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APPLICATION NOTE

MONOCLONAL ANTIBODY CHARACTERIZATION BY CE-SDS: MAURICE VERSUS LABCHIP

INTRODUCTION

Biologics, including monoclonal antibodies, vaccines, hormones, growth factors and polypeptides, among others, are sensitive to the manufacturing process. They are varied in their origin, makeup, effector function and delivery and therefore require thorough formulation development including characterization, quantitation and preservation. Biologics often compete with small-molecule drugs for similar therapeutic targets, particularly for cancer, type 2 diabetes mellitus and rheumatoid arthritis. However, because they are not chemically synthesized the way small-molecule drugs are, precision during manufacturing is essential to avoid variations in the product protein. Quality control is critical to maintaining not only the product's quality but also its safety.

Accurate reporting of a biologic's critical quality attributes (CQA) is required for its commercialization. In the case of monoclonal antibodies (mAb), one such CQA deals with mAb fragmentation and post-translational modifications like glycosylation, both of which are often inherent in the complex composition of a protein therapeutic[1]. To accurately analyze size/fragment heterogeneity, one should evaluate several parameters specific to the instrument's detection performance such as sensitivity, linearity, precision and resolution. Also, ease-of-use, reliability and sustainability are increasingly demanded in the industry.

The means for assessing mAb fragmentation and size migration have evolved from gel (SDS-PAGE) to capillary electrophoresis (CE-SDS). CE-SDS is a high-precision, high-throughput analytical technology equipped with quantitative data integration that has effectively reduced turnaround time during screening and development. In this application note, we compare Maurice™ against PerkinElmer's LabChip® GXII Touch, a chip-based electrophoretic separation system. Under reduced and non-reduced conditions, we evaluate CE-SDS separation using a reference mAb from the National Institute of Standards and Technology (NIST). Maurice and LabChip are scrutinized on linearity, sensitivity, precision, reproducibility and resolution, with the technological approach, workflow and data quality outlined for easy comparison.

INTRODUCTION TO MAURICE CE-SDS

Maurice (ProteinSimple, PN 090-000) CE-SDS enables direct, label-free separation of protein samples within a wide molecular weight range of 10 kDa to 240 kDa using a capillary made of bare fused silica. The protocol involves placing a vial of running buffer inside the CE-SDS cartridge, loading your samples and reagents and simply pressing Start. Samples are electrokinetically injected into the cartridge capillary based on their defined location in the batch and subsequently electrophoresed within. The peaks are directly detected via ultraviolet (UV) absorbance at 220 nm and plotted on an electropherogram (e-gram). The pre-assembled, ready-to-use cartridge simplifies the workflow and affords automated separation of up to 48 samples per batch, guaranteeing 100 injections per cartridge. All assay steps happen in the ready-to-go CE-SDS cartridge, which streamlines the entire process. You can pre-program batch and method parameters, monitor your run in real-time and analyze data using Compass for iCE software that is 21 CFR 11 compliant for use in regulated environments.

INTRODUCTION TO LABCHIP GXII TOUCH

The LabChip GXII Touch system (PerkinElmer, PN CLS138160) uses microchip capillary electrophoresis-based separation. The molecular sizing separation ranges from approximately 14 kDa to 300 kDa. In general, antibodies are first labeled with a fluorescent dye and detected using laser-induced fluorescence. Analysis of each sample is fast, occurring in 40 seconds. Samples can be analyzed directly from a 96- or 384-well plate on a robotic stage. A vacuum pulls the sample plug from the well, up the sipper and into the prepared assay LabChip. Once there, the sample is mixed with a dye and marker, and cross-injection voltages between specific wells inject a small volume of sample into the separation channel. The sample then moves through the sieving matrix where it is electrophoretically separated by size and finally de-stained to remove fluorescence background by applying a negative voltage to the end wells of the chip. The protein micelles remain bright in the presence of a laser and the detected fluorescence is plotted on an electropherogram.

MATERIALS AND METHODS

NIST mAb

Maurice and LabChip were evaluated using NIST mAb reference material (RM 8671, Lot 14HB-D-002)[2]. This homodimer is a representative test molecule for the evaluation of therapeutic protein characterization technologies. It is a recombinant humanized IgG1κ that contains a high abundance of N-terminal pyroglutamination, C-terminal lysine clipping and heavy chain (HC) glycosylation and a low abundance of post-translational modifications like methionine oxidation, deamidation and glycation[2].

