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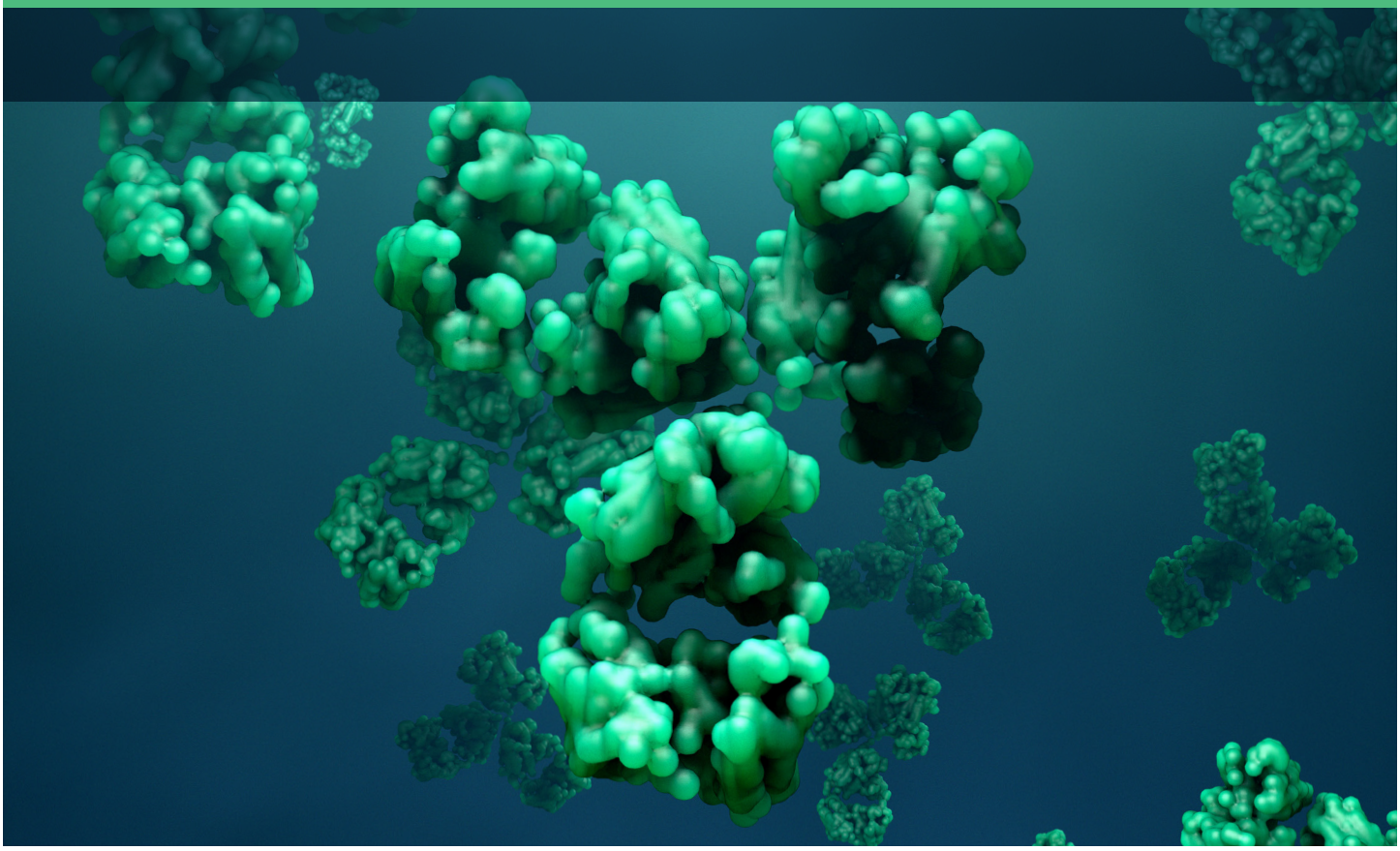
Antibody Analysis Using the Agilent ProteoAnalyzer System

Application Compendium



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Antibody analysis with automated CE-SDS

As antibody based therapeutics continue to grow in complexity and importance, having reliable, easy to use analytical tools is essential for confident decision making throughout development and characterization. Scientists need solutions that deliver robust, reproducible, and high-throughput results while fitting seamlessly into busy laboratory workflows.

The Agilent ProteoAnalyzer system (Figure 1) is a high-throughput, automated parallel capillary electrophoresis (CE) platform designed to simplify protein analysis. By combining standardized capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) methods, sensitive detection, and automated analysis, the ProteoAnalyzer system provides laboratories with consistent, high resolution results and minimal hands on time. This enables scientists to focus more on interpreting data and less on managing complex analytical workflows.

Figure 1. The Agilent ProteoAnalyzer system.

For antibody analysis, the ProteoAnalyzer system supports CE-SDS under both reduced and nonreduced conditions, providing a comprehensive view of antibody structure and integrity. These capabilities enable rapid and reliable assessment of critical quality attributes (CQAs), such as molecular weight, purity, fragmentation, and size heterogeneity in monoclonal antibodies (mAbs). As antibody modalities evolve, the same high throughput, automated approach also provides a strong foundation for the analysis of more complex molecules, including antibody-drug conjugates (ADCs).

This application compendium highlights examples of how the ProteoAnalyzer system is used for antibody analysis. The included publications demonstrate best practices, showcase method performance, and compare complementary detection and analytical approaches. Together, these studies illustrate how the ProteoAnalyzer system helps deliver reliable, actionable insights that support antibody research, development, and analytical confidence.

Denaturing protein analysis with the Agilent ProteoAnalyzer system

Ensuring the production or extraction of high-quality monoclonal antibodies is crucial for biopharmaceutical development and manufacturing. 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 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 CE-SDS. Rapid and consistent sizing and quantification results can be achieved for both reduced and nonreduced protein samples.

The ProteoAnalyzer system analyzes up to 12 samples simultaneously (Figure 2) in one run in approximately 30 minutes using the Agilent Protein Broad Range P240 kit. 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 system is designed to facilitate precise and accurate measurements of proteins and to detect impurities while requiring only 1 µL of sample. With capabilities well suited for protein applications in biotherapeutics and synthetic biology workflows, the robust ProteoAnalyzer system enables scientists to efficiently perform various protein quality and quantity assessments.

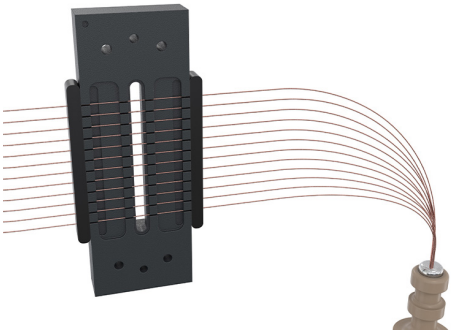


Figure 2. The Agilent ProteoAnalyzer 12-capillary array separates 12 protein samples in parallel in as little as 30 minutes.

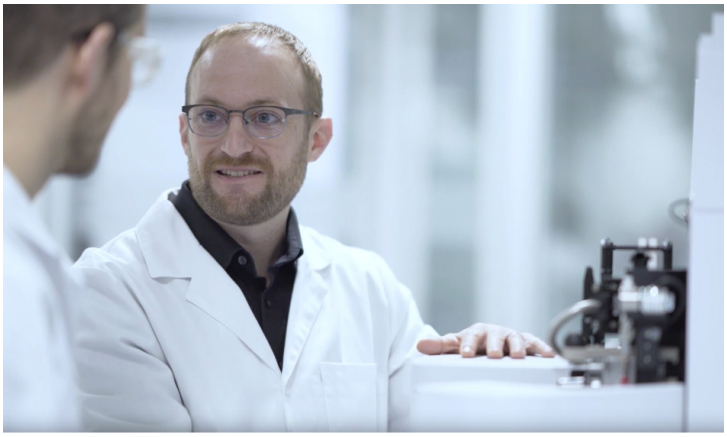
Table 1. Agilent Protein Brand Range P240 kit specifications. LM: lower marker; UM: upper marker; MWR: molecular weight resolution.

Analytical Specifications	Method	Protein Broad Range P240 Kit
Sizing Range	LM only	10–240 kDa
	LM and UM	10–200 kDa
Typical Sizing Accuracy (Percent Error)	LM only	< 15% for BSA, CAII
	LM and UM	< 10% for BSA, CAII
Typical Sizing Resolution		< 10% MWR between 15 to 150 kDa (based on ladder)
Sizing Precision	LM only	< 8% CV for BSA, CAII, GREMLIN-1, and NISTmAb (using reduced conditions)
		< 10% CV for intact NISTmAb using nonreduced conditions
	LM and UM	< 5% CV for BSA, CAII, GREMLIN-1, and NISTmAb (using reduced conditions)
Quantitative Range		2–2,000 ng/µL for BSA in PBS
Sensitivity (Signal-to-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

Interview with Kyle Luttgeharm, Product Manager, Agilent Technologies

1. What are the key analytical challenges scientists face when characterizing antibodies, and how do those challenges evolve as formats become more complex?

   Any time you manufacture a product using a biological system, both process- and product-related impurities need to be characterized. These impurities include not only host cell proteins, but also fragments and clipped antibody species usually resulting from proteolytic cleavage. As the antibody product becomes more complicated, so do the chances of impurity formation.



Dr. Kyle Luttgeharm, Product Manager for the Agilent ProteoAnalyzer system.

2. How was the ProteoAnalyzer system designed to support antibody analysis workflows, and what gaps was it intended to address?

   Size heterogeneity has long been analyzed by electrophoresis methods. However, traditional methods such as SDS-PAGE and single capillary electrophoresis are inherently limited in throughput. The manual nature of SDS-PAGE also increases data variation. The ProteoAnalyzer system was designed to provide automated higher-throughput CE-SDS to address the throughput bottleneck, while also generating high-quality digital data.

3. Why does CE-SDS remain such an important technique for antibody characterization?

   Capillary electrophoresis was first commercially introduced in the 1990s and has continued to advance since. As biologic therapeutics were starting to be introduced, CE-SDS was widely recognized for its ability to provide automated high-resolution separations for size heterogeneity analysis. CE-SDS ultimately became the standard technique for analyzing critical quality attributes, such as monomeric purity, percent glycosylation, and percent thioether. As the technology has developed, easy-to-use systems like the ProteoAnalyzer have been released that allow for greater accessibility to CE-SDS.

4. Why are reference materials like the NIST monoclonal antibody important for antibody analysis, and how are they typically used on the ProteoAnalyzer system?

   Reference standards such as the NISTmAb are extremely important during both the development of new systems and for the validation of existing systems. During development of the ProteoAnalyzer, the NISTmAb allowed us to ensure that the system provided accurate data. Users of the ProteoAnalyzer system also use reference standards to ensure that their individual systems and reagents are suitable for sample analysis on any given day. To ensure the instrument is working properly, the best practice is to use a standard for a system suitability test prior to sample analysis each day.

5. How does the ProteoAnalyzer system support the analysis of more complex antibody formats, such as antibody-drug conjugates?

   The ProteoAnalyzer Protein Broad Range kit provides high-resolution separations coupled with a 3-log quantitative linear dynamic range, allowing for confidence in sample analysis. While the default methods provided are a good starting point, we also recognize that, as samples become more complicated, no method is a one-size-fits-all solution. The ProteoAnalyzer system was designed with flexibility in mind by allowing for optimization of both the injection and electrophoresis conditions so that scientists can tailor the method to their individual needs.released that allow for greater accessibility to CE-SDS.

6. What would you say is the biggest advantage of using the ProteoAnalyzer system for antibody analysis? And what do customers say they value most?

   I would say ease of use and throughput, and customers agree. The system and its consumables were designed to require minimal hands-on time and maximum walk-away capabilities while providing the high-resolution, high-sensitivity separations expected from a CE-SDS system. The result is a sample preparation workflow requiring only 10 minutes of hands-on time and a system that can be loaded for overnight runs and left for the day—with confidence that the data will be ready for you in the morning. Customers are constantly amazed at how simple it is to prepare the instrument and samples.

NIST mAb as a Benchmark for Technology Performance

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Analysis of NIST Antibody on the Agilent ProteoAnalyzer System

Introduction

The National Institute of Standards and Technology (NIST), a branch of the US Department of Commerce, develops reference materials to foster the innovation and standardization of technology in various domains. One of these materials is a monoclonal antibody (mAb) called NISTmAb, which has been thoroughly characterized by multiple analytical techniques [1,2,3]. The antibody is composed of two heavy chains and two light chains, connected by interchain disulfide linkages. However, lower molecular weight fragment impurities can be generated during manufacturing, storage, and handling, yielding combinations of heavy and light chains that are smaller than the monomer. Electrophoresis is a standard method used to separate and visualize the pattern of these fragments, and provides information about the mAb size, concentration, composition, and purity. For complete characterization of mAb purity and glycan occupancy, a high-resolution separation system is required that can analyze the mAb under both reduced and nonreduced conditions.

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. Agilent, a leading provider of solutions for mAb characterization, now offers a novel technology that delivers a fast, automated, and higher-throughput alternative to conventional single capillary CE-SDS methods: The Agilent ProteoAnalyzer system[4]. In this technical overview, the characterization of the NISTmAb assessed with the ProteoAnalyzer was compared to the published NIST data obtained using traditional single capillary CE-SDS technology[1,2].

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Experimental

NISTmAb (Sigma p/n NIST8671, aliquot from Reference Material 8671, Lot 14HB-D-002)[1] was prepared in PBS at a concentration of 2,000 ng/µL under both reducing and nonreducing conditions according to the Agilent Protein Broad Range P240 kit (p/n 5191-6640) manual[5]. The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes. The reduced and nonreduced antibodies were analyzed across multiple capillaries of an Agilent ProteoAnalyzer system[4] with the ProteoAnalyzer Brand Range Kit LM only method. For nonreduced conditions, the sample injection was decreased to 7 kV 6 seconds for optimal results. Analysis of the samples from the ProteoAnalyzer was compared to the known specifications of the NISTmAb from datasheets and published results[1,2].

Results and discussion

Visual representation of NISTmAb

Characterization of the NISTmAb with traditional single capillary CE-SDS is described in the published NIST datasheet[1]. The results of the NISTmAb analyzed with the ProteoAnalyzer were thus compared to the NIST datasheet and published results[2] to evaluate the instrument.

Figure 1 shows representative examples of the published nonreduced NISTmAb compared to the results achieved by the ProteoAnalyzer. Analysis with either method uses a marker for sample alignment and quantitation (single-capillary CE-SDS: 10 kDa peak; ProteoAnalyzer: 6 kDa Lower Marker (LM)). The main peak, or monomer, of the NISTmAb and several smaller fragment peaks can be identified with both the single capillary CE-SDS method and the ProteoAnalyzer. The smaller peaks that can be visualized in the nonreduced sample include the light chain (L or LC), heavy chain (H or HC), and combinations of the two (HC:LC, HC:HC, HC:HC:LC). Although not described in the NISTmAb datasheet,

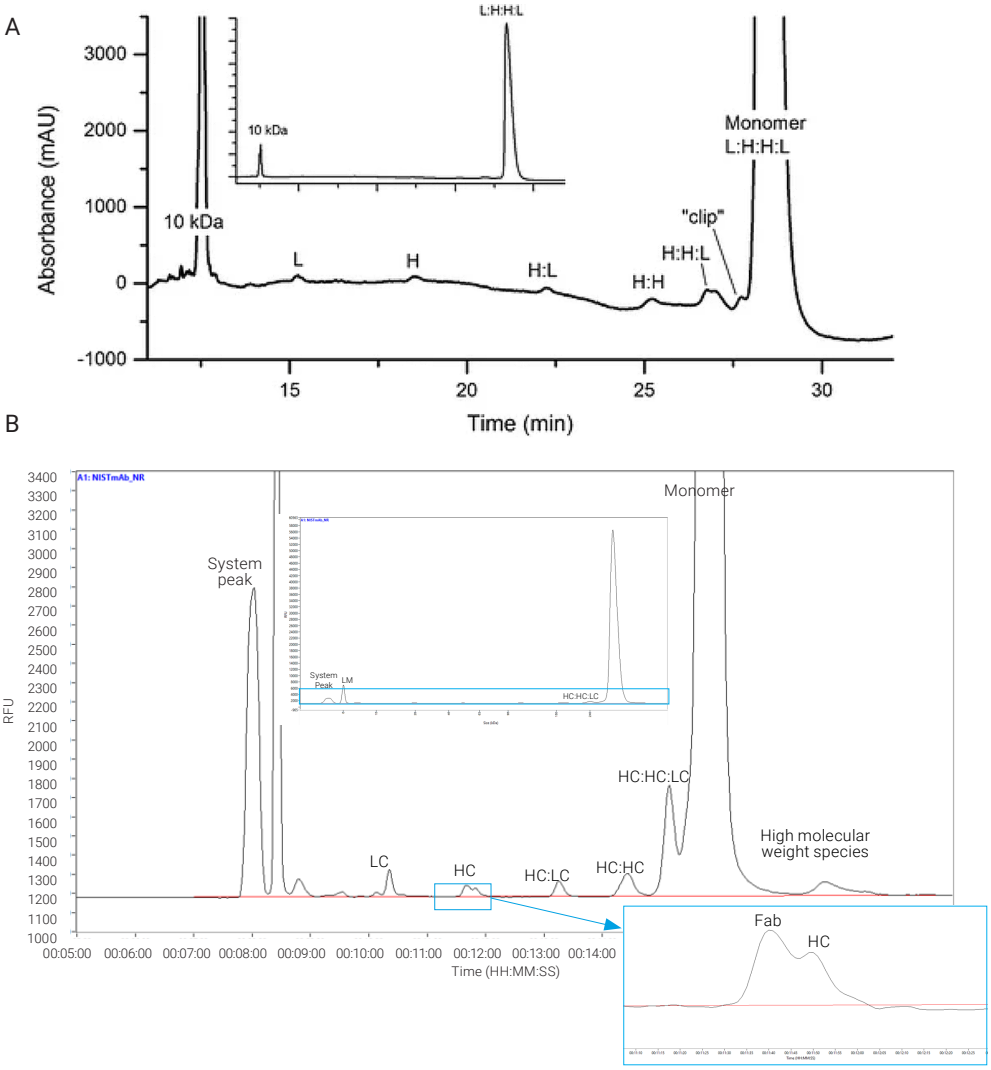

Figure 1. Nonreduced NISTmAb. A) This figure has been reproduced from Turner et al.[2] B) Results of the nonreduced NISTmAb using the Agilent ProteoAnalyzer system.

