APPLICATION NOTE

Characterization of NISTmAb with microfluidic CE-SDS using the protein clear HR assay.

Introduction

Capillary electrophoresis (CE), more specifically CE-SDS, is becoming an increasingly popular and routinely used method for monitoring and documenting critical quality attributes for monoclonal antibody therapeutic products, due to its automated operation, on-column detection, great resolving power, and protein quantification capability. Such characterization is frequently used in the development and manufacture of biopharmaceutical products, to meet FDA documentation requirements.

The LabChip® GXII Touch™ protein characterization system features microfluidic CE-SDS (µCE-SDS) technology for analytical characterization of biopharmaceutical products. It offers an automated alternative to traditional SDS-PAGE methods, by streamlining the multiple, manual steps of slab gel electrophoresis, while providing the throughput and data quality essential to biotherapeutic workflows.

In this application note, the characterization of a reference monoclonal antibody from the National Institute of Standards and Technology (NIST) using LabChip GXII Touch protein characterization system is compared to those published by NIST using the SCIEX® PA-800 Plus system.

For research purposes only. Not for use in diagnostic procedures.

LabChip® GXII Touch™ Protein Characterization System

NISTmAb

The reference material (RM 8671) from the National Institute of Standards and Technology (NIST), here referred to as NISTmAb, is a recombinant humanized IgG that contains a high abundance of N-terminal pyroglutamination, C-terminal lysine clipping, and heavy chain glycosylation. It is a 150 kDa antibody protein that was donated by MedImmune, the global biologics division of AstraZeneca, and subject to industry-standard downstream processing to ensure its purity. Each vial of NIST RM 8671 contains 800 µL of the NISTmAb at a concentration of 10 mg/mL. Its attributes have been comprehensively studied by NIST, as well as more than 100 collaborating companies, regulatory agencies, and research institutes worldwide. NIST provides characterization results of RM 8671, and each lot comes with its own investigation report, providing a thorough profile of the reference protein, including details on size, concentration, composition, structure, purity, stability, and other attributes. In their report, the physicochemical attributes of NISTmAb are measured using CE-SDS.

The NISTmAb serves as a common benchmark reference standard for analytical characterization of biopharmaceutical products, facilitates the assessment of existing analytical methods, and promotes faster adoption of new technologies. Thus, it has been accepted as the ideal standard protein for universal system suitability testing, ensuring the accuracy of monoclonal antibody (mAb) assay and method development within the biopharmaceutical industry.

Experimental design

The RM 8761 is from Lot 14HB-D-002, and was stored at -80°C before use. The concentration of NISTmAb was first verified in-house using the DropletQuant™ spectrophotometer to measure UV absorbance against its stock concentration of 10 mg/mL described in the NIST report. NISTmAb was then diluted to a concentration of 1 mg/mL in 1X PBS buffer for subsequent evaluation. Measurements were performed in triplicate. The DropletQuant spectrophotometer is an automated UV/Vis spectrophotometer used to measure absorption of samples on a microfluidic chip, which can run up to 96 samples at once without intervention.

For non-reduced samples, 5 µL of 1 mg/mL NISTmAb was mixed in a 100 µL PCR tube with 35 µL of non-reducing sample buffer that was prepared by combining 700 µL Protein Express™ sample buffer with 24.3 µL of iodoacetimide at 250 mM concentration. For reduced samples, 5 µL of 1 mg/mL NISTmAb was mixed with 35 µL of reducing sample buffer that was prepared by combining 700 µL Protein Express sample buffer with 24.3 µL of DTT at 1M concentration. A reference ladder was also prepared separately by diluting the stock protein ladder in Milli-Q® water at 1:10 ratio. All samples except for the ladders were incubated at 70 °C for 10 minutes for denaturation. 70 µL of Milli-Q® water was then added to each sample vial and samples were thoroughly mixed by pipetting up and down. 100 µL of each prepared sample was transferred to a 96-well plate and placed into the LabChip GXII Touch protein characterization system for analysis. A high resolution microfluidic chip which serves as the protein separation device was prepared as described per manufacturer's instructions.

To better assess the percent purity and identity of NISTmAb, the Protein Clear™ HR assay was used. The assay features IntelliChip™ technology and contains a VeriMAb™ reference standard to calibrate the optimal running conditions for the assay. This ensures that the measurement of percent purity is accurate and precise across different instruments and chips. The calibration is a two-step process. In the first step, the current of the separation buffer within the microchannel is monitored and tuned to allow consistent migration time of the VeriMAb reference standard. Then the applied separation and de-staining currents are measured and optimized at the onset of each run to ensure that the measured percent purity aligns with the known value of the VeriMAb reference standard. These running conditions are locked and applied to subsequent sample runs. In this study, both the non-reduced and reduced NISTmAb samples were replicated four times for statistical analysis.