SAMPLE PREPARATION: MAURICE AND LABCHIP GXII TOUCH

REDUCED CE-SDS

A 2-mg/mL NIST mAb sample was spiked with different concentrations of recombinant human thyroid receptor-interacting protein-4 (rhTRIP-4; R&D Systems, Custom PN 631895) and recombinant *Escherichia coli* Methionine Aminopeptidase/METAP, CF, (reMAP; R&D Systems, PN 1628-ZN) onsite at ProteinSimple. The spiked 2-mg/mL NIST mAb sample was snap-frozen on dry ice and shipped to a collaborator within the biopharmaceutical industry where researchers used Maurice 1X Sample Buffer to obtain the following final concentrations of spiked proteins: 20 µg/mL, 5 µg/mL, 1 µg/mL and 0.33 µg/mL. Unspiked NIST mAb was used at a final concentration of 1 mg/mL in all samples. Next, 2 µL of Maurice 25X Internal Standard and 2.5 µL of 14.2 M β-ME were added to each sample. All samples were denatured at 70 °C for 10 minutes and stored on ice. For analysis on Maurice, samples were mixed by vortex and spun down, then a 50-µL aliquot was transferred to a sample vial and spun in a centrifuge at 1000 x g for 10 minutes. For analysis on LabChip, a 44-µL sample was transferred to the sample plate and spun in a centrifuge at 1200 x g for two minutes.

NON-REDUCED CE-SDS

A 2-mg/mL NIST mAb sample was spiked with different concentrations of recombinant Botulinum Neurotoxin Type B Light Chain, CF (rBoNT/B-LC; R&D Systems, PN 5420-ZN) onsite at ProteinSimple. The spiked 2-mg/mL NIST mAb sample was snap-frozen on dry ice and shipped to a collaborator within the biopharmaceutical industry where researchers used Maurice 1X Sample Buffer to obtain the following final concentrations of spiked proteins: 5 µg/mL, 3 µg/mL, 1 µg/mL, 0.33 µg/mL and 0.11 µg/mL. Unspiked NIST mAb was used at a final concentration of 1 mg/mL in all samples. Next, a 2-µL aliquot of Maurice 25X Internal Standard and 2.5 µL of freshly prepared 250 mM iodoacetamide were added. All samples were denatured at 70 °C for 10 minutes and stored on ice. Samples were mixed by vortex and spun down, then a 50-µL sample was transferred to a sample vial and spun in a centrifuge at 1000 x g for 10 minutes

SAMPLE LABELING: LABCHIP GXII TOUCH

During sample preparation, the LabChip system requires additional chip-washing and sample-labeling steps for detection by laser-induced fluorescence. First, the Protein Express Dye Solution (PerkinElmer, PN CLS 960008) is added to the Gel Matrix (PN CLS920002) in a spin filter and spun in a centrifuge to create a blue colored Gel-Dye Solution. Second, Gel Matrix is added to a spin filter and spun in a centrifuge to create a Destain Solution to reduce background signal in the assay. All reagents and the ladder are pipetted into the assay LabChip wells following the reagent chip-well placement guide provided in the assay user guide[3]. During the assay, the Gel-Dye non-covalently binds to both protein-SDS complexes and SDS-micelles within the sample directly in the capillary for detection by the instrument.

SAMPLE RUNNING: MAURICE AND LABCHIP GXII TOUCH

For Maurice, sample vials were placed in the instrument along with a CE-SDS cartridge containing a new CE-SDS Running Buffer Top vial following the manufacturer's default protocol and recommendations. All samples were electrokinetically injected for 20 seconds at 4600 V and separated for 25 minutes (for reduced samples) or 35 minutes (for non-reduced samples) at 5750 V. Direct detection and batch data analysis was performed using Compass for iCE software. For LabChip GXII Touch, samples were run using default injection and separation conditions according to the manufacturer's instructions for the HT Protein Express assay.

DATA ANALYSIS

The CE-SDS data generated by both Maurice and LabChip were exported from each platform and imported into Empower™ 3 Software (Waters Corporation) for analysis. The **Traditional Algorithm** was selected, and the peak width and threshold were adjusted to follow the peak integration example used in "NIST mAb: Structural Variant Analysis of RM 8671 by Capillary Electrophoresis"[4]. For this comparison, the "clip" integrated non-reduced CE-SDS electropherogram example was included with the monomer peak to eliminate the need for manual integration during processing. The spiked proteins were integrated at baseline where possible. The peak area and percent peak area data were then exported from Empower 3 for analysis using Microsoft Excel. The linearity data was obtained from the averaged peak area data (n=3) and plotted and displayed using Microsoft Excel. The percent relative standard deviation (RSD) was calculated as 100 × standard deviation/average of 15 replicates.