Turner, A., Yandrofski, K., Telikepalli, S. et al. Development of orthogonal NISTmAb size heterogeneity control methods. Anal Bioanal Chem 410, 2095–2110 (2018). https://doi.org/10.1007/s00216-017-0819-3

the HC when analyzed on the ProteoAnalyzer partially resolves two peaks, indicative of a disulfide-linked fragment antigen binding (Fab) region[6]. Additionally, high molecular weight (HMW) aggregates, when present, are visualized to the right of the monomer and have previously been identified as multimers[6].

The same NISTmAb sample was also run under reduced conditions on the ProteoAnalyzer, and the electropherogram compared to the standards from the NIST datasheet and publications[1,2] (Figure 2). Both systems were able to easily visualize the light chain and heavy chain. The smaller peak to the left of the heavy chain is representative of the nonglycosylated heavy chain (NGHC), and to the right is the thioether peak (or nonreduced species, NRS). Analysis of the reduced NISTmAb with the ProteoAnalyzer used the optional Upper Marker (UM) for better alignment and thus higher sizing precision of the sample peaks. Assessment of the NISTmAb by the ProteoAnalyzer provides highly comparable results to the traditional single capillary CE-SDS, providing users with the confidence to use either system for their analytical methods.

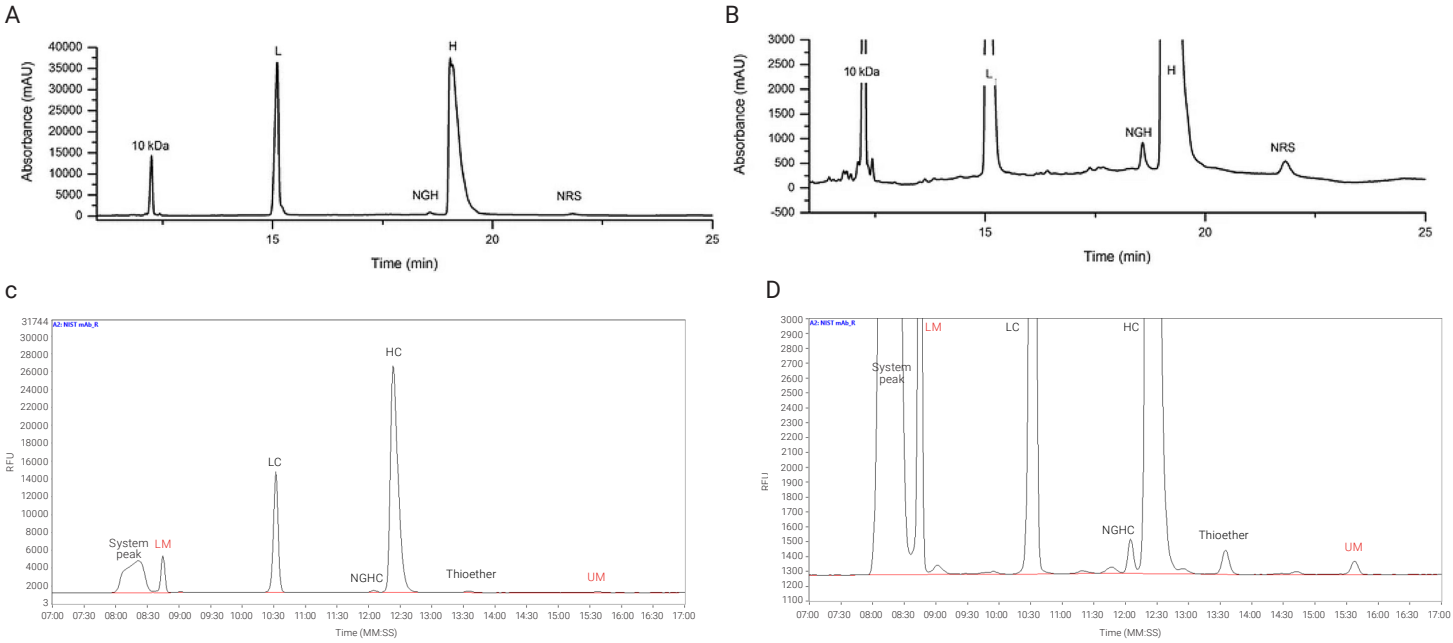

Figure 2. Reduced NISTmAb. (NRS = nonreduced species). A,B) This figure has been reproduced from Turner et al.[2] C,D) Results of the reduced NISTmAb using the Agilent ProteoAnalyzer system.

Turner, A., Yandrofski, K., Telikepalli, S. et al. Development of orthogonal NISTmAb size heterogeneity control methods. Anal Bioanal Chem 410, 2095–2110 (2018). https://doi.org/10.1007/s00216-017-0819-3

Table 1. Critical Quality Attributes of purity for the NISTmAb were determined by the Agilent ProteoAnalyzer system and compared to the published NIST datasheet[1]. ProteoAnalyzer: Nonreduced n = 11; Reduced n = 33.

	NIST Datasheet[1]		ProteoAnalyzer system	
	Size heterogeneity (%)	Combined standard uncertainty (%)	Size heterogeneity (%)	%CV
Monomeric Purity (nonreduced)	98.47	1.03	98.18	0.09
Thioether (reduced)	0.30	0.03	0.40	6.35
Glycan Occupancy (reduced)	99.39	0.07	99.30	0.02

Critical quality attributes of NISTmAb

A crucial aspect in determining the quality and efficacy of monoclonal antibodies is the Critical Quality Attributes (CQAs) of monomeric purity, glycan occupancy, and percent thioether (NRS). Accurate and reliable analysis is required for appropriate assessment of biopharmaceutical products. To evaluate the ProteoAnalyzer system, the monomeric purity, glycan occupancy, and percent thioether CQAs of the NISTmAb were calculated using established equations (Figure 3) and compared to the published NISTmAb data using single capillary CE-SDS[1,2].

$$\text{Monomeric Purity}(\%) = \frac{\text{Conc}_{\text{monomer}}}{\text{Conc}_{\text{monomer}} + \sum\text{Conc}_{\text{fragments}}} \times 100$$

$$\text{Glycan Occupancy}(\%) = \frac{\text{Conc}_{\text{HC}}}{\text{Conc}_{\text{HC}} + \text{Conc}_{\text{NGHC}}} \times 100$$

$$\text{Thioether}(\%) = \frac{\text{Conc}_{\text{thioether}}}{\text{Conc}_{\text{LC}} + \text{Conc}_{\text{HC}} + \text{Conc}_{\text{NGHC}} + \text{Conc}_{\text{thioether}}} \times 100$$

Figure 3. Equations used for calculating the Critical Quality Attributes of purity for the NIST mAb. (Conc = the concentration (ng/ul) of the designated peak as determined by the Agilent ProteoAnalyzer system and ProSize data analysis software.)

A measurement of nonreduced protein quality is the purity of the monomer peak. The published datasheet for the NISTmAb reports a monomeric purity of 98.47%[1]. In contrast, analysis with the ProteoAnalyzer results in a monomeric purity of 98.18%, only a 0.29% difference from the reported single capillary CE-SDS data (Table 1).

For the reduced form, two CQAs are displayed in the published datasheet: the thioether amount, which is 0.30%, and the glycan occupancy, at 99.39%[1]. Analysis of the NISTmAb with the ProteoAnalyzer results in a thioether measurement of 0.40% and a glycan occupancy of 99.30% (Table 1). The thioether measurement is comparable to the published data, while the glycan occupancy is incredibly close, with only a 0.09% difference between the two sources.

A crucial aspect of glycan occupancy assessment is the ability to resolve the NGHC and HC fragments from each other. As shown in Figure 2D, the ProteoAnalyzer offers excellent resolution of the NGHC and HC peaks, with an average R value of 1.60, meeting the kit specifications of an NGHC/HC R ≥ 1 for NISTmAb under reduced conditions. This is highly reproducible between capillaries, with 4.5%CV (n = 33). Together, this data and a comparison to the published NIST datasheet highlight that the ProteoAnalyzer allows for excellent separations and CQA assessment of both the nonreduced and reduced NISTmAb.

Conclusion

The well-characterized NISTmAb is used as a model protein for analysis of new and emerging analytical technologies. In this technical overview, the previously published single capillary CE-SDS separations and purity calculations using previously established formulas[1,2] were compared to the Agilent ProteoAnalyzer system. The system automates sample analysis and allows for higher throughput, running up to 12 samples in parallel, and has the ability to program up to eight runs at a time without user intervention. Data is displayed as either a digital gel or electropherogram, along with a peak table supplying information about the concentration of each fragment within the sample. Evaluation of the NISTmAb demonstrated that the ProteoAnalyzer provides robust separations and percent purity values that correlate well with the published reference data and can be used with confidence in analytical workflows.

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Application Note

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Accurate mAb Sizing Using the NIST mAb as a Ladder for the Agilent ProteoAnalyzer System

Authors

Kyle Luttgeharm and Whitney Pike
Agilent Technologies, Inc.

Abstract

Accurate sizing of monoclonal antibodies (mAbs) is essential for quality control in biopharmaceutical development. However, sizing can be challenging due to the complex secondary structure of intact mAbs. This application note demonstrates how integration of the NIST mAb (RM 8671) as a ladder within the Agilent ProteoAnalyzer system workflow can help achieve accurate sizing analysis of nonreduced mAbs. Analysis of several representative mAb samples using the NIST mAb as a ladder resulted in significantly improved sizing accuracy and precision with the ProteoAnalyzer system.

Introduction

Accurate analysis of monoclonal antibodies (mAb) is critical for ensuring product consistency, detecting structural variants, and meeting regulatory standards in biopharmaceutical development. The Agilent ProteoAnalyzer system is an automated capillary electrophoresis platform designed to accelerate and simplify protein quality assessment.[1] The ProteoAnalyzer supports a wide range of sample types, including crude lysates,[2] purification fractions,[3] and mAbs.[4] The system delivers high-resolution separation across a broad sizing range from 10 to 240 kDa. Up to 12 samples can be analyzed in parallel within 30 minutes, streamlining workflows.

During sample preparation for the ProteoAnalyzer, proteins are labeled with fluorescent dye. Once loaded onto the system, the labeled proteins undergo electrophoretic separation. During this time, fluorescence intensity and migration time are measured to determine both protein quantity and molecular weight. The system is integrated with the Agilent ProSize data analysis software that automatically aligns the sample data with a known size ladder and markers, enabling accurate calculation of protein size and concentration.

Accurate sizing analysis with electrophoresis therefore relies on the migration rate of the sample, which can be heavily influenced by the secondary structure of the protein. Denaturation of protein samples with SDS eliminates the noncovalent secondary structures. However, due to their covalent nature, disulfide bonds remain intact, maintaining a degree of secondary structure. For example, intact mAbs are composed of two heavy chains and two light chains that are linked by disulfide bonds to form a Y-shaped structure. This configuration allows for binding to specific antigens, making them valuable tools for diagnostics and therapeutics. However, the Y-shaped structure of the intact mAb is maintained during denatured nonreduced analysis and can cause slower migration through a gel matrix compared to single linear polypeptides. As a result, intact nonreduced mAbs may have a larger apparent size than expected in relation to ladders.

To correct for this discrepancy in sizing, a ladder with a similar secondary structure to the sample can be used. The NIST mAb (RM 8671) serves as a highly characterized reference material developed to support analytical standardization. Its defined structure and fragmentation profile make it particularly well-suited for use as a sizing ladder in antibody electrophoretic workflows.[5] This application note demonstrates the integration of the NIST mAb as a ladder into the ProteoAnalyzer workflow, enabling improved sizing accuracy for intact and fragmented mAbs.

Methods

Several antibodies of interest were analyzed with the Agilent ProteoAnalyzer system using the Agilent Protein Broad Range P240 kit (p/n 5191-6640) under nonreduced conditions. Samples tested include: Infliximab, Trastuzumab (Intact and HHL fragment), SILu™Lite SigmaMAb Rituximab Monoclonal Antibody (Sigma-Aldrich p/n MSQC17), and USP mAb Reference Standards (Monoclonal IgG1, mAb 001 (p/n 1445539), mAb 002 (p/n 1445547), and mAb 003 (p/n 1445595). The Agilent ProSize data analysis software was used to size each sample using both the Agilent P240 Broad Range Ladder (p/n 5191-6648), supplied with the kit, and the NIST mAb (Agilent p/n 5191-5744) at a concentration of 1,500 ng/µL in 1x PBS.

The NIST mAb can be used as a ladder in ProSize by entering the known sizes of the intact mAb and fragment impurities into the "Calibration Ladder" section of the calibration curve (Figure 1). The sizes of the ladder fragments have been previously determined by capillary zone electrophoresis-mass spectrometry (CZE-MS) to be: 23 (light chain, LC), 50 (heavy chain, HC), 73 (HC:LC), 101 (HC:HC), 124 (HC:HC:LC), and 148 kDa (Intact mAb).[5]

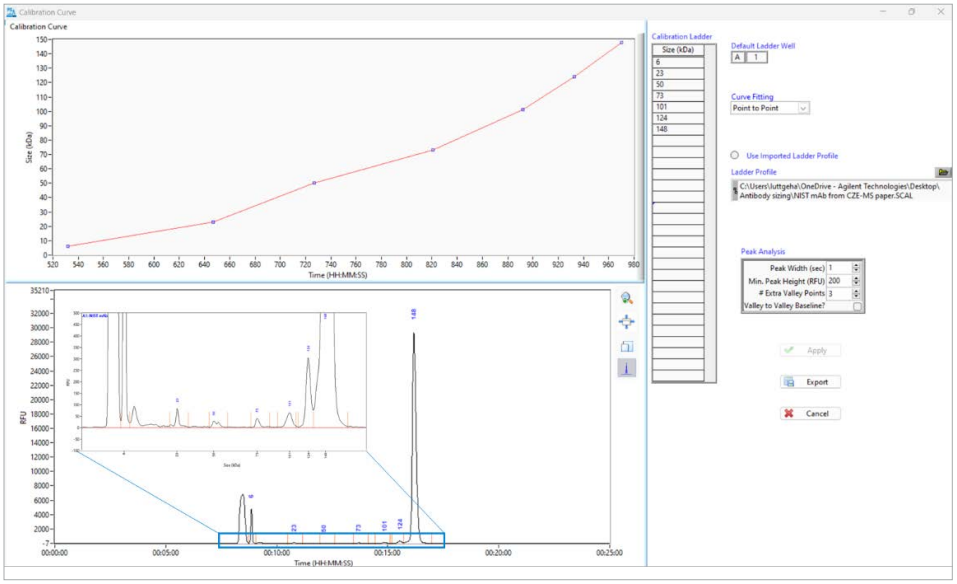

Figure 1. Representative calibration curve of the NIST mAb as the calibration ladder for the Agilent ProteoAnalyzer system.

Results and discussion

Electrophoretic analysis relies on comparing the migration rate of the sample to known standards that form a ladder. The secondary structures of disulfide-containing samples, such as mAbs, can impact how they migrate through a gel, necessitating a ladder that will migrate similarly to the sample. When analyzed with the Protein Broad Range P240 kit for the ProteoAnalyzer system, the sizing of many mAbs does not appear as expected.

To accommodate the complex structure of nonreduced mAbs, the fragments of the NIST mAb were used as the ladder for sizing analysis on the ProteoAnalyzer. Several mAb samples were analyzed with both the P240 ladder and the NIST mAb as a ladder for comparison. Samples assessed include three USP reference standards: Trastuzumab (Intact monomer and HHL fragment), Infliximab, and Rituximab, each of which display a large monomeric peak and several small impurity fragments. As an example, Figure 2 shows the electropherogram results of the (A) Trastuzumab monomer and the (B) HHL fragment when analyzed on the ProteoAnalyzer. The primary peaks in these samples have expected sizes of 150 and 125 kDa, respectively. Analysis with the P240 ladder results in sizes much larger than expected, at approximately 250 and 175 kDa. When analyzed with the NIST mAb as a ladder, the samples are much closer to the expected sizes, on average 144 and 114 kDa, respectively. The sizing measurements of the Trastuzumab monomer and fragment when using the NIST mAb were highly precise and accurate for both samples, with less than 3 %CV and less than 8% error, as shown in Table 1.

Similar results were achieved for the three USP samples, which have theoretical sizes of 147, 150, and 146 kDa, respectively. Analysis with the P240 ladder resulted in apparent average sizes of 306, 299, and 313 kDa, respectively. In contrast, analyzing the samples with the NIST mAb as ladder resulted in highly accurate sizing of 150, 146, and 150 kDa. Sizing of the USP mAbs using the NIST mAb as a ladder showed good precision with less than 5 %CV, and excellent accuracy as shown by percent errors of less than 3% for each sample (Table 1).

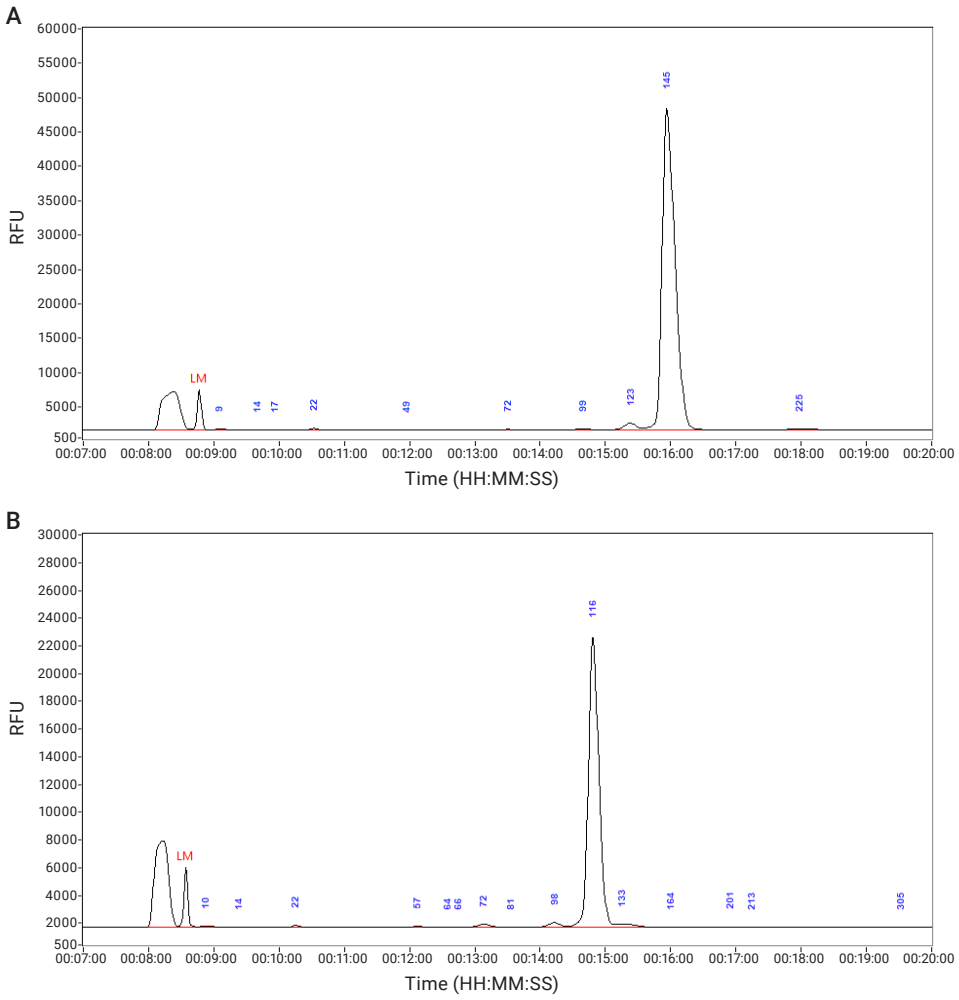

Figure 2. Electropherogram results of the Trastuzumab (**A**) monomer and (**B**) HHL fragment when analyzed on the Agilent ProteoAnalyzer system and sizing analysis performed with the NIST mAb as a ladder.

