Application Note

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

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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]

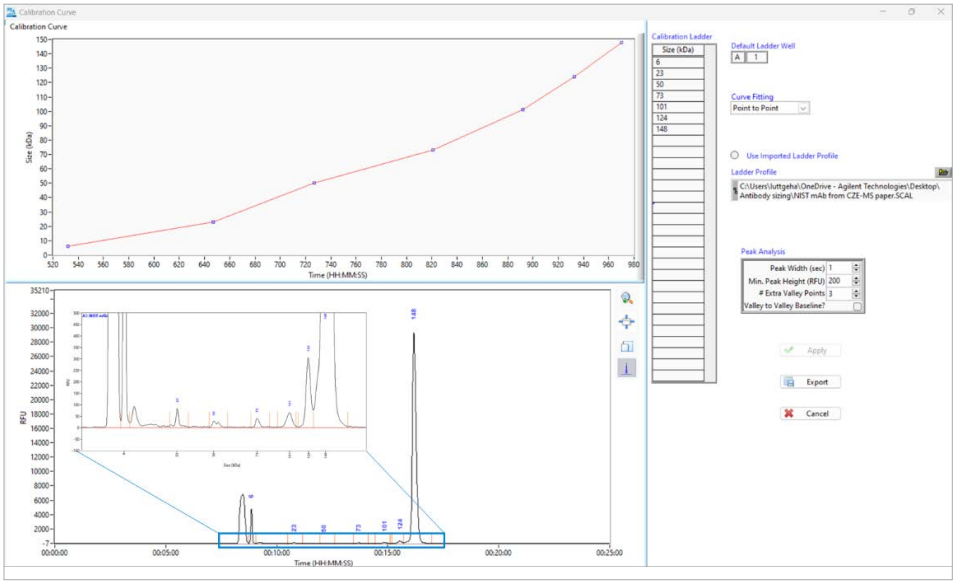

Figure 1. Representative calibration curve of the NIST mAb as the calibration ladder for the Agilent ProteoAnalyzer system.

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).