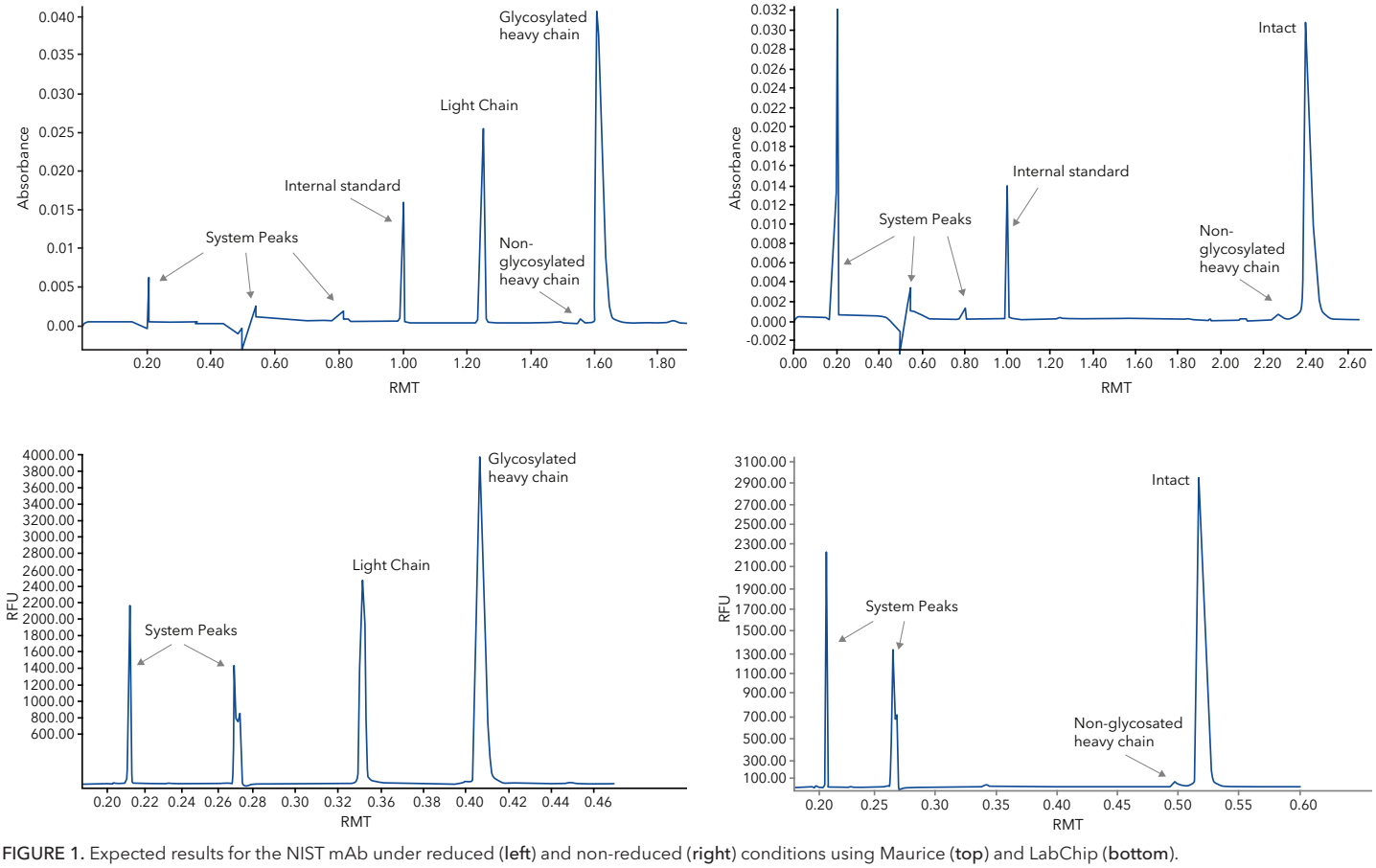


FIGURE 1. Expected results for the NIST mAb under reduced (**left**) and non-reduced (**right**) conditions using Maurice (**top**) and LabChip (**bottom**).

RESULTS

DETECTION OF mAb FRAGMENTS

Detecting the finger-print fragmentation of a mAb is critical for determining both manufacturing and product stability consistency. We first confirmed the ability of Maurice and LabChip to detect and resolve molecular fragments of a normal NIST mAb. Both instruments efficiently resolved non-glycosylated HC (NGHC) from the HC of NIST mAb; the very small NGHC can be seen in reduced samples (**FIGURE 1, left side**). In non-reduced samples, Maurice and LabChip were able to detect the intact mAb monomer and NGHC (**FIGURE 1, right side**).

SENSITIVITY COMPARISON OF MAURICE AND LABCHIP

Maurice and LabChip both offer low-end sensitivity to detect and visualize impurities, and the limit of detection (LOD) and limit of quantitation (LOQ) were used to evaluate assay sensitivity for both instruments. Additionally, the signal-to-noise ratio (S/N ratio) for impurities was determined. This parameter allows for the use of the same calculation method to compare different instruments where the detection approach may be different. LOD and LOQ were calculated in Empower 3 for both Maurice and LabChip.

Under reduced sample conditions (**FIGURE 2, left**), the dominant light chain (LC) and HC peaks plus the major LC impurities or spikes (rhTRIP-4, spike 1; reMAP, spike 2) are detected by both Maurice (**FIGURE 2, top**) and LabChip (**FIGURE 2, bottom**), but notably better resolution between the LC peak and two spikes were observed with Maurice. On Maurice, the LOD was 1.5 µg/mL and 1.9 µg/mL for Spike 1 and Spike 2, respectively, whereas the LOQ was 11.5 µg/mL (Spike 1) and 9.6 µg/mL (Spike 2). Using LabChip, the LOD and LOQ could only be determined for Spike 1 (0.5 µg/mL and 1.7 µg/mL, respectively) due to poor resolution between Spike 2 and the LC peak. This poor resolution may result from incomplete de-staining, differences in separation matrix, or the shorter separation distance in the microfluidic channel of the LabChip. The labeling method LabChip uses does detect lower LOD and LOQ concentrations, as evidenced by the values recorded for Spike 1 (**TABLE 1**), versus label-free Maurice. However, sample labeling does add additional steps to your protocol and may not be advantageous for all purposes. Moreover, NGHC was detected by both but with obvious better separation on Maurice (**FIGURE 2, top**). The non-reducible thioether-linked species (HL-Thio) is detected on both platforms and represented in each e-gram.

