Technical Overview

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Best Practices for Protein Analysis with the Agilent ProteoAnalyzer System

Introduction

Reliable quality control (QC) of protein samples is essential to many workflows, including protein characterization and product release. Among the many different attributes of proteins, the size, purity, and relative concentration of proteins are assessed with electrophoretic separations using sodium dodecyl sulfate (SDS) to denature the samples and help create a consistent mass: charge ratio to facilitate separation based on size. Traditionally, this type of protein analysis is performed using polyacrylamide gel electrophoresis with SDS (SDS-PAGE), a lengthy process that requires large amounts of hands-on time. To automate protein analyses, the Agilent ProteoAnalyzer system uses parallel capillary electrophoresis with SDS (CE-SDS)[1,2]. The system separates proteins in discrete capillaries, allowing for the analysis of 12 samples at the same time. Between runs, the capillaries are automatically cleaned, rejuvenated, and filled with fresh separation gel to provide consistent results for a wide variety of protein sample types, including purified antibodies, membrane proteins, fermentation supernatant, and crude lysates, among many others.

The Agilent Protein Broad Range P240 kit can be used for high-resolution separations of protein samples ranging in size from 10 to 240 kDa. Samples can be analyzed under reduced or nonreduced conditions, and are prepared using a rapid covalent labeling method. The streamlined workflow quickly labels proteins with a fluorescent dye. Samples are voltage injected on the ProteoAnalyzer and the proteins are electrophoretically separated within individual capillaries. The labeled proteins are detected by the system using a sensitive charge-coupled device camera that provides a 3-log dynamic range for detection of minute levels of impurities with low levels of background noise. The entire process takes less than an hour, with 20 minutes of sample preparation and 30 minutes of running time. Data is seamlessly integrated with Agilent ProSize data analysis software, providing unambiguous results, reducing time spent analyzing gels, and allowing for simple data archiving. This technical overview describes best practices for protein analysis, including sample handling and storage, running conditions, and data analysis, with the automated CE-SDS ProteoAnalyzer system.

Methods

Commercially available bovine serum albumin (BSA) (Sigma p/n A7906 or NEB p/n B9000S) was diluted in 1x PBS unless otherwise stated and prepared under reducing conditions according to the labeling workflow described in the Agilent Protein Broad Range P240 kit quick guide[3].

NISTmAb (Sigma p/n NIST8671, aliquot from Reference Material 8671, Lot 14HB-D-002)[4] was prepared in PBS at a concentration of 2,000 ng/µL under both reducing and nonreducing conditions. The samples were covalently labeled according to the kit quick guide, with an incubation temperature of 70 °C for 10 minutes for optimal results[5].

Samples were assessed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit (p/n 5191-6640) using the LM-only and the LM and UM methods[1]. Analytical specifications of the kit are listed in Table 1. For NISTmAb prepared under nonreduced conditions, the sample injection was decreased to 7 kV for 6 seconds for optimal results[5].

Results and discussion

Buffer compatibility

Buffers and additives can affect the appearance of the dye front and the migration of a sample. The ProteoAnalyzer user guide contains a list of buffers which have been shown to have minimal impact when compared to PBS (Table 2). To demonstrate the effect that different buffers can have on migration, the protein ladder was prepared in a variety of buffers and analyzed on the ProteoAnalyzer system using the LM-only method. Representative examples of the resulting electropherograms and the digital gel images are shown in Figure 1.

Table 1. Agilent Protein Broad Range P240 kit specifications. LM: lower marker; UM: upper marker. MWR: molecular weight resolution.

Analytical Specifications		ProteoAnalyzer Protein Broad Range P240 kit
Sizing Range	LM only	10 to 240 kDa
	LM and UM	10 to 200 kDa
Typical Sizing Accuracy (% Sizing Error)	LM only	< 15% for BSA, CAII (using reduced conditions)
	LM and UM	< 10% for BSA, CAII (using reduced conditions)
Typical Resolution		< 10% molecular weight resolution between 15 to 150 kDa (based on ladder) R ≥ 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 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
Quantification Reproducibility		<15 %CV for 20 – 2,000 ng/µL BSA <25 %CV for 2 – 20 ng/µL BSA

Table 2. Sample buffer compatibility list for the Agilent Protein Broad Range P240 kit.

