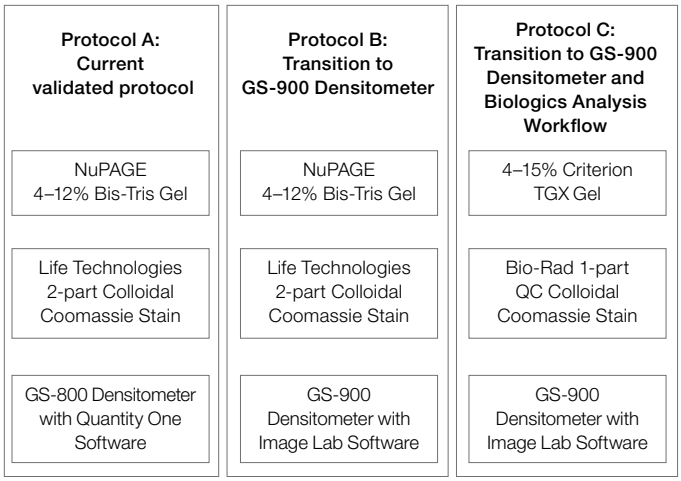



**Fig. 1. Experimental design. A**, an established QC lab protocol that uses NuPAGE 4–12% Bis-Tris Gels and Life Technologies' two-part Colloidal Coomassie Stain; imaging and analysis are performed using the GS-800 Densitometer with Quantity One Software. **B**, the same protocol as in **A**, except that imaging and analysis are performed using the GS-900 Densitometer with Image Lab Software. Comparison of protocols A and B is the basis for the comparability study required for transitioning to the GS-900 System. **C**, a complete update to the established protocol in **A**: it uses 4–15% Criterion TGX Gels and the Bio-Rad one-part QC Colloidal Coomassie Stain, and gels are imaged and analyzed using the GS-900 Densitometer with Image Lab Software. Comparison of protocols A and C is the basis for a study that changes all parameters of a protein assay.

## Experimental Design

To gain cGMP approval, a comparability test must compare the following characteristics: accuracy, precision (both repeatability and intermediate precision), specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, and range. The recommended methodologies for defining each characteristic are described in the *Validation of Analytical Procedures: Text and Methodology Q2 (R1)* document and are summarized in the appendix. With appropriate experimental design, data to evaluate these characteristics can be generated with as few as two sets of samples, minimizing the amount of work needed. For a comparability study, the characteristics and pass criteria (for example, the sample must be greater than 90% pure by percent band) are those established for the previous cGMP-approved protocol.

The first sample set should be a validated target protein that reflects the purity of a representative lot, as defined by the percent band (the volume of a given band divided by the total volume of all bands) or percent lane (the volume of a given band divided by the total volume in the lane). The linearity, LOD, LOQ, and range are determined from a dilution series of this sample, in which sample amounts range from saturation to below the LOD. Within this range, there will be a region in which the band quantitation (volume measured in OD) is linear with respect to the amount of protein loaded and the percent purity will be constant. This region, in which both conditions are satisfied, defines the range over which the results (percent band or percent lane) are accurate and precise. This region should include a minimum of ±20% of the target protein load and will define the valid range of the protocol. In this study, a dilution series of 90–95% pure reduced bovine serum albumin (BSA) was used.

The second sample set should consist of stressed samples that contain impurities. In this study, four samples were generated: a second lot of reduced BSA, a BSA sample that has undergone partial degradation, a BSA sample containing aggregates, and a BSA sample with impurities (approximately 70% impurities by percent lane). These samples were characterized in three different amounts, covering the target load and both the high and low limits of the range, defined from the analysis of the first sample set. These samples further define the accuracy, precision, and specificity of the assay for detecting protein impurities.

In this study, these sample sets were separated by SDS-PAGE, stained with a colloidal Coomassie Stain, imaged with a calibrated densitometer, and analyzed by three different protocols, as outlined in Figure 1. One set of experiments was performed using the same samples, gels, and stain and imaged using either the GS-800 or GS-900 System to demonstrate the equivalent performance of the densitometers (Figure 1, protocols A and B). Another set of experiments was then performed using the same samples but with a different protocol (different gels, electrophoresis system, and stain) to demonstrate the equivalence of the two protocols and illustrate how multiple changes to a protocol can be easily characterized (Figure 1, protocols A and C).