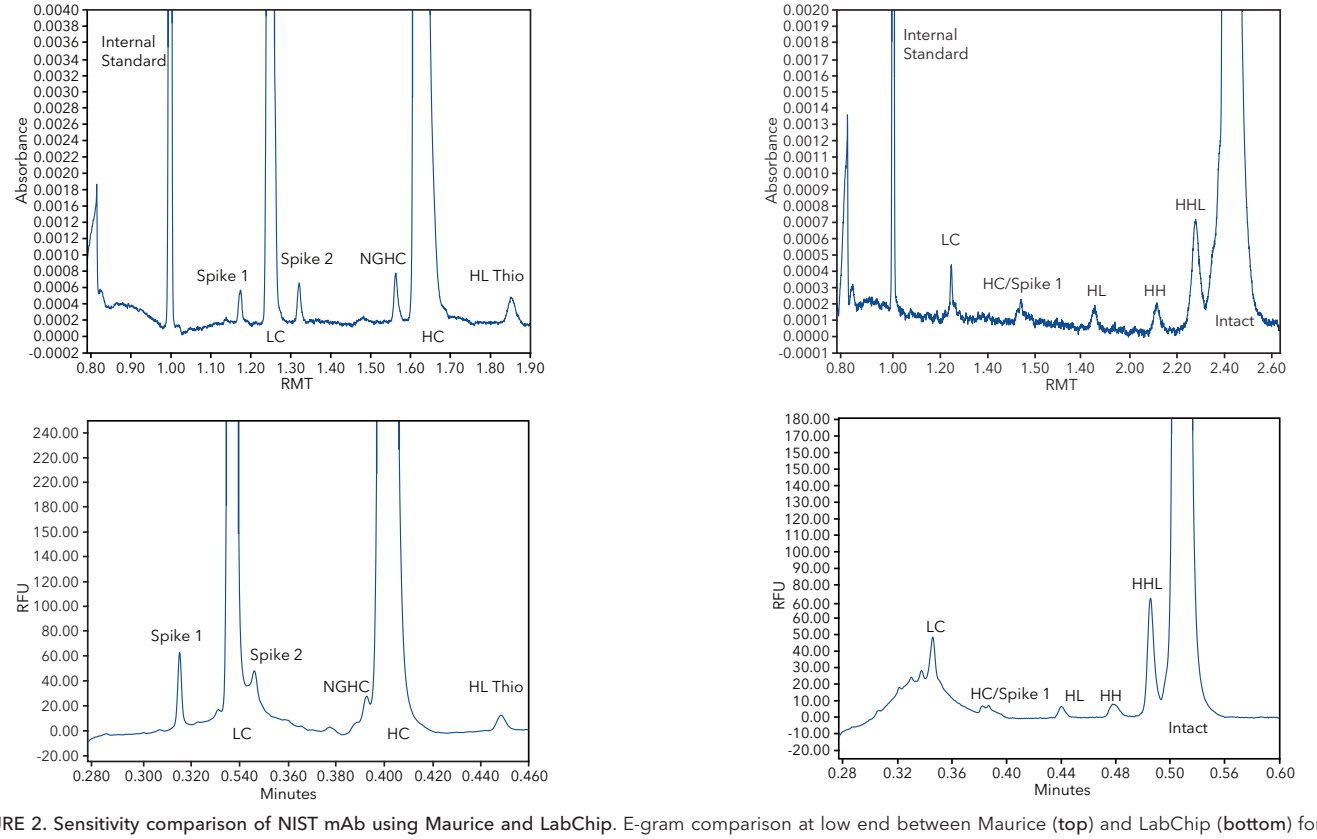


FIGURE 2. Sensitivity comparison of NIST mAb using Maurice and LabChip. E-gram comparison at low end between Maurice (**top**) and LabChip (**bottom**) for reduced conditions (**left**) using a final concentration of 5 µg/mL of spiked proteins (Spike 1 and Spike 2). E-gram comparison at low end between Maurice and LabChip under non-reduced conditions (**right**) using a final concentration of 1 µg/mL of spiked protein (Spike 1).

When the non-reduced CE-SDS assays (**FIGURE 2, right**) were performed using both Maurice (**FIGURE 2, top**) and LabChip (**FIGURE 2, bottom**), the dominant peak was, as expected, intact NIST mAb. The residual LC and HC peaks and the HC impurity or spike (rBoNT/B-LC) are detected by both instruments, with visual separation between the HC and spike being comparable between instruments. The LOD and LOQ for the detection of the rBoNT/B-LC (Spike 1) by Maurice in the non-reduced assay was determined to be 3.7 µg/mL and 20.1 µg/mL, respectively. Whereas the LOD and LOQ for LabChip were 1.3 µg/mL and 8.1 µg/mL, respectively. Again, LabChip did detect lower LOD and LOQ concentrations under non-reduced conditions, as evidenced by the values represented in **TABLE 2** for the HC/Spike peak.

