

Analysis of Model Proteins Using the Agilent ProteoAnalyzer System and a μ CE-SDS System

Introduction

Quality control (QC) of protein samples is crucial for workflows like protein characterization. Key quality attributes such as size, purity, and relative concentration of proteins are typically assessed through electrophoretic separations. Such measurements are essential for verifying the success of protein extraction and purification processes. To obtain reliable protein QC results, choice of instrument is a critical factor.

To streamline QC in protein analysis, multiple automated SDS electrophoresis instruments have been developed. The Agilent ProteoAnalyzer system is a capillary electrophoresis sodium dodecyl sulfate (CE-SDS) instrument. The ProteoAnalyzer is designed to facilitate precise and accurate measurements of up to 12 protein samples in parallel, and up to 96 total samples without user interaction. Between runs, the capillaries are automatically cleaned and filled with fresh gel, ensuring consistent results across diverse protein sample types. Using the Agilent Protein Broad Range P240 kit, the system accommodates sample sizes ranging from 10 to 240 kDa. Using a covalent labeling method, reduced and nonreduced samples can be assessed.

An alternative instrument for protein QC, hereafter referred to as System G, uses chip based microfluidic capillary electrophoresis with sodium dodecyl sulfate (μ CE-SDS). μ CE-SDS allows for fast separation times due to its small channel size and efficient heat dissipation, though it can only perform one separation at a time. System G offers multiple protein assays, including specific analyses like charge variant and N-Glycan analysis, as well as general protein analysis.

In this technical overview, an assay for high-resolution protein purity assessment was used on System G to compare with the Broad Range P240 protein kit on the ProteoAnalyzer. The comparison includes sizing accuracy, sizing precision, quantification, and resolution of different model protein samples, such as bovine serum albumin (BSA), carbonic anhydrase II (CAII) from bovine, and NIST monoclonal antibody (mAb).

Table 1. Comparison of specifications between the Agilent Broad Range P240 kit for the Agilent ProteoAnalyzer system and System G with its associated kit.

Specification	ProteoAnalyzer System	System G
Sizing Range	10 – 240 kDa (lower marker only), 10 – 200 kDa (lower marker and upper marker)	14 – 250 kDa
Sizing Accuracy	LM only: for BSA, CAII (using reduced conditions) < 15% ; LM and UM: for BSA, CAII (using reduced conditions) < 10 %	Not reported
Sizing Precision	LM only: < 8% CV for BSA, CAII, GREMLIN-1, and NIST mAb (using reduced conditions), < 10% CV for intact NIST mAb (using non-reduced conditions); LM and UM: < 5% CV for BSA, CAII, GREMLIN-1 and NIST mAb (using reduced conditions)	< 2% CV
Quantitative Range	2 – 2,000 ng/μL for BSA in PBS	10 – 1,000 ng/μL
Sensitivity	1 ng/μL for BSA, CAII in PBS	5 ng/μL
Quantification Reproducibility	< 25 %CV for 2 – 20 ng/μL, < 15 %CV for 20 – 2,000 ng/μL	Not reported
Analysis Time	11 samples + 1 ladder: 30 min	65 sec/sample
Resolution	< 10% molecular weight resolution between 15 – 150 kDa (based on ladder); R ≥ 1 NIST mAb NGHC/ HC (using reduced conditions)	Resolution > 1.0

Experimental

Commercially available bovine serum albumin (BSA) (Sigma, part number A7906 or NEB, part number B9000S) and carbonic anhydrase II from bovine erythrocytes (CAII) (Sigma, part number C2273) were prepared in Dulbecco's Phosphate Buffered Saline, Sigma D8537 (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) at concentrations of 1 to 2,000 ng/μL under reduced conditions. Dilutions of BSA and CAII were assessed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit (part number 5191-6640). The same samples were also analyzed on System G according to the manufacturer's instructions.

NIST mAb (Sigma part number NIST8671, aliquot from Reference Material 8671, Lot 14HB-D-002) was prepared in PBS at a concentration of 2,000 ng/μL under reduced and non-reduced conditions according to the Protein Broad Range P240 kit manual. The samples were covalently labeled by incubating them with the supplied reagents at 70 °C for 10 minutes, followed by analysis on the ProteoAnalyzer. The same sample was also analyzed on System G according to the manufacturer's instructions.