Low Impact: 50% to 300% signal compared to PBS*	Comment
200 mM Tris-HCl, pH 8.0	
50 mM Citrate buffer pH 4	
50 mM Acetate buffer pH 5.2	
50 mM MES pH 6	
50 mM MOPS pH 7	
50 mM HEPES, pH 8.0	
50 mM $NaHCO_3$ pH 8.5	
7 M urea 2 M thiourea	
5% 2-Mercaptoethanol	
20 mM DL-Dithiothreitol (Cleland's Reagent, DTT)	
50 mM $MgCl_2$	
100 mM KCl	Higher concentrations of potassium are not recommended due to the low solubility of potassium dodecyl sulfate
1% Triton X-100	
1% Tween 20	
4% CHAPS	High CHAPS concentrations affect sizing, the use of Ladder diluted in sample buffer is recommended
1% NP-40	
1% SDS	
300 mM NaCl	If the salt concentration in the sample is higher than 300 mM, a buffer exchange to a lower iconic strength buffer is recommended
Strong Impact: < 50% signal compared to PBS*	
50 mM Tris(2-carboxyethyl)phosphine (TCEP)	Incompatible with fluorescent dye

** Phosphate Buffered Saline: 30 mM Tris-HCl, 26 mM NaH_2PO_4, 41 mM Na_2HPO_4, 79 mM NaCl, pH 8.5*

Reducing agents

Reagent	
10 mM DL-Dithiothreitol (Cleland's Reagent, DTT)	Recommended
5% 2-Mercaptoethanol	3- to 5-fold signal decrease in comparison to 10 mM DTT
50 mM Tris(2-carboxyethyl)phosphine (TCEP)	Incompatible with fluorescent dye

Examination of the peaks when prepared in PBS compared to other buffers highlights the migratory effects caused by the different buffers. For example, as shown in the overlay image of ladders prepared in different buffers, 4% CHAPS causes the peaks to migrate faster, while 300 and 1,000 mM NaCl both cause the peaks to migrate slower (Figure 1A). Additionally, different buffers can impact the shape and peak height of the dye front that appears prior to the lower marker (LM), as seen in Figure 1A.

The migratory patterns of the ladder peaks are indicative of sample migration effects that can arise during assessment. For example, since sample sizing is determined by comparing the migration time of the sample to that of the peaks in a ladder, the apparent sizing of a sample may be impacted by which buffer the sample and ladder are prepared in. To demonstrate this, BSA was prepared in a variety of buffers and analyzed with a ladder prepared in PBS. When BSA and ladder are both prepared in PBS, BSA sizes at 69-70 kDa on the ProteoAnalyzer system using the LM-only method. When the BSA is prepared in 4% CHAPS, but the sizing ladder is in PBS, the faster migration of the BSA results in a smaller apparent size than expected (Figure 1B). Alternately, preparing BSA with 300 and 1,000 mM NaCl caused slower migration times, and thus larger than expected sample sizing. When sample and ladder are prepared in the same buffer, migratory effect is diminished, and sizing is closer to the expected size of 66 kDa (Figure 1C). For best results, it is recommended to dilute the ladder in the same buffer as the sample as described in the kit quick guide[3].