LINEARITY COMPARISON OF MAURICE AND LABCHIP

Under reduced conditions, both systems were able to detect molecular fragments that correspond to LC, NGHC, HC and HL-Thio in 1 mg/mL NIST mAb. Using Maurice, the NGHC peak was clearly separated from the HC peak (**FIGURE 3A**), whereas with LabChip, the NGHC peak was difficult to clearly resolve from the HC peak (**FIGURE 3B**). The samples were spiked with several concentrations (from 20 µg/mL to 0.33 µg/mL) of rhTRIP-4 (Spike 1) and reMAP (Spike 2). rhTRIP-4 (Spike 1) was baseline resolved at 5 µg/mL with a small peak detectable at 1 µg/mL and reMAP (Spike 2) was baseline resolved at 5 µg/mL with a small peak detectable at 0.33 µg/mL on Maurice. On LabChip, rhTRIP-4 (Spike 1) was baseline resolved at 1 µg/mL with a small peak detectable at 0.33 µg/mL, whereas reMAP (Spike 2) could not be baseline resolved. At concentrations of 1 µg/mL and below, the reMAP

(Spike 2) could not be resolved from the LC peak. The relationship between the spiked protein peak area and its concentration was evaluated by linear regression with an R^2 value of 0.99 (reMAP, Spike 1) and 0.99 (rhTRIP-4/Spike 2) for Maurice and 0.99 (rhTRIP-4, Spike 1) and 0.94 (reMAP, Spike 2) for LabChip (**FIGURE 3C, D**), indicating good linear correlation between this concentration range.

For non-reduced assay conditions, the linearity of impurity detection was determined by running 1 mg/mL of NIST mAb spiked with dilutions of rBoNT/B-LC (from 15 µg/mL to 0.11 µg/mL). Both systems detected molecular fragments that correspond to LC, HC, HL, HH and HHL fragments, along with the intact mAb monomer and the internal standard peak corresponding to 10 kDa (**FIGURE 4A, C**). The spiked protein migrated with the HC fragment. The rBoNT/B-LC spike was first detected at 0.33 µg/mL using Maurice and at 1 µg/mL using LabChip. The relationship between the HC/spike peak area and protein concentration was evaluated by linear regression with an R^2 value of 0.97 for Maurice and 0.87 for LabChip.

	SPIKES (µg/mL)	
SAMPLE (REDUCED)	LOD (S/N=3)	LOQ (S/N=10)
Maurice Spike 1	1.5	11.5
Maurice Spike 2	1.9	9.6
LabChip Spike 1	0.5	1.7
LabChip Spike 2	N/A	N/A

TABLE 1. LOD and LOQ data comparison for reduced samples on Maurice versus LabChip.

	SPIKES (µg/mL)	
SAMPLE (NON-REDUCED)	LOD (S/N=3)	LOQ (S/N=10)
Maurice Spike 1	3.7	20.1
LabChip Spike 1	1.3	8.1

TABLE 2. LOD and LOQ data comparison for non-reduced samples on Maurice versus LabChip.