## Methods

### Sample Preparation

UltraPure BSA was solubilized using 1x phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, and 2 mM potassium phosphate at pH 7.4) to create a 5 mg/ml stock solution. To prepare the reduced BSA sample, the stock solution was diluted to a final concentration of 0.5 mg/ml in 1x LDS Laemmli Sample Buffer or 1x NuPAGE LDS Sample Buffer with 355 mM 2-mercaptoethanol (βME). The degraded and reduced BSA samples were prepared by heating an aliquot of the 0.5 mg/ml reduced BSA in 1x Laemmli Sample Buffer at 95°C for 5 min and then diluting them to target concentration with the respective sample buffer. Aggregated nonreduced BSA was generated by heating an aliquot of the 5 mg/ml BSA stock solution in 1x PBS at 65°C for 5 min, then diluting it to 0.5 mg/ml in 1x sample buffer. Finally, the BSA sample with *E. coli* cell lysate was made by diluting the 5 mg/ml BSA stock solution to 0.5 mg/ml BSA with 0.625 mg/ml *E. coli* lysate. The *E. coli* cell lysate was prepared by solubilizing the lyophilized lysate in 1x PBS to 10 mg/ml prior to mixing with the BSA stock solution. Samples were diluted into the appropriate sample buffer (NuPAGE LDS Sample Buffer for the Bis-Tris gels and Bio-Rad Laemmli LDS Sample Buffer for the Criterion TGX Gels).

### SDS-PAGE

SDS-PAGE was performed using two different electrophoresis systems. NuPAGE Bis-Tris 4–12% Gels were run with 1x MOPS buffer with NuPAGE Antioxidant in the XCell SureLock Mini-Cell at 200 V for 50 min (aggregated samples were run using nonreducing conditions without the antioxidant). 4–15% Criterion TGX Gels were run in 1x Tris/glycine/SDS buffer in the Criterion Cell at 300 V for 20 min. Samples were loaded at a constant volume of 20 µl, with empty lanes containing 20 µl of 1x sample buffer. All gels included Precision Plus Protein™ All Blue Protein Standard.

### Staining

Two G-250 colloidal Coomassie staining systems, which are more sensitive and have a greater linear range than R-250 formulations, were used. For staining with the Life Technologies Colloidal Blue Staining Kit, the Bis-Tris gels were first incubated with gentle shaking in a fixing solution of 50% methanol and 10% acetic acid for 15 min. The gels were then incubated with shaking for 10 min in staining solution (55 ml deionized water, 20 ml methanol, and 20 ml stainer A solution without stainer B solution). Next, 5 ml stainer B solution was added and the gels were stained overnight (16–18 hr) at room temperature with gentle agitation. The staining solution was decanted, and 100 ml deionized water was added to destain the gel. The water was changed hourly for 3 hr, after which the gel was imaged.

For staining with the Bio-Rad QC Colloidal Coomassie Stain, the Criterion TGX Gels were incubated in a fixing solution of 40% ethanol and 10% acetic acid with gentle agitation for 15 min. The fixing solution was then decanted, the gel was lightly rinsed with water, and 100 ml stain was added. The gel was stained overnight (16–18 hr) at room temperature with gentle agitation. The staining solution was decanted, and 100 ml deionized water was added to destain the gel. The water was changed hourly for 3 hr, after which the gel was imaged.

### Imaging and Analysis

For a comparability study, imaging and analysis should be performed in a similar manner to the previously validated assay. For example, purity (percent band or percent lane) and analysis parameters (lane height and width, rolling disk size for background subtraction, etc.) should be defined consistently between methods. In this study, we evaluate comparability using both definitions of purity given a consistent set of parameters as defined by our model validated assay (protocol A).

Images were acquired using either a GS-800 Calibrated Densitometer and Quantity One Software or a GS-900 Calibrated Densitometer using Image Lab Software. Images were acquired at 63.5 µm resolution using a red channel transmissive scan standard for Coomassie-stained gels.