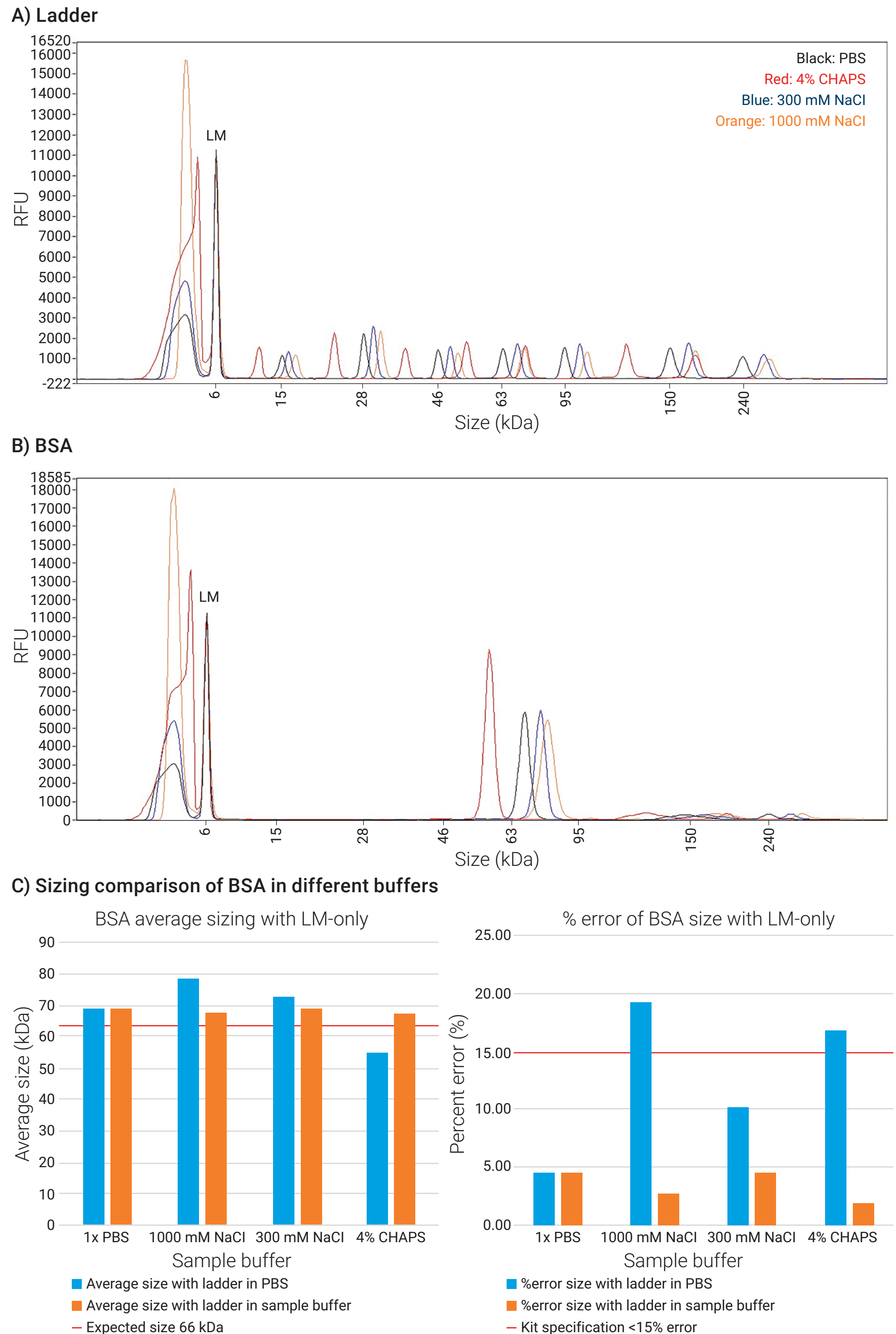


Figure 1. Overlays of A) ladder and B) BSA were prepared in different buffers and analyzed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit using the LM-only method to demonstrate the impact that the sample buffer can have on sample migration. C) BSA prepared in different buffers was analyzed using ladder prepared in PBS or the same sample buffer for comparison. For best results, the sample and ladder should both be prepared in the same buffer.

The Protein Broad Range P240 kit for the ProteoAnalyzer has also been validated for use with a provided optional upper marker for improved alignment and therefore better sizing accuracy. To demonstrate this, BSA and ladders prepared in different buffers were also analyzed using the LM and UM method and compared to the sizing analysis from the LM-only method. As shown by the overlay images of the ladder and BSA in Figure 2, the sample peaks prepared in PBS and NaCl are more similar in size to each other, while the samples prepared in 4% CHAPS were closer to the expected size but still slightly smaller than the PBS preparation. The sizing comparison in Table 3 demonstrates that use of the LM-only exacerbates the migratory effects of a sample buffer, while the LM and UM method can be used for better alignment to minimize this effect.