Results

Visualization of system peaks

The ProteoAnalyzer presents electrophoretic separations as both a digital gel image and an electropherogram. When visualizing the electropherogram, a few peaks, known as system peaks, will appear in every run, but are not a part of the sample. With the ProteoAnalyzer, as shown in Figure 1A, the leftmost signal represents the dye front and is followed by the 6 kDa lower marker (LM) used for sizing alignment and sample quantitation. The dye front appears before the lower marker, preventing its interference with the sample analysis region.

Similarly, System G also provides results in the form of a digital gel image and an electropherogram. The electropherogram in Figure 1B shows the LM as the leftmost peak at 1.4 kDa, followed by a cluster of system peaks. These peaks can interfere with the detection of protein samples less than 14 kDa.

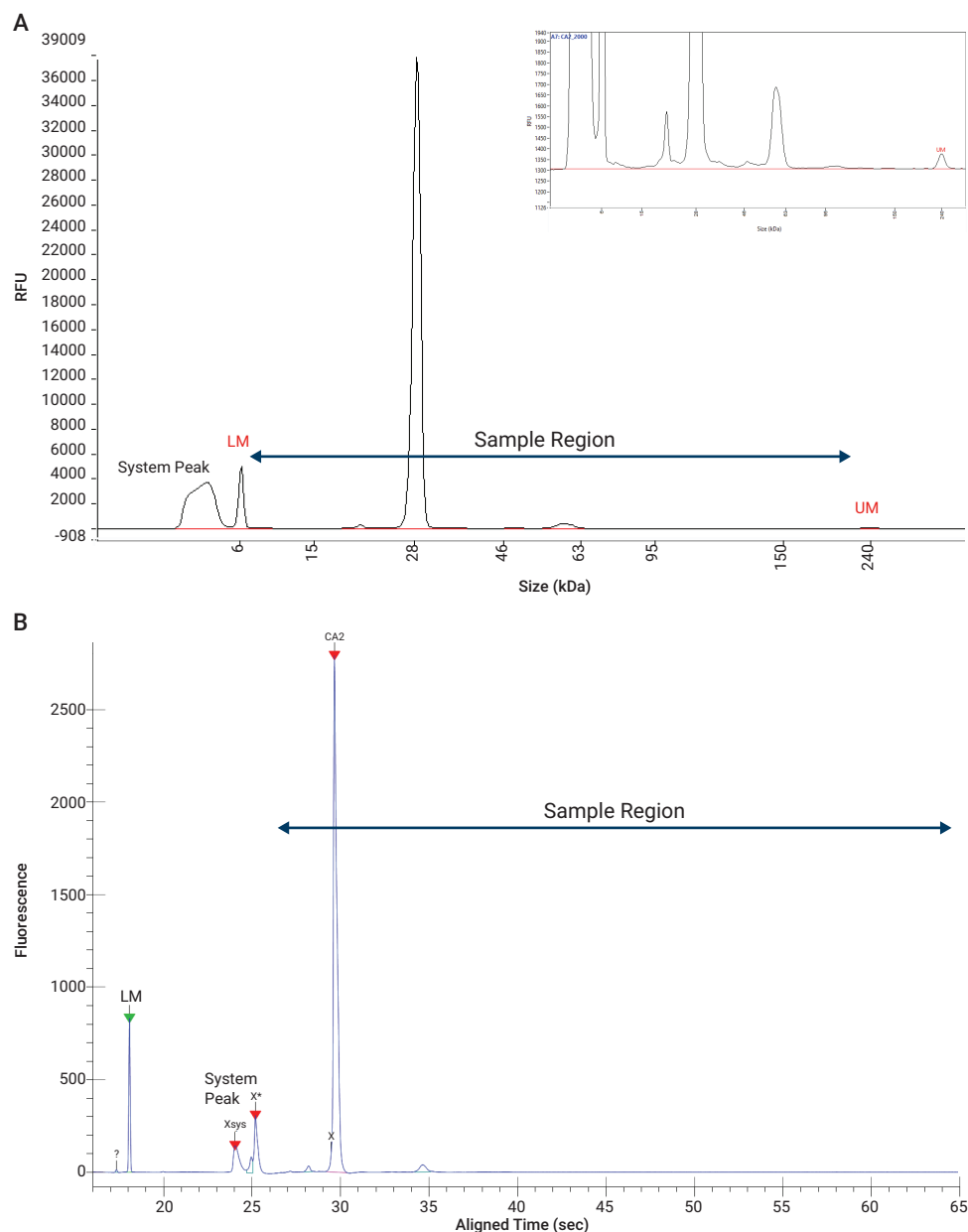


Figure 1. Representative analysis of CAII highlighting the differences in the electropherogram results between (A) the Agilent ProteoAnalyzer system and (B) System G.

Detection range

To evaluate the detection capabilities of the ProteoAnalyzer and System G, 10x serial dilutions of BSA and CAII, ranging from 2 to 2,000 ng/μL, were assessed. Additionally, a 1 ng/μL concentration was tested to demonstrate the detection sensitivity of the ProteoAnalyzer system. The ProteoAnalyzer detected both BSA and CAII across the entire concentration range. Although the specifications of System G state a detection