To test equivalence between different protocols, identical parameters in both Quantity One and Image Lab Software were used. For image analysis, the lane frame was defined from the bottom of the loading well to the bottom of the gel. The lane width was defined as 7 mm (lane widths are commonly defined between 50–100% of the well width). Precision Plus Protein All Blue Protein Standards were used to determine the molecular weight of the sample bands using a point-to-point semi-log regression method. For this study, a rolling disk background subtraction method was used with a rolling disk size of 10 mm in Image Lab Software or 39 pixels in Quantity One Software. To convert disk size from Quantity One to Image Lab Software or vice versa, the following equations were used:

*From Quantity One to Image Lab Software:*

Quantity One disk size (in pixels) = (Image Lab disk size (in mm) x 1,000/ Image Lab scanner resolution)/4

*For Image Lab to Quantity One Software:*

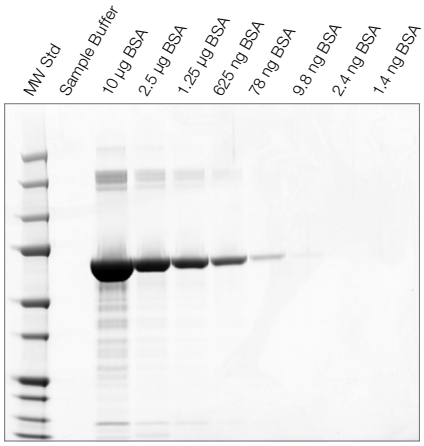
Image Lab disk size (in mm) = (Quantity One disk size (in pixels) x 4)/ (1,000/Image Lab scanner resolution)

For band detection with Image Lab Software, a sensitivity setting of 100 was used. If an apparent band was not detected by the software, the band was manually added if it had a volume equal to or greater than the LOD volume threshold and appeared consistently across all three gel replicates. Artifact bands were manually deleted if the detected band had a volume below the LOD threshold and did not appear consistently across all three replicates. Manual adjustments to the band height (lane profile width) were made to ensure consistent analysis between lanes. The band volumes were fit with a linear least squares method.

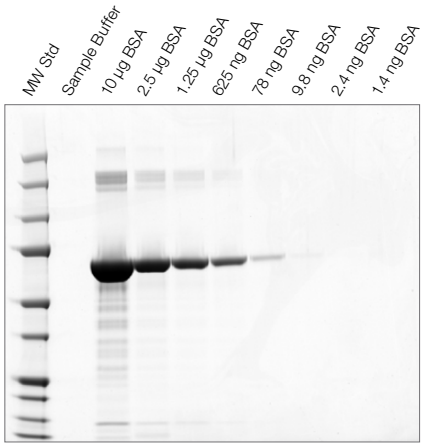
## Results

To determine the linearity, range, LOD, and LOQ of protocols A–C, samples from a dilution series of BSA (~90–95% pure) were run in triplicate on both NuPAGE Bis-Tris Gels stained with Life Technologies' Colloidal Coomassie Stain and on Criterion TGX Gels stained with the Bio-Rad QC Colloidal Coomassie Stain. To compare the performance of the GS-800 and GS-900 Systems, the NuPAGE Gels were imaged on both instruments and analyzed using the respective software (Quantity One for the GS-800 images and Image Lab for the GS-900 images) (Figure 1, protocols A and B). Bio-Rad's TGX Gels were imaged and analyzed using the GS-900 System and Image Lab Software (Figure 1, protocol C). From these data, the band volume and percent purity (both as a percent band and as a percent lane) of BSA were measured for each lane, averaged across the three gels (n = 3), and the band volume was fit with a least squares linear regression. Using the three samples in the target range (2.5 µg, 1.25 µg, and 625 ng) across the three gels (n = 9), the molecular weight of the BSA was determined (Table 1 and Figure 2).

