

Comparing SDS-PAGE with Maurice CE-SDS for Protein Purity Analysis

Introduction

The accurate and quantitative analysis of product purity is a fundamental component of effective development of a biotherapeutic protein. But without consistent techniques and high-quality, reproducible results, it is difficult to correctly assess the product's efficacy and/or safety, potentially delaying, even jeopardizing, its approval.

For years, scientists have used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a part of lot release, stability testing, batch-to-batch consistency and purity analyses. But the gel-based technique is self-limiting in terms of sensitivity, reproducibility and its semi-quantitative nature. These shortcomings have led to the emergence of capillary electrophoresis (CE-SDS) as a more appropriate, analytical and instrument-based replacement that is increasingly being adopted in biopharmaceutical workflows. CE-SDS possesses high separation power, affords accurate quantitation and can be automated to streamline purity analyses of both intact and reduced biotherapeutic proteins. In this application note, we compare Maurice™ CE-SDS with SDS-PAGE in parallel, using a standard and a commercially available therapeutic monoclonal antibody (mAb) under varying conditions. The comparative results highlight the advantages of Maurice CE-SDS over SDS-PAGE for routine product purity characterization.



How Maurice CE-SDS Works

Maurice is a fully automated system with an easy-to-follow CE-SDS workflow: just pop in one of the pre-assembled CE-SDS cartridges, drop in a few reagents, add your sample vials or a 96-well plate and hit start. Maurice performs size-based CE-SDS on proteins varying in size from 10 kDa to 270 kDa. Typical separation time for reduced samples is just 25 minutes and for non-reduced samples, 35 minutes, running up to 48 samples per batch.

During the run, SDS-coated proteins are electrokinetically injected into the cartridge capillary based on their defined location in the batch protocol within Compass for iCE software. After sample injection, the cartridge is positioned such that both ends of the capillary are immersed in running buffer. An electric field is established between the electrodes from a power source, commencing the migration of proteins through a matrix. The peaks are directly detected via UV absorbance at 220 nm and plotted on an electropherogram (e-gram). You can preprogram batch and method parameters, monitor your run in real-time and easily analyze data using Compass

for iCE software that is compliant with the Food and Drug Administration (FDA) Title 21 Code of Federal Regulations Part 11 (21 CFR Part 11).

Materials and Methods

INFLIXIMAB

Infliximab is a mAb against tumor necrosis factor-alpha. It has several approved uses, including treatment of rheumatoid arthritis, Crohn's disease, ulcerative colitis and psoriatic arthritis, as indicated by the FDA¹. The infliximab molecule used in this application note was donated by a pharmaceutical collaborator. Infliximab samples were prepared as described in the SDS-PAGE and Maurice CE-SDS sample preparation and running sections.

NIST mAb

NIST mAb reference material (RM