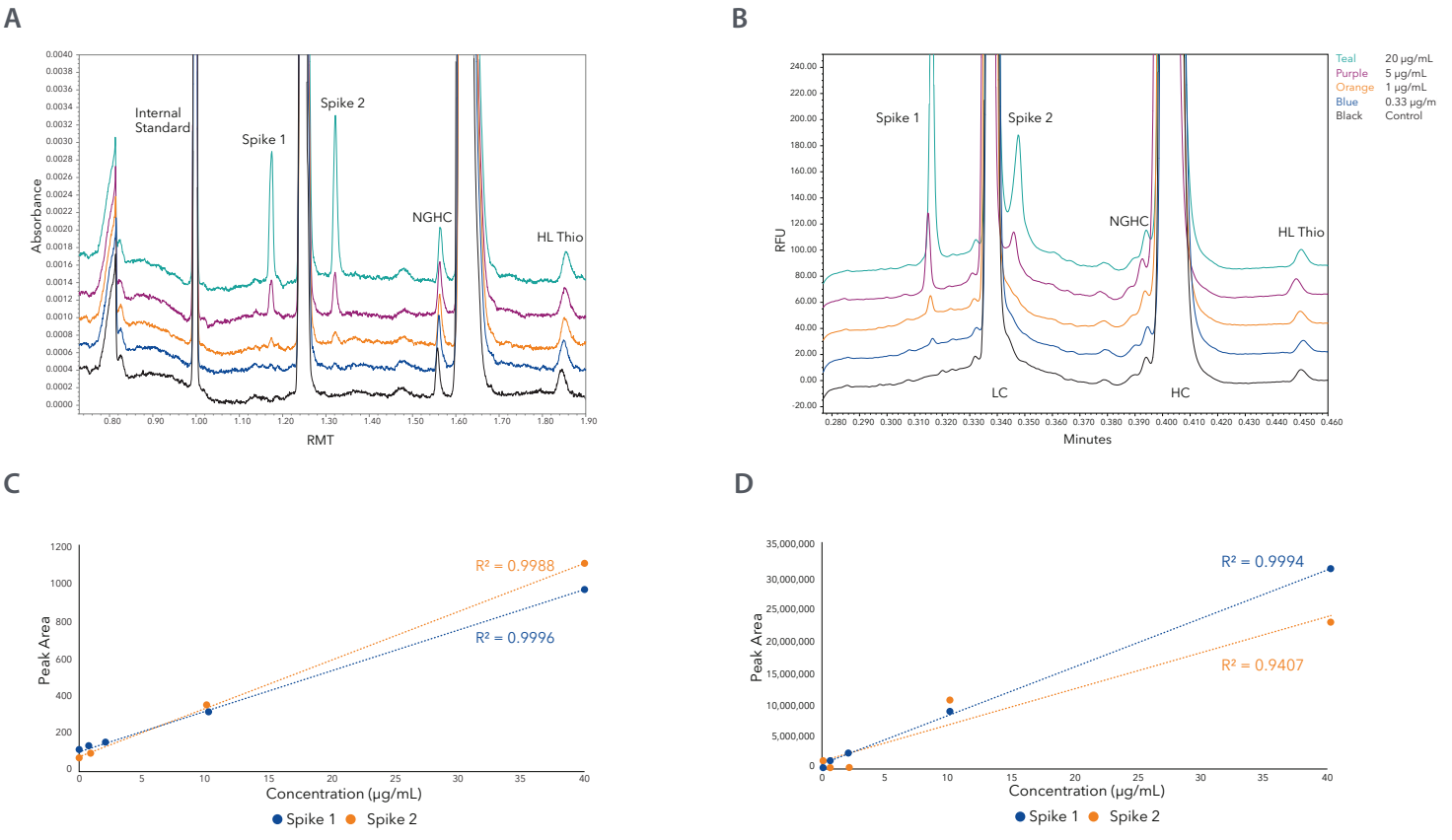


FIGURE 3. Linearity under reduced assay conditions was determined by analyzing several concentrations of rhTRIP-4 and reMAP (from 20 µg/mL to 0.33 µg/mL) in the presence of 1 mg/mL NIST mAb on Maurice (**A**) and LabChip (**B**). Linear regression analysis of the spike peak areas is shown for Maurice (**C**) and LabChip (**D**).

