Technical Overview

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Protein Sizing and Quantification

With the Agilent ProteoAnalyzer System

Figure 1. The Agilent ProteoAnalyzer system.

Introduction

Ensuring the production or extraction of high-quality protein molecules is crucial for numerous industries. Quality assurance of proteins encompasses two main aspects: confirming the accuracy of the manufactured products, and identifying any impurities present in the samples. The Agilent ProteoAnalyzer system (Figure 1) is an automated quality control instrument designed to provide accurate and precise quality measurements of protein samples. The system analyzes proteins based on migration time and fluorescence intensity using parallel capillary electrophoresis with sodium dodecyl sulfate (CE-SDS). Rapid and consistent sizing and quantification results can be achieved for both reduced and non-reduced protein samples.

The ProteoAnalyzer system analyzes up to 12 samples simultaneously in one run in approximately 30 minutes. Accurate sizing can be achieved for both small and large samples, ranging in size from 10 to 240 kDa. Samples can be detected over a wide concentration range, from 2 to 2,000 ng/µL (Table 1). The ProteoAnalyzer is a robust system, well-suited for protein applications in biotherapeutics and synthetic biology workflows, enabling scientists to efficiently perform various protein quality and quantity assessments using the Agilent Protein Broad Range P240 kit.

This technical overview highlights the reproducibility of protein sizing and quantification with the ProteoAnalyzer. Sizing accuracy and precision is demonstrated using BSA and CAII. The samples will also be used to provide an overview of how the system calculates both relative and absolute quantification of proteins.

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 1x PBS (30 mM Tris-HCl, 26 mM NaH_2PO_4, 41 mM Na_2HPO_4, 79 mM NaCl, pH 8.5) at concentrations of 2 to 2,000 ng/µL under reduced conditions. Concentration was confirmed with Nanodrop using the default BSA settings and an extinction coefficient of 0.575 for CAII. Dilutions of BSA and CAII were assessed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit.

Results and discussion

The ProteoAnalyzer system employs capillary gel electrophoresis (CGE) for protein analysis. During sample preparation, a fluorescent dye is covalently bound to the proteins in the sample. The fluorescent intensity of the protein is measured as a function of its migration time during separation. The intensity and time are then used to evaluate the quantitation and molecular weight of the proteins within a sample. Standards are used to calculate the size and concentration of the sample. The standards include a ladder composed of known protein sizes, and a set of markers.

A lower marker (LM) of known size and concentration is added to each sample for alignment and quantitation. An optional upper marker (UM) can be added for improved alignment and thus, higher sizing precision. Samples are automatically analyzed using the Agilent ProSize data analysis software, which reports the molecular weight and relative concentration of the proteins in a sample.

Sizing of protein samples

For size determination of proteins, the sample and ladder wells are aligned using the 6 kDa lower marker (LM), and the peak migration time is compared to the known sizes of the ladder, ranging from 10 to 240 kDa. The optional 240 kDa upper marker (UM) provides higher precision for samples smaller than 200 kDa. Figure 2 shows the sizing ladder used for the kit, prepared with either the LM only (Figure 2A) or analyzed with the LM & UM method (Figure 2B).