Mass concentration of NISTmAb reference standard

The mass concentration of proteins (C) is usually determined by measuring absorbance at 280 nm (A_{280}) using a UV-Visible spectrophotometer, and the concentration of proteins is then evaluated according to the following equation:

$$C = \frac{A_{280}}{\varepsilon \cdot b}$$

where ε is the extinction coefficient and is equal to 1.42 mg/mL ▪ cm according to the NIST report. The result was then corrected for non-glycosylated protein by dividing by the glycan mass correction factor of 0.977.

There was no statistical difference between the measurements taken using the DropletQuant spectrophotometer and those reported by NIST. Thus, the stock concentration of NISTmAb was considered to be 10 mg/mL for the continuation of this study.

Table 1. The absorbance of NISTmAb measured in triplicate by the DropletQuant spectrophotometer in comparison to that from the NIST report.

	Measurement 1 (mg/mL)	Measurement 2 (mg/mL)	Measurement 3 (mg/mL)	Average measurement (mg/mL)	Standard deviation
DropletQuant® (n=3)	10.023	10.053	10.053	10.043	0.017
Published data from NISTmAb data sheet (n=10)				10.003	0.0176

Microfluidic CE-SDS (µCE-SDS) Analysis of Non-reduced NISTmAb Reference Standard

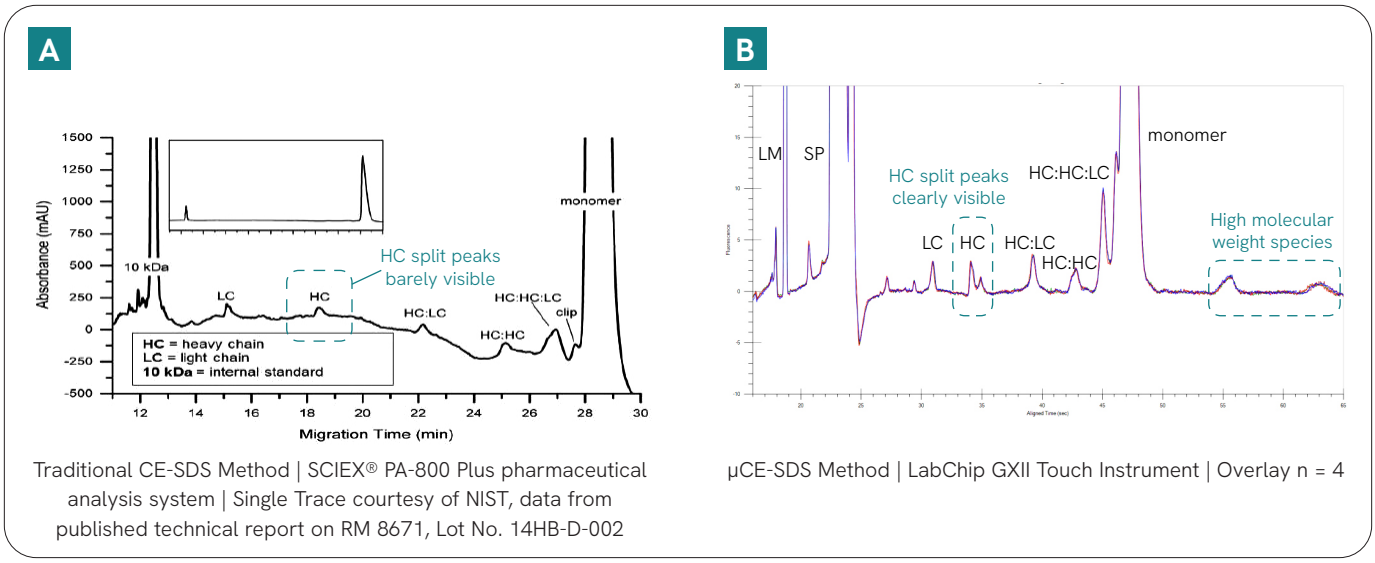
The results of the non-reduced NISTmAb characterized by µCESDS using the Protein Clear HR assay with the LabChip GXII Touch protein characterization system was compared to the published NIST datasheet using traditional CE-SDS technology (SCIEX® PA-800 Plus pharmaceutical analysis system). The SCIEX® PA-800 Plus pharmaceutical analysis system was equipped with a photodiode array (PDA) UV detector. The results from both instruments were compared in Figure 1.