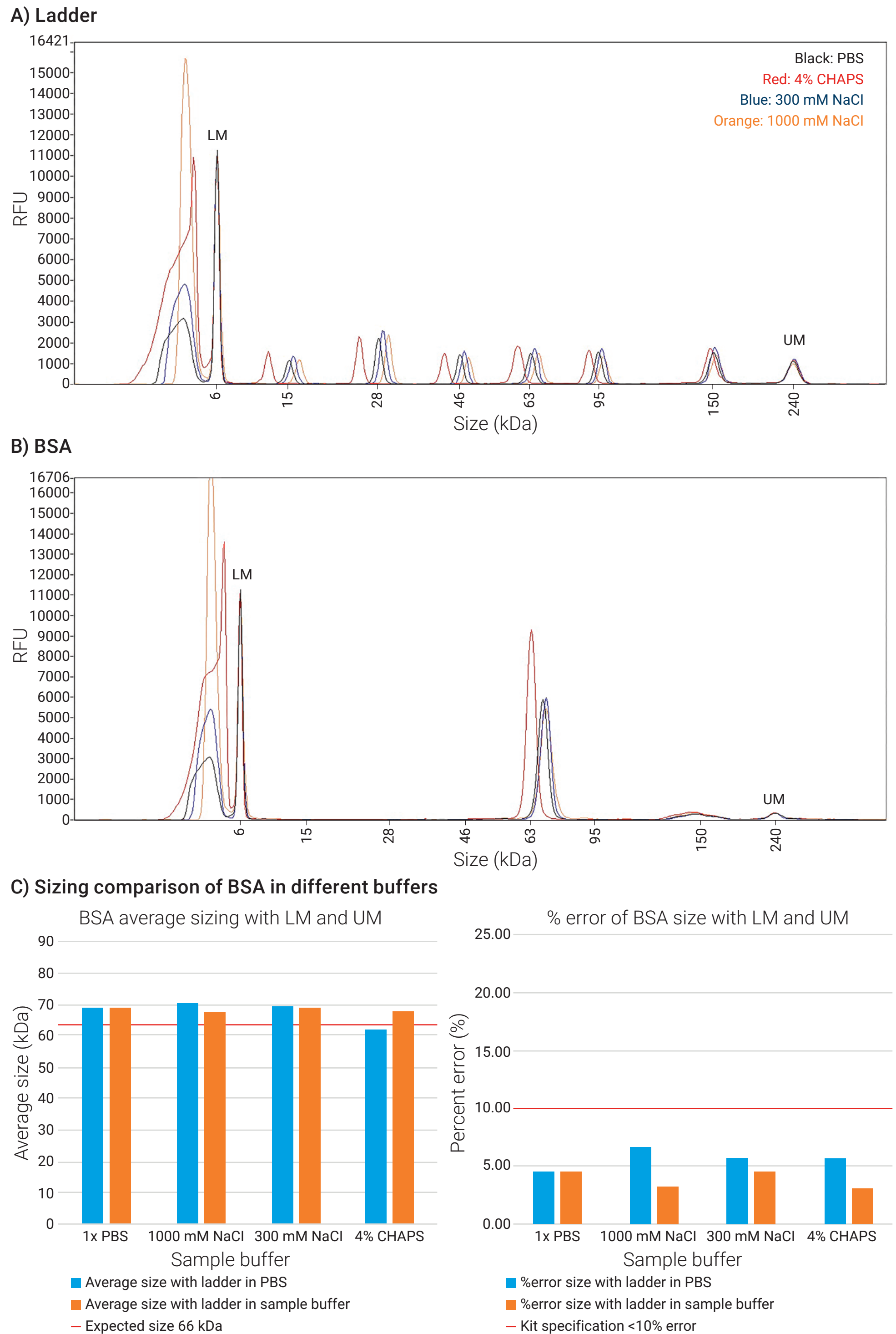


Figure 2. Overlays of A) ladder and B) BSA were prepared in different buffers and analyzed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit using the LM and UM method to demonstrate the impact that the sample buffer can have on sample migration. C) BSA prepared in different buffers was analyzed using ladder prepared in PBS or the same sample buffer for comparison. For best results, the sample and ladder should both be prepared in the same buffer.

Table 3. Comparison of BSA sizing when prepared in different buffers and analyzed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit using A) the LM-only method and B) the LM and UM method.

A) LM-only method

	Average Size (kDa)		%CV		Percent Error (expected 66 kDa)	
Sample Buffer	Ladder in PBS	Ladder in sample buffer	Ladder in PBS	Ladder in sample buffer	Ladder in PBS	Ladder in sample buffer
1x PBS	69.00	69.00	2.68	2.68	4.55	4.55
1000 mM NaCl	78.75	67.75	1.31	3.03	19.32	2.65
300 mM NaCl	72.75	69.00	4.97	1.90	10.23	4.55
4% CHAPS	54.88	67.25	3.29	4.26	16.86	1.89

B) LM and UM method

	Average		%CV		Percent Error (expected 66 kDa)	
Sample Buffer	Ladder in PBS	Ladder in sample buffer	Ladder in PBS	Ladder in sample buffer	Ladder in PBS	Ladder in sample buffer
1x PBS	69.00	69.00	0.00	0.00	4.55	4.55
1000 mM NaCl	70.38	68.13	0.74	0.94	6.63	3.22
300 mM NaCl	69.75	69.00	1.01	0.00	5.68	4.55
4% CHAPS	62.25	68.00	0.74	1.36	5.68	3.03

Injection conditions

The controller software for the ProteoAnalyzer system provides users with predefined methods for protein analysis, but also provides the flexibility to adjust settings such as injection and separation time and voltage. Since different proteins may label with different efficiencies, this allows for optimization of the analysis for specific protein sample types and preparations. For example, in the image shown in Figure 3A, the sample was first analyzed with the LM-only method for the Protein Broad Range P240 kit, which uses a 10-second injection time (blue trace). The height of the sample peak was over 80,000 RFU, which is higher than the allowed tolerance for accurate analysis of the sample, and is indicated by the presence of a warning flag in the ProSize data analysis software (Figure 3B). The same sample was reanalyzed with a lower injection time of six seconds to optimize sample analysis and avoid oversaturation of the main peak (black trace). For best results, the height of the main peak should not exceed approximately 50,000 RFU.