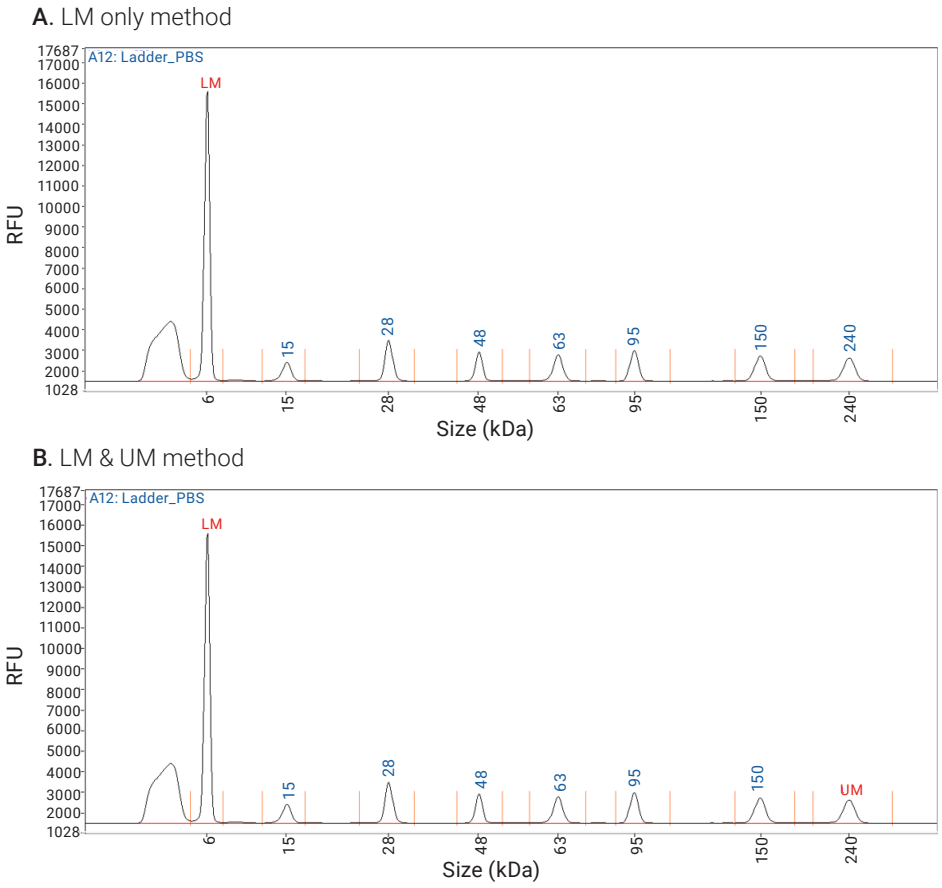


Figure 2. Protein Broad range ladder analyzed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit using the A) LM only method and B) LM & UM method.

Table 1. Agilent Protein Broad Range P240 kit specifications. LM: Lower Marker; UM: Upper Marker.

Analytical Specifications		ProteoAnalyzer Protein Broad Range P240 kit
Sizing Range	LM only LM and UM	10 to 240 kDa 10 to 200 kDa
Typical Sizing Accuracy (% Sizing Error)	LM only LM and UM	< 15% for BSA, CAII < 10% for BSA, CAII
Typical Resolution		< 10% molecular weight resolution between 15 to 150 kDa (based on ladder) R≥1 NIST mAb NGHC/HC (using reduced conditions)
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)
Quantitative Range		2 ng/µL to 2,000 ng/µL for BSA in PBS
Sensitivity (Signal/Noise > 3)		1 ng/µl for BSA, CAII in PBS