sensitivity of 5 ng/μL, BSA was only detected from 20 to 2,000 ng/μL. CAII was detected from 2 to 2,000 ng/μL. The ProteoAnalyzer was able to detect both sample types down to 1 ng/μL, providing higher sample detection sensitivity across the concentration ranges.

Sizing

Accurately determining the size of a protein sample is an essential quality control step. The sizing performance of the ProteoAnalyzer system and System G was assessed using BSA and CAII. Analysis of BSA on the ProteoAnalyzer resulted in an overall average size of 68.6 kDa, while CAII had an overall average size of 28.6 kDa. When measured with System G, BSA showed an overall average size of 75.9 kDa, and CAII had an overall average size of 30.9 kDa. The sizing difference between systems was 10% when assessing BSA and 8% when assessing CAII.

Sizing accuracy was determined by calculating the percent error from the measured sizes of BSA and CAII compared to their expected sizes. BSA has an expected size of 66 kDa, and CAII has an expected size of 29 kDa. This allowed for a comparison of the accuracies between systems at each concentration across the 10x serial dilution (Figures 2A and 3A).

The ProteoAnalyzer demonstrated impressive accuracy, with an average percent error across all concentrations of 3.9% for BSA and 2.1% for CAII, well within the kit specification of less than 15% error. In contrast, System G exhibited lower accuracy, as shown by higher percent errors, averaging 14.1% for BSA and 6.4% for CAII (Figure 2B) across all concentrations.