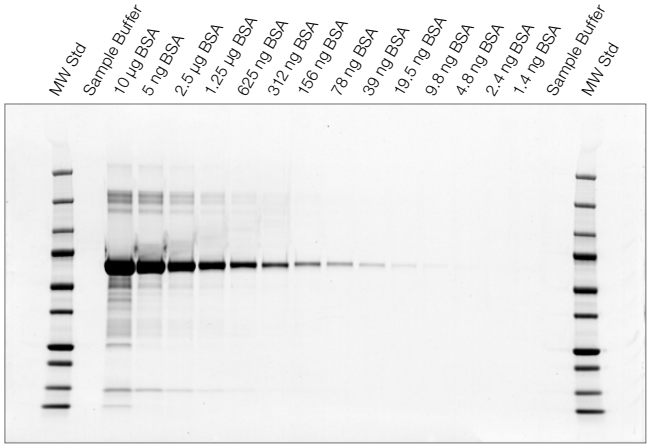
To further test the accuracy, precision, and specificity of the protocols, a second lot of reduced BSA and three different stressed samples (reduced and degraded, non-reduced and aggregated, and reduced with cell lysate) were run at three different concentrations bracketing the target load for the assay. These samples covered different ranges of purity and contained



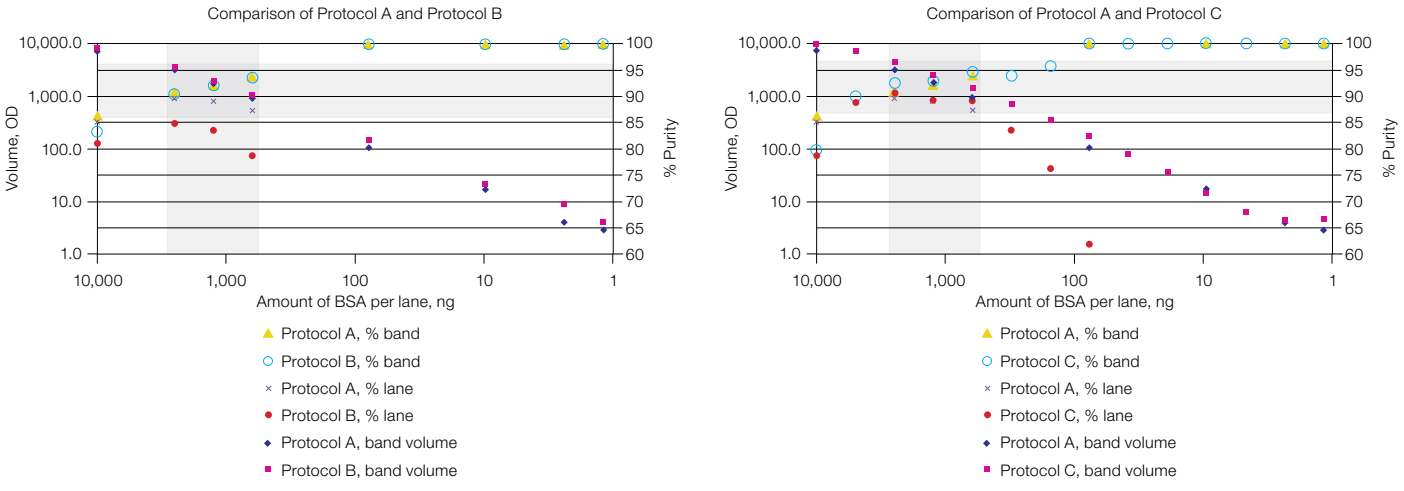

Protocol A (original protocol)




Protocol B (change to GS-900 Densitometer)




Protocol C (change entire protocol)




MW Std = Precision Plus Protein All Blue (5 µl)

**Fig. 2. SDS-PAGE and analysis of the BSA dilution series analyzed with different protocols.** Gels resulting from the separation of the BSA dilution series using protocols A–C are shown. The shaded regions in these plots highlight the range in which the percent purity is constant (% band is ±1%) and volume is linear ($R^2$ ≥0.99). To best present the four orders of magnitude spanned by the dilution series, the volume and amount of BSA loaded per lane are both plotted on a logarithmic scale and, therefore, do not show the deviation from linearity at BSA amounts greater than 2,500 ng of BSA. Since Criterion TGX Gels (18 wells) have more wells per gel than NuPAGE Gels (10 wells), the entire dilution series could be run on a single gel.

different contaminants (low and high molecular weight) to compare the performance of the different protocols and the specificity of the stain to different protein contaminants. From these data, the percent band and percent lane of the dominant band was determined (Table 2). In the case of the reduced sample with cell lysate, only the percent lane was calculated due to the complexity of the sample.