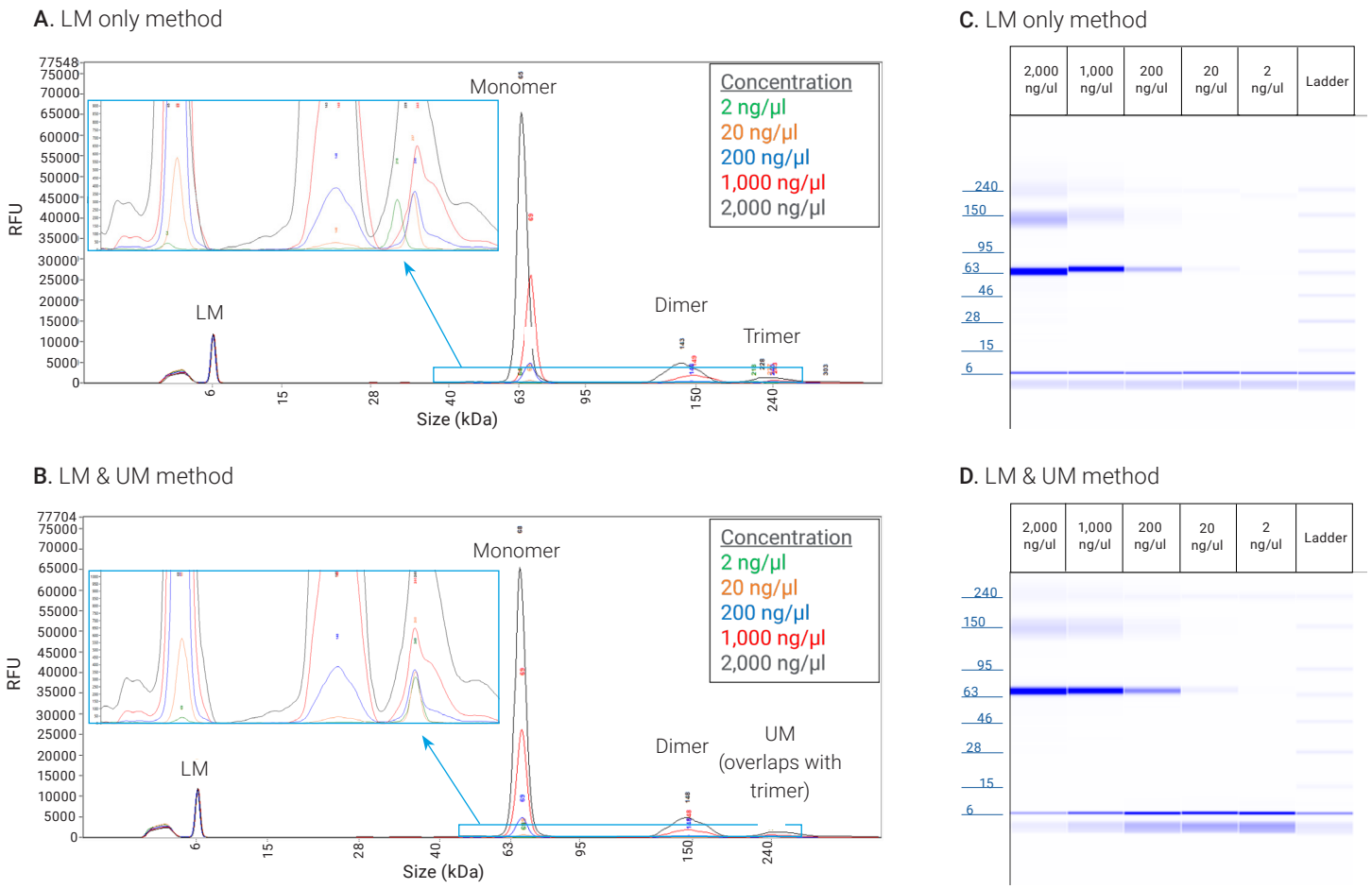
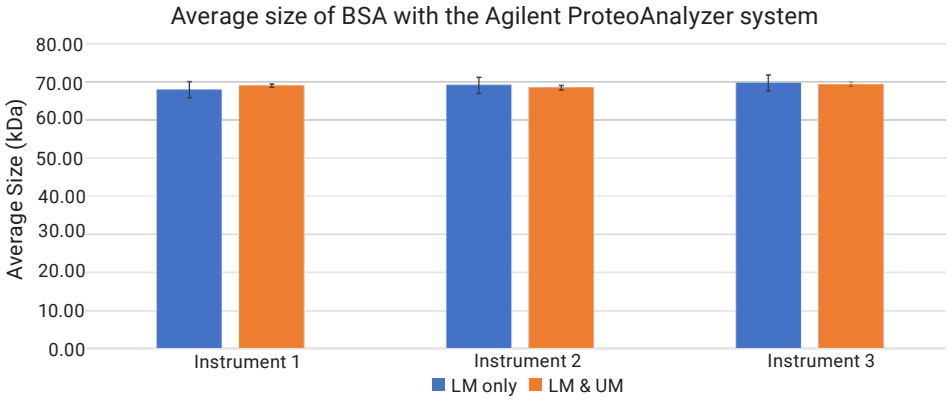


Figure 3. Overlay of serial dilution of BSA analyzed on the Agilent ProteoAnalyzer system using A) LM only and B) LM & UM methods. Comparison of the digital gel images from both the C) LM only and D) LM & UM methods. The LM only method is ideal for larger samples, while the addition of the UM provides enhanced alignment, and therefore better sizing accuracy and precision.