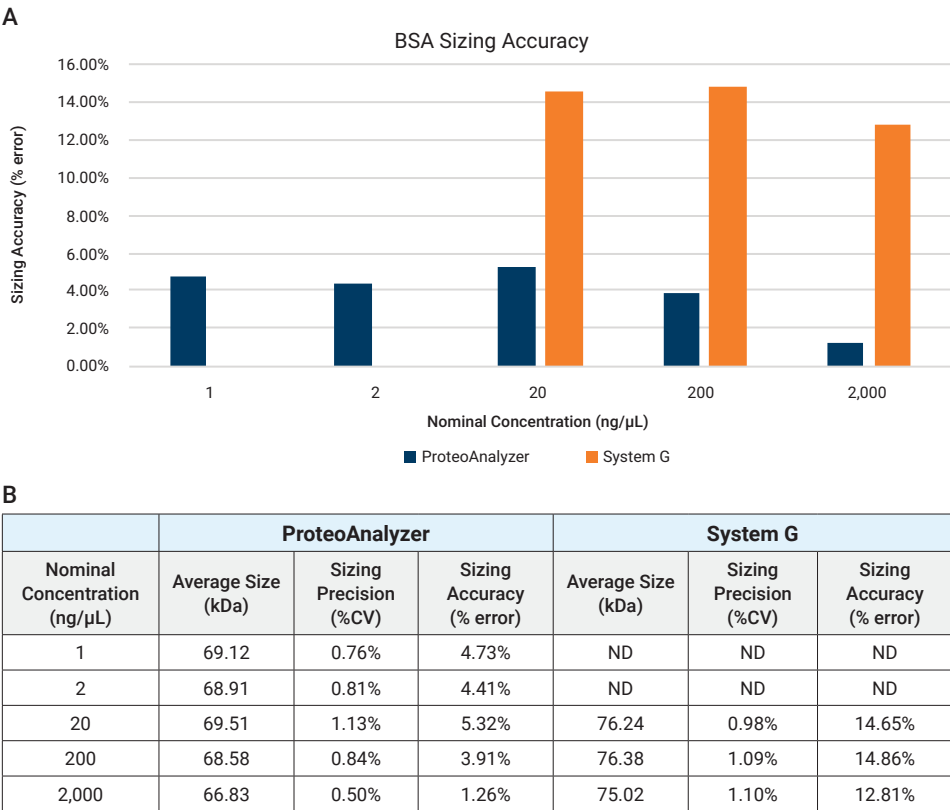


Figure 2. (A) Average percent error for sizing of BSA from the Agilent ProteoAnalyzer system and System G. (B) Comparison of average size, precision shown as %CV, and percent error for a 10x serial dilution of BSA using the ProteoAnalyzer system and System G. Sample was not detected (ND) at 1 and 2 ng/μL using System G. (n = 9).

Sizing precision was compared between each system by calculating the %CVs from the average sizes of the proteins at each concentration across the 10x serial dilution. The ProteoAnalyzer demonstrated a precision of 1.3 %CV or less for BSA and 2.46 %CV or less for CAII, well below the kit specification of 8 %CV. Analysis with System G resulted in a precision of 1.10 %CV or less for BSA and 1.15 %CV or less for CAII, within the kit specifications of less than 2 %CV. Both systems exhibited excellent precision with low %CVs across the tested samples, highlighting their reliability in protein size measurements.