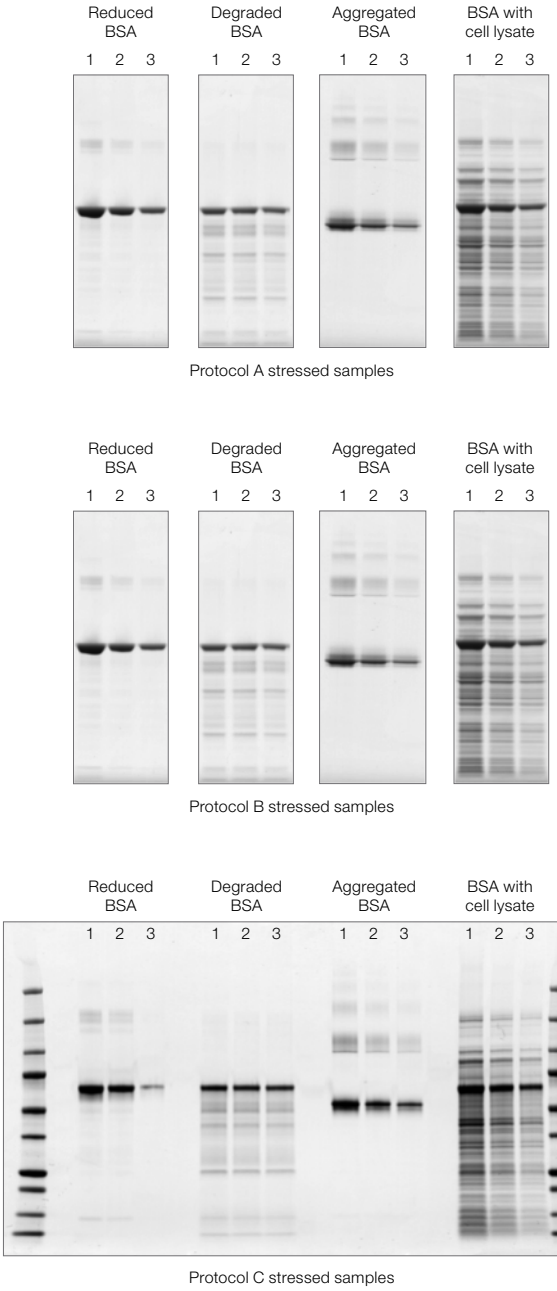
All three protocols performed similarly across all seven characteristics (Table 1 and Table 2). The linear ranges (2.5 µg to 2.4 ng) and coefficients of correlation (all greater than 0.99) were indistinguishable, with minor differences in the slope and y-intercept. BSA amounts above 2.5 µg were excluded from the linear fit because of a reduction in the coefficient of correlation below 0.99. The LOD and the LOQ (as defined in the appendix) had relatively large errors as they are, by definition, the limits of the assay and are statistically equivalent. For the dilution series used in this experiment, the values presented in Table 1

**Table 1. Comparison of performace characteristics from a dilution series of BSA from different protocols.** All characteristics are reported as defined in the appendix. Fit parameters were generated from data over the specified range. Percent band and percent lane were calculated from lanes with 2.5 µg, 1.25 µg, and 625 ng BSA using data from 3 gels (n = 9), the region over which the percent purity was determined to be constant. The molecular weight of BSA is 66.5 kD. All errors are reported as a 95% confidence interval (CI, two standard deviations).