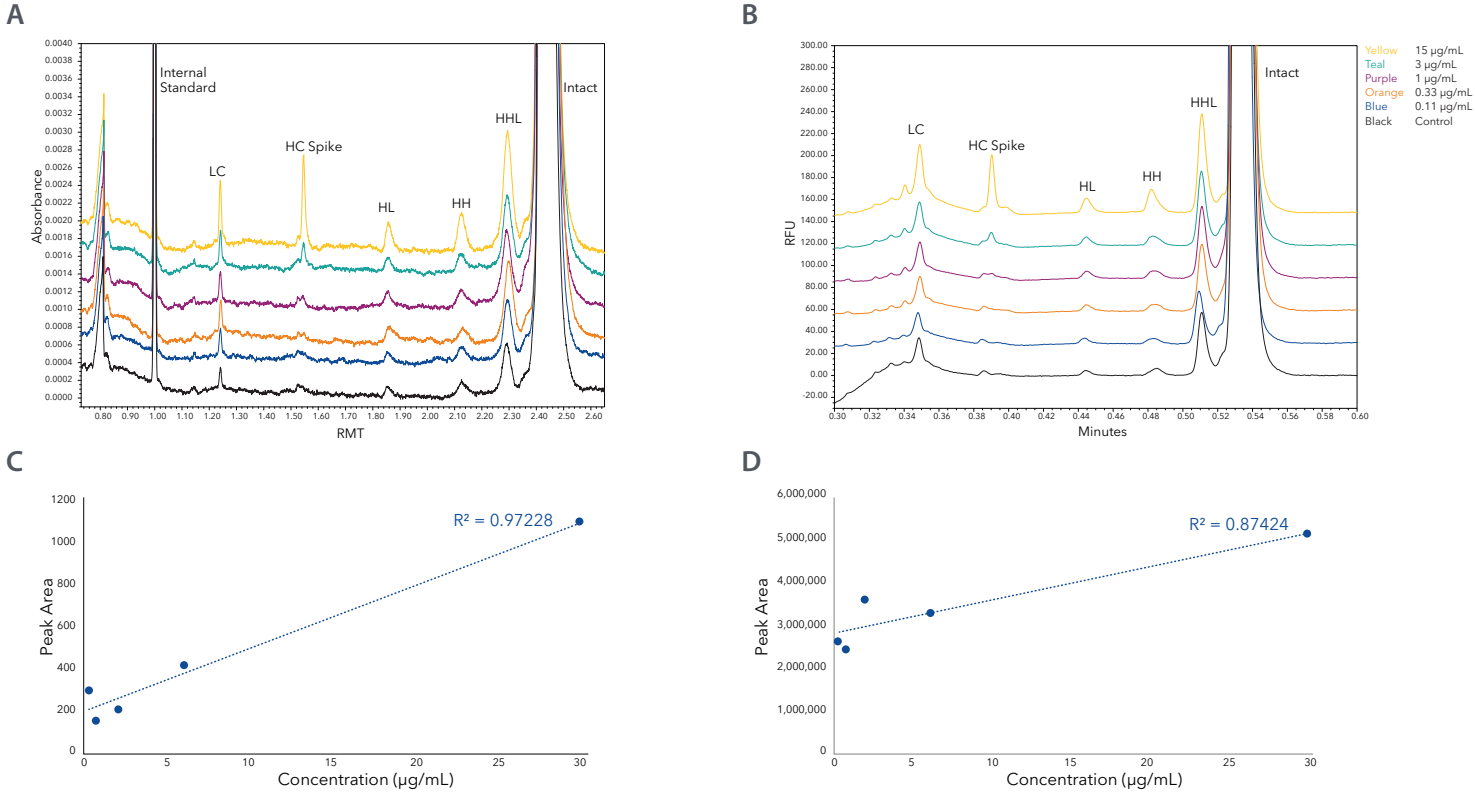


FIGURE 4. Linearity under non-reduced assay conditions was determined by analyzing several dilutions of rBoNT/B-LC (from 15 µg/mL to 0.11 µg/mL) in 1 mg/mL NIST mAb on Maurice (**A**) and LabChip (**B**). Linear regression analysis of the HC/spike peak area is shown for Maurice (**C**) and LabChip (**D**).

PRECISION COMPARISON OF MAURICE AND LABCHIP

Experimental precision was measured in terms of experimental reproducibility. Under reduced conditions, we performed an experimental reproducibility comparison between the two instruments using the RSD of the percent area of the LC, NGHC, HC and HL-Thio peaks. The provided spiked samples were prepared in triplicates by biopharma researchers on their site where one injection for each triplicate (n=3) was analyzed to evaluate prep-to-prep variation. Overall, the percent peak area RSD values showed that Maurice was almost two times more precise than LabChip. Percent RSD measurements for the non-reducible HL-Thio are similar (Maurice, 16.9% vs. LabChip, 14.2%). **FIGURE 5A, B** depicts the e-grams for the three samples as an overlay image for Maurice and LabChip, respectively. **TABLE 3** is a combined summary of the results.

For non-reduced conditions, the RSD of the percent purity of the LC, HL, HH, HLL and the intact NIST mAb were calculated. The provided spiked samples were prepared in triplicates by biopharma researchers on their site where one injection for each triplicate (n=3) was analyzed to evaluate prep-to-prep variation. In this case, percent peak area RSD values are similar, with LabChip having improved precision over Maurice for reproducibility of intact NIST mAb (LabChip, 0.8% vs. Maurice, 1.5%). The e-gram overlays are shown in **FIGURE 6 A, B** for Maurice and LabChip, respectively, and the results are summarized in **TABLE 4**.

RESOLUTION COMPARISON OF MAURICE AND LABCHIP

Reproducibility, precision and ease of quantitation are all dependent on how good the resolution of separation for your instrument is. For the data generated herein, the resolution value was determined using the USP standard equation in Empower 3 software. On Maurice, the resolution between the NGHC and HC for the reduced sample was 1.97. However, a resolution number could not be determined from the LabChip data because the peak width could not be calculated due to a poor baseline and, therefore, poor NGHC resolution (**FIGURE 5B**). This may be a limitation inherent in the shorter microfluidic channel design of the LabChip instrument in addition to possible incomplete de-staining resulting in only partial removal of fluorescent micelles at the end of a run.

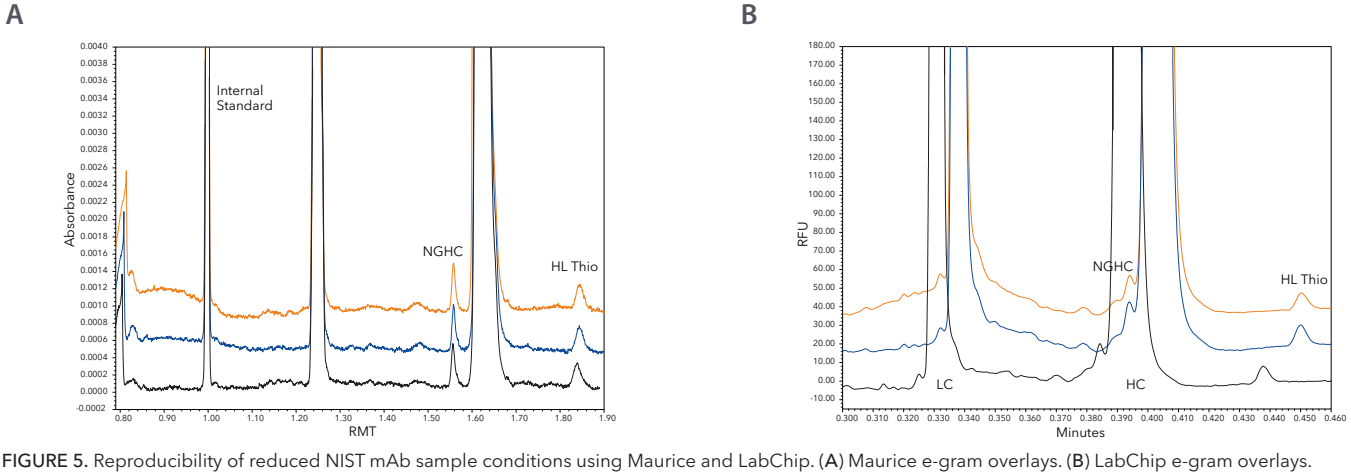
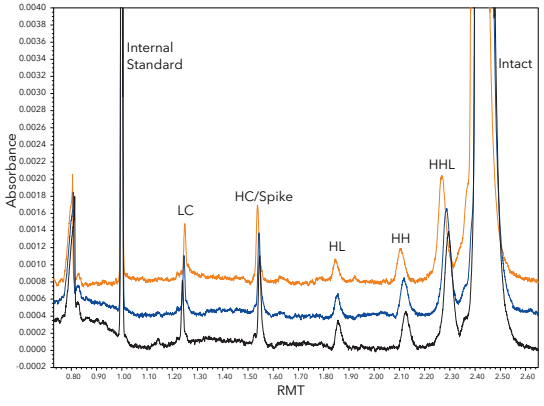