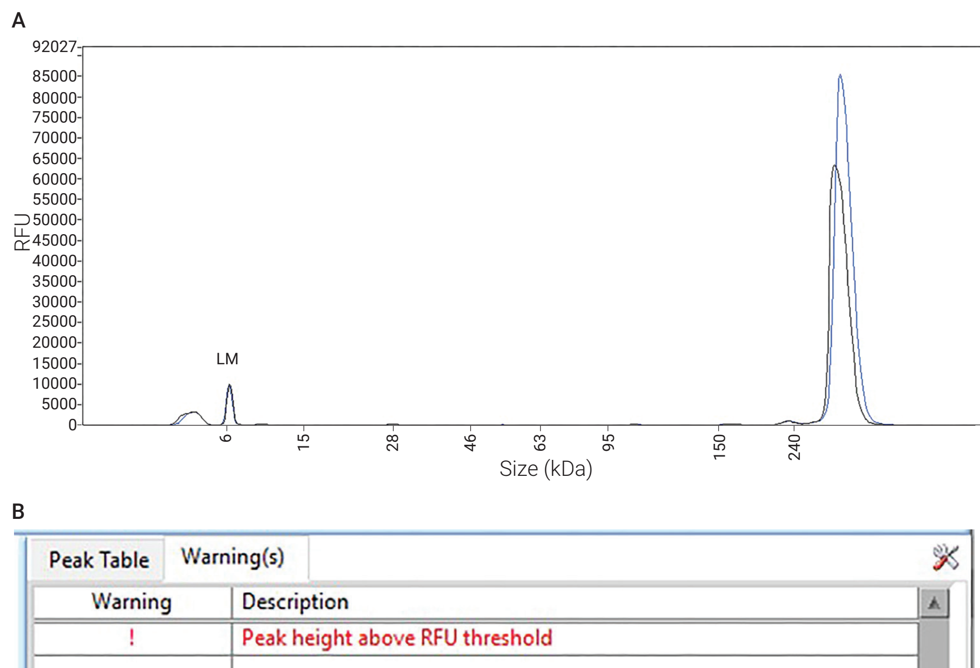


Figure 3. Injection time optimization on the Agilent ProteoAnalyzer system. A) Overlay of BSA injected for different times to optimize analysis conditions. Blue trace: ten second injection; Black trace: six second injection. B) The blue trace has a peak height of > 80,000 RFU, indicated by the warning flag in the ProSize data analysis software.

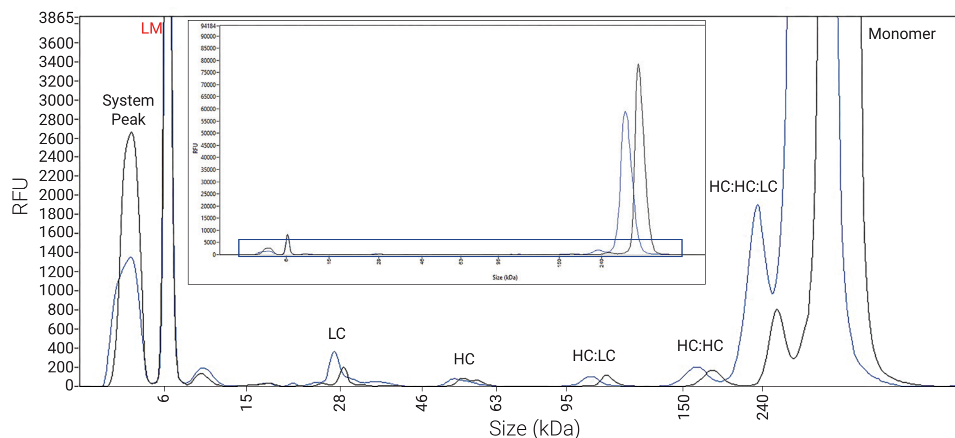
Incubation temperature

Purity assessment of samples can be impacted by many factors, including storage, handling, and temperature. The ProteoAnalyzer provides a starting point for the analysis of many different proteins, but sample preparation and separation parameters should be optimized for sensitive samples such as monoclonal antibodies (mAb). For example, the kit quick guide for the Protein Broad Range kit uses a 10 minute, 85 °C incubation for the labeling reaction.