| Characteristic | Protocol A | Protocol B | Protocol C |
|---|---|---|---|
| Range | 2.5 µg to 2.4 ng | 2.5 µg to 2.4 ng | 2.5 µg to 2.4 ng |
| Linearity ($R^2$) | 0.99 | 0.99 | 0.99 |
| Slope | 1.28 ± 0.04 | 1.35 ± 0.06 | 1.76 ± 0.05 |
| Intercept | 44 ± 45 | 78 ± 65 | 69 ± 48 |
| LOD (ng) | 2 ± 2 | 7 ± 6 | 4 ± 4 |
| LOQ (ng) | 3 ± 2 | 13 ± 12 | 9 ± 9 |
| % Band | 92 ± 4 | 92 ± 4 | 93 ± 2 |
| % Lane | 89 ± 2 | 82 ± 8 | 90 ± 2 |
| Molecular Weight (kD) | 68.7 ± 4.2 | 67.3 ± 3.4 | 66.7 ± 2.0 |

**Table 2. Comparison of performance characteristics (accuracy and precision) from stressed BSA samples from different protocols.** All characteristics are reported as defined in the appendix. The average percent purity with 95% CI (two standard deviations) are reported for all stressed samples (average from three concentrations run in triplicate) (three different gels, n = 9). Percent purity is reported for the dominant band (BSA) as either the percent band or as the percent lane.

| | % Purity | | |
|---|---|---|---|
| Sample | Protocol A | Protocol B | Protocol C |
| Reduced (% band) | 89 ± 6 | 89 ± 2 | 92 ± 2 |
| Reduced (% lane) | 84 ± 11 | 76 ± 11 | 88 ± 4 |
| Reduced, degraded (% band) | 61 ± 4 | 58 ± 2 | 62 ± 6 |
| Reduced, degraded (% lane) | 59 ± 6 | 51 ± 4 | 60 ± 5 |
| Non-reduced, aggregated (% band) | 84 ± 2 | 82 ± 2 | 82 ± 2 |
| Non-reduced, aggregated (% lane) | 82 ± 4 | 75 ± 7 | 79 ± 2 |
| Reduced with cell lysate (% lane) | 38 ± 8 | 32 ± 6 | 36 ± 10 |




Reduced BSA: 1 = 2.5 µg BSA, 2 = 1.25 µg BSA, 3 = 625 ng BSA
Degraded BSA: 1 = 1.2 µg BSA, 2 = 1.0 µg BSA, 3 = 800 ng BSA
Aggregated BSA: 1 = 2.5 µg BSA, 2 = 1.25 µg BSA, 3 = 625 ng BSA
BSA with cell lysate: 1 = 2.5 µg BSA, 2 = 1.25 µg BSA, 3 = 625 ng BSA

**Fig. 3. SDS-PAGE and analysis of the stressed BSA samples analyzed with different protocols.** Gels resulting from the separation of the stressed BSA using protocols A–C are shown. For each sample type (reduced, degraded, aggregated, and with cell lysate), three different load amounts were loaded to span the linear range of the assay. Since Criterion TGX Gels (18 wells) have more wells per gel than NuPAGE Gels (10 wells), the samples shown in protocols A and B are from representative gels.

translate to routine detection of 2.4 ng BSA in each of the three methods, and to reproducible quantitation of BSA above 9.8 ng to within ±20%, with a 95% CI.

In the region from the maximum load of 2.5 µg down to 625 ng, the purity defined by the mean percent band (the volume of a given band divided by the total volume of all identified bands) for each protocol were within a single standard deviation of each other and were statistically equivalent. For a target load of 1.25 µg BSA, the percent band was within 1% of the 2.5 µg and 625 ng load (±50% of target load), allowing for robust purity determination. At loads above 2.5 µg BSA, the BSA band began to saturate while signals from impurities continued to increase, resulting in a decrease in the measured relative purity of the dominant band. At amounts lower than 625 ng, the percent band quickly approached 100% as the signal from the impurities dropped below the LOD.

The purity as measured by the percent lane (the volume of a given band divided by the total volume of the lane) was consistently lower than the purity measured by percent band and had greater variability.