Table 1. Sizing comparison of representative therapeutic antibodies and reference standards on the Agilent ProteoAnalyzer system using the Agilent Protein Broad Range P240 Ladder and the NIST mAb as a ladder (N = >3).

		Size with Protein Broad Range P240 Ladder		Size with NIST mAb as a Ladder		
Sample	**Theoretical Size (kDa)**	**Average size (kDa)**	**Precision (%CV)**	**Average size (kDa)**	**Precision (%CV)**	**Accuracy (%error)**
Trastuzumab monomer	150	254.67	0.82	144.33	0.40	3.33
Trastuzumab HHL	125	176.33	4.26	113.67	2.21	7.20
Infliximab	149	280.33	3.07	145.74	1.72	2.18
Rituximab	147	307.92	2.91	153.64	2.33	5.00
USP001	147	305.95	5.10	149.95	3.83	2.00
USP002	150	298.73	3.96	145.87	3.03	2.80
USP003	146	313.00	6.07	149.48	4.92	2.40

To further assess sizing with the ProteoAnalyzer using the NIST mAb as a ladder, the Infliximab mAb was analyzed at several concentrations across the range of the kit. Figure 3A shows an electropherogram overlay of the sample at 1,000, 1,500, and 2,000 ng/µL. The average size of the sample remained consistent across the concentration range tested (Figure 3B). Using the NIST mAb, the sample showed an average size of 146 kDa across all concentrations, very similar to the theoretical size of 149 kDa. The measurements were highly precise and accurate, with only 1.72 %CV and 2.18% error (Table 1).

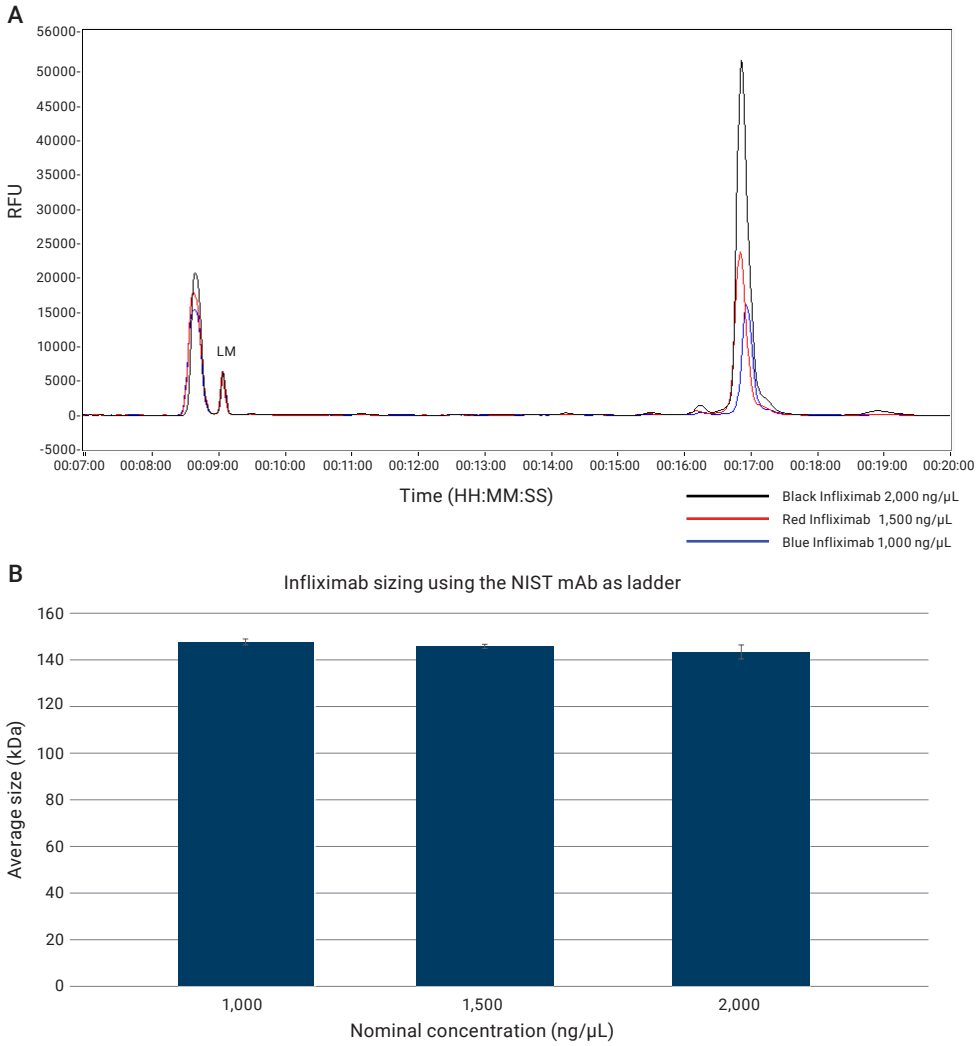

Figure 3. (**A**) Electropherogram overlay and (**B**) sizing of the Infliximab mAb at varying concentrations across the detection range of the Agilent ProteoAnalyzer system using the NIST mAb as a ladder.

To demonstrate the reliability of the ProteoAnalyzer using the NIST mAb as a ladder, multiple replicates of the Rituximab mAb were analyzed across several runs. Figure 4A shows an electropherogram overlay of six replicates of the sample, illustrating the variability in migration rate of the primary peak from well to well. The ProSize software corrects for these slight differences by aligning the lower markers from each well. When the sample was analyzed with the P240 ladder across the replicates, the sample sized from 294 to 330 kDa. In contrast, with the NIST mAb as ladder the Rituximab sized at 148 to 160 kDa (Figure 4B). The precision was excellent, with a variation of only 2.3 %CV. In comparison to the theoretical size of 147 kDa, analysis with the NIST mAb as a ladder showed good sizing accuracy with only 5% error (Table 1).

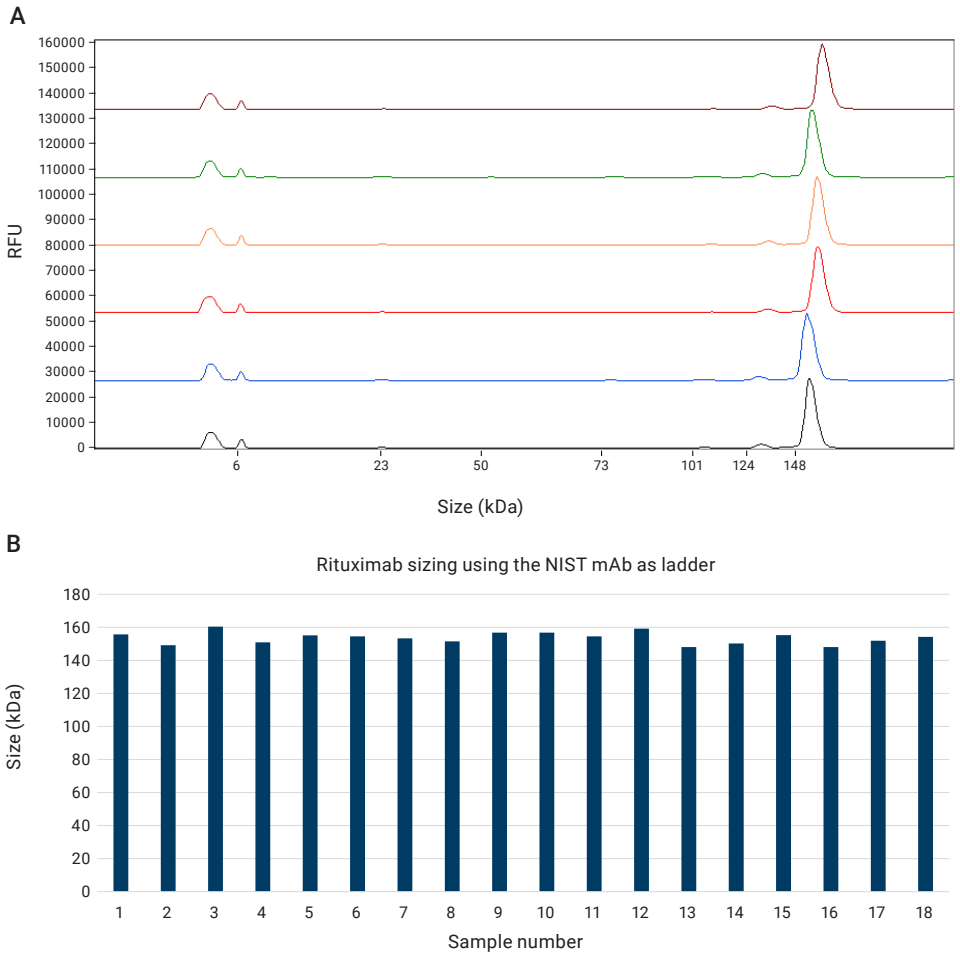

Figure 4. Rituximab was analyzed on the Agilent ProteoAnalyzer system using the NIST mAb as a sizing ladder in multiple replicates. (**A**) Electropherogram overlay of six wells within a single run. (**B**) Size of 18 replicates demonstrating the sizing precision of the system (n = 6 replicates per row * 3 rows).

Conclusion

Accurate molecular sizing of mAbs is essential for biopharmaceutical development, structural characterization, and regulatory compliance, making precision in analytical platforms increasingly important. This application note demonstrates the use of the NIST mAb as a sizing ladder for capillary electrophoresis of intact mAbs using the Agilent ProteoAnalyzer system. Compared to the P240 ladder, which overestimates nonreduced mAb sizes, using the NIST mAb as a ladder significantly improved sizing accuracy of nonreduced mAbs. Additionally, sizing nonreduced mAbs with the NIST mAb as a ladder shows lower variation across replicates, indicating excellent precision and reproducibility. The integration of the NIST mAb as a ladder into electrophoretic workflows for nonreduced mAb analysis offers a robust solution for overcoming structural migration discrepancies, supporting more reliable characterization in biopharmaceutical research and quality control.

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Application Note

Genomics

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Complementary Methodologies for Analysis of NISTmAb Impurities

Authors

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Abstract

Size heterogeneity is a critical quality attribute (CQA) of monoclonal antibodies (mAb) as both aggregation and degradation can impact the safety and efficacy of therapeutic antibodies. Size-based impurities consist of low molecular weight (LMW) degradation products and high molecular weight (HMW) aggregates. To fully characterize mAb size heterogeneity, both Size Exclusion Chromatography (SEC) and Capillary Electrophoresis-sodium dodecyl sulfate (CE-SDS) are used, with each providing key insights into mAb monomeric purity. SEC uses native conditions that allow for characterization of HMW aggregates, while CE-SDS provides high-resolution separations of LMW species. In this application note, the NISTmAb reference standard is used to compare monomeric purity measurements by SEC and CE-SDS while highlighting the complementary nature of these techniques.

Introduction

The presence of both HMW and LMW impurities can alter the effectiveness or safety profile of therapeutic antibodies. As a result, regulatory agencies mandate a thorough characterization of therapeutic antibodies as part of the approval process for new biologics and quality control release criteria[1]. Several CQAs are therefore assessed during the development and production of biotherapeutic monoclonal antibodies. One key CQA used as a measure of sample integrity is size heterogeneity, or the percent monomeric purity. Monoclonal antibodies require precise analytical techniques for size heterogeneity characterization. To aid in this assessment, two techniques, SEC and CE-SDS offer complementary, yet unique insights into the impurity composition of these antibodies[1,2].

SEC separations occur in native environments allowing for the separation of HMW multimeric mAb aggregates from the monomeric mAb and LMW mAb fragments. HMW aggregates elute first and are composed of multimeric species. Depending on the SEC system used, resolution of the different aggregate species, such as the dimer and trimer, may be possible. The monomeric mAb elutes as the largest main peak, followed by the LMW species. The LMW species generally elute as a single peak and are composed of partial mAb fragments. SEC excels in analyzing soluble noncovalent HMW aggregates of monoclonal antibodies. However, resolution of individual LMW fragment species is typically not possible[1,2].

In contrast, CE-SDS is performed using denaturing conditions which break apart noncovalent aggregates. This allows for high resolution separations of the LMW fragments when analyzed under nonreducing conditions, but prevents its use for HMW aggregate characterization. Nonreducing CE-SDS is used to determine monomeric percent purity and quantitation of each individual LMW fragment. By preparing the mAb sample in the presence of a reducing agent, such as Dithiothreitol (DT T), the same method can be used to assess heavy and light chain relative abundance, heavy chain glycan occupancy, and nonreducible species content of the mAb[1,2,3]. These CQAs are unable to be determined by SEC analysis. The choice between SEC and CE-SDS hinges on the specific requirements of the analysis, with each offering different, yet complementary insights about the integrity of the antibody.

Agilent provides both SEC and CE-SDS solutions for size heterogeneity analysis, including high-quality biocompatible ultrahigh performance liquid chromatography (UHPLC) instrumentation, sub-2 µm column technology, and the ProteoAnalyzer system. The biocompatible Agilent 1290 Infinity II Bio LC system perfectly copes with the high salt concentrations often found in SEC buffers, providing confident results at the lowest maintenance cost. To enable optimal performance, a combination of sub-2 µm columns and a UHPLC instrument with dead volumes as low as possible is preferred. Large dead volumes destroy the resolution obtained by these columns due to dispersion effects[4]. Complementing this is the Agilent ProteoAnalyzer system, which provides high-resolution CE-SDS analysis of 12 samples in parallel. In this application note, the NIST mAb standard is analyzed on the Agilent 1290 Infinity II Bio LC for SEC analysis and Agilent ProteoAnalyzer system for CE-SDS purity analysis, highlighting the orthogonal nature of the two techniques. In particular, the ability of each technology to provide unique data in relation to HMW and LMW impurities is examined.

Experimental

CE-SDS analysis

NISTmAb (Sigma p/n NIST8671, aliquot from Reference Material 8671, Lot 14HB-D-002)[1] was prepared in PBS at a concentration of 2,000 ng/µL under both reducing and nonreducing conditions according to the Agilent Protein Broad Range P240 (p/n 5191-6640) manual[5]. The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes. The reduced and nonreduced antibodies were analyzed across multiple capillaries of an Agilent ProteoAnalyzer system[6] with the Agilent Protein Broad Range P240 kit LM Only method. For nonreduced conditions, the sample injection was decreased to 7 kV 6 seconds for optimal results. Analysis of the samples from the ProteoAnalyzer was compared to the known specifications of the NISTmAb from datasheets and published results[1,7].

UHPLC SEC analysis

Instrument

Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)

Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)

Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger

Agilent 1290 Infinity II Diode Array Detector (G7117B), equipped with a biocompatible light sensitive sample flow cell, 10 mm, 1 µL

Additional parts

Agilent 1290 Infinity II Bio Ultra Low Dispersion kit (G7132A#006)

Software

Agilent OpenLab, version 2.5

LC method

- **Solvent:** 150 mM sodium phosphate pH 7.0
- **Flow rate:** 0.1 mL/min isocratic separation
- **Column temperature:** 30 °C
- **Sample temperature:** 4 °C
- **Injection volume:** 1 µL
- **Detection:** 280 nm – 20 Hz

Column

Agilent AdvanceBio SEC 1.9 µm column, PEEK Lined 2.1 x 150 mm – 620 bar.

Solvents and chemicals

A phosphate buffer mobile phase was prepared by dissolving sodium phosphate dibasic and sodium phosphate monobasic in fresh ultrapure water obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge. The pH was adjusted to 7.0 with phosphoric acid at 25 °C. All UHPLC chemicals were purchased from Sigma, USA. The NISTmAb was prepared in the mobile phase at a concentration of 2 mg/mL.

Results and discussion

Analysis of the NISTmAb by SEC clearly separated the HMW, monomer, and LMW species. The HMW species split into two populations, the dimer and trimer (Figure 1). As expected, the LMW species all co-eluted as a single peak. The monomeric purity of the NISTmAb by SEC was found to be 97.83%, compared to the NISTmAb reference material sheet value of 96.63%. The HMW species were found to compose a total of 2.05%, with the LMW species representing 0.12% (Table 1). These values were comparable to the NISTmAb Reference Material Sheet[7].

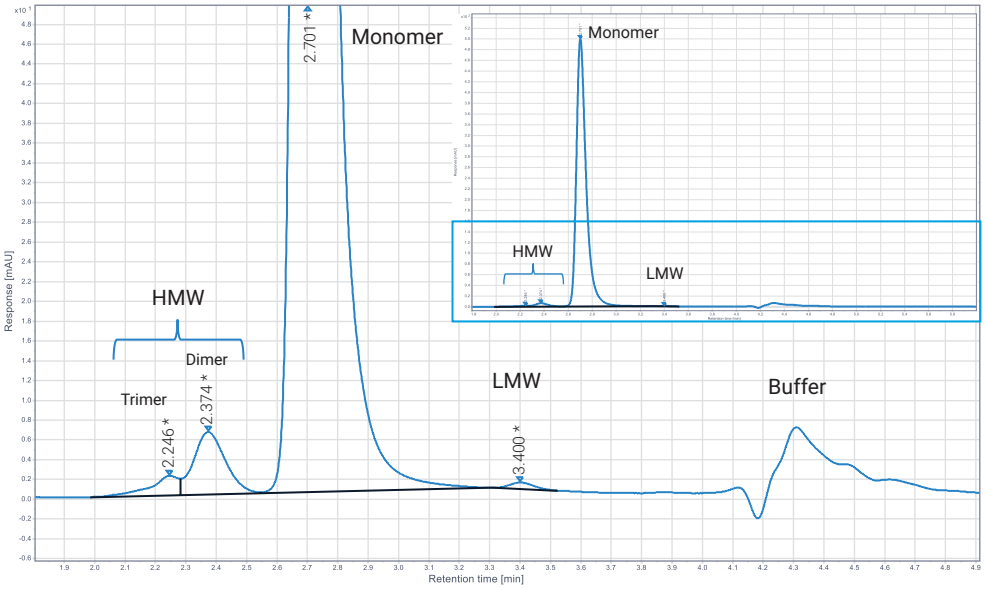

Figure 1. Results of SEC NISTmAb separation using the Agilent 1290 Infinity II Bio LC system.