As Figure 1 illustrates, smaller fragment peaks were clearly identified using both techniques in addition to the main peak; associated with the monomer, low-abundant peaks corresponding to light chain (LC), heavy chain (HC), HC:LC, HC:HC, HC:HC:LC. The low abundance split peak closely-associated to the proximal side of the dominant monomer peak, that may arise from partial de-glycosylation of the non-reduced antibody, was also clearly detected, indicating the resolving power of both techniques.

It is worth noting that with µCE-SDS, a peak was visualized for the heavy chain fragment, which may be due to various isoforms. The split peak was not identified in the NIST datasheet using traditional CE-SDS. In this case, µCE-SDS technique has higher resolution than CE-SDS, which may be due to the extremely small sample plug size created by the unique microfluidic chip platform. In addition, the baseline for µCE-SDS is visibly flatter and smoother than with CE-SDS, facilitating peak calling and more accurate peak area evaluation.

Additional peaks past the dominant monomer peak were identified with µCE-SDS; commonly associated with high molecular weight species, such as aggregates. These peaks were not reported in the CE-SDS NIST datasheet.

Analysis of each sample using the LabChip GXII Touch protein characterization system (µCE-SDS) was performed in 65 seconds, as compared to 30 minutes using tradtional CE-SDS methods.



Traditional CE-SDS Method | SCIEX® PA-800 Plus pharmaceutical analysis system | Single Trace courtesy of NIST, data from published technical report on RM 8671, Lot No. 14HB-D-002

µCE-SDS Method | LabChip GXII Touch Instrument | Overlay n = 4

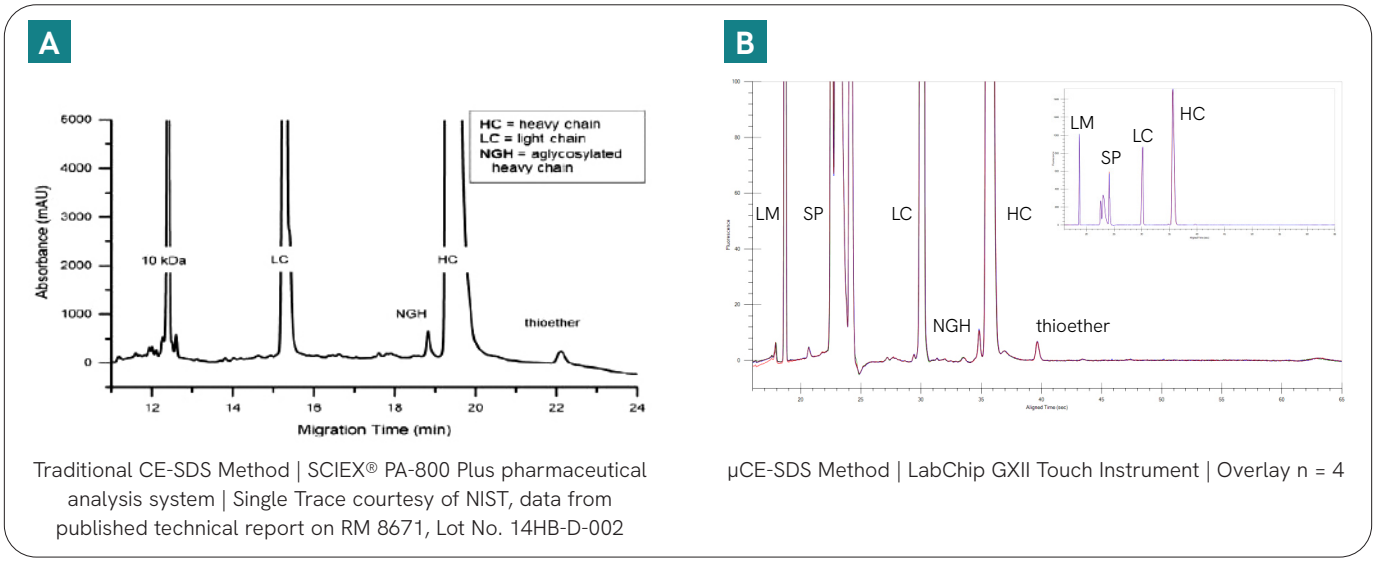
Figure 1: Comparison of non-reduced NISTmAb (Lot No. 14HB-D-002) characterized using µCE-SDS and traditional CE-SDS separation analysis techniques. Data from the LabChip GXII Touch protein characterization system was analyzed using the LabChip GX Reviewer software. Lower Marker (LM) is used to normalize and align sample data. System Peak (SP) is not used in calculations.

Microfluidic CE-SDS (µCE-SDS) analysis of reduced NISTmAb reference standard

The reduced form of the NISTmAb reference standard was compared (Figure 2) using both separation techniques. Reduced samples were run using the Protein Clear HR assay on the LabChip GXII Touch protein characterization system. Results from SCIEX® PA 800 Plus pharmaceutical analysis system results were obtained from the published NIST datasheet.