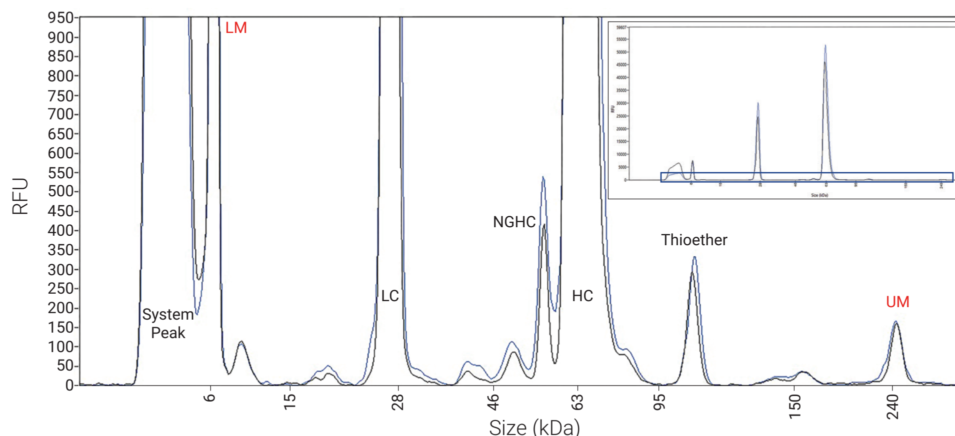
To provide guidance for optimizing the labeling conditions used with the ProteoAnalyzer system, different incubation temperatures were tested for analysis of the NISTmAb. Due to its extensive characterization, the NISTmAb is a well-established standard within the biopharmaceutical industry and is widely used for evaluating analytical methods for mAb quality assessment. In the datasheet and publications describing the characterization of the NISTmAb, the labeling temperature used with other CE-SDS methods was decreased to 70 °C for optimal results[4,6]. Based on this previous work we compared the results of labeling the NISTmAb at 70 and 85 °C on the ProteoAnalyzer to the NIST Reference Material Sheet[4].

Electropherogram overlays of the nonreduced (Figure 4A) and reduced (Figure 4B) NISTmAb prepared with these different incubation temperatures show slight differences. For example, in the nonreduced NISTmAb, incubation at 70 °C compared to 85 °C results in higher peak heights of the monomer.

A) Nonreduced NISTmAb



B) Reduced NISTmAb



C) NISTmAb data analysis

	NIST Datasheet		ProteoAnalyzer, 70 °C (nonreduced N = 11; reduced N = 33)		ProteoAnalyzer, 85 °C (nonreduced N = 3; reduced N = 5)	
	Size heterogeneity (%)	Combined standard uncertainty (%)	Size heterogeneity (%)	%CV	Size heterogeneity (%)	%CV
Monomeric Purity (nonreduced)	98.47	1.03	98.18	0.09	94.43	0.31
Thioether (reduced)	0.30	0.03	0.40	6.35	0.42	5.64
Glycan Occupancy (reduced)	99.39	0.07	99.30	0.02	98.93	0.03

Figure 4. Overlay of NIST mAb analyzed on the Agilent ProteoAnalyzer system under A) nonreduced and B) reduced conditions. Blue trace: 85 °C; Black trace 70 °C: C) NISTmAb analysis using the ProteoAnalyzer was compared to the data from the published NIST reference materials[4].

Close examination of the light chain (LC), heavy chain (HC), and combinations of the two highlight slight differences when the temperature is changed. While the HC, HC:LC, and HC:HC peaks are the same, the LC and HC:HC:LC peaks are larger when prepared at 85 °C. It can be inferred that the higher temperature causes a LC to separate from the monomer, causing an increase in the LC and HC:HC:LC impurity peaks. While subtle in appearance, these changes can impact the monomeric percent purity values, as shown in Figure 4C.

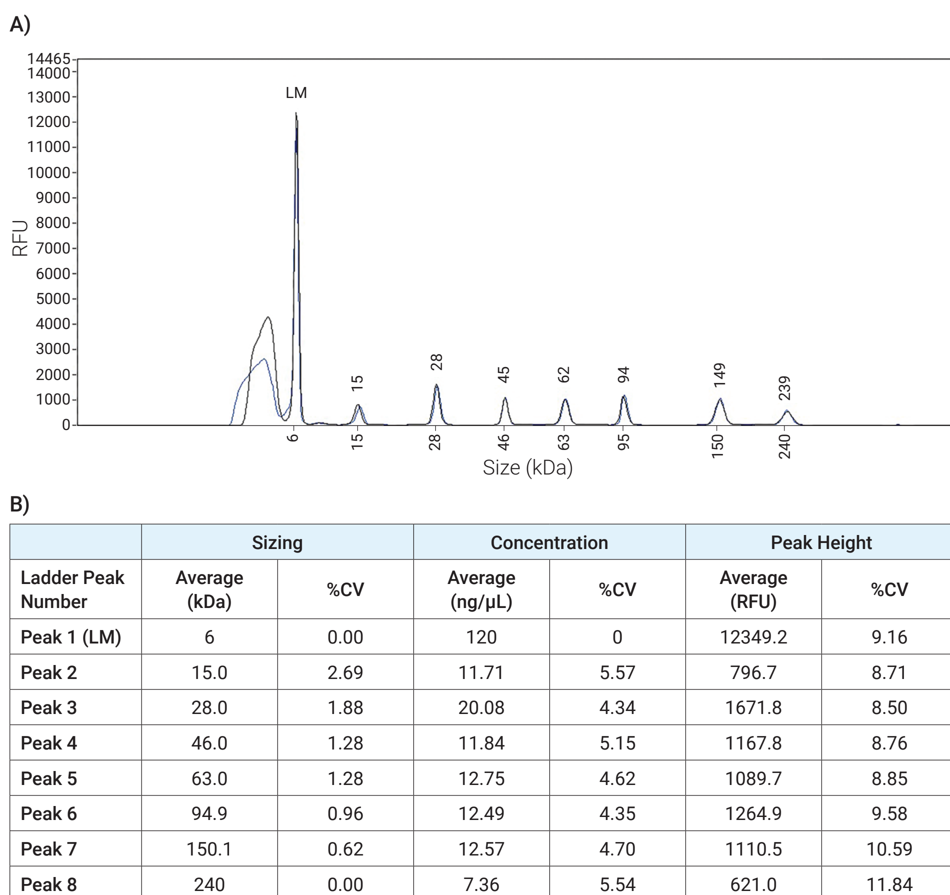
Similarly, analysis of the reduced NISTmAb with different temperatures shows small, yet significant changes in the electropherogram (Figure 4B). In particular, the incubation temperature can affect the resolution between the nonglycosylated heavy chain (NGHC) and HC peaks. The resolution between these two peaks is closer to the baseline and shows a higher R value when the sample was prepared with a 70 °C incubation temperature (average R = 1.60) than when at 85 °C (average R = 1.36). The higher resolution achieved at 70 °C allows the analysis software to define the area of the peaks more accurately, leading to better quantification of each. This provides a more accurate representation of the low-level impurities present in the sample.