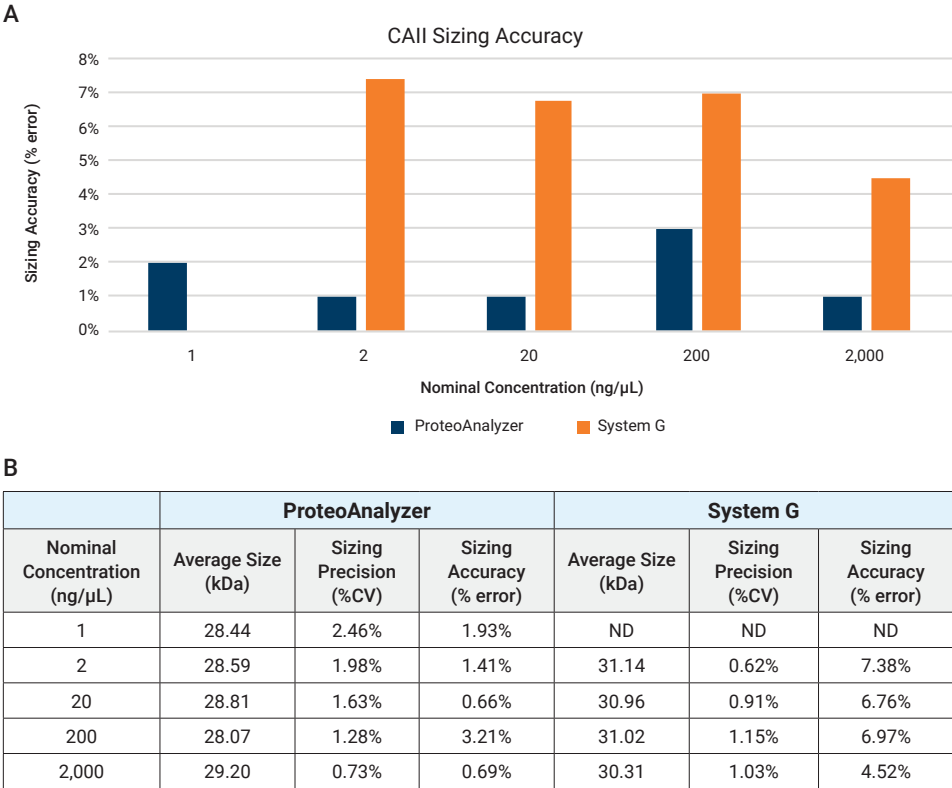


Figure 3. (A) Average percent error for sizing of CAII from the Agilent ProteoAnalyzer system and System G. (B) Comparison of average size, precision, and accuracy of CAII with the ProteoAnalyzer system and System G. The sample could not be detected (ND) at 1 ng/μL using System G. (n = 9).

Concentration

Reliable assessment of a sample's relative concentration is another crucial part of quality control. Figure 4 illustrates the linear correlation between the measured and nominal concentrations of BSA and CAII on the ProteoAnalyzer and System G. Linearity for each system was assessed using R^2 values, where higher R^2 values indicate better linearity. The ProteoAnalyzer measured samples in a concentration range from 1 to 2,000 ng/ μ L with an R^2 of 1.0 for both BSA (Figure 4A) and CAII (Figure 4C), indicating a strong linear relationship. System G measured BSA samples from 20 to 2,000 ng/ μ L, resulting in an R^2 of 0.9997 (Figure 4B), and CAII samples from 2 to 2,000 ng/ μ L with an R^2 of 0.9953 for CAII (Figure 4D). Both systems showed high degrees of linearity.

The precision of each instrument's quantification measurements is demonstrated by the %CV calculations as shown in Table 2, where a lower %CV indicates higher precision. For protein samples, the ProteoAnalyzer kit specifications state that precision should be less than 25 %CV for samples from 2 to 20 ng/ μ L and less than 15 %CV for samples from 20 to 2,000 ng/ μ L. BSA measured on the ProteoAnalyzer had a calculated precision of 17.7 %CV or less for 1 and 2 ng/ μ L concentrations, and 9% or less for concentrations from 20 to 2,000 ng/ μ L. For CAII samples, the ProteoAnalyzer showed a %CV of 18.2% for the 1 ng/ μ L sample and 7.1% or less for concentrations from 2 to 2,000 ng/ μ L, demonstrating good overall precision.

The analytical specifications for System G do not include concentration precision. When tested, the system had a %CV of 2.6% or less for all BSA concentrations tested from 20 to 2,000 ng/ μ L, indicating good precision. However, for CAII samples, System G had a %CV of 22% or less for all concentrations tested (2 to 2,000 ng/ μ L).

Overall, System G showed good precision for BSA but much lower precision for CAII, while the ProteoAnalyzer demonstrated good precision across the concentration ranges of both model proteins tested. These linearity and precision results demonstrate that the ProteoAnalyzer delivers reliable concentration measurements with consistent performance, making it a dependable choice for thorough analysis.

Table 2. Comparative analysis of the average quantification measurements and precision (%CV) for BSA and CAII using the Agilent ProteoAnalyzer system and System G. Samples not detected (ND). (n=9).

Nominal Concentration	BSA				CAII			
	ProteoAnalyzer		System G		ProteoAnalyzer		System G	
	Average Concentration (ng/ μ L)	Quantitative	Average Corrected Area	Precision (%CV)	Average Concentration (ng/ μ L)	Precision (%CV)	Average Corrected Area	Precision (%CV)
1	0.51	17.7%	ND	ND	0.55	18.2%	ND	ND
2	0.92	17.4%	ND	ND	1.08	6.8%	1.35	22.0%
20	9.61	8.7%	14.57	2.40%	11.31	5.2%	27.11	2.0%
200	100.68	9.0%	248.06	2.60%	111.00	7.1%	402.27	3.6%
2,000	1,037.29	2.5%	2,160.56	2.20%	1,140.57	5.9%	2,399.89	3.6%