The non-glycosylated heavy chain (NGHC), heavy chain (HC), and a small peak after the HC that corresponds to the thioester of the reduced NISTmAb reference standard were clearly resolved using both methodologies (Figure 2). However, a low abundant peak to the distal shoulder of the HC was only detected using microfluidic CE-SDS.

It should be noted that characterization of each sample using the LabChip GXII Touch protein characterization system (µCE-SDS) was performed in 65 seconds, as compared to 24 minutes using traditional CE-SDS methods.



Traditional CE-SDS Method | SCIEX® PA-800 Plus pharmaceutical analysis system | Single Trace courtesy of NIST, data from published technical report on RM 8671, Lot No. 14HB-D-002

µCE-SDS Method | LabChip GXII Touch Instrument | Overlay n = 4

Figure 2. Comparison of reduced NISTmAb (Lot No. 14HB-D-002) characterized using traditional CE-SDS (Panel A) and CE-SDS (Panel B) separation analysis techniques. Data from the LabChip GXII Touch protein characterization system was analyzed using the LabChip GX Reviewer software. Lower Marker (LM) is used to normalize and align sample data. System Peak (SP) is not used in calculation.

Absolute Percent Purity Comparison between µCE-SDS and Traditional CE-SDS Analysis

Accurate and reproducible assessment of protein purity of monoclonal antibodies is critical to ensure quality, safety, and efficacy (QSE) of biopharmaceutical products. We evaluated the results for percent purity for both platforms, shown in Table 2.

The major peak of the non-reduced NISTmAb form is associated with monomer and percent purity, reflecting the manufacturing quality and overall half-life stability. The measured percent purity was nearly identical using both techniques: 98.52% and 98.47% for µCE-SDS and traditional CE-SDS from the NIST datasheet, respectively.

Measurement precision (CV) of each technique was also evaluated. The µCE-SDS method has a CV of 0.05% when measuring the monomer of non-reduced sample, while CE-SDS has a CV of 0.8%, slightly higher than that of µCE-SDS.

The glycosylation of biotherapeutic products is associated with efficacy and immunogenicity, and is usually monitored during manufacturing quality control. For the reduced NISTmAb, glycan occupancy was measured to be 99.64% by µCE-SDS, and 99.39% by CE-SDS with CVs of 0.01% and 0.003%, respectively.

Table 2. Comparison of species abundance for NISTmAb (Lot No. 14HB-D-002) using µCE-SDS and traditional CE-SDS separation techniques.

		Non-reduced		Reduced		
Technique	**Instrument**	**Monomer Purity (%)**	**CV (%)**	**Glycan Occupancy (%)**	**CV (%)**	**Number of Replicates**
µCE-SDS	LabChip GXII Touch	98.52%	0.05%	99.64%	0.010%	4
Traditional CE-SDS	SCIEX® PA 800 Plus	98.47%	0.80%	99.39%	0.003%	3

Conclusions

Microfluidic CE (µCE-SDS) is a highly automated, high-throughput, rapid analytical technique for the identification, molecular weight sizing, and purity assessment of monoclonal antibodies. The LabChip GXII Touch protein characterization system is a powerful analytical tool for microfluidic CE-SDS electrophoretic separation of proteins, offering multiple assays to accelerate protein characterization. This application note highlights the LabChip Protein Clear HR assay with its IntelliChip design and VeriMAb reference standards to improve percent purity assessment, molecular weight sizing, and reproducibility.

In this study, we compared µCE-SDS and CE-SDS methods by characterization of NISTmAb, a reference standard monoclonal antibody product that is commercially available and rigorously characterized from NIST. We evaluated the mAb under both non-reducing and reducing conditions. Data showed equivalent characterization between µCE-SDS and CE-SDS methods under both conditions. All low abundance fragments detected by CE-SDS were also completely identified using the LabChip GXII Touch instrument. There was no statistical difference between methods when measuring the percent purity of main non-reduced NISTmAb with both techniques providing highly reproducible results, CV <1%.

Increasing throughput and reducing the time required for this assessment is critical for the success in both R&D and downstream protein production. The LabChip GXII Touch instrument is able to characterize the NISTmAb in 65 seconds versus 24-30 minutes using traditional CE-SDS methods to obtain equivalent results.

In conclusion, the LabChip Protein Clear HR assay provides high inter- and intra-run reproducibility in percent purity and molecular weight sizing, comparable to that of traditional capillary electrophoresis, but with unparalleled throughput and sample run time.

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