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.

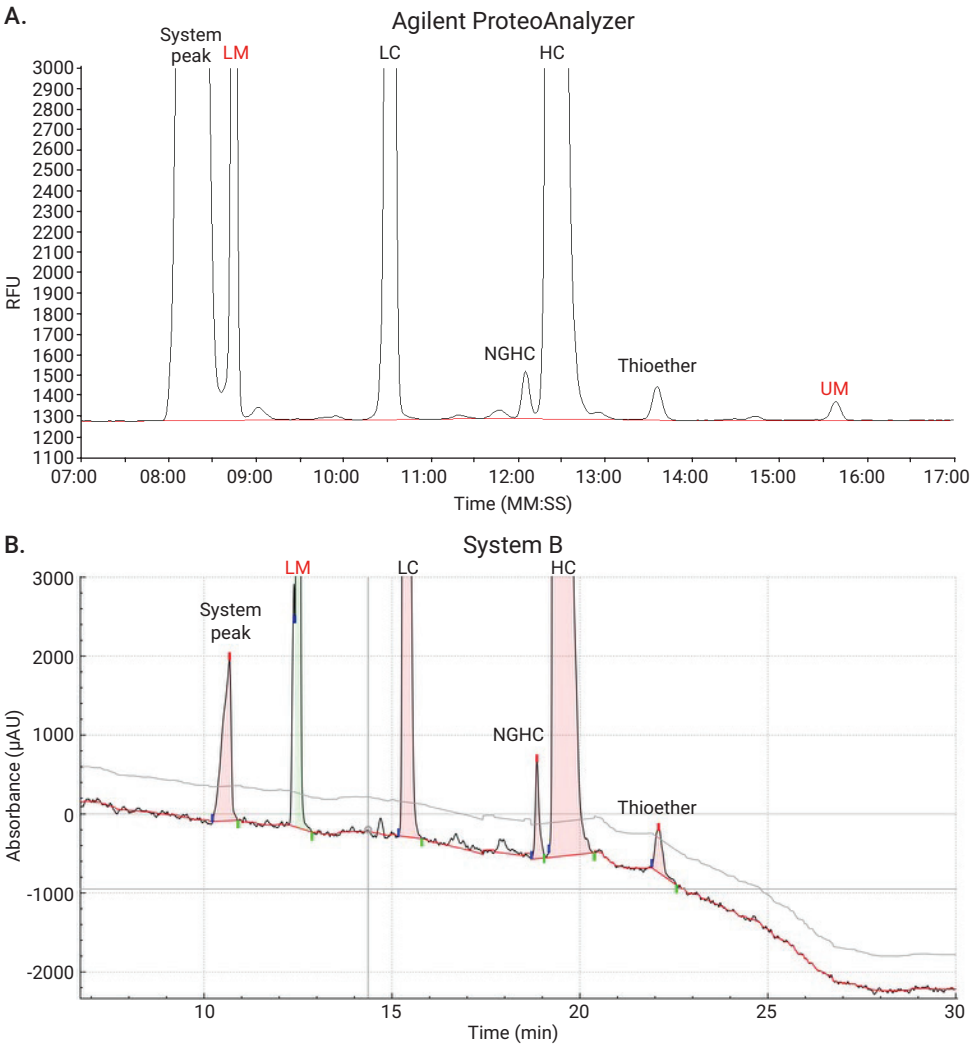

Figure 1. Reduced NISTmAb electropherograms using the (A) Agilent ProteoAnalyzer system and (B) System B. LM = lower marker; LC = light chain; NGHC = nonglycosylated heavy chain; HC = heavy chain; UM = upper marker.

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.

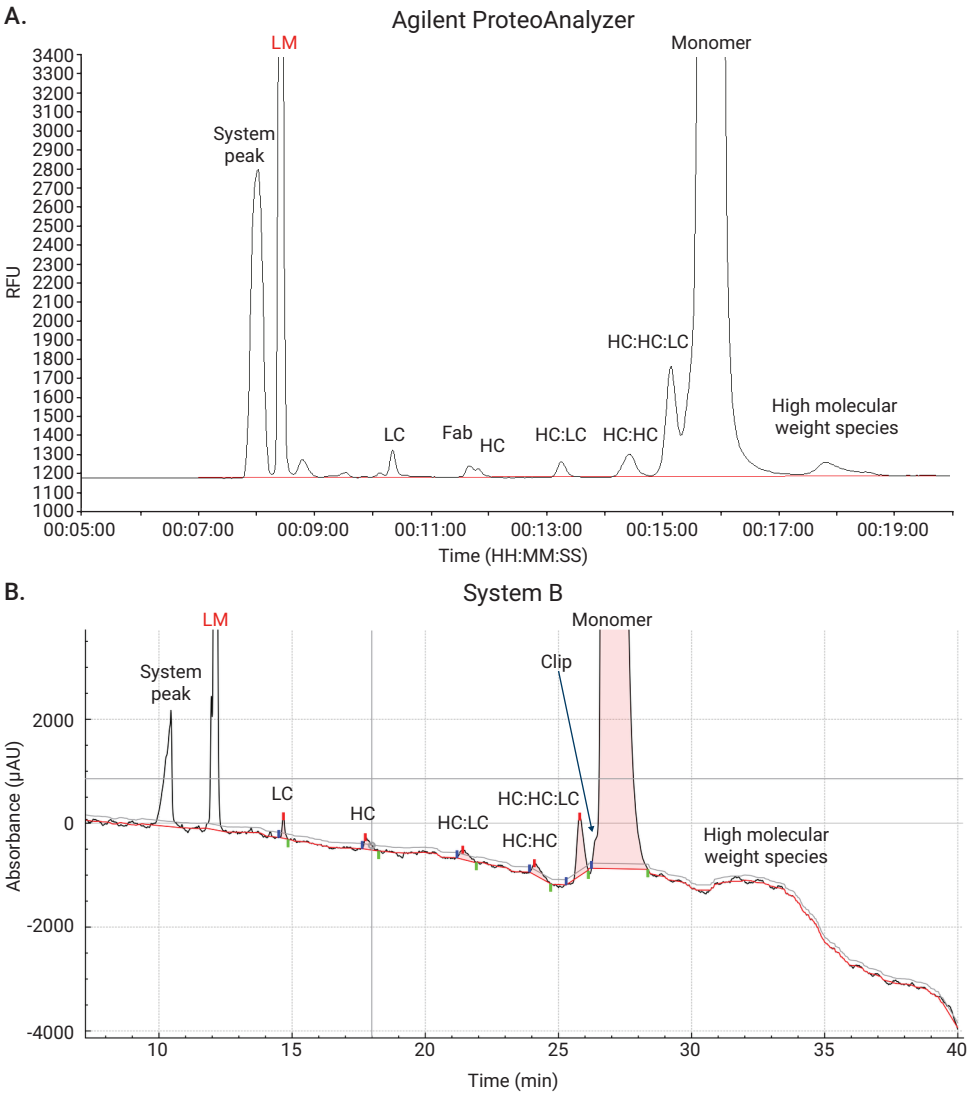

Figure 2. Nonreduced NISTmAb electropherograms using the (A) Agilent ProteoAnalyzer system and (B) System B. LM = lower marker; LC = light chain; HC = heavy chain.

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.

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Published in the USA, July 24, 2025
5994-8534EN

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