As shown in Figure 4C, analysis of the monomeric purity, thioether amount, and glycan occupancy of the NISTmAb when using an incubation temperature of 70 °C was highly comparable to the data presented in the NISTmAb datasheet, and matched more closely than the data for the sample when prepared at 85 °C.

Number of injections

The capillary array used with the ProteoAnalyzer is available in a 12 capillary format to analyze a single row of a 96-well plate with each run. Multiple rows of a plate can be programmed for subsequent runs without user assistance. Due to sample evaporation, the recommended number of samples to load onto the instrument at a time is 96, or 8 runs. However, depending on local lab environments such as humidity levels, up to three 96-well plates may be loaded on the instrument at once.

To demonstrate that three plates can be prepared and successfully analyzed on the ProteoAnalyzer, 24 runs were analyzed in succession over 14 hours, with each row consisting of 12 wells of ladder prepared as a master mix. An overlay of the ladders from runs 1 and 24 show that the size and peak height of each fragment did not significantly change between runs (Figure 5A). This is further demonstrated through analysis of each of the ladder peaks across all 24 runs, shown in Figure 5B. The percent CV of the average size of each peak is less than 3, well within the kit specifications and highlighting the excellent sizing precision that can be achieved by the ProteoAnalyzer system.



	Sizing		Concentration		Peak Height	
Ladder Peak Number	**Average (kDa)**	**%CV**	**Average (ng/µL)**	**%CV**	**Average (RFU)**	**%CV**
Peak 1 (LM)	6	0.00	120	0	12349.2	9.16
Peak 2	15.0	2.69	11.71	5.57	796.7	8.71
Peak 3	28.0	1.88	20.08	4.34	1671.8	8.50
Peak 4	46.0	1.28	11.84	5.15	1167.8	8.76
Peak 5	63.0	1.28	12.75	4.62	1089.7	8.85
Peak 6	94.9	0.96	12.49	4.35	1264.9	9.58
Peak 7	150.1	0.62	12.57	4.70	1110.5	10.59
Peak 8	240	0.00	7.36	5.54	621.0	11.84

Figure 5. Overlay of subsequent runs on the Agilent ProteoAnalyzer system, showing minimal variation of the peaks between runs 1 (black trace) and 24 (blue trace). B) Average size, concentration, and peak height of each of the ladder peaks, with low variability between the runs, indicated by low %CV (N = 264).

Plate storage

To examine the stability of labeled sample over time, BSA and ladder were prepared for analysis with the ProteoAnalyzer system and stored at room temperature or 4 °C for 10 days in both light and dark conditions. The samples were analyzed on the ProteoAnalyzer at days 0, 3, 7, and 10. The samples stored at room temperature had evaporated by day 10 and were not analyzed past day 7. As shown in the electropherogram overlays of BSA in Figure 6, after as little as three days of storage, the sample peak heights were significantly decreased and the width of the peak increased. This causes a slight size shift of the sample and could impact percent purity calculations. Thus, for the most accurate analysis, it is recommended to analyze protein samples on the ProteoAnalyzer within one day of sample preparation.