BSA sizing

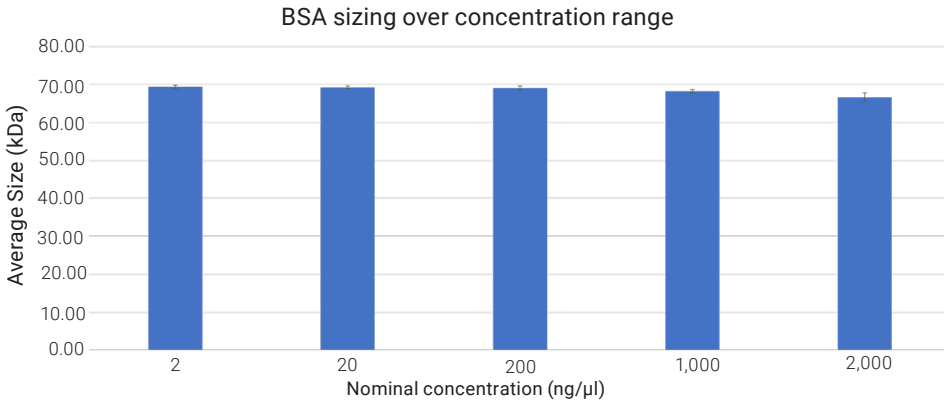
An overlay of BSA when analyzed with the LM only compared to the LM & UM method demonstrates the alignment that is achieved when the UM is used (Figure 3). The monomer, dimer, and trimer peaks of the BSA sample can all be observed when analyzed with the LM only (Figure 3A and C). Analysis with the LM & UM method results in better alignment, as indicated by the precise overlap of the monomeric peaks. However, the trimer peak overlaps with the UM (Figure 3B and D). Still, analysis of the monomeric peak with either method results in similar average sizes, %CV, and percent error, all well within the kit specifications (Figure 4).

To assess the reproducibility of the ProteoAnalyzer, two-fold serial dilutions of BSA protein were analyzed on three instruments in multiple replicates. Representative analysis of the sample at 200 ng/ul is shown in Figure 4. Sizing of the monomeric peak of the BSA remains consistent across multiple instruments and across the concentration range of the kit, from 2 to 2,000 ng/µL. The average size of the monomeric peak of the BSA sample was 68.5 kDa when analyzed at different concentrations with the LM & UM method (Figure 5). The accuracy was excellent, with a percent error of 3.6% or less at each concentration. Additionally, the %CV at each concentration was 1.8 %CV or less, highlighting the precision of the system. Both the sizing accuracy and precision were well within the kit specifications of 10% error and 5 %CV for BSA using the LM & UM method.



	Average Size (kDa)		Percent Error		% CV	
	LM only	LM & UM	LM only	LM & UM	LM only	LM & UM
Instrument 1	67.99	69.11	1.47	3.14	3.09	0.67
Instrument 2	69.20	68.55	3.29	2.31	3.01	0.82
Instrument 3	69.84	69.41	4.24	3.60	2.91	0.81

Figure 4. Reproducibility of the Agilent ProteoAnalyzer system is demonstrated across three instruments with both the LM only and LM & UM methods for sizing of BSA at 200 ng/µL. Instruments 1 and 3: n=14; instrument 2: n=21.



Nominal Concentration	Average Size (kDa)	Percent Error	%CV
2 ng/µl	69.38	3.6	0.7
20 ng/µl	69.24	3.3	0.5
200 ng/µl	69.02	3.0	0.8
1,000 ng/µl	68.25	1.9	0.6
2,000 ng/µl	66.62	-0.6	1.8

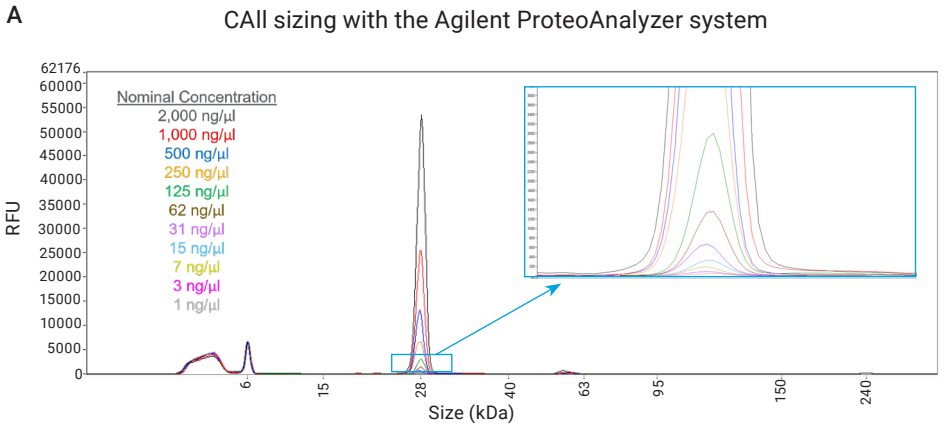
Figure 5. Sizing reproducibility of BSA over a serial dilution analyzed on the Agilent ProteoAnalyzer system with the LM & UM method. 2, 20, and 1,000 ng/µL: n=28; 200 and 2,000 ng/µL: n=49.