The signal arising from the lane volume in percent lane analysis is inherently greater than the sum of the band volumes in percent band analysis. Consequently, the ratio of a given band to the lane volume (that is, percent lane) is always less than the ratio of the same band to the sum of the band volumes (that is, percent band). Likewise, percent lane will always have greater variability than percent band. While more variable, the percent lane measurements for each protocol were consistent and within error of each other. Below 625 ng of BSA, the percent lane quickly approaches zero as the volume of the dominant band decreases relative to the entire lane volume.

The additional complexity of the stressed samples provided a more stringent test of the accuracy and precision and further demonstrated the equivalence of the three protocols. The percent purity (either percent band or percent lane) values for a given sample over the tested range were statistically equivalent (Table 2) across the three protocols. This demonstrates that all three protocols resolve, stain, and detect a variety of protein contaminants equivalently and, therefore, have similar specificities.

## Conclusion

The experimental design presented here can be used to efficiently generate data to define the seven characteristics required for cGMP approval of changes to a protocol. Further, this experimental design provides the framework for measuring the intermediate precision of the protocol (that is, user-to-user variability, instrument-to-instrument variability, etc.). With the replacement of the GS-800 Densitometer with the GS-900, such a comparability study will be required and provides an opportunity to make other changes that can be validated to enable improvement of current protocols.

We also observed that the new Biologics Analysis Workflow (protocol C) was faster and more efficient compared to the other two protocols. First, the larger format of Criterion TGX Gels (18 wells compared to 10) allowed more samples (dilutions) to be run on a single gel; a twofold dilution series could be run uninterrupted from 10 µg down to 1.4 ng. This provided greater resolution across the dilution range, thereby improving determination of the linearity and range, allowing for fewer iterations to bracket the desired range for the study. (For the entire comparability study, the Biologics Analysis Workflow (protocol C) required half as many gels as protocols A or B, 6 gels as opposed to 12 gels.) The Criterion Gels also ran faster (one-third time) than the NuPAGE Gels, and the Bio-Rad one-part QC Colloidal Coomassie Stain and protocol was easier to use (no mixing/assembly were required, and thus fewer steps to document). The Biologics Analysis Workflow (protocol C) is a more efficient workflow, and documenting these changes for the regulatory agencies did not require significantly more work over that already required for the transition to use the GS-900 Densitometer.

## Appendix: Definition of Characteristics

**Accuracy:** For a new protocol, accuracy can be demonstrated using previously characterized samples by a previously validated method and reporting the difference between the mean and the accepted true value together with the confidence intervals. Impurities must be defined relative to the major analyte, and in SDS-PAGE they can be represented as either a percent band or as the percent lane, depending on the sample type. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range; for example, three concentrations and three replicates each of the total analytical procedure.

**Precision:** Precision consists of defining the reproducibility or variability of a protocol. Precision should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range; for example, three concentrations and three replicates each of the total analytical procedure. The standard deviation, relative standard deviation (coefficient of variation), and confidence interval should be reported for each type of precision investigated.

**Specificity:** Specificity can be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure, for example, the previously approved procedure or another validated independent procedure. In this case, the impurity profiles should be compared. In instances where samples of the impurities can be obtained, known concentrations should be spiked into known concentrations of the target sample and evaluated. For the purpose of SDS-PAGE analysis of a biological product, impurities resulting from stressed samples cannot be readily obtained.

Therefore, the analysis of a previously characterized stressed sample that contains impurities as a result of nonspecific degradation or aggregation can be used for the comparison to the new protocol.

**Linearity:** Linearity should be evaluated across a range centered on the specified target amount to be used in the assay. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or by separate weighing of synthetic mixtures of the drug product components using the proposed procedure. The data should be plotted and analyzed by a regression line fit by the method of least squares and a plot of the data and fit along the correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be reported. For the establishment of linearity, a minimum of five concentrations that cover a range from the reporting level of the impurities (that is, the LOD) to a minimum of 120% of specified target amount to be used in the assay is recommended.