Data analysis with ProSize

After sample runs are completed with the ProteoAnalyzer system, data is processed using the corresponding Agilent ProSize data analysis software[7]. The data is shown as an electropherogram that allows for a detailed assessment of the sample, including small degradation products. Additionally, the data is translated into a digital gel image which allows for easy and quick visual assessment of sample bands, analogous to an SDS-PAGE gel[2]. The software includes features to aid analysis, such as smear integration and a peak table to provide size, concentration, and purity information of the samples.

ProSize uses a proprietary algorithm to identify changes in the detected fluorescence. Users can specify the minimum values to define the peak width and minimum peak height. The peak width values are represented in seconds and are used to determine the threshold of what constitutes a peak along the horizontal axis. Higher values widen the peak start and end points, while smaller values define sharp peak start and end points. The minimum peak height is the threshold used to determine if a signal is composed of a high enough relative fluorescence unit (RFU) on the vertical axis to be integrated as a sample peak. Peaks with RFU values below the minimum value will not be selected for integration. The data from the ProteoAnalyzer will be automatically analyzed with preset values, but the software provides users the flexibility to easily adjust the values for specific sample types.

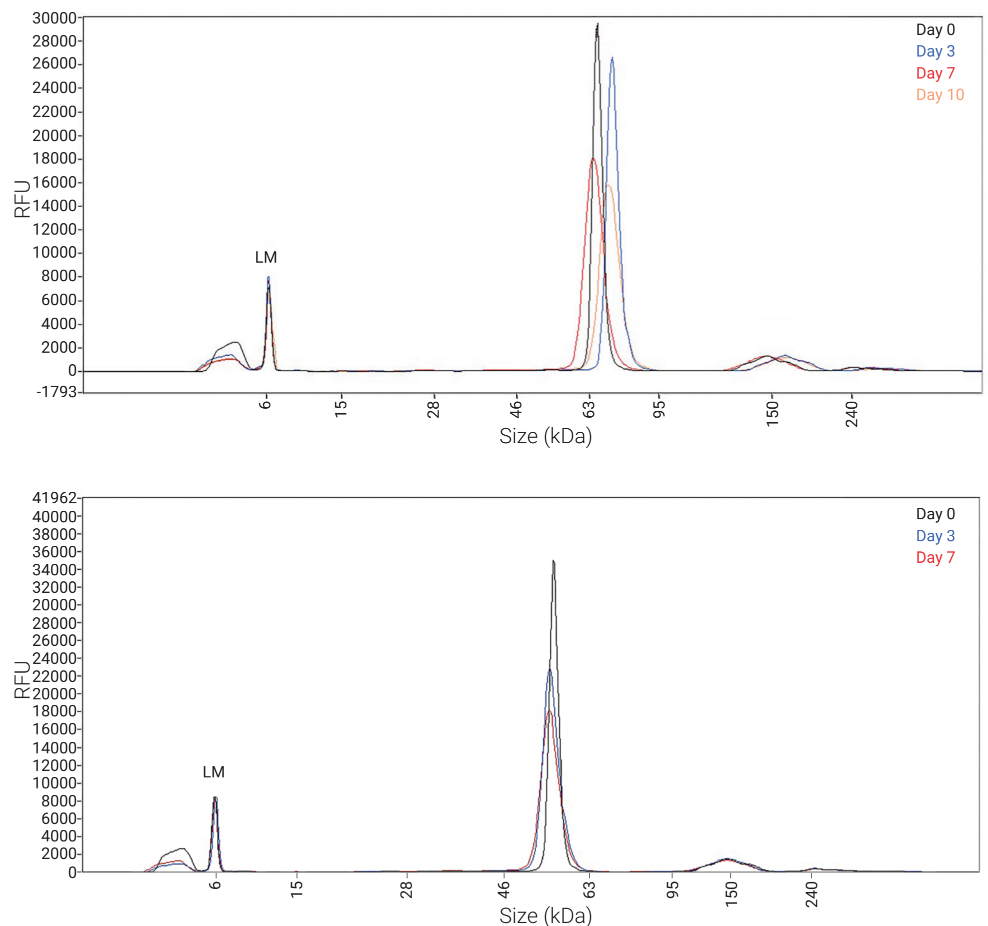


Figure 6. BSA was prepared for analysis on the Agilent ProteoAnalyzer system and stored under a variety of storage temperatures to examine stability of labeled samples over time. Storage at both A) 4 °C and B) room temperature under either light or dark conditions showed a decrease in sample peak height after just three days of storage, indicating that samples should be assessed on the ProteoAnalyzer system within one day of sample preparation for best results.

Conclusion

This technical overview describes some of the best practices for protein sizing, quantification, and integrity analysis with the Agilent ProteoAnalyzer system, including sample handling and method optimization. The system offers the ability to analyze many different proteins, from 10 to 240 kDa in size, over an expansive concentration range from 2 to 2,000 ng/µl. 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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