Table 1. Critical quality attributes of purity for the NISTmAb were determined by SEC using the Agilent 1290 Infinity II Bio LC system and compared to the NIST datasheet[7]. 1290 Infinity II Bio LC: N = 10.

	NIST Datasheet SEC		Agilent SEC	
	Size heterogeneity (%)	Combined standard uncertainty (%)	Size heterogeneity (%)	%CV
Monomeric Purity (nonreduced)	96.63	0.15	97.83	0.04
High Molecular Weight	3.17	0.15	2.05	1.99
Low Molecular Weight	0.20	0.008	0.12	3.00

When the NISTmAb was analyzed by nonreduced CE-SDS, the LMW fragments were clearly resolved into the individual constituents, followed by a large peak representing the mAb monomer (Figure 2). To the right of the mAb monomer is a small peak, representative of the HMW species that were not denatured by SDS and heat. The monomeric purity was calculated as previously described[1,8] and found to be 98.18% with CE-SDS by the ProteoAnalyzer, compared to 98.47% as reported in the NISTmAb Reference Material Sheet. The remaining 1.82% of the sample was composed of the LMW fragments, including the Light Chain (LC), Heavy Chain (HC), fragment antigen binding (Fab) region, HC:LC, HC:HC, and HC:HC:LC impurities (Table 2). The ProteoAnalyzer values were consistent with those reported in the NIST Material Reference Sheet[7].

The monomeric purity observed by SEC and CE-SDS were found to be only 0.86% different, while the LMW fragment percentage values differed significantly. SEC found the LMW impurities to be 0.15%, while CE-SDS found these species to compose 1.82% of the total sample. The under-quantification of LMW species by SEC compared to CE-SDS was consistent with values previously reported by NIST, and may be the result of SDS releasing fragments held together by noncovalent interactions[1].

While some HMW material was detected by CE-SDS, the presence of SDS breaks up aggregates, preventing its use for HMW material quantification[1,2]. The HMW species was not included in the CE-SDS monomeric purity calculation in accordance with the measured and recorded analytes reported by NIST[1]. Analysis of reduced samples by CE-SDS can also be used to determine the percent glycosylation and thioether content. The ProteoAnalyzer system has previously been shown to provide values for these CQAs that are consistent with those reported in the NIST Material Reference Sheet[7].

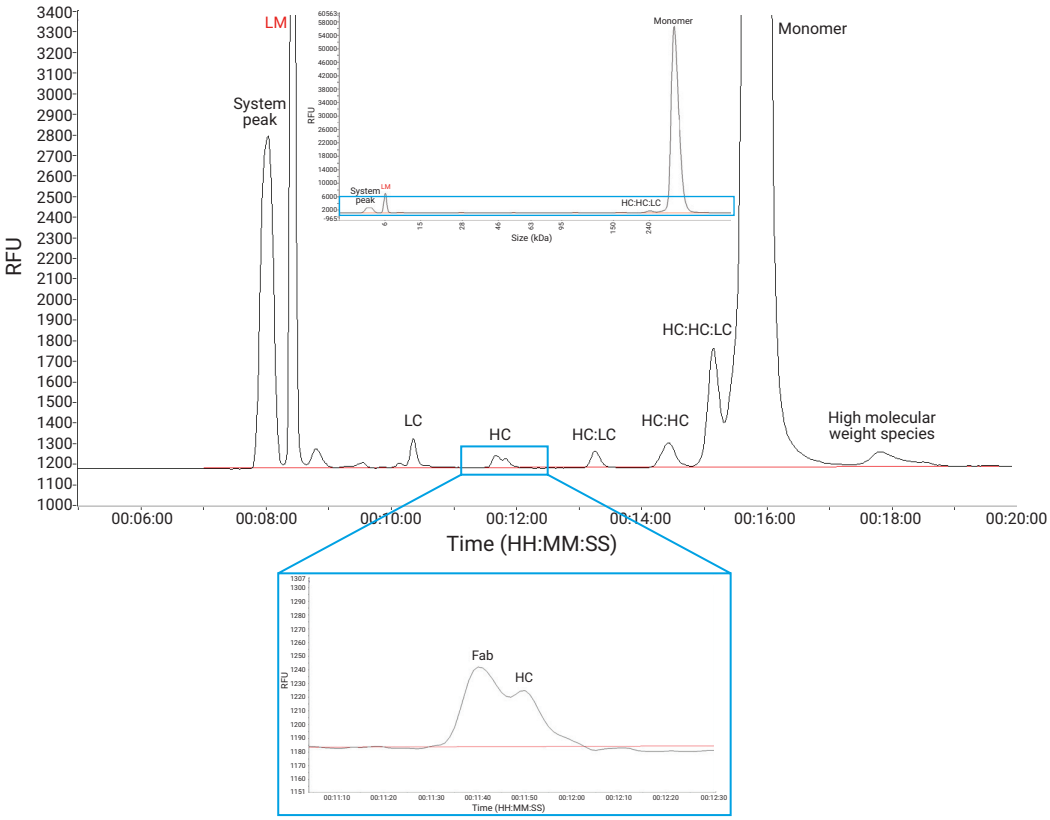

Figure 2. Results of the nonreduced NISTmAb using the Agilent ProteoAnalyzer system.

Table 2. Critical quality attributes of purity for the NISTmAb were determined by the Agilent ProteoAnalyzer system and compared to the NIST datasheet[7]. ProteoAnalyzer: N = 11.

	NIST Datasheet CE-SDS		ProteoAnalyzer CE-SDS	
	Size heterogeneity (%)	Combined standard uncertainty (%)	Size heterogeneity (%)	%CV
Monomeric Purity (nonreduced)	98.47	1.0	98.18	0.09
High Molecular Weight	Not calculated	Not calculated	Not calculated	Not calculated
Low Molecular Weight	Not calculated	Not calculated	1.82	5%

Conclusion

The data presented here demonstrates the orthogonality of SEC and CE-SDS when performing mAb monomeric purity assessments. Monomeric purity assessments by SEC provide accurate quantification of HMW aggregates, but can under-quantify low molecular weight species as shown in the NIST mAb Reference Material Sheet and confirmed here. This is because SEC is more robust for measuring monomer and HMW species, but the quantitation of LMW species can vary depending on the mAb studied[2]. On the other hand, CE-SDS provides improved resolution of closely related size-variants and is better suited for accurately quantifying LMW impurities. For precise quantification of low molecular weight antibody fragments, CE-SDS is the preferred method, while SEC is the preferred method for quantification of HMW aggregates. Combining both techniques provides the best overall characterization of mAbs, as described by organizations such as the USP[2]. Agilent offers reliable solutions for both of these technologies with the Agilent 1290 Infinity II Bio LC and Agilent ProteoAnalyzer systems.

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Advanced Applications: ADC Analysis

Application Note

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Antibody-Drug Conjugate (ADC) Analysis with the Agilent ProteoAnalyzer System

Author

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Agilent Technologies, Inc.

Abstract

This application note demonstrates the use of the Agilent ProteoAnalyzer system for antibody-drug conjugate (ADC) analysis by capillary electrophoresis with sodium dodecyl sulfate (CE-SDS). The high resolution delivered by the Agilent ProteoAnalyzer system enables precise sizing and assessment of heterogeneity of these samples. Representative figures and quantitative data from cysteine- and lysine-conjugated ADCs confirm the accuracy and reproducibility of the system for ADC characterization and quality control.

Introduction

Antibody-drug conjugates (ADCs) are targeted biopharmaceuticals composed of a monoclonal antibody (mAb) connected to a drug through a stable linker. Factors such as drug-to-antibody ratio (DAR), conjugation strategy, and linker chemistry significantly influence ADC therapeutic efficacy, safety, and biophysical properties. Advanced analytical techniques like capillary electrophoresis with sodium dodecyl sulfate (CE-SDS), hydrophobic interaction chromatography (HIC), and mass spectrometry are essential for characterizing ADC critical quality attributes (CQAs) such as heterogeneity, purity, DAR, and payload location.

CE-SDS is used in ADC analysis to determine molecular weight, size heterogeneity, and purity. The technology can provide high resolution for closely related size variants, making it ideal for monitoring antibody fragments dissociable by SDS. The electrophoretic profile depends on the drug conjugation sites, such as cysteine or lysine residues (Figure 1). Additionally, different linker chemistries can impact the antibody structure, and therefore the separation profile, by forming nondisulfide covalent crosslinks between light chains and heavy chains during the ADC manufacturing process.[1,2]

The Agilent ProteoAnalyzer system automates CE-SDS analysis of both reduced and nonreduced proteins and enables efficient workflows by separating up to 12 samples in parallel.[3] While accurate sizing of antibody samples can be difficult, the system allows for the use of custom size calibration ladders to correct for migration differences caused by secondary structures. Previous work has shown that the NIST mAb and its associated antibody fragments can be used as a ladder when analyzing mAbs[4] and is used here for sizing nonreduced ADCs. This application note demonstrates the capabilities of the ProteoAnalyzer system for comprehensive ADC analysis, with examples for both cysteine- and lysine-conjugated ADCs.

Experimental

Commercially available ADC samples and corresponding naked mAb standards were obtained and prepared for analysis with the Agilent ProteoAnalyzer system. Samples include: SiLu SigmaMAb Universal Antibody Standard Human (Sigma, p/n MSQC4-1MG), SigmaMAb Antibody Drug Conjugate (ADC) Mimic (Sigma, p/n MSQC8-0.5MG), Trastuzumab, Trastuzumab deruxtecan (MedChem Express, p/n HY-138298A), and Trastuzumab emtansine (T-DM1) (MedChem Express, p/n HY-P9921). Samples were reconstituted to 10 mg/mL and further diluted to 1.5 mg/mL in nuclease free water. Sample concentration was confirmed by NanoDrop (settings: Protein A280; curve type: other E1%; extinction coefficient: 14.3).

Each sample was then prepared under both reducing and nonreducing conditions according to the Agilent Protein Broad Range P240 kit (p/n 5191-6640) manual.[5] The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes).[6] The reduced and nonreduced antibodies were analyzed across multiple capillaries of a ProteoAnalyzer system with the ProteoAnalyzer Broad Range Kit lower marker (LM) only method. For nonreduced conditions, the sample injection was decreased to 7 kV, 6 seconds for optimal results.[6] Sizing analysis of the nonreduced samples was performed using the NIST mAb as the ladder.[4]

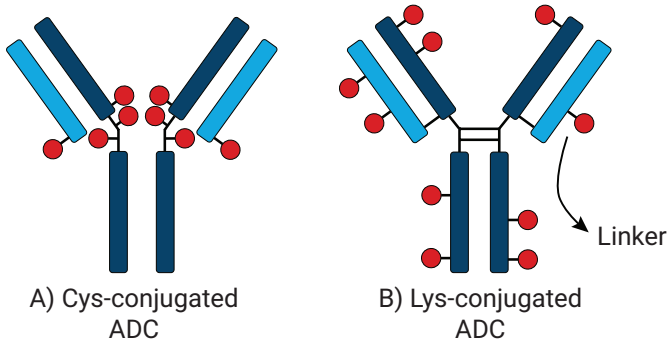


Figure 1. Schematic representation of (A) cysteine- and (B) lysine-conjugated antibody-drug conjugates (ADCs). Red circles indicate the potential drug conjugation sites.

Results and discussion

Reduced CE-SDS analysis of cysteine-conjugated ADCs

CE-SDS analysis of cysteine-conjugated ADCs under reduced conditions is expected to show a light chain (LC) and heavy chain (HC). In addition to these peaks, partial separation of the LC without a payload (L0) and an LC with one payload (L1) may also be observed. Electrophoretic separation of the L0 and L1 depends on the size and properties of the payloads and antibody.[7,8]

Analysis of the SigmaMAb ADC Mimic and its naked counterpart, the SigmaMAb Antibody Standard, was performed using the ProteoAnalyzer under reduced conditions. The standard is an IgG1 monoclonal antibody, with an expected profile of two major peaks, indicative of the LC and HC (Figure 2A). The ADC Mimic is the SigmaMAb Standard linked to dansyl fluorophores by way of an LC-SMCC crosslinker. The electrophoretic profile of the ADC shows the expected LC and HC, with separation of the LC into two peaks, consistent of an L0 and L1 payload (Figure 2B).

Another example of a cysteine-conjugated ADC is Trastuzumab deruxtecan, consisting of the mAb Trastuzumab covalently linked to the topoisomerase I inhibitor deruxtecan. The Trastuzumab mAb and the Trastuzumab deruxtecan ADC showed similar profiles, with prominent peaks for both the LC and HC, and a small NGHC, as shown in Figure 2C–D. While the ProteoAnalyzer could not resolve the differences between the conjugated and unconjugated forms, separation of the LC, NGHC, and HC peaks still enables calculations of CQAs such as percent glycosylation and percent thioether.

Sizing and concentration analysis of triplicate replicates of both the mAbs and the cysteine-conjugated ADC showed highly reproducible results. All samples had a sizing precision of less than 2.5% CV and quantification precision of less than 10% CV (Table 1).

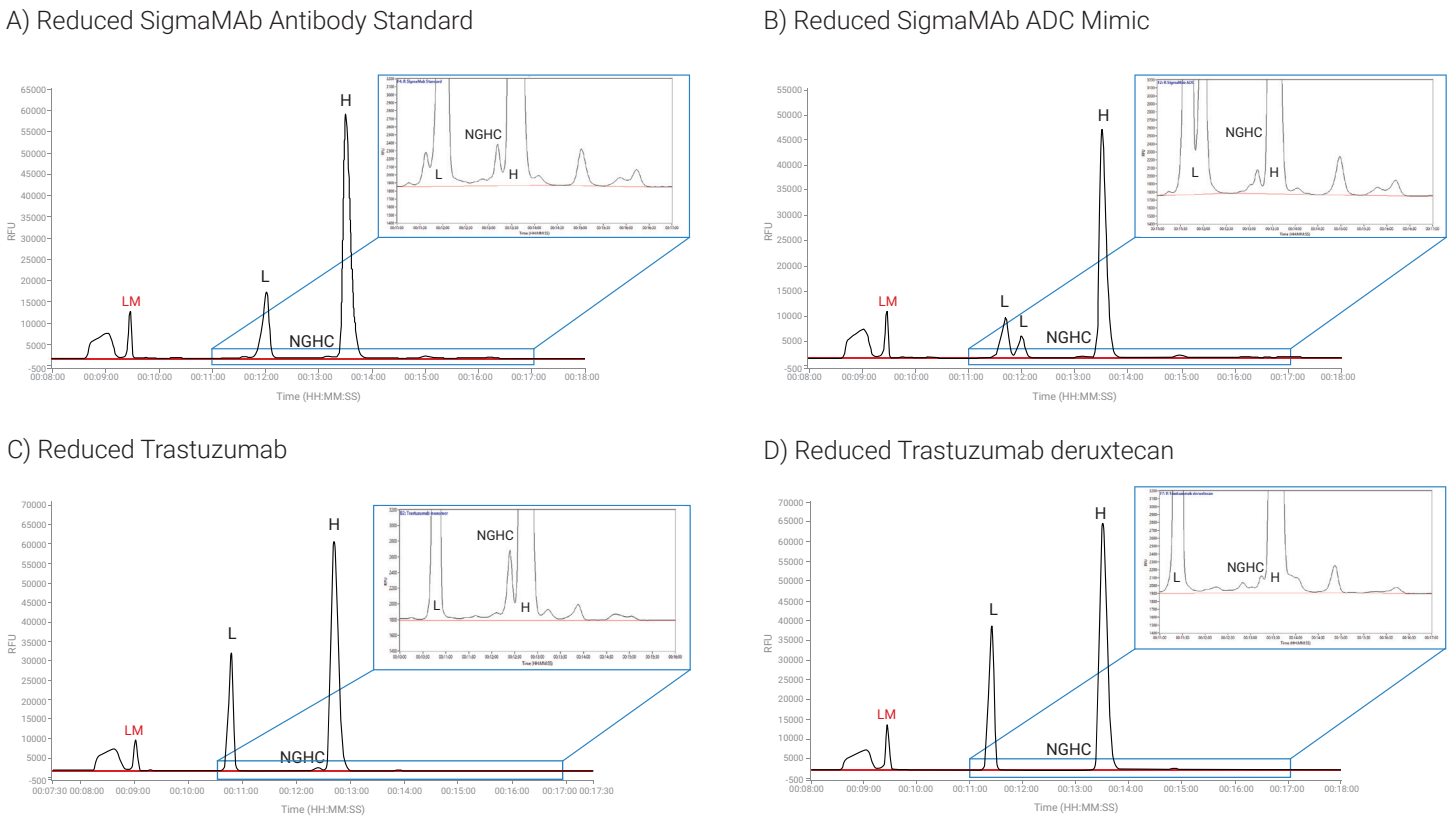


Figure 2. Reduced analysis of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit.

Table 1. Average sizing and concentration of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan under reduced conditions using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit (n=3).

			Size (kDa)		Concentration (ng/µL)	
	mAb chains	Appx. size	Average	%CV	Average	%CV
R SigmaMAb Standard	L	25	36.87	0.31	239.65	1.54
	NGHC		56.97	0.51	7.40	9.25
	H	50	62.60	0.55	830.24	0.99
R SigmaMAb ADC	L	25	32.80	0.61	190.98	0.41
	L	25	37.77	0.40	96.09	0.90
	NGHC		58.63	0.43	6.92	1.93
	H	50	65.77	0.84	846.18	1.44
R Trastuzumab	L	25	25.80	1.69	523.95	0.28
	NGHC		54.17	2.32	18.44	1.37
	H	50	60.03	2.11	1267.31	2.77
	L	25	27.05	0.26	431.20	0.58
R Trastuzumab deruxtecan	NGHC		57.90	0.49	2.59	7.84
	H	50	62.25	0.34	869.34	0.85

Nonreduced CE-SDS analysis of cysteine-conjugated ADCs

Under nonreducing CE-SDS conditions, cysteine-conjugated ADCs show characteristic fragments such as the LC and HC, as well as heavy-light (HL), heavy-heavy (HH), and heavy-heavy-light (HHL) chains. The ADC dissociates in the presence of SDS, based on the number and location of drugs linked to the antibody. The presence of the drug prevents the interchain disulfide bond from reforming.[9] An illustration of the potential fragments that can be expected with cysteine-conjugated IgG1 ADCs is shown in Figure 3. ADC samples were analyzed on the ProteoAnalyzer system to demonstrate the ability of the system to assess cysteine-conjugated ADC samples. Representative examples of the electrophoretic profiles of the SigmaMAb and Trastuzumab ADCs under nonreduced conditions are shown in Figure 4.