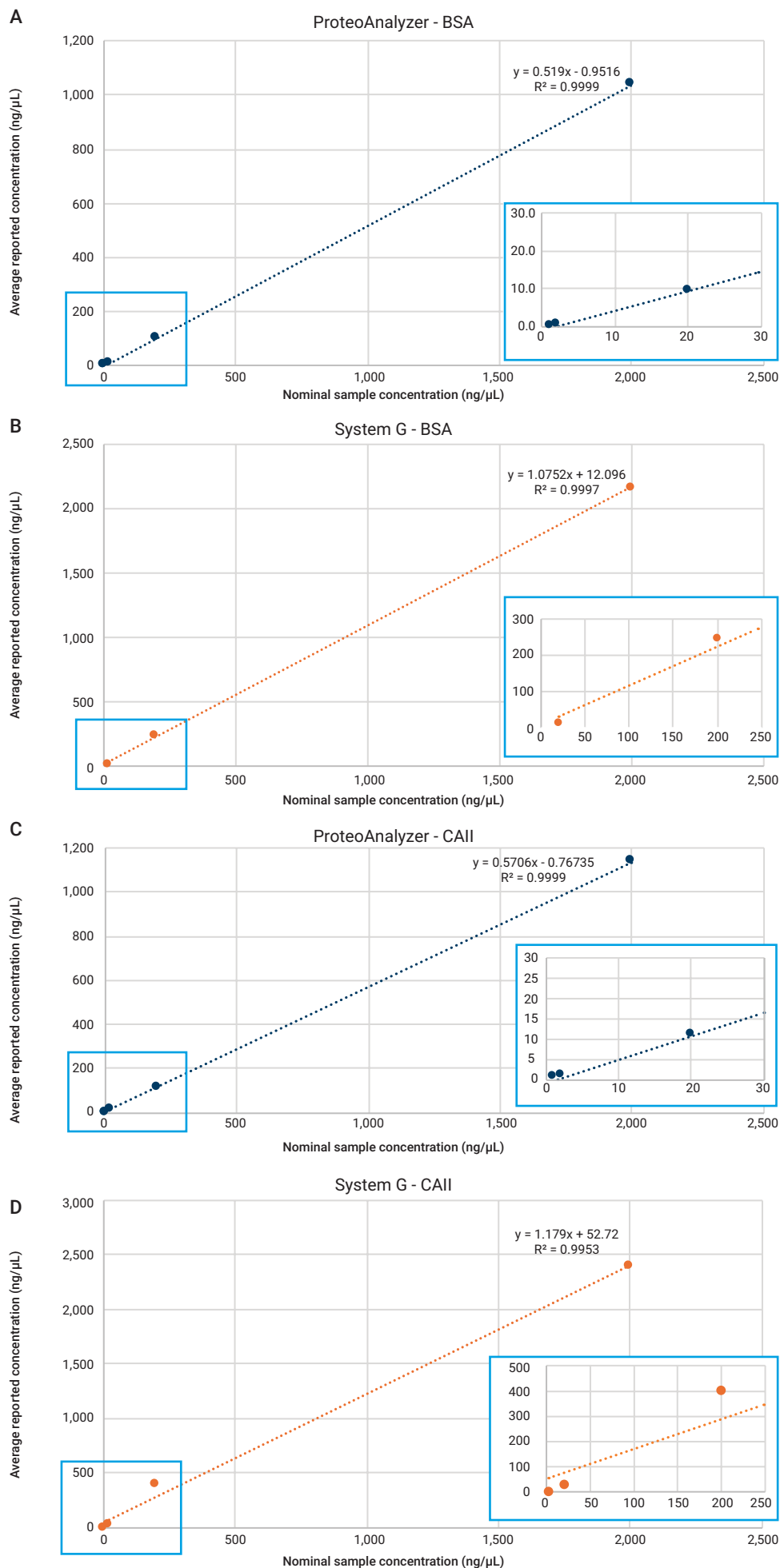


Figure 4. Comparisons of linearity between the Agilent ProteoAnalyzer system and System G relative quantifications. Shown are correlations between the nominal concentration of each dilution against the measured concentrations for both (A) BSA on the ProteoAnalyzer and (B) BSA on System G, along with (C) CAII on the ProteoAnalyzer and, (D) CAII on System G.