**Limit of detection (LOD):** LOD is the lowest amount of substrate that can be detected with a specified confidence interval that is defined by two parameters: the rate of false positives and the rate of false negatives. The false positive rate is determined relative to the noise in the system, which can be defined by measuring the mean and standard deviation of a blank. From this, a threshold can be determined to give a desired rate of false positives. For a blank that has a normal distribution (Gaussian distribution), a threshold of the mean signal of the blank plus 3.3 standard deviations would result in a 0.1% false positive rate. In the case of a stained gel, the blank is a lane loaded with only sample buffer and no protein. Specifically, the noise is measured by integrating the volume over the area of a typical band after background subtraction using a rolling disk method. Using this threshold, the mean signal from a quantity at the LOD would be distributed around this threshold, resulting in a false negative rate of 50%. The method for determining LOD is outlined below.

**Limit of quantitation (LOQ):** LOQ is the amount that can be consistently measured with a specified precision. Typically, the LOQ is set with a threshold ten standard deviations above the mean noise. The method for determining LOQ is outlined below.

**Range:** The range is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. In the case of an impurities test using SDS-PAGE, the range should cover a region of at least ±20% of the specified target over which the percent band or percent lane is consistent, within the precision of the measurement.

**Table 3. Summary of definitions and recommendations of performance characteristics.**

| Validation Characteristic | Description | Methodology |
|---|---|---|
| Accuracy | Difference between the mean and accepted true value, defined by the mean value and standard deviation | Nine determinations over a minimum of three concentrations covering the specified range (three concentrations/ three replicates) |
| Precision | Reproducibility as defined by the standard deviation, coefficient of variation, and confidence interval | Nine determinations over a minimum of three concentrations covering the specified range (three concentrations/ three replicates) |
| Specificity | | No specified recommendation |
| Linearity | Defined by the fit of the method of least squares along the correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares | Minimum of five different concentrations/three replicates |
| Limit of detection | Lowest amount of target that can be detected with a specified confidence interval, which is defined by two parameters: the rate of false positives and the rate of false negatives | No specified recommendation |
| Limit of quantitation | Lowest amount of target that can be quantified within a specified confidence interval | No specified recommendation |
| Range | The limits over which acceptable degrees of linearity, accuracy, and precision are achieved within a specified confidence interval | Minimum of five different concentrations/three replicates |

### Determination of the Limit of Detection (LOD) and the Limit of Quantitation (LOQ)

cGMP regulatory agencies recommend a rigorous definition of the LOD and recommends several alternative methods by which to define this threshold. The FDA-recommended method used in this study takes the average signal measured from a blank to define the threshold for the LOD and LOQ. For SDS-PAGE, this signal is the average integrated volume after background subtraction over the area of a typical band in a blank lane loaded only with sample buffer.

To define the threshold for the LOD, two parameters must first be defined: the rate of false positives and the rate of false negatives. The rate of false positives is determined in relation to the noise in the system, which can be defined by measuring the mean and standard deviation from a blank. Given the normal distribution of the noise, a threshold can be chosen that will define the rate of false positives. For example, a threshold set at two standard deviations above the mean signal would give a 5% false positive rate while a threshold set at three standard deviations above the

mean noise would give a 0.2% false positive rate. Using this threshold to set the LOD in turn defines the rate of false negatives. As the signal is centered on this threshold, in half the experiments the signal will vary below the threshold and therefore be undetected, giving a 50% false negative rate.

For these protocols, the average noise for a typical band was calculated by dividing the entire integrated volume of the blank lane after background subtraction with a rolling disk by the number of typical bands per lane. Typical band width (0.02 $R_f$) was determined by measuring the lane profile width of bands close to the LOD. The integrated volume was divided by 50 (0.020 $R_f$/1.0 $R_f$) and was calculated for three different lanes across three different gels (n = 9). These values were used to calculate the average and standard deviation of the average volume noise. cGMP regulatory agencies recommend setting the threshold to 3.3 standard deviations above the mean noise for a false positive rate of 0.1%. Using this threshold and the slope of the line from the linear fits, the LOD for BSA was calculated as a volume threshold for each protocol, which translated into an equivalent LOD of 2.4 ng for all protocols.

The LOQ threshold was set to 10 standard deviations above the mean volume of a typical band in a blank lane. This corresponded to ~9.8 ng of BSA. Above this amount, the band volume could be quantitated within 20%, with a 95% CI for all protocols.



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