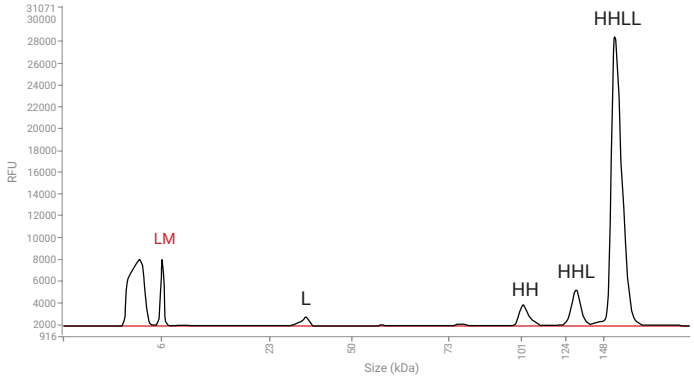
Drug to Antibody Ratio	Position of Payload		Fragments formed under NR conditions					
0	DAR0		mAb					
2	$DAR2_f$			HHL				L
	$DAR2_h$		mAb					
4	$DAR4_{ff}$				HH			2L
	$DAR4_{fh}$			HHL				L
	$DAR4_{hh}$					2HL		
6	$DAR6_{ffh}$				HH			2L
	$DAR6_{fhh}$					HL	H	L
8	DAR8						2H	2L

Figure 3. Expected fragments of positional isomers from cysteine-conjugated IgG1 ADCs.

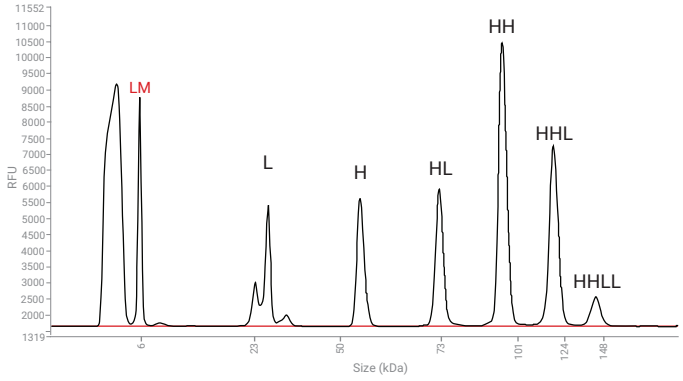
The SigmaMAb Standard shows four prominent peaks. When sized with the NIST mAb as ladder, the most prominent peak had an average size of 156 kDa, close to the expected molecular weight of the intact mAb (HHLL) at approximately 150 kDa. The other three peaks displayed average sizes of 35, 103, and 131 kDa, representative of the L, HH, and HHL species, respectively (Figure 4A, Table 2). The SigmaMAb ADC Mimic displays multiple peaks with sizes consistent with the L, H, HL, HH, HHL, and HHLL species (Figure 4B). There are three distinct peaks within the area of the L chain, likely representative of the different payload locations. The average size and concentration of each peak is shown in Table 2.

The Trastuzumab and the Trastuzumab deruxtecan ADC are shown in Figure 4C–D. The monomeric Trastuzumab sample shows a large peak at approximately 150 kDa, indicative of the intact mAb, or the HHLL. Smaller peaks with sizes similar to the L, H, HL, HH, and HHL chains are also evident. Comparison of the size of these peaks to those in the Trastuzumab deruxtecan provides confidence in the peak assignment of the sample. Upon nonreduced analysis, the ADC shows two large peaks at 22 and 56 kDa, consistent with an increased amount of an L and an H chain. The three smaller peaks to the right of the electropherogram had average sizes of 73, 100, and 126 kDa, indicative of the HL, HH, and HHL fragments. Together, these examples demonstrate that cysteine conjugated Ig1 ADCs can be successfully analyzed using the ProteoAnalyzer system.

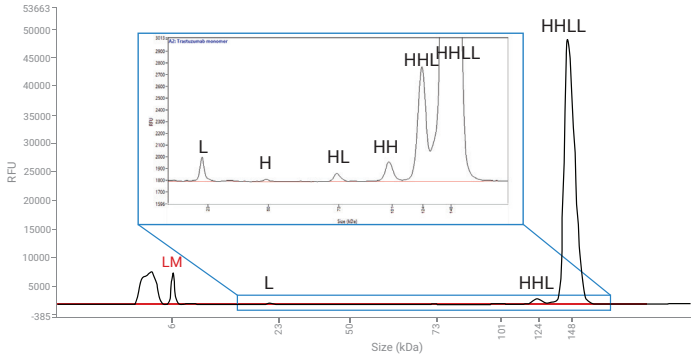
A) Nonreduced SigmaMAb Antibody Standard



B) Nonreduced SigmaMAb ADC Mimic



C) Nonreduced Trastuzumab



D) Nonreduced Trastuzumab deruxtecan

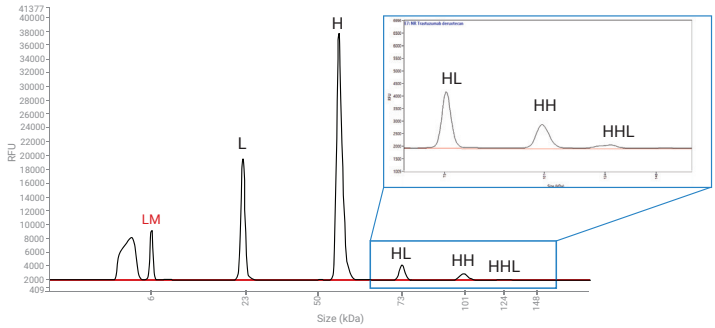


Figure 4. Nonreduced analysis of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder.

Table 2. Average sizing and concentration of (A) SigmaMAb Antibody Standard, (B) SigmaMAb ADC Mimic, (C) Trastuzumab, and (D) Trastuzumab deruxtecan under nonreduced conditions using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder (n=3).

			Size (kDa)		Concentration (ng/µL)	
	mAb chains	**Appx. size**	**Average**	**%CV**	**Average**	**%CV**
NR SigmaMAb Standard	L	25	35.43	1.55	29.79	3.38
	HH	100	103.03	3.23	60.92	3.68
	HHL	125	131.60	3.23	94.53	2.16
	HHL (intact)	150	156.77	3.06	822.93	0.56
NR SigmaMAb ADC	L	25	24.73	2.07	35.05	3.44
			28.63	1.72	79.74	2.03
			34.63	2.05	13.51	5.97
	H	50	56.53	1.67	104.77	0.89
	HL	75	76.10	2.94	105.56	0.46
	HH	100	100.20	3.34	213.74	0.26
	HHL	125	127.67	4.27	136.89	0.31
	HHLL (intact)	150	154.77	3.84	25.37	5.77
NR Trastuzumab	L	25	21.77	0.53	6.46	0.68
	H	50	48.80	0.35	0.83	9.47
	HL	75	72.13	0.21	2.34	6.45
	HH	100	98.93	0.21	6.06	4.95
	HHL	125	122.50	0.67	34.38	1.89
	HHLL (intact)	150	144.20	0.42	1641.45	0.55
NR Trastuzumab deruxtecan	L	25	22.37	0.52	280.83	0.97
	H	50	56.53	0.51	662.96	1.94
	HL	75	73.30	1.21	41.45	2.36
	HH	100	100.07	1.01	22.56	2.26
	HHL	125	126.03	1.55	4.37	4.60

Lysine-conjugated ADC analysis

Lysine-conjugated ADCs present different analytical considerations compared to their cysteine-conjugated counterparts. In nonreduced CE-SDS analysis, lysine-conjugated ADCs retain their cysteine disulfide bonds, resulting in electrophoretic profiles that closely resemble those of naked mAbs. This similarity allows for straightforward assessment of monomeric purity and overall molecular integrity.

A representative example is Trastuzumab emtansine (T-DM1), an ADC generated by linking the anti-tubulin drug DM1 to lysine residues of Trastuzumab by way of an SMCC linker. The manufacturing process involves first attaching the linker to form the intermediate TmAb-MCC, followed by conjugation of DM1 to the linker. The intermediate can form nondisulfide covalent linkages between HCs and LCs, which can be detected by CE-SDS. The drug load distribution for T-DM1 per antibody ranges from 0 to 8, with an average drug-to-antibody ratio (DAR) of approximately 3.5.

CE-SDS analysis of Trastuzumab emtansine under nonreducing conditions with the ProteoAnalyzer displays a major peak at ~150 kDa, consistent with the intact mAb and similar to the naked Trastuzumab control (Figure 5). Additionally, the electropherogram profile did not show significant fragmentation. Analysis of the monomeric structure allows for reliable monomeric purity CQA assessment of the ADC using the ProteoAnalyzer.

Under reduced conditions, CE-SDS analysis shows the expected LC, HC, as well as higher molecular weight species (Figure 6). These higher molecular weight fragments, not observed in the naked Trastuzumab control, were sized at approximately 75, 100, and 125 kDa using an imported nonreduced NIST mAb as ladder (Table 3). Their presence suggests the formation of nondisulfide covalent linkages between HC and LC during manufacturing as previously described.[2]

It is important to note that for lysine conjugated ADCs, the analytical sensitivity of the ProteoAnalyzer system may be affected by the DAR. If all lysine residues are conjugated to payloads, detection of the ADC may be compromised due to a lack of available lysines for dye binding.

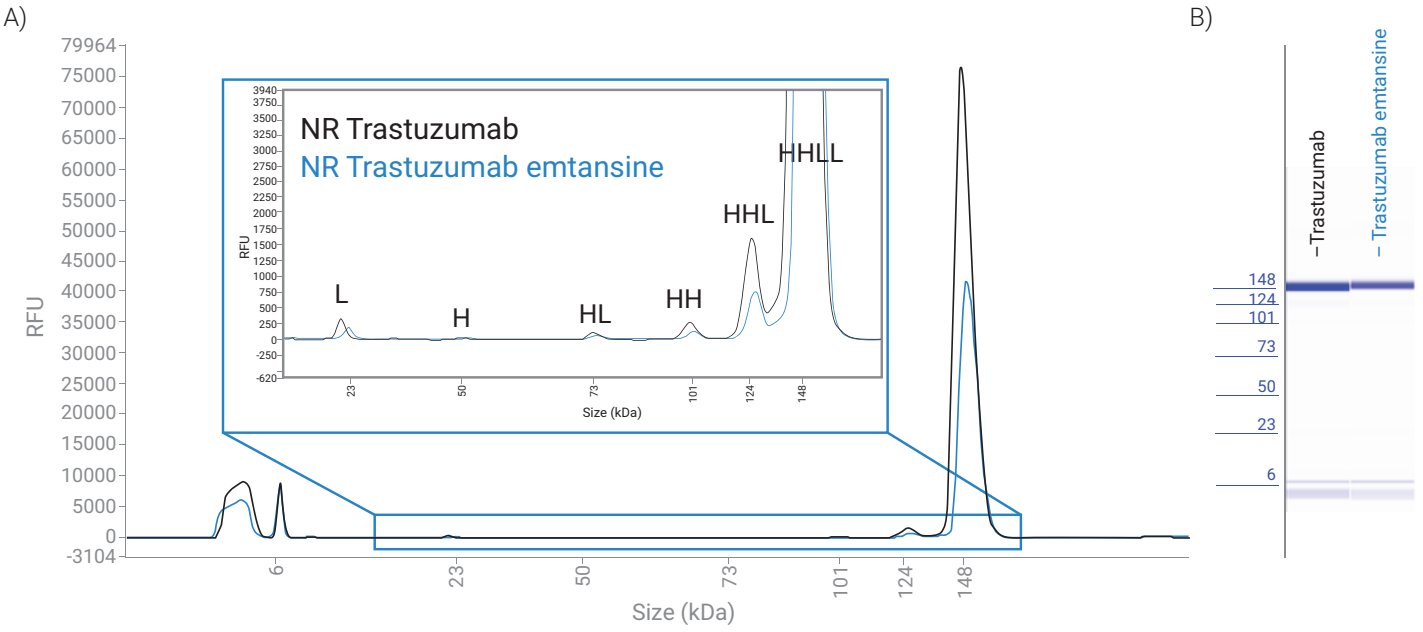


Figure 5. (A) Electropherogram overlay and (B) gel image of nonreduced CE-SDS analysis of Trastuzumab control (black trace) and Trastuzumab emtansine (blue trace) using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder.

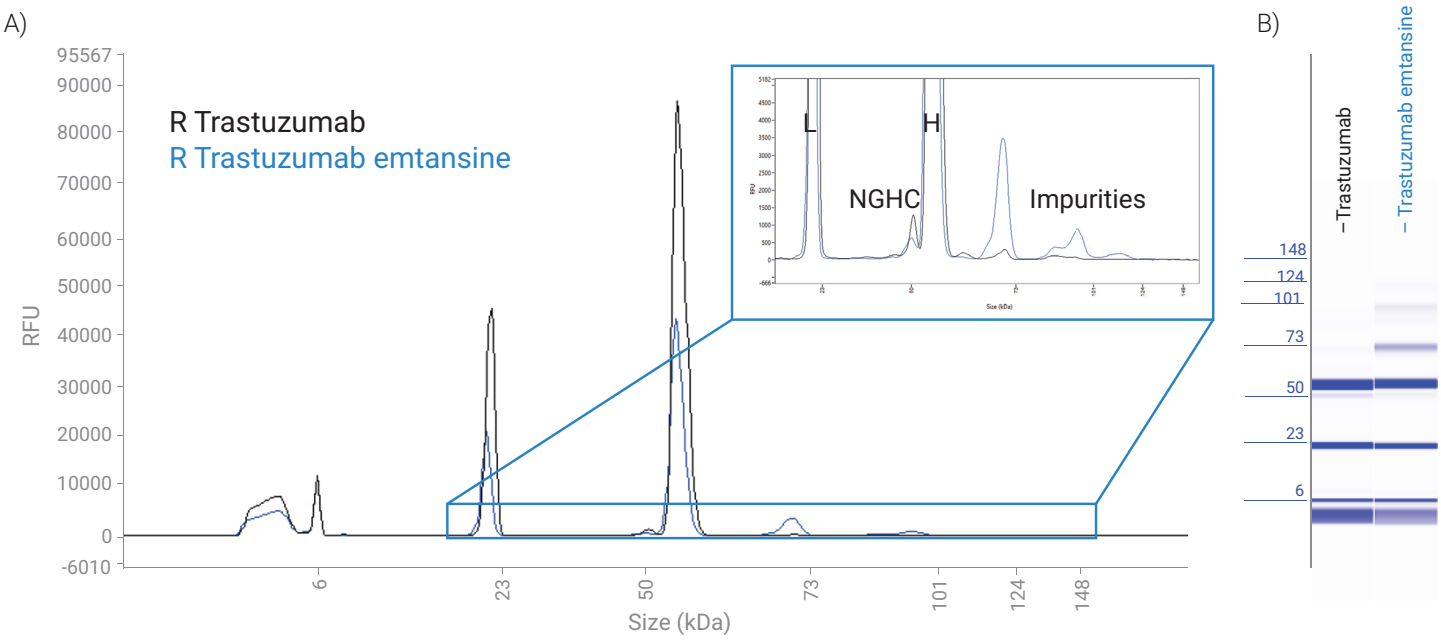


Figure 6. (A) Electropherogram overlay and (B) gel image of reduced CE-SDS analysis of Trastuzumab control (black trace) and Trastuzumab emtansine (blue trace) using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder.

Table 3. Average sizing and concentration of Trastuzumab emtansine under nonreduced and reduced conditions using the Agilent ProteoAnalyzer system and the Agilent Protein Broad Range P240 kit with NIST mAb as the ladder (n=3).

			Size (kDa)		Concentration (ng/µL)	
	mAb chains	Appx. size	Average	%CV	Average	%CV
NR Trastuzumab emtansine	L	25	22.30	1.35	3.79	5.69
	H	50	50.70	1.71	0.45	5.24
	HL	75	72.63	0.65	1.41	3.43
	HH	100	100.03	0.74	2.78	1.42
	HHL	125	123.83	1.02	17.03	2.06
	HHLL (intact)	150	146.97	0.89	965.88	1.44
	Impurity		225.70	0.94	12.69	2.64
R Trastuzumab emtansine	L	25	21.50	0.47	267.45	0.26
	NGHC		51.33	0.22	12.70	1.68
	H	50	55.13	0.21	652.78	0.71
	HL	75	71.03	0.16	69.48	0.44
	Impurities		88.60	0.20	7.49	3.10
	Impurities		97.43	0.41	21.04	3.88
	Impurities		115.27	0.70	5.29	1.99

Conclusions

The Agilent ProteoAnalyzer system provides detailed characterization of cysteine-conjugated ADCs by CE-SDS analysis. Under reduced conditions, the electropherograms display distinct peaks for the LC, HC, and, in some cases, partially separated LC species with and without payloads (L0 and L1). These results highlight the heterogeneity introduced by drug conjugation and demonstrate the system's precision in sizing and quantification.

Nonreduced CE-SDS analysis further distinguishes characteristic fragments such as LC, HC, HL, HH, and HHL chains, providing insight into the positional isomers. This data can be combined with HIC for full characterization of the average DAR and payload location. The presence and relative abundance of these fragments are consistent with expected molecular weights and conformations, confirming the system's capability to resolve complex ADC mixtures. Together, these results illustrate that the ProteoAnalyzer system offers robust and reproducible analysis of cysteine-conjugated ADCs, supporting both purity assessment and structural characterization.

CE-SDS analysis of lysine-conjugated ADCs such as Trastuzumab emtansine demonstrates that nonreduced profiles closely resemble those of unconjugated mAbs, allowing for monomeric purity assessment. Reduced profiles reveal unique crosslinked species, providing insight into the effects of conjugation chemistry on antibody structure. For complete characterization, CE-SDS should be complemented with additional analytical methods, such as HIC.[10]

Overall, this application note demonstrates that the ProteoAnalyzer system provides detailed, reproducible, and insightful analysis for both cysteine- and lysine-conjugated ADCs. The ability of the system to resolve, size, and quantify fragments supports both purity assessment and structural characterization, making it a valuable tool for ADC development and quality control.