CAII sizing

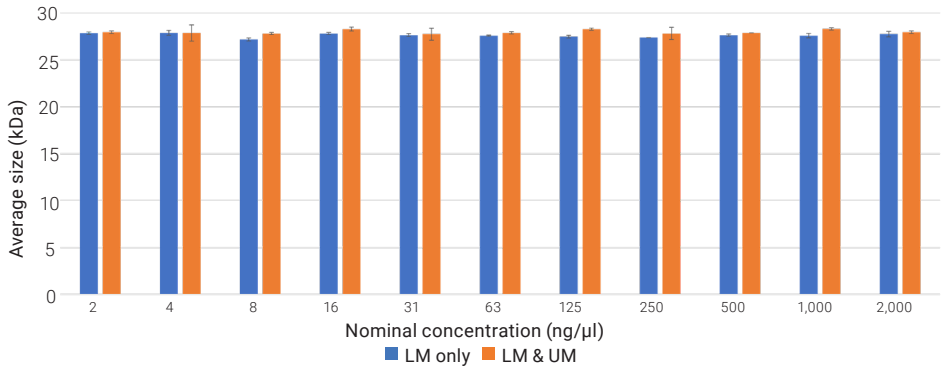
In another example, the size of CAII was assessed across the concentration range of the kit (Figure 6). When sized with the LM & UM method, the average size of the CAII sample was 28.0 kDa across all concentrations. The sizing accuracy was excellent, with a percent error of 5% or less at each concentration, compared to the expected size of 29 kDa. Additionally, the average %CV among all concentrations was 1.4 %CV, highlighting the precision of the system. Both the sizing accuracy and precision were well within the kit specifications of 10% error and 5 %CV for CAII using the LM & UM method (Figure 6).

Together, the analysis of BSA and CAII demonstrates that the ProteoAnalyzer system provides flexible analysis methods that allow for accurate and precise sizing of proteins up to 240 kDa over a broad concentration range.

A



B



C

	Average Size (kDa)		Percent Error		%CV	
Nominal Concentration (ng/µl)	LM only	LM & UM	LM only	LM & UM	LM only	LM & UM
2	27.83	27.93	4.37	3.68	0.42	0.41
4	27.87	27.87	4.94	2.41	0.91	3.06
8	27.17	27.80	4.71	3.91	0.55	0.41
16	27.80	28.27	5.63	4.14	0.42	0.72
31	27.63	27.73	5.29	2.53	0.56	2.27
63	27.57	27.87	4.94	3.91	0.21	0.41
125	27.47	28.27	4.71	4.37	0.55	0.42
250	27.37	27.80	4.14	2.53	0.00	2.27
500	27.63	27.87	6.32	4.14	0.43	0.00
1,000	27.57	28.30	3.91	3.91	0.83	0.41
2,000	27.73	27.93	4.02	3.68	1.04	0.41

Figure 6. The Agilent ProteoAnalyzer system was used to assess the sizing of CAII across the quantitative range of the kit. A) Electropherogram overlay of different concentrations. B) Sizing with both the LM only and LM & UM methods is shown for comparison. C) Tables show the percent error and %CV for each concentration tested. n=3.

Sample concentration

The ProteoAnalyzer measures the concentration of a sample by comparing the area of a peak to internal standards – either the lower marker for relative quantitation, or with user-defined calibration standards for absolute quantitation. The system provides a 3-log quantitative dynamic range, with a broad sample concentration range from 2 to 2,000 ng/µL.

Relative quantification

During sample preparation, a fluorescent dye is covalently bound to the proteins in the sample. The fluorescence intensity of the dye-bound protein is proportional to its concentration. The ProSize data analysis software automatically analyzes the data from the ProteoAnalyzer and reports the area under the curve. Knowing the peak areas of both the protein sample and the lower marker allows for calculation of the protein's relative concentration in comparison to the known marker concentration. These values are precise and can be compared to values obtained from other capillaries. However, since dye labeling efficiency depends on the amino acid composition, these relative concentrations will differ from the absolute mass of protein present.