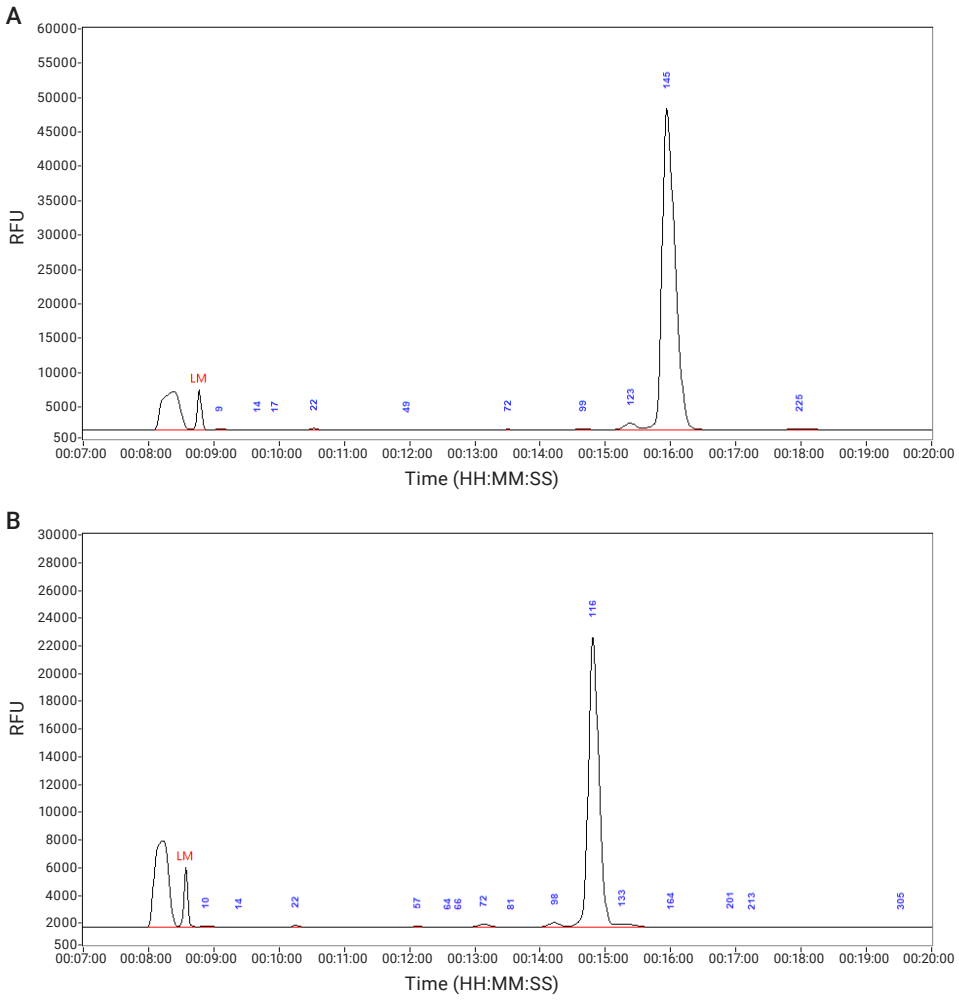

Figure 2. Electropherogram results of the Trastuzumab (**A**) monomer and (**B**) HHL fragment when analyzed on the Agilent ProteoAnalyzer system and sizing analysis performed with the NIST mAb as a ladder.

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).