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Application Note

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Determining Antibody-Drug Conjugate Positional Isomer Distribution with Nonreduced CE-SDS and HIC

Using the Agilent ProteoAnalyzer system and the Agilent 1290 Infinity III Bio LC

Authors

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Abstract

Antibody-drug conjugates (ADCs) require characterization of both drug-to-antibody ratio (DAR) and drug conjugation location, because each influences drug efficacy. Hydrophobic interaction chromatography (HIC) robustly resolves hydrophobic payloads to quantify DAR distributions, but does not provide positional information. Alternately, nonreduced capillary electrophoresis with sodium dodecyl sulfate (CE-SDS) reports fragmentation patterns that reflect payload placement at interchain disulfides but cannot directly assign DAR. Here, we combine HIC and nonreduced CE-SDS data, applying a previously described system-of-equations approach to estimate the relative abundance of positional isomers in a cysteine-conjugated ADC population.

The SigmaMAb ADC mimic was analyzed by CE-SDS using the Agilent ProteoAnalyzer system to determine the relative levels of the light chain (L), heavy chain (H), HL, HH, HHL, and intact mAb species. HIC analysis of the ADC using the Agilent 1290 Infinity III Bio LC with an Agilent AdvanceBio HIC column was used to determine the relative amounts of the DAR0, DAR2, DAR4, DAR6, and DAR8 species. Analysis of the coupled dataset revealed that the dominant species in the sample was a DAR4, with drugs located at both the F_{ab} disulfides, accounting for 41.5%. Additional major species included $DAR2_f$ (19.3%) and $DAR6_{fhh}$ (22.9%), while $DAR2_h$ and $DAR6_{ffh}$ were not observed. This integrated workflow using Agilent CE-SDS and Agilent 1290 Infinity III Bio LC systems enables the rapid estimation of ADC positional isomer composition using widely adopted methods, providing actionable structural insight without additional specialized assays.

Introduction

Antibody-drug conjugates (ADCs) have emerged as potent biopharmaceuticals that allow the delivery of payloads to specific tissues. ADCs have been widely adopted for the targeted delivery of cytotoxic cancer biopharmaceuticals directly to cancerous cells, reducing systemic side effects. The drug payloads are conjugated to antibodies with a stable linker attached to an amino acid. Generally, the linkers target reactive amino acids such as lysine and cysteine. The conjugation strategy used influences the drug-to-antibody ratio (DAR) and the potential locations of the drug. Since both DAR and drug location impact therapeutic efficacy, full characterization of an ADC requires determination of both parameters.

Canonical IgG1 monoclonal antibodies (mAbs) have four interchain reducible disulfides: one at each of the light chain (L) and heavy chain (H) junctions, referred to as the Fab (f) location, and two at the H:H junction, referred to as the hinge (h) location (Figure 1). Conjugation of the therapeutic to these cysteine residues by disulfide reduction allows for greater control of the DAR than conjugation to lysine. Each reduced disulfide bond allows for the addition of two drugs. While this allows for consistent DAR values, the exact location of the drug cannot be controlled. The addition of the drug breaks the disulfides that covalently link the light and heavy chains, allowing SDS to denature the antibody into individual light and heavy chains. Depending on the number and location of the drugs, different antibody fragments are formed in the presence of SDS (Figure 1).

Most therapeutic payloads are hydrophobic in nature, which allows hydrophobic interaction chromatography (HIC) to separate the DAR0, DAR2, DAR4, DAR6, and DAR8 species. This has resulted in the wide adoption of HIC for DAR determination.[1] However, positional information is not captured by HIC. In contrast, CE-SDS allows for quantitation of the fragments produced by addition of the drug payload, providing insights into drug location,[2] but does not allow for determination of the DAR. Previous work has demonstrated that, by combining HIC DAR data with the positional information from nonreduced CE-SDS, it is possible to mathematically determine the amounts of each positional isomer in the sample using a system-of-equations approach.[3]

The Agilent 1290 Infinity III Bio LC is specifically designed for conditions used in bio chromatography, such as the high-concentration buffers used for HIC analysis, to avoid potential corrosive damage to the system. The Agilent ProteoAnalyzer system automates CE-SDS separations of reduced and nonreduced samples using a 12-channel parallel capillary array, enabling efficient workflows. In this application note, the data from nonreduced CE-SDS using the ProteoAnalyzer system and HIC using the 1290 Infinity III Bio LC were applied to a previously described system of equations (Equations 1.1–1.9) to determine the amount of each positional isomer in the SigmaMAb ADC mimic.

Drug-to-Antibody Ratio	Positional Isomer		Fragments					
0	DAR0		mAb					
2	$DAR2_f$			HHL				L
	$DAR2_h$		mAb					
4	$DAR4_{ff}$				HH			2L
	$DAR4_{fh}$			HHL				L
	$DAR4_{hh}$					2HL		
6	$DAR6_{ffh}$				HH			2L
	$DAR6_{fhh}$					HL	H	L
8	DAR8						2H	2L

Figure 1. Antibody fragments produced under nonreduced denaturing CE-SDS analysis of cysteine-conjugated ADC.

Experimental

The SigmaMAb Antibody Drug Conjugate Mimic (Sigma, part number MSQC8-0.5MG) was used for all analyses.

CE-SDS using the Agilent ProteoAnalyzer system

The SigmaMAb ADC mimic was reconstituted to 10 mg/mL and further diluted to 1.5 mg/mL in nuclease-free water. Sample concentration was confirmed by NanoDrop (settings: Protein A280; Curve type: other E1%; Extinction coefficient: 14.3). The sample was then prepared under nonreducing conditions according to the Agilent Protein Broad Range P240 kit (part number 5191-6640) manual.[4] The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes[5] and analyzed across multiple capillaries on an Agilent ProteoAnalyzer system with the ProteoAnalyzer Broad Range kit LM Only method. For optimal results, the sample injection was decreased to 7 kV 6 seconds.[5] Sizing analysis was performed using NISTmAb as the ladder.[6]

Agilent ProSize data analysis software was used to determine the percent total of the mAb, HHL, HH, HL, H, and L chains.

Hydrophobic interaction chromatography

Software

Agilent OpenLab version 2.8 or later was used to determine peak areas for calculating the average DAR, as well as the relative percentages of the DAR0, DAR2, DAR4, DAR6, and DAR8 species.

The Agilent 1290 Infinity III Bio LC system comprised the following modules:

- Agilent 1290 Infinity III Bio Flexible Pump (G7131A)
- Agilent 1290 Infinity III Variable Wavelength Detector (G7114B) equipped with a biocompatible micro flow cell, 3 mm, 2 µL
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity III Bio Multisampler (G7137A) with option no. 101

Column

Agilent AdvanceBio HIC column, 3.5 µm, 4.6 × 100 mm (part number 685975-908)

Buffer and solvent preparation

A. 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

B. 50 mM phosphate buffer at pH 7

C. Isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled to 2 L using ultrapure water. The pH value was checked and adjusted, if necessary, to pH 7 (Buffer B). Ammonium sulfate (198.21 g; total concentration 1.5 M) was added to an empty amber-colored 1 L bottle and filled to 1 L using the prepared phosphate buffer (Buffer A). The pH value was checked and adjusted, if necessary, to pH 7 (the addition of high amounts of salt can alter pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

Chemicals

All solvents were LC grade. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium sulfate were obtained from Sigma-Aldrich (Steinheim, Germany).

Table 1. Chromatographic conditions* used in this study.

Parameter	Value
Buffers and Solvents	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 C) Isopropanol
Gradient	Gradient: 0 min 55% A, 40% B, 5% C 25 min 0% A, 75% B, 25% C Stop time: 35 min Post-time: 10 min
Flow Rate	0.400 mL/min
Temperature	25 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 15 µL Sample temperature: 10 °C Needle wash: 3 s in water

*When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method, as high amounts of salt can change the compressibility of the solvent. Using the preconfigured solvent tables enables the best pump performance.

Samples

The previously mentioned reconstituted unmodified SigmaMAb control and SigmaMAb ADC mimic, each at a concentration of 10 mg/L, were used as samples and diluted with 50% Buffer A (Table 1).

System-of-equations analysis

SciPy's lsq_linear python was used to solve the system of equations (Equations 1.1–1.9) for $DAR2_f$, $DAR2_h$, $DAR4_{ff}$, $DAR4_{fh}$, $DAR4_{hh}$, $DAR6_{ffh}$, and $DAR6_{fhh}$ (variables in bold) using least-squares minimization, with the variable values constrained to be greater than or equal to zero. The relative percentages for the individual antibody fragments (as determined by CE-SDS) and the DAR (as determined by HIC) were used as the input values. Because the CE-SDS analysis uses a dye that covalently labels primary amines, equations were corrected by determining the relative number of lysine residues in the specific antibody fragment in relation to the intact mAb.

Equations 1.1-1.9

$$\text{mAb} = (DARO + \boldsymbol{DAR2_h}) \times \frac{2 \times LysH + 2 \times LysL}{2 \times LysH + 2 \times LysL}$$

$$\text{HHL} = (\boldsymbol{DAR2_f} + \boldsymbol{DAR4_{hh}}) \times \frac{2 \times LysH + LysL}{2 \times LysH + 2 \times LysL}$$

$$\text{HH} = (\boldsymbol{DAR4_{ff}} + \boldsymbol{DAR6_{fhh}}) \times \frac{2 \times LysH}{2 \times LysH + 2 \times LysL}$$

$$\text{HL} = (2 \times \boldsymbol{DAR4_{ff}} + \boldsymbol{DAR6_{fhh}}) \times \frac{LysH + LysL}{2 \times LysH + 2 \times LysL}$$

$$\text{H} = (\boldsymbol{DAR6_{ffh}} + 2 \times DAR8) \times \frac{LysH}{2 \times LysH + 2 \times LysL}$$

$$\text{L} = (\boldsymbol{DAR2_f} + 2 \times \boldsymbol{DAR4_{ff}} + \boldsymbol{DAR4_{fh}} + 2 \times \boldsymbol{DAR6_{ffh}} + \boldsymbol{DAR6_{fhh}} + 2 \times \boldsymbol{DAR8}) \times \frac{LysH}{2 \times LysH + 2 \times LysL}$$

$$1 = \frac{\boldsymbol{DAR2_f} + \boldsymbol{DAR2_h}}{DAR2}$$

$$1 = \frac{\boldsymbol{DAR4_{ff}} + \boldsymbol{DAR4_{fh}} + \boldsymbol{DAR4_{hh}}}{DAR4}$$

$$1 = \frac{\boldsymbol{DAR6_{ffh}} + \boldsymbol{DAR6_{fhh}}}{DAR6}$$

Results and discussion

Reduced CE-SDS analysis of cysteine-conjugated ADCs

Nonreduced CE-SDS analysis of the SigmaMAb ADC mimic produces a fragmentation pattern consistent with L, H, HL, HH, HHL, and intact HHLL antibody species. The relative concentration of each peak is used to determine the percentage of each fragment relative to the total (Table 2, Figure 2).

Table 2. Relative percentage of each antibody fragment as determined by CE-SDS analysis using the Agilent ProteoAnalyzer system.

Antibody Fragment	Relative Percent
L	18.0%
H	14.7%
HL	14.8%
HH	29.9%
HHL	19.2%
HHLL (intact mAb)	3.6%

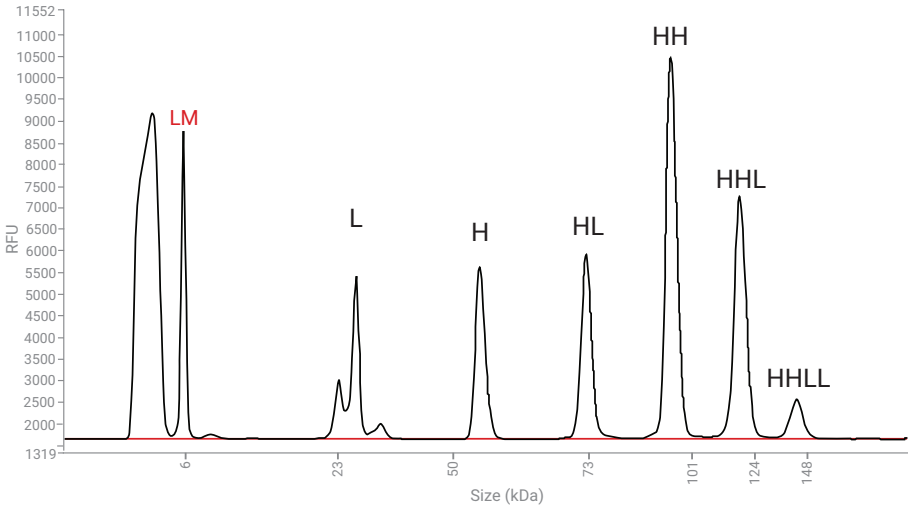

Figure 2. CE-SDS analysis of the SigmaMAb ADC mimic using the Agilent ProteoAnalyzer system under nonreducing conditions.

HIC was used to determine the overall DAR value according to Equation 2. The SigmaMAb ADC mimic has an average DAR of 4.04, consistent with the value of 4.4 reported in the certificate of analysis (CoA). Additionally, peak areas are used to determine the percentage of each DAR species (Table 3, Figure 3).

Equation 2

$$\text{Average DAR} = \sum \frac{\text{LC peak area x Number of drugs}}{\text{Total LC peak area}}$$

The values from Tables 2 and 3, along with the number of lysines in the L and H chains as reported by Sigma in the SigmaMAb data sheet, were used to solve the system of equations for the different positional DAR isomers (Table 4). Replicate computations with the same inputs return the same solutions, indicating that the algorithm only identifies a single best solution (Table 4).

Using the system of equations, 41.5% of the ADC population is composed of DAR4, with the payloads located at the two Fab locations ($DAR4_{ff}$ species). Summation of $DAR4_{ff}$, $DAR4_{fh}$, and $DAR4_{hh}$ values determined by the system of equations indicates that 48.1% of the ADC is composed of DAR4 species. This is consistent with the HIC data, which indicate that 48.3% of the ADC has a DAR of 4. The next major species determined by the system of equations are $DAR2_f$ and $DAR6f_{hh}$ at 19.3% and 22.9%, respectively. Notably, the $DAR2_h$ and $DAR6_{ffh}$ species are consistently absent. The total amounts of DAR2 and DAR6 calculated by the system of equations is consistent with the HIC values of 19.5% and 22.2%, respectively.

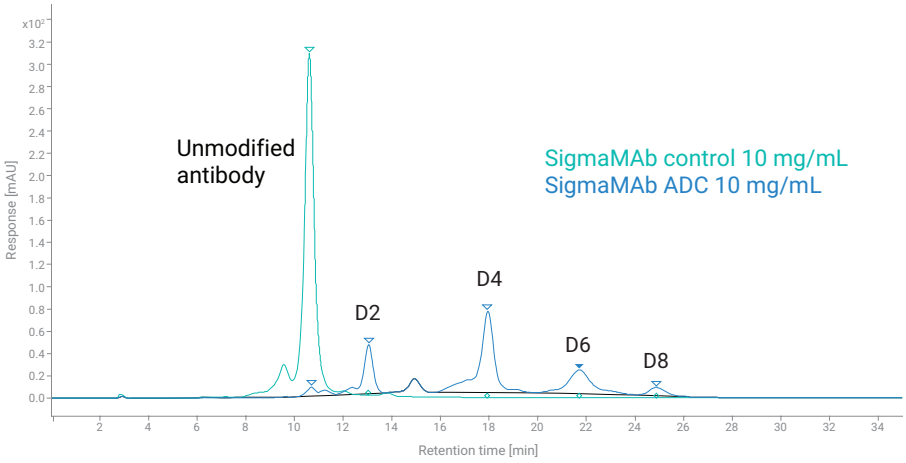

Figure 3. HIC analysis of the unmodified SigmaMAb control and the SigmaMAb ADC mimic using the Agilent 1290 Infinity III Bio LC. The DAR0, DAR2, DAR4, DAR6, and DAR8 peak areas are used to determine the relative amount of each individual species, as well as the average DAR.

Table 3. Relative percentage of each DAR species as determined by HIC using the Agilent 1290 Infinity III Bio LC.

Drug-to-Antibody Ratio (DAR)	Relative Percent
0	5.2%
2	19.5%
4	48.3%
6	22.2%
8	4.8%

Table 4. Relative percentages of each ADC species as determined by solving the system of equations using CE-SDS and HIC data.

ADC Species	Relative Percent
DAR0	5.2%
$DAR2_f$	19.3%
$DAR2_h$	0.0%
$DAR4_{ff}$	41.5%
$DAR4_{fh}$	3.1%
$DAR4_{hh}$	3.6%
$DAR6_{ffh}$	0.0%
$DAR6_{fhh}$	22.9%
DAR8	4.8%

Conclusions

The method presented here combines analytical techniques commonly used during ADC analysis to computationally determine the positional isomers of the ADC population. This allows for quick estimation of the relative ADC isomer abundance without the need for additional techniques. The Agilent ProteoAnalyzer system and the Agilent 1290 Infinity III Bio LC provide highly reproducible CE-SDS and HIC analyses for characterization of ADCs.

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Foundations of Protein Analysis on the ProteoAnalyzer

Application Note

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Fluorescence vs. UV Detection: Comparing Two CE-SDS Systems Using NISTmAb

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Abstract

Fluorescence and UV detection are commonly used in protein capillary electrophoresis. This study compares these detection methods with two different capillary electrophoresis systems: the Agilent ProteoAnalyzer system, which uses fluorescence, and a similar system that uses UV. Both detection technologies were employed to assess the same National Institute of Standards and Technology monoclonal antibody reference material standard (NISTmAb). In this application note, both detection methods provided similar data. Additionally, the results were comparable to the NISTmAb datasheet, confirming accuracy of the results.

Introduction

Electrophoresis is used to separate proteins and assess their quality. In capillary electrophoresis (CE), technologies including fluorescence and UV absorbance are used to detect proteins. Each detection technology has advantages and disadvantages. Fluorescence detection involves the covalent binding of a dye molecule to specific amino acids. Once the dye is bound, the labeled protein fluoresces under a specific light source, such as a light emitting diode (LED) or a laser. The detector is selective for protein fluorescence, resulting in a low-noise baseline and clear peaks. Alternatively, ultraviolet (UV) detection measures the absorbance of UV light by amino acids as they pass through the detection area. UV detection is sensitive to aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine, due to their strong absorbance in the 260–280 nm UV range. UV detection is also sensitive to other UV-absorbing compounds present in the sample. Additionally, the intensity of UV light emitted by the lamp can vary based on its temperature. When combined, these factors can create a noisy or sloping baseline.