To demonstrate the relative quantification performance of the system, a serial dilution of BSA was prepared in PBS and analyzed across multiple ProteoAnalyzer instruments. The systems were highly reproducible, providing consistent relative quantification of the monomeric peak for each dilution tested. The %CV for the lowest concentration tested, 2 ng/µL, was between 12 and 14% on each instrument. For all other concentrations from 20 to 2,000 ng/µL, the %CV was less than 4 %CV. The quantification reproducibility was well below the kit specifications of 25 %CV for samples from 2 to 20 ng/µL and 15 %CV for samples from 20 to 2,000 ng/µL (Figure 7). Combined, the instruments displayed a perfect linear correlation for relative quantification across the concentration range, with an R^2 value of 1.0 (Figure 8).

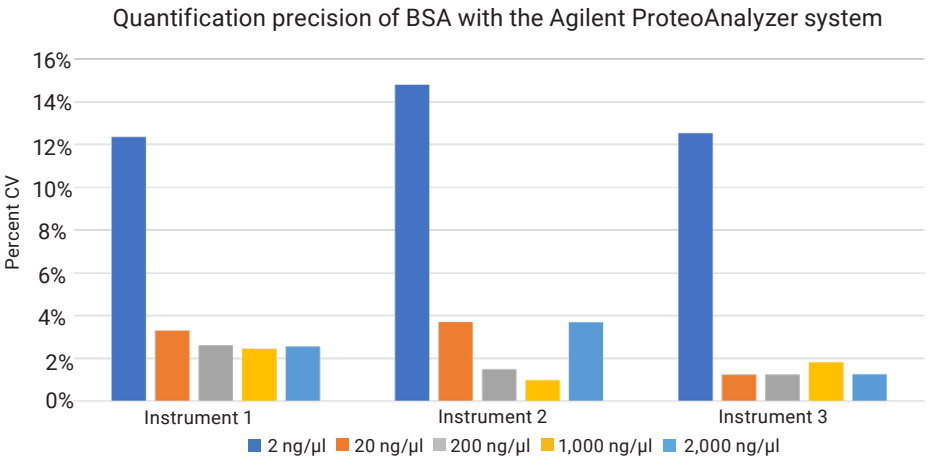


Figure 7. Quantification precision passes kit specifications at all concentrations tested for BSA analyzed with the Agilent ProteoAnalyzer system. n=8.

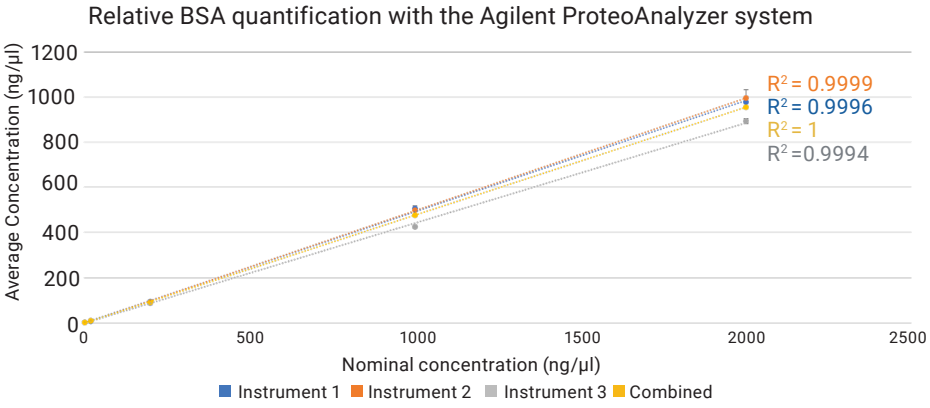


Figure 8. The Agilent ProteoAnalyzer system was used to analyze the relative quantification of BSA across the quantitative range of the kit. The linearity of the concentration is shown for the data collected with three individual instruments. The data from all three instruments combined (yellow) showed a perfect correlation between the nominal and average relative concentration measured by the ProteoAnalyzer. n=8.