Impurities

Accurate and reliable analysis is required for appropriate assessment of biopharmaceutical products. Evaluating critical quality attributes (CQAs) such as monomeric purity, glycan occupancy, and percent thioether (non-reducible species, NRS) is a crucial aspect in determining the quality and efficacy of monoclonal antibodies. Using a 2,000 ng/μL aliquot of the NIST mAb sample, the results from each system were used to calculate these specific CQAs using established equations.^{1,2} These metrics were then compared between the ProteoAnalyzer system, System G, and the published NIST mAb datasheet (Table 3).

The monomeric purity measured by the ProteoAnalyzer was slightly lower than the datasheet value but still highly similar, indicating a high level of purity. System G reported a slightly lower monomeric purity compared to the ProteoAnalyzer. For glycan occupancy, both systems showed values comparable to the datasheet, with System G slightly higher than the datasheet value and the ProteoAnalyzer being slightly under it. This finding suggests that both systems are reliable in measuring glycan occupancy.

Regarding percent thioether, the ProteoAnalyzer system reported a higher value than the datasheet, indicating a slightly higher presence of thioether bonds. System G's value was closely aligned to the datasheet, suggesting minimal deviation. Overall, the results from both systems are comparable to the values in the NIST datasheet, demonstrating their reliability in measuring these CQAs.

Table 3. The critical quality attributes (CQAs) of monomeric purity, glycan occupancy, and percent thioether were assessed using data from the NIST datasheet and calculated values from the NIST mAb sample. These assessments were conducted using the Agilent ProteoAnalyzer system and System G.

	Monomeric Purity			Glycan Occupancy			% Thioether		
	NIST Datasheet	ProteoAnalyzer	System G	NIST Datasheet	ProteoAnalyzer	System G	NIST Datasheet	ProteoAnalyzer	System G
Average	98.47%	97.98%	97.03%	99.39%	99.26%	99.43%	0.30%	0.43%	0.29%
%CV	-	0.31%	1.35%	-	0.02%	0.04%	-	4.40%	8.00%

Resolution

High-resolution analysis of the nonglycosylated heavy chain (NGHC) and heavy chain (HC) fragments in mono clonal antibodies is necessary for accurate assessment of glycan occupancy. Resolution (R) measures the ability to separate two peaks, such as the NGHC and HC. Higher R values indicate better resolution.³

Using the NIST mAb as a representative sample, the ProteoAnalyzer offers excellent resolution of the NGHC and HC peaks with a calculated average R value of 1.54, indicating baseline resolution (Figure 5A). This result met the kit specifications of an NGHC/HC $R \geq 1$ for NIST mAb under reduced conditions. This high resolution is reproducible between capillaries, with a calculated precision of 5.9 %CV.

System G also showed good resolution (Figure 5B) and precision for the resolution between the NGHC and HC peaks. System G had a calculated average R value of 1.18, which was within the kit specification of an R greater than 1, and had a calculated %CV of 3.2%. Both systems performed well, but the ProteoAnalyzer provided higher resolution and similar reproducibility compared to System G.