FIGURE 5. Reproducibility of reduced NIST mAb sample conditions using Maurice and LabChip. (**A**) Maurice e-gram overlays. (**B**) LabChip e-gram overlays.

REDUCED SAMPLE	% PEAK AREA				% PEAK AREA RSD			
	LC	NGHC	HC	HL-THIO	LC	NGHC	HC	HL-THIO
Maurice	24.87	0.54	73.17	0.61	1.1	5.8	0.9	16.9
LabChip	28.02	0.43	70.47	0.16	2.0	18.3	1.6	14.2

TABLE 3. Reproducibility data summary of Maurice versus LabChip for reduced samples.

A



B

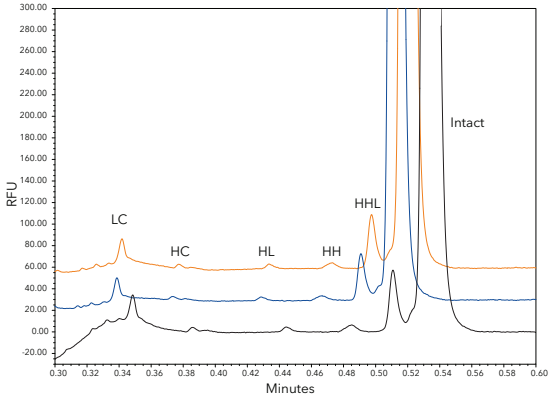


FIGURE 6. Reproducibility of non-reduced NIST mAb sample conditions using Maurice and LabChip. (A) Maurice e-gram overlays. (B) LabChip e-gram overlays.

NON-REDUCED SAMPLE	% PEAK AREA					% PEAK AREA RSD				
	LC	HL	HH	HLL	INTACT	LC	HL	HH	HL	INTACT
Maurice	0.38	0.34	0.53	2.20	96.04	34.1	39.4	38.3	22.1	1.5
LabChip	0.76	0.24	0.32	1.55	96.78	22.0	32.2	42.7	16.6	0.8

TABLE 4. Reproducibility data summary of Maurice versus LabChip for non-reduced samples.

CONCLUSIONS

In this study, we compared CE-SDS results on Maurice and PerkinElmer's LabChip GXII Touch using the NIST mAb, a humanized IgG1κ monoclonal antibody released by NIST. Both systems could detect and resolve mAb fragments, but further investigation into the sensitivity, linearity, precision and resolution quality attributes between instruments revealed Maurice to provide clearer separation between the dominant HC and LC peaks and nearby impurities (spikes). Further, the linearity of detection, expressed as the R^2 value, was overall better for Maurice versus LabChip. In terms of precision (experimental reproducibility), Maurice proved to be almost two times more precise than LabChip under reduced conditions. Finally, whereas the resolution between the NGHC and HC for the reduced sample could be calculated from the data generated on Maurice (1.97), it could not be for the data produced on LapChip. Because integration of the reduced NGHC and HC is commonly used to determine glycan occupancy, it is very important to have baseline resolution, of which Maurice is capable and LabChip is not.

With Maurice, you can assess the purity and identity of therapeutic mAb by way of CE-SDS using a much simpler workflow that results in higher quality data. Although enabling a higher sample throughput per run, LabChip requires several manual steps that are prone to error, such as sample labeling, detector optimization, interface cleaning and building and washing of assay LabChips. Maurice eliminates these preliminary steps and can have you running a batch in about 10 minutes of hands-on time. Taken together, Maurice not only offers a more simplified way of doing CE-SDS, he also generates better quality data than you may currently be collecting. Maurice grants you superior confidence in your protein sample identity and purity required for meeting regulatory guidelines and getting to market faster.

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