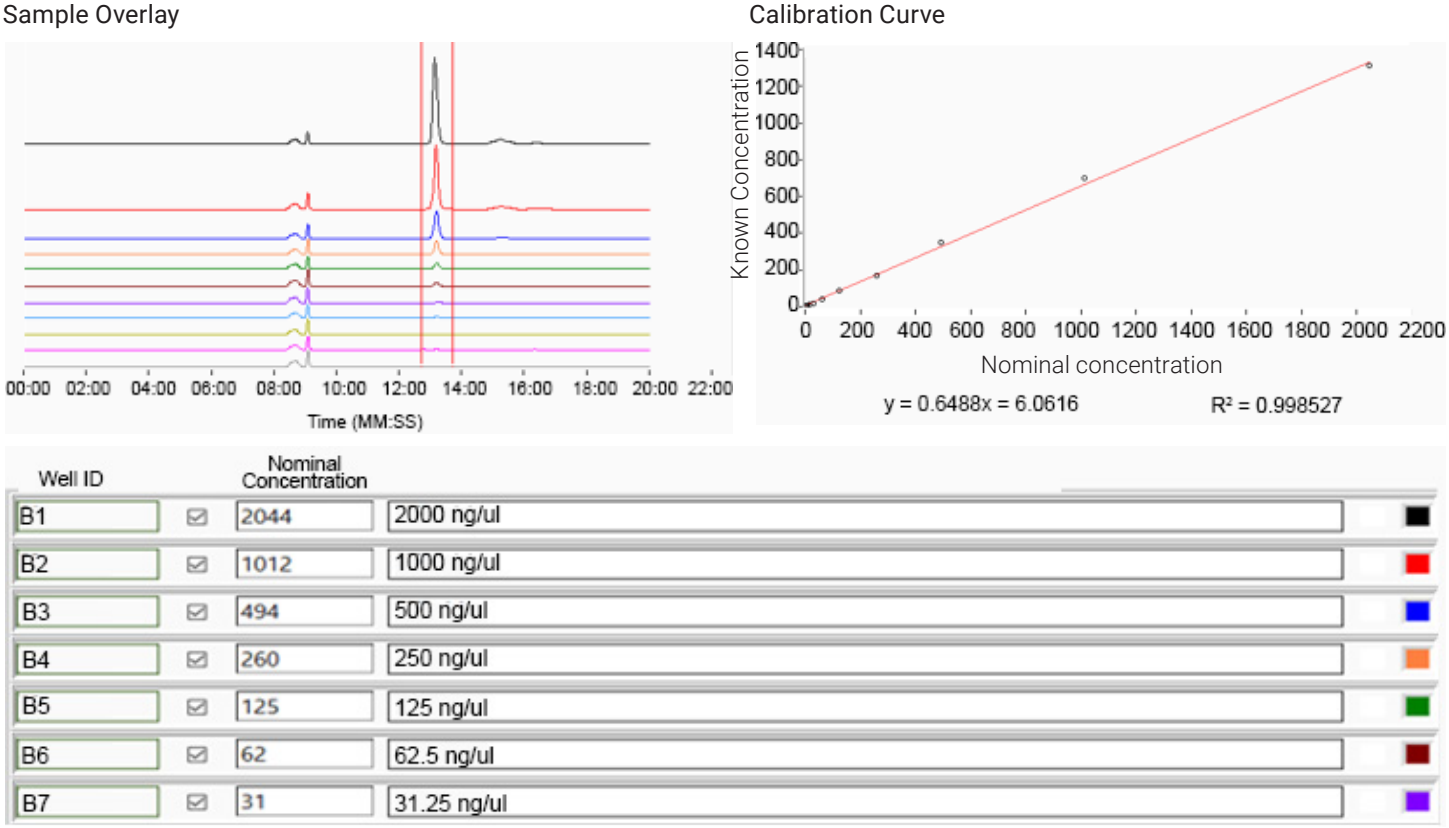
Absolute quantification

To adjust for proteins with different labeling efficiencies, a correction factor can be used to calculate the absolute concentration of the target proteins. To do this, a calibration can be performed by analyzing a dilution series of the target protein. The nominal concentration of each dilution and the observed concentrations from the ProteoAnalyzer are used to create a calibration curve. This curve is then used as the correction factor

to convert the relative quantification to an absolute quantification (Figure 9A). Once created, a calibration curve can be saved for importing into future runs.