The Agilent ProteoAnalyzer system is a capillary electrophoresis sodium dodecyl sulfate (CE-SDS) instrument that uses LED-induced fluorescence (LEDIF) detection for protein quality control.[1,2] This system enables the precise and accurate measurement of up to 12 protein samples in parallel and up to 96 total samples, without user interaction. Sample separation is completed in 30 minutes, and results are automatically analyzed with the Agilent ProSize data analysis software. Using the Agilent Protein Broad Range P240 kit, the ProteoAnalyzer system accommodates protein samples ranging in size from 10 to 240 kDa. Samples can be assessed under reduced or nonreduced conditions and are prepared using a rapid, easy-to-follow, covalent labeling method. The labeling method involves mixing the sample with a master mix containing dye, a lower marker, buffer, and reducing or nonreducing reagents, followed by heating.

LEDIF with the ProteoAnalyzer was compared to UV detection using an alternate system, System B, which is a CE instrument designed to use either LIF or UV for sample detection. This system can process up to 8 samples simultaneously and up to 96 total samples, without user interaction. System B requires 55 minutes for sample separation and automatically analyzes the results in its analysis software. With the associated protein analysis kit, proteins ranging from 10 to 225 kDa can be analyzed under reduced and nonreduced conditions. Samples are prepared by mixing with an optional lower marker and a reducing or nonreducing reagent, followed by heating.

This application note provides a comparison between fluorescence detection using LEDIF on the ProteoAnalyzer system and UV detection on System B. Analyzing the National Institute of Standards and Technology monoclonal antibody reference material standard (NISTmAb) allowed for comparison of both detection methods. Each detection technology was compared visually using electropherograms and by analyzing mAb critical quality attributes (CQAs), including monomeric purity, percent glycan occupancy, and percent thioether. Nonglycosylated heavy chain/heavy chain (NGHC/HC) resolution was also compared between the two detection technologies. The results demonstrated that fluorescence provides data comparable to that of UV detection.

Experimental

NISTmAb (Sigma, part number NIST8671; aliquot from Reference Material 8671, Lot 14HB-D-002)[3] was prepared in phosphate buffered saline (PBS) under both reduced and nonreduced conditions, following the Agilent Protein Broad Range P240 kit (part number 5191-6640) manual.[1] The samples were covalently labeled by incubation with the supplied reagents at 70 °C for 10 minutes. The reduced and nonreduced antibodies were analyzed across multiple capillaries on the Agilent ProteoAnalyzer system using the Protein Broad Range kit lower and upper marker method, or the lower marker (LM)-only method.[1,2] For nonreduced conditions, the sample injection was decreased to 7 kV for 6 seconds to achieve optimal results, as previously described.[4]

Sample preparation for System B was performed using the reagents provided in the associated protein kit. Samples were diluted with SDS-MW sample buffer to a volume of 95 µL and a concentration of 1,000 ng/µL. In a fume hood, 2 µL of the 10 kDa internal standard was added, followed by 5 µL of 250 mM iodoacetamide solution for nonreduced conditions or 2-mercaptoethanol for reduced conditions. Samples were vortexed and centrifuged for 1 minute at 300 g, then sealed and heated at 70 °C for 10 minutes. After cooling to room temperature for 3 minutes, samples were loaded on System B and electrokinetically injected at –5.0 kV for 20 seconds. The samples were then separated using –15.0 kV for 30 minutes (reduced) or 40 minutes (nonreduced). Table 1 shows the reported kit specifications.

Table 1. Comparison of specifications for the Agilent Protein Broad Range P240 kit used on the Agilent ProteoAnalyzer system and the kit used on System B.

Specifications	Agilent ProteoAnalyzer System and Protein Broad Range P240 Kit	System B and Associated Kit
Sizing Range	10–240 kDa (lower marker only); 10–200 kDa (lower marker and upper marker)	10–225 kDa (sizing standard)
Sizing Accuracy	< 15% for BSA, CAII (lower marker only; using reduced conditions), < 10% for BSA, CAII (lower marker and upper marker; using reduced conditions)	Not reported
Sizing Precision	< 8% CV for BSA, CAII, GREMLIN-1, and NISTmAb (lower marker only; using reduced conditions), < 10% CV for intact NISTmAb (lower marker only; using nonreduced conditions), < 5% CV for BSA, CAII, GREMLIN-1, and NISTmAb (lower marker and upper marker; using reduced conditions)	Not reported
Quantitative Range	2–2,000 ng/µL for BSA in PBS	Not reported
Sensitivity	1 ng/µL for BSA, CAII in PBS	Not reported
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	8 samples: 55 minutes (reduced), 65 minutes (non-reduced)
Resolution	< 10% molecular weight resolution between 15 and 150 kDa (based on ladder); R ≥ 1 NISTmAb NGHC/HC (using reduced conditions)	Not reported

Results and discussion

Visual representation of fluorescence and UV detection

The ProteoAnalyzer system uses a covalent dye that binds to proteins and fluoresces when exposed to an LED (Figures 1A and 2A). An advantage of fluorescence detection is that the detector only perceives the distinct signal of the fluorescent dye, resulting in a near-flat, low-noise baseline. In Figures 1A and 2A, the ProteoAnalyzer electropherogram exhibits a system peak as the first signal. This peak represents the unconjugated dye front and appears before sample peaks to avoid interference. The next peak is a 6 kDa lower marker, followed by a sample detection region. Using the NISTmAb standard as an example, various peaks are observed in the sample detection region for both reduced (Figure 1A) and nonreduced (Figure 2A) conditions, as previously described.[5] The flat baseline from fluorescence detection makes it easy to identify high molecular weight (HMW) aggregates in the nonreduced analysis, as illustrated by the last peak on the electropherogram in Figure 2A. These have previously been identified as covalently bound oligomers.[4]

System B detects samples by measuring UV light absorption. While this method does not require a dye-labeling reaction to detect samples, other UV-absorbing compounds in the gel or sample can generate baseline noise. Additionally, a warmup period is required for UV lamps to provide a more stable baseline. For example, System B requires a warm-up period of 30 minutes. The electropherograms for reduced (Figure 1B) and nonreduced (Figure 2B) conditions show one system peak starting from the left side. Following the system peak, a peak corresponding to an optional 10 kDa lower marker, which can be mixed with the sample during preparation, is observed. Like the ProteoAnalyzer system, System B identifies the same peaks of the reduced and nonreduced NISTmAb. In these examples, the baseline slopes downward to the right. This slant is characteristic of UV detection and may cause issues with peak calling.

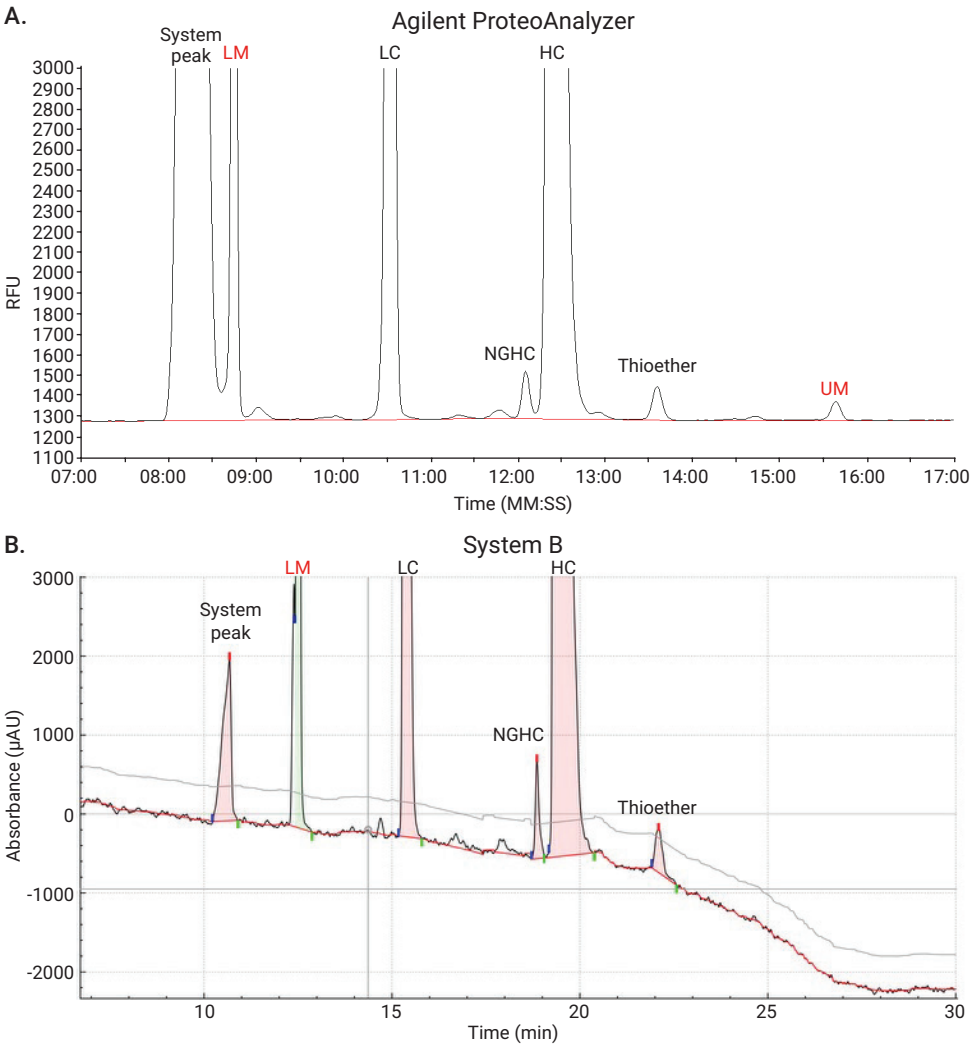

Figure 1. Reduced NISTmAb electropherograms using the (A) Agilent ProteoAnalyzer system and (B) System B. LM = lower marker; LC = light chain; NGHC = nonglycosylated heavy chain; HC = heavy chain; UM = upper marker.

At the far right of the electropherogram for the nonreduced sample, HMW species are identified on the ProteoAnalyzer system (Figure 2A) but appear as noise or as an unstable baseline on System B (Figure 2B). System B analysis software requires manual integration of this peak due to the sloping baseline. As a result, the ambiguity of the HMW peak could result in this impurity not being included in the analysis.

In summary, although UV detection is quick and easy, it can struggle with clear identification of sample peaks due to baseline noise. In contrast, fluorescence detection with the ProteoAnalyzer system requires more sample preparation but offers a cleaner, more stable baseline, facilitating easier data interpretation as well as enhanced clarity and sensitivity of the results. This makes fluorescence detection a valuable method for protein analysis.

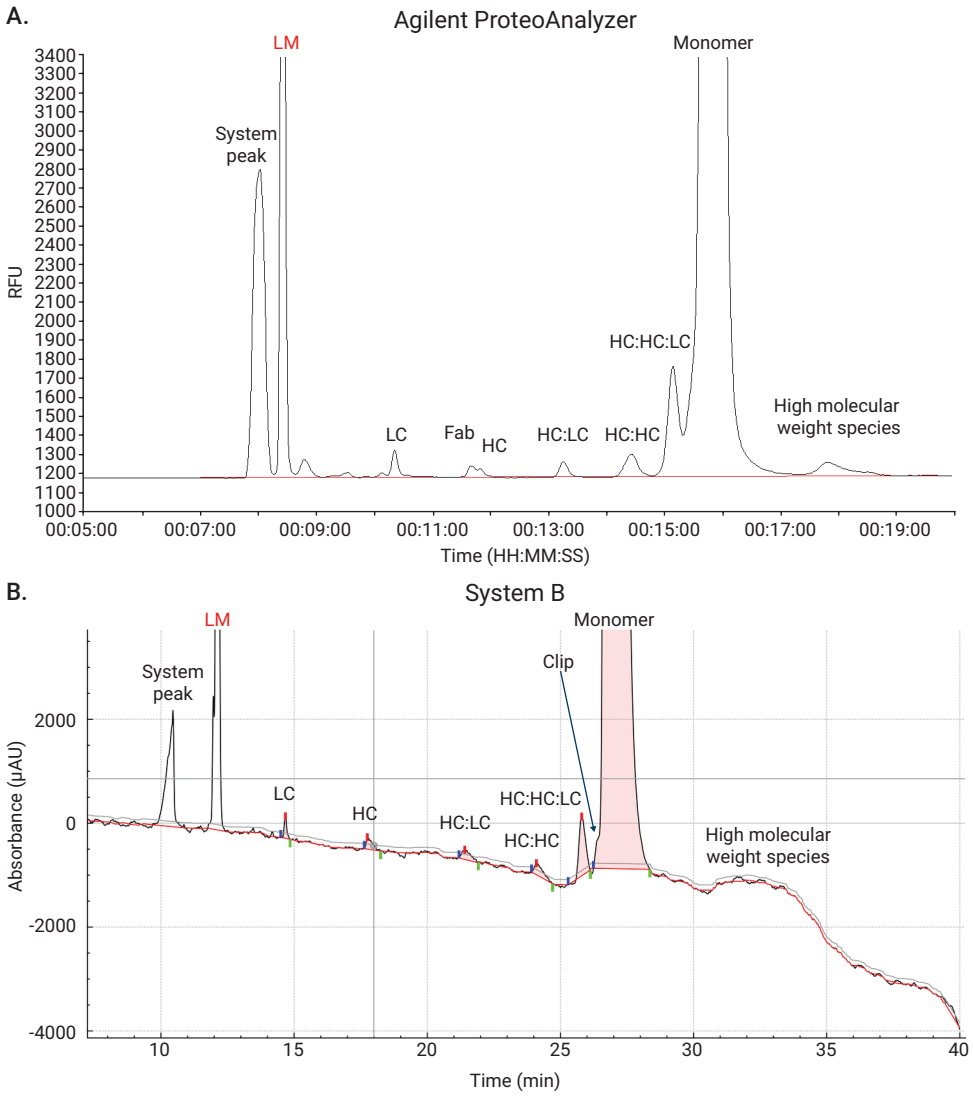

Figure 2. Nonreduced NISTmAb electropherograms using the (A) Agilent ProteoAnalyzer system and (B) System B. LM = lower marker; LC = light chain; HC = heavy chain.

Comparison of critical quality attributes for fluorescence and UV detection

Both fluorescence and UV detection systems offer quantitative analysis capabilities for evaluating proteins. Assessing mAbs requires accuracy and reliability, particularly when evaluating CQAs such as monomeric purity, glycan occupancy, and percent thioether. To compare fluorescence detection (LEDIF) and UV detection, both systems were used to calculate the CQAs of the NISTmAb standard using established equations. Results were compared between systems and against the NIST datasheet.[5]

Fluorescence detection using the ProteoAnalyzer system found the nonreduced monomeric purity to be 98.18%, while UV detection by System B found the monomeric purity to be 98.32%. Each system provides reliable measurements and high precision (%CV ≤ 0.19%). Additionally, both systems have calculated monomeric purity values close to the NIST datasheet value (98.47%), demonstrating the accuracy of the fluorescence and UV methods (Table 2).

Glycan occupancy and percent thioether are both calculated using reduced analysis. Fluorescence detection on the ProteoAnalyzer system measures glycan occupancy at 99.30%, while UV detection on System B reports a similar value of 99.26%—both closely aligning with the NIST datasheet value of 99.39%. Furthermore, both systems exhibit the same precision (%CV = 0.02%). For percent thioether, fluorescence detection and UV detection measure average values of 0.40% and 0.41%, respectively. These values are close to the percent thioether value published in the NIST datasheet (0.30%) and demonstrate good precision (%CV ≤ 6.35%). The results demonstrate that both fluorescence and UV detection methods provide accurate and precise measurements of glycan occupancy and percent thioether (Table 2).

Accurate glycan occupancy calculations require resolution of the NGHC and HC fragments. Both the ProteoAnalyzer system and System B show excellent resolution factors (R) for the NGHC and HC peaks, with an average R value of 1.60 for each system. This value meets the ProteoAnalyzer kit specification for an NGHC/HC R ≥ 1 for NISTmAb under reduced conditions. Both system results are reproducible, with %CV ≤ 4.5% (Table 2).

Together, the data shows that fluorescence detection on the ProteoAnalyzer and UV detection on System B yield comparable results across all measured CQAs and confirms the accuracy of each technique when compared against the NISTmAb reference datasheet. With excellent resolution of NGHC and HC fragments and strong reproducibility, either detection method can support accurate and consistent CQA analysis of monoclonal antibodies.

Table 2. Critical quality attributes (CQAs) for NISTmAb were calculated from analysis on the Agilent ProteoAnalyzer system and System B. Results were compared to the published NIST datasheet[1]. (ProteoAnalyzer: Nonreduced n = 11; Reduced n = 33. System B: Nonreduced n = 3; Reduced n=3)

	ProteoAnalyzer System		System B		NIST Datasheet		ProteoAnalyzer vs. System B
	Average	%CV	Average	%CV	Average	Combined Standard Uncertainty	Percent Difference
Monomeric Purity	98.18%	0.09%	98.32%	0.19%	98.47%	1.0%	**0.14%**
Thioether (Reduced)	0.40%	6.35%	0.41%	2.92%	0.30%	0.03%	**0.00%**
Glycan Occupancy (Reduced)	99.30%	0.02%	99.26%	0.02%	99.39%	0.07%	**0.04%**
Resolution NGHC/HC	1.60	4.5%	1.60	0.39%	–	–	**0.00%**

Conclusion

In this application note, fluorescence detection using the Agilent ProteoAnalyzer system and UV detection using System B were compared through analysis of the industry standard NISTmAb. Both systems effectively detected and resolved the primary and impurity peaks of the mAb. Similar results were obtained for monomeric purity, glycan occupancy, and percent thioether, demonstrating the comparability of analytical performance and supporting the use of fluorescence detection as a viable alternative to UV detection for NISTmAb analysis.

References

1. Agilent Protein Broad Range P240 Kit. *Agilent Technologies quick guide*, publication number D0031125, **2023**.
2. Dependable Denaturing Protein Electrophoresis: Agilent ProteoAnalyzer system. *Agilent Technologies brochure*, publication number 5994- 6716EN, **2023**.
3. **National Institute of Standards and Technology (NIST), U.S. Department of Commerce**. *Reference Material 8671: NISTmAb, Humanized IgG1κ Monoclonal Antibody, Lot 14HB-D-002. Reference Material Information Sheet*; NIST: Gaithersburg, MD. https://tsapps.nist.gov/srmext/certificates/8671.pdf.
4. Analysis of NIST Antibody on the Agilent ProteoAnalyzer System. *Agilent Technologies technical overview*, publication number 5994-6960EN, **2024**.
5. Turner, A.; Yandrofski, K.; Telikepalli, S.; King, J.; Heckert, A.; Filliben, J.; Ripple, D.; Schiel, J. E. Development of Orthogonal NISTmAb Size Heterogeneity Control Methods. *Anal. Bioanal. Chem*. **2018**, *410* (8), 2095–2110. DOI: 10.1007/s00216-017-0819-3.