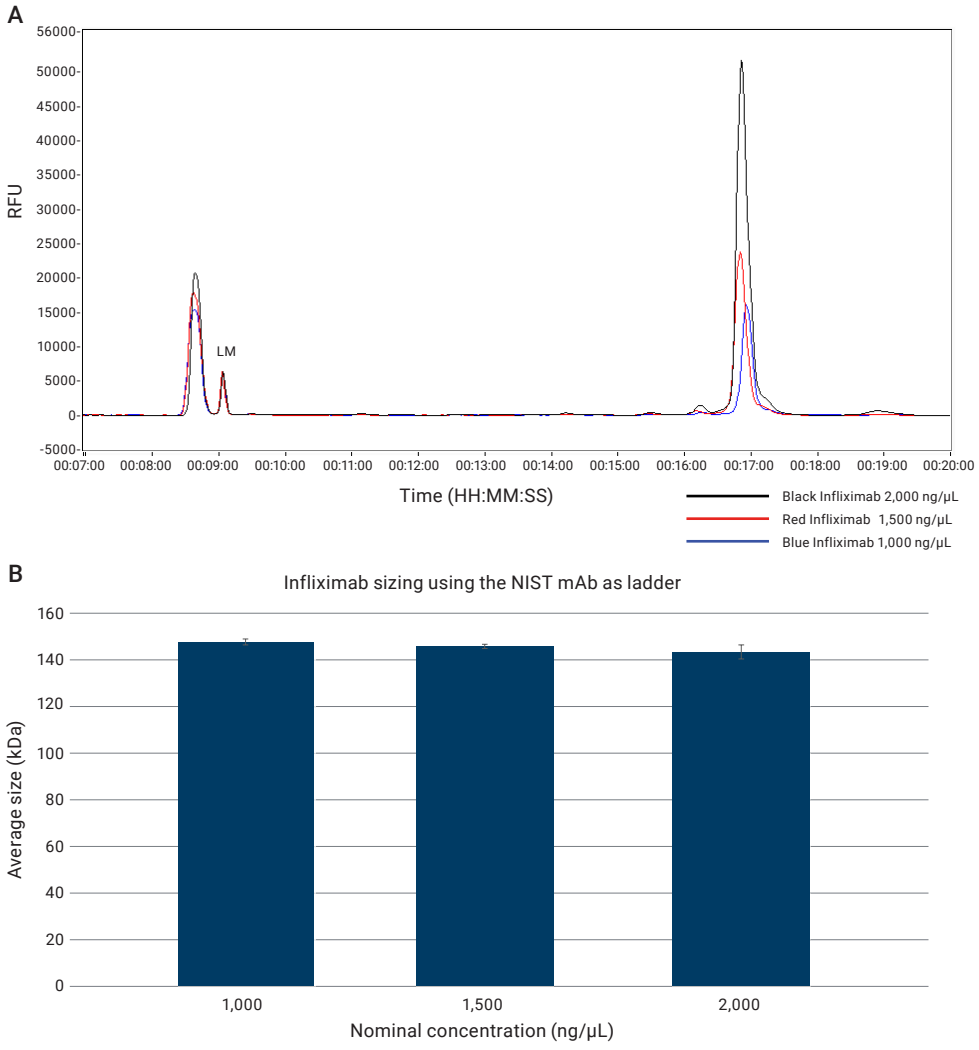

Figure 3. (**A**) Electropherogram overlay and (**B**) sizing of the Infliximab mAb at varying concentrations across the detection range of the Agilent ProteoAnalyzer system using the NIST mAb as a ladder.

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).

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.

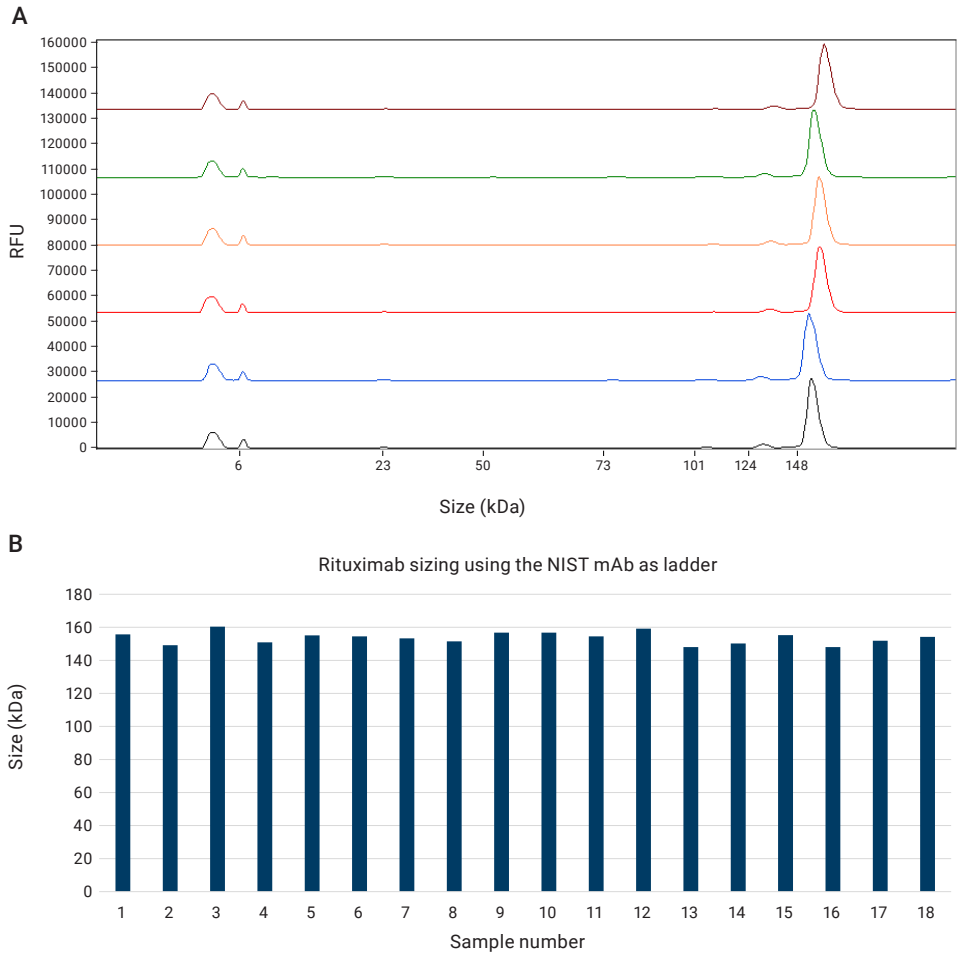

Figure 4. Rituximab was analyzed on the Agilent ProteoAnalyzer system using the NIST mAb as a sizing ladder in multiple replicates. (**A**) Electropherogram overlay of six wells within a single run. (**B**) Size of 18 replicates demonstrating the sizing precision of the system (n = 6 replicates per row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

References

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