This ability to create calibrated concentration curves within the ProSize data analysis software allows for absolute quantification of samples with the ProteoAnalyzer. To demonstrate this, a serial dilution of BSA from 2 to 2,000 ng/µL was analyzed on the ProteoAnalyzer, and the nominal concentration confirmed by UV spectroscopy. A calibration curve was generated from the data using ProSize (Figure 9A). The calibration curve was applied in the analysis of two BSA samples at random concentrations and compared to the concentration obtained by UV spectroscopy. The percent error of both samples was greater than 40% when measured by relative quantification using the ProteoAnalyzer. When applying the BSA calibration curve generated by the ProteoAnalyzer for absolute quantification, the percent error decreased to less than 15% for both samples (Figure 9B).

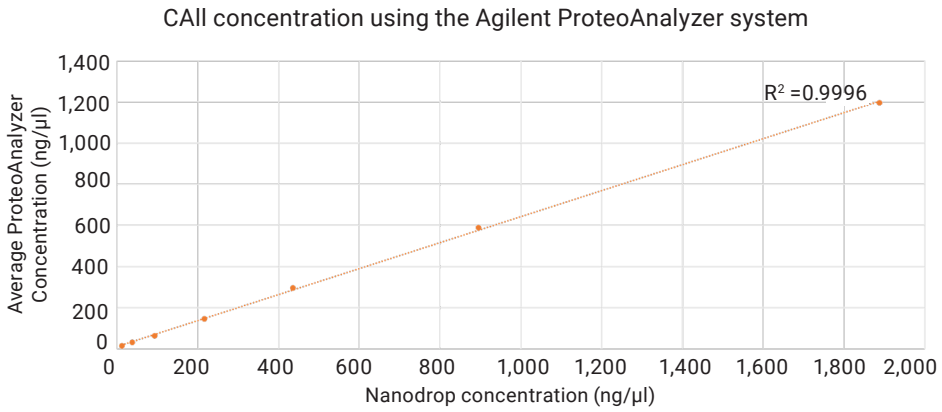
A



B

	Relative Quantification			Absolute Quantification			Nanodrop
	Average Concentration (ng/µl)	Percent Error	%CV	Average Concentration (ng/µl)	Percent Error	%CV	Average Concentration (ng/µl)
Sample A	467.45	43.68	2.70	711.12	14.32	2.73	830
Sample B	48.75	40.91	2.86	65.79	20.26	3.27	82.5

Figure 9. The Agilent ProteoAnalyzer system reports the absolute quantification of protein samples. A) A serial dilution of BSA was analyzed on the ProteoAnalyzer and the data were used to generate a calibration curve using the Agilent ProSize data analysis software. B) The calibration curve was used to calculate the absolute quantification of two unknown concentrations of a BSA sample. Relative quantification and Nanodrop are shown for comparison; the percent error is based on the reported concentration from the Nanodrop. n=4.



ProteoAnalyzer System		Nanodrop
Average concentration (ng/µl)	%CV	Average concentration (ng/µl)
1195.87	7.17	1887.73
589.70	1.77	894.70
296.23	2.26	435.85
146.29	6.28	216.20
63.77	1.99	93.15
31.81	4.77	37.95
14.96	9.18	12.08
7.89	6.75	NA
4.39	11.45	NA
2.50	24.36	NA

Figure 10. Quantification of CAII serial dilution using the Agilent ProteoAnalyzer system. n=3.

Quantification of CAII

To further demonstrate the quantitative capabilities of the ProteoAnalyzer, a two-fold serial dilution of a CAII sample was analyzed and compared to the nominal concentration. The nominal concentration was determined by Nanodrop, using an extinction coefficient for the specific protein. As shown in Figure 10, the ProteoAnalyzer and Nanodrop showed excellent linear correlation, with an R^2 of 0.9996 when analyzed with the LM & UM method.

The flexibility of the ProteoAnalyzer system allows for either absolute or relative quantification of different proteins, as demonstrated by the BSA and CAII examples described in this paper.

Conclusion

This technical overview highlights the reproducibility of protein sizing and quantification with the Agilent ProteoAnalyzer system and demonstrates the technical specifications of the Agilent Protein Broad Range P240 kit using BSA and CAII proteins. The ProteoAnalyzer can be used for quality control of proteins, including size, concentration, and integrity of the sample. The system offers the ability to analyze many different proteins, from 10 to 240 kDa in size, over an expansive concentration range. The accurate and precise quality measurements achieved by the ProteoAnalyzer are ideal for many applications, such as biotherapeutics and synthetic biology workflows.

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