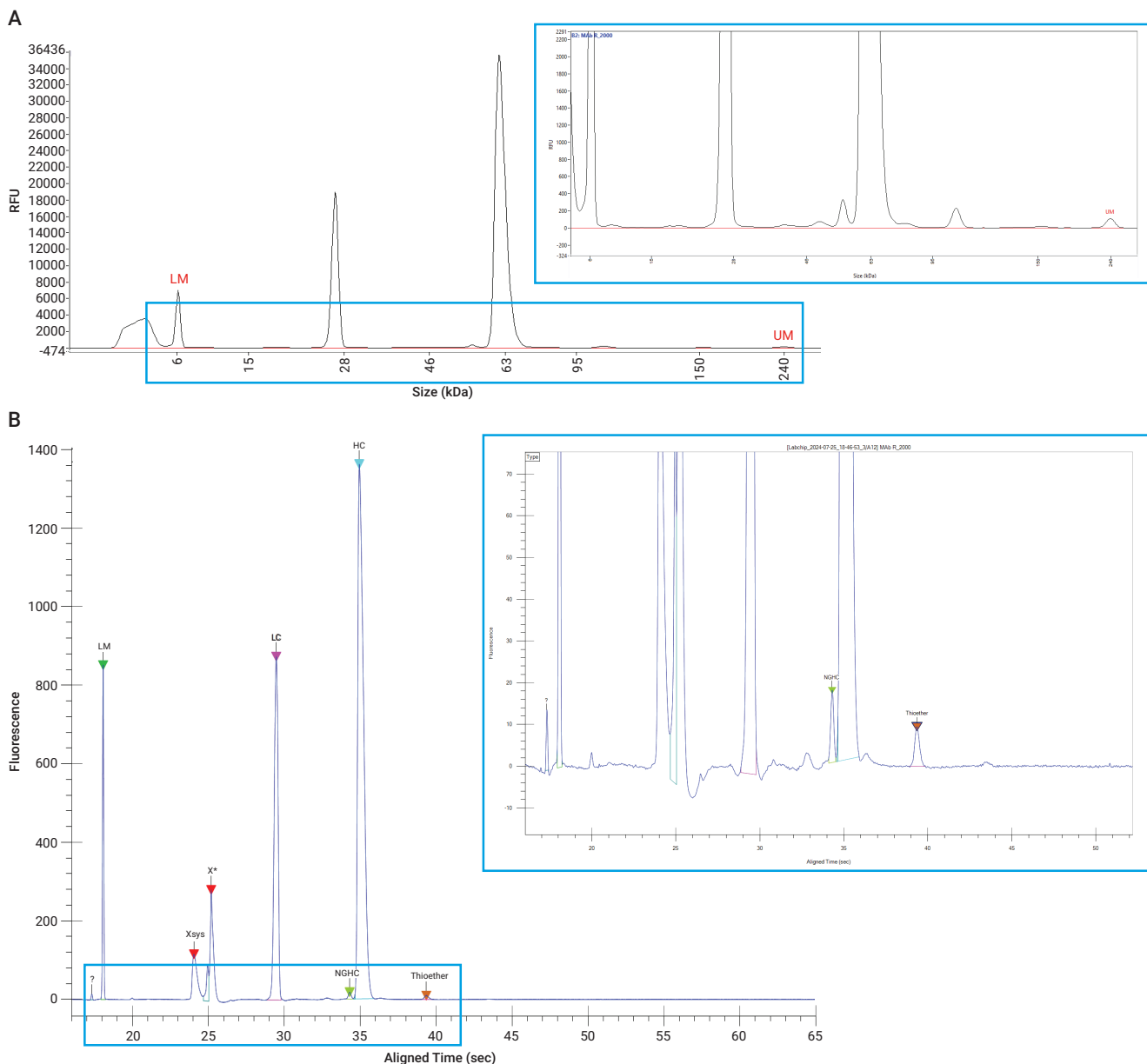


Figure 5. Electropherogram results representing a reduced NIST mAb run using the (A) Agilent ProteoAnalyzer system and (B) System G.

Conclusion

This technical overview highlights the capabilities of the Agilent ProteoAnalyzer system compared to a μ CE-SDS system (System G) in assessing sizing accuracy, sizing precision, quantification reproducibility, impurity detection, and resolution. The results showed that the ProteoAnalyzer system excelled in sizing accuracy and detection sensitivity compared to System G. Both systems demonstrated high degrees of linearity and precision in concentration assessment, and both showed excellent resolution capabilities. The ability of the ProteoAnalyzer to detect lower concentrations and achieve excellent resolution further emphasizes its superior performance.

References

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2. National Institute of Standards and Technology; U.S. Department of Commerce. *Reference Material 8671: NISTmAb, Humanized IgG1k Monoclonal Antibody, Lot 14HB-D-002. Reference Material Information Sheet*. Nist.gov. <https://tsapps.nist.gov/srmext/certificates/archives/8671.pdf>.
3. Highly Resolved Separation of DNA Fragments on the Agilent 5200 Fragment Analyzer System. *Agilent Technologies Application Note*, publication number 5994-0517EN, **2019**.

www.agilent.com/genomics/proteoanalyzer

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PR7001-4569

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Published in the USA, July 8, 2025
5994-8459EN