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5994-8534EN

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 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 (using reduced conditions) < 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].

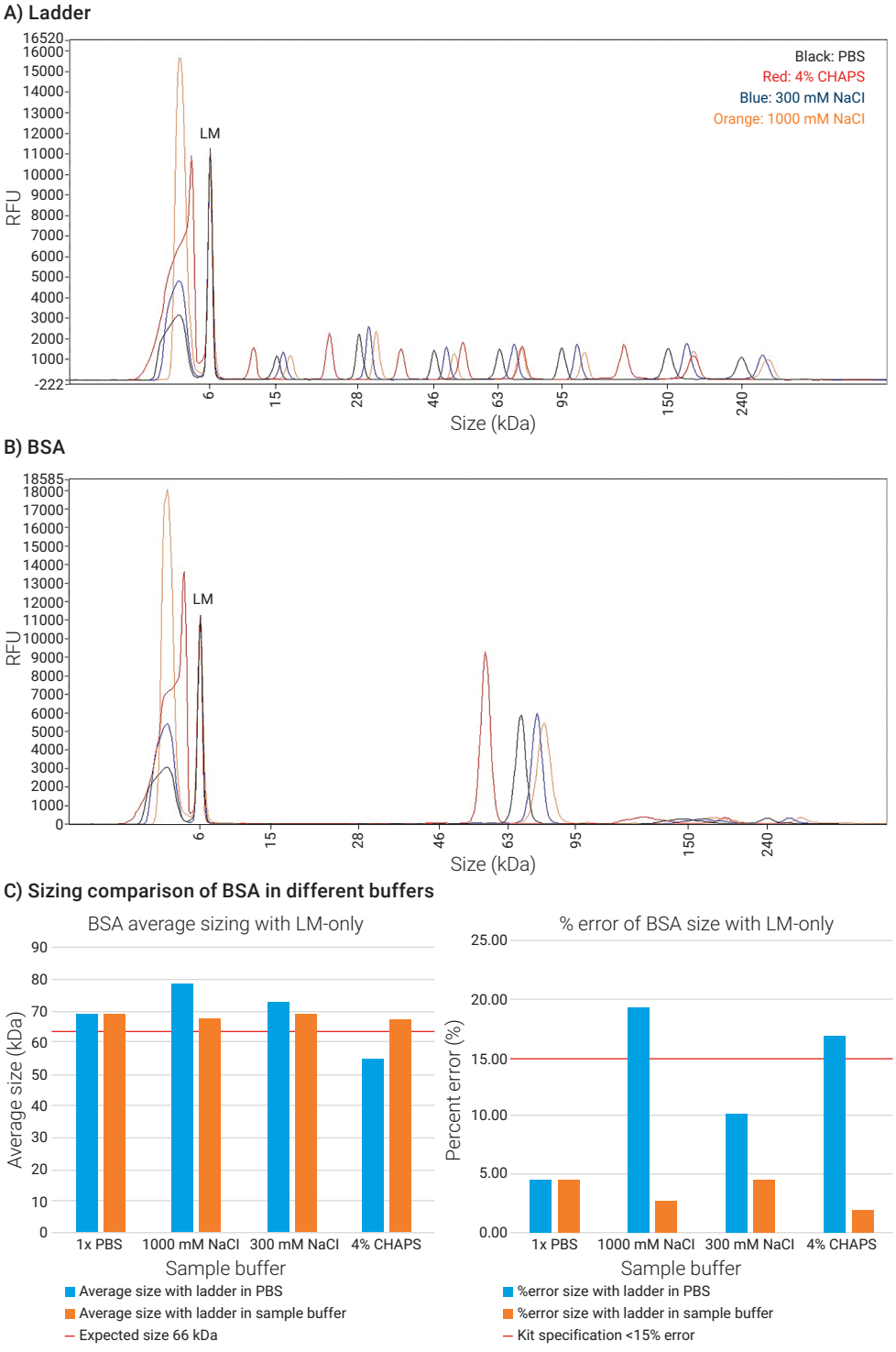


Figure 1. Overlays of A) ladder and B) BSA were prepared in different buffers and analyzed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit using the LM-only method to demonstrate the impact that the sample buffer can have on sample migration. C) BSA prepared in different 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.

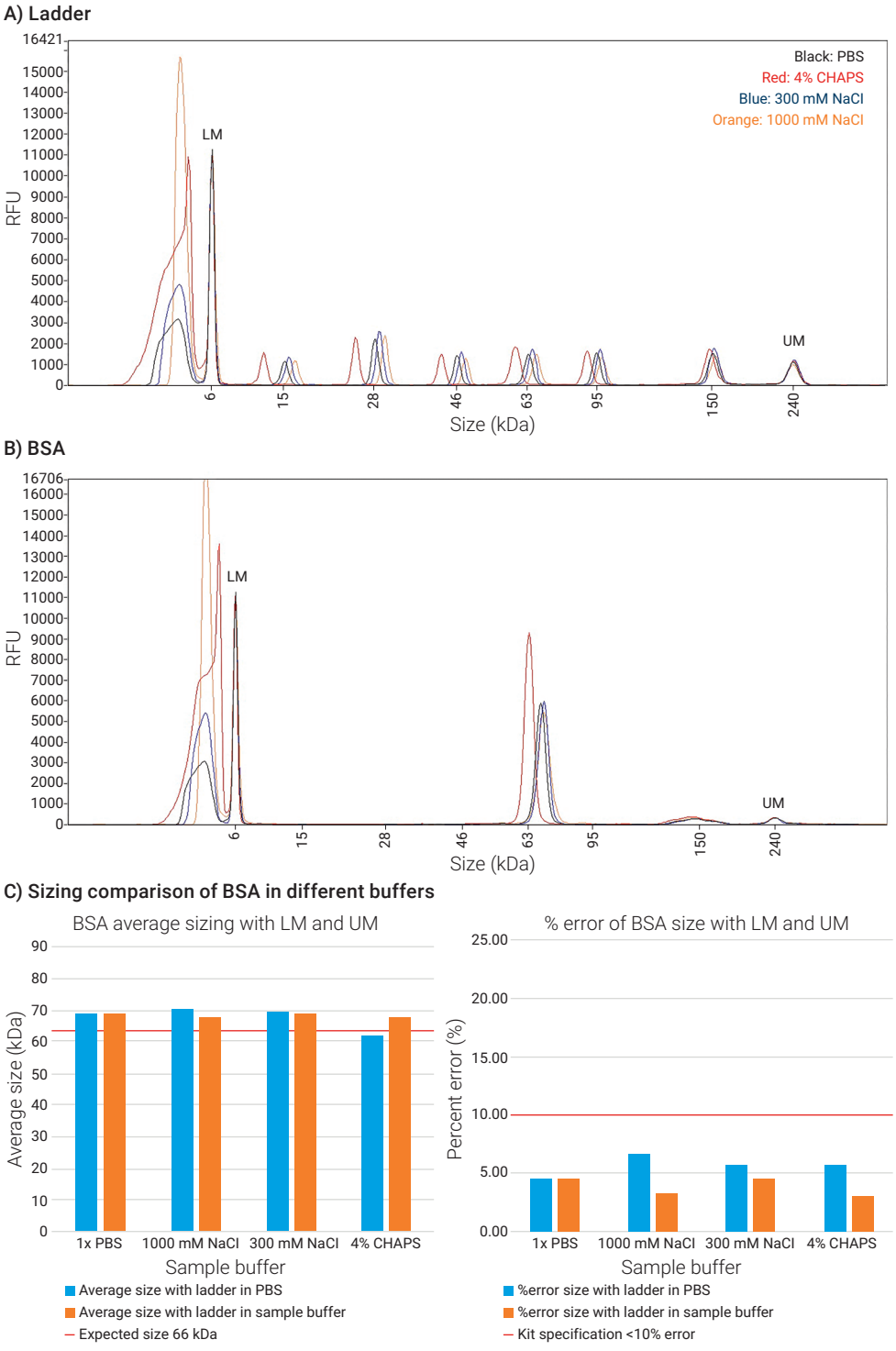


Figure 2. Overlays of A) ladder and B) BSA were prepared in different buffers and analyzed on the Agilent ProteoAnalyzer system with the Agilent Protein Broad Range P240 kit using the LM and UM method to demonstrate the impact that the sample buffer can have on sample migration. C) BSA prepared in different 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

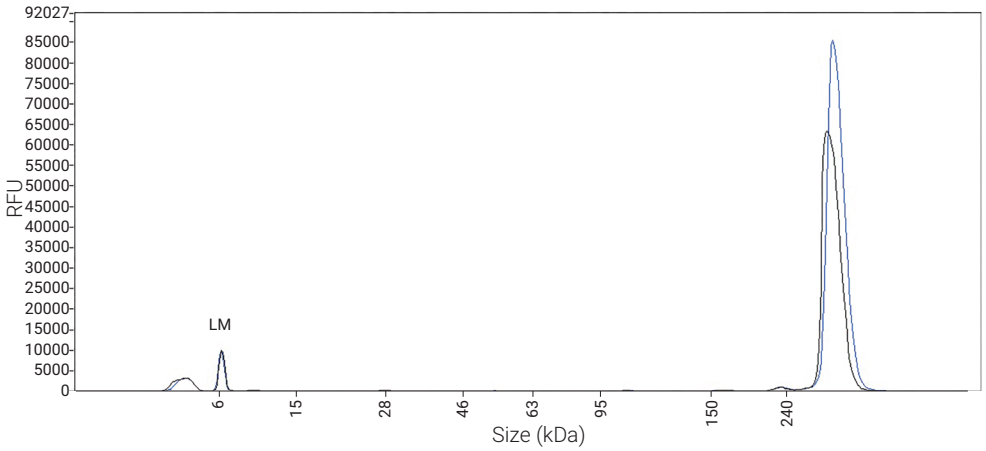
B) LM and UM method

	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.

A



B

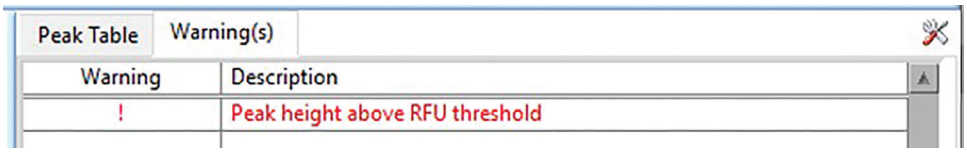


Figure 3. Injection time optimization on the Agilent ProteoAnalyzer system. A) Overlay of BSA injected for different times to optimize analysis conditions. Blue trace: ten second injection; Black trace: six second injection. B) The blue trace has a peak height of > 80,000 RFU, indicated by the warning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

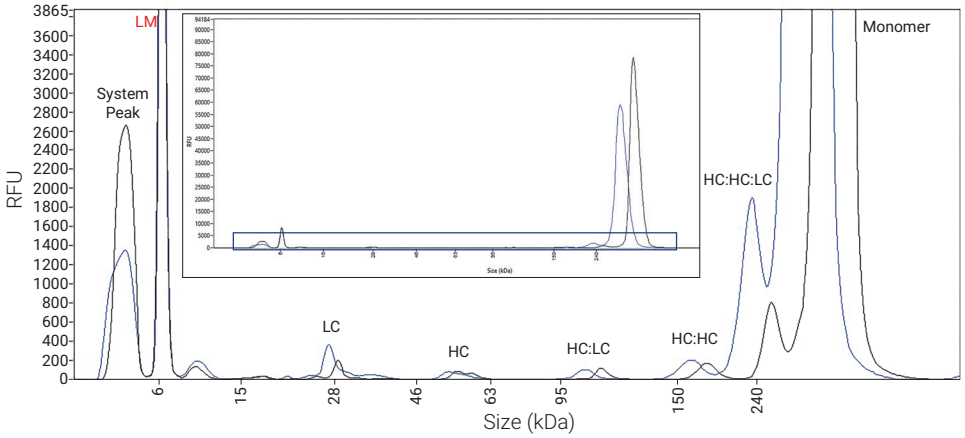
Incubation temperature

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.

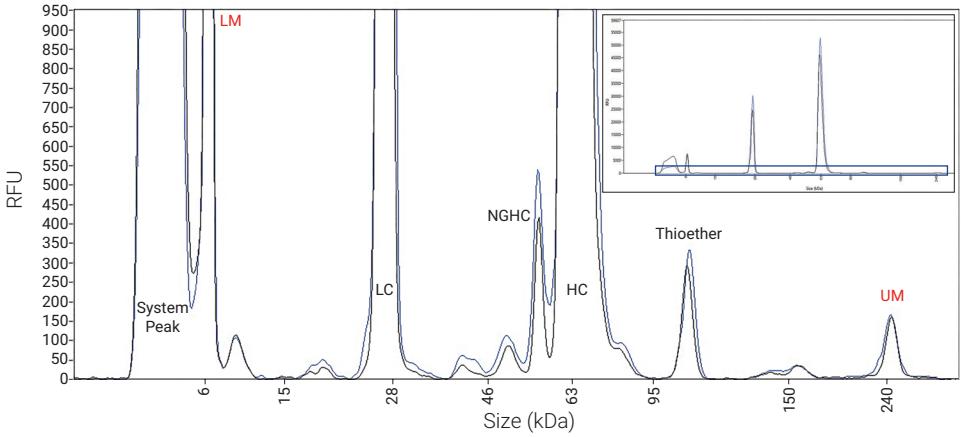
To provide guidance for optimizing the labeling conditions used with the ProteoAnalyzer system, different incubation temperatures were tested for analysis of the NISTmAb. Due to its extensive characterization, the NISTmAb is a well-established standard within the biopharmaceutical industry and is widely 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.

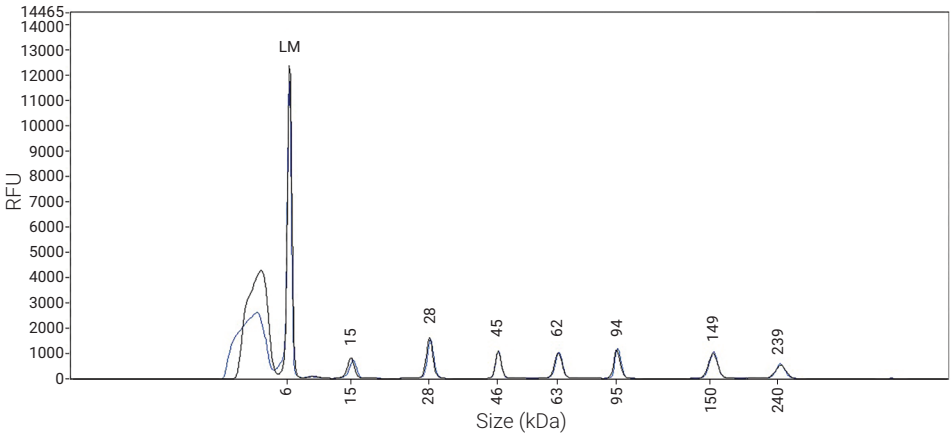
As shown in Figure 4C, analysis of the monomeric purity, thioether amount, and glycan occupancy of the NISTmAb when using an incubation temperature of 70 °C was highly comparable to the data presented in the NISTmAb datasheet, and matched more closely than the data for the sample when prepared at 85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

A)



B)

	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.

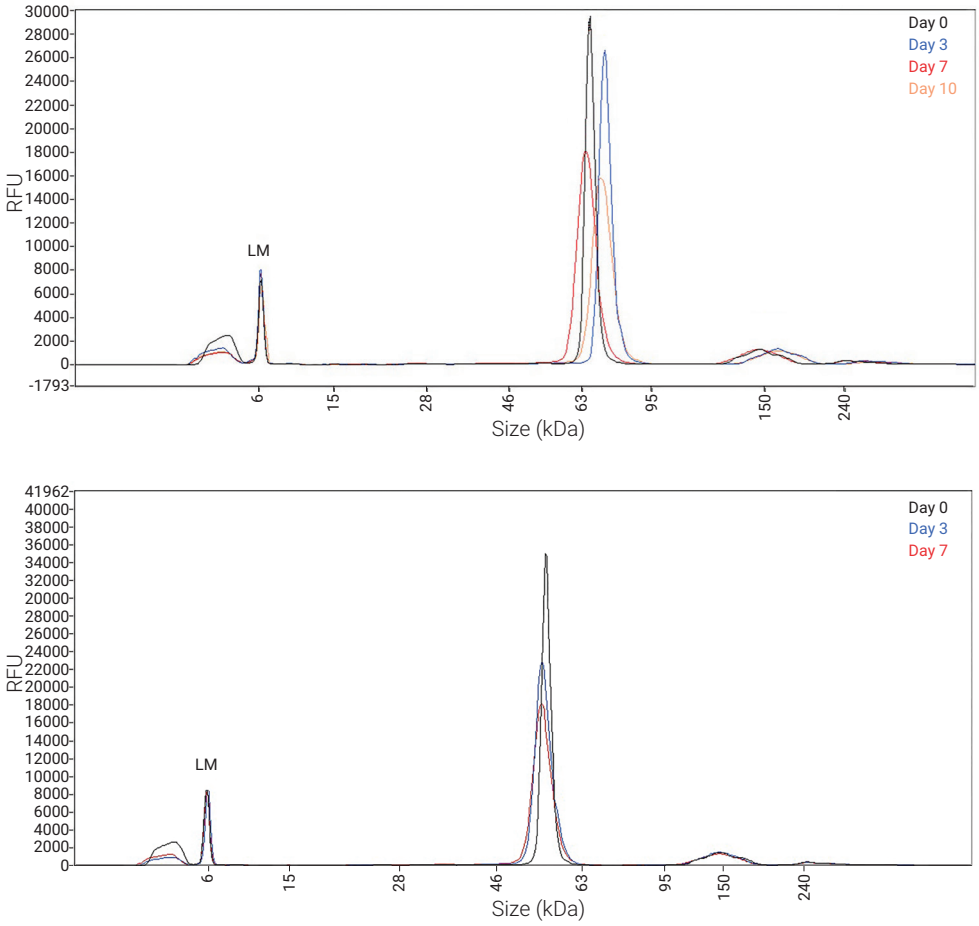


Figure 6. BSA was prepared for analysis on the Agilent ProteoAnalyzer system and stored under a variety of storage temperatures to examine stability of labeled samples over time. Storage at both A) 4 °C and B) room temperature under either light or dark conditions showed a decrease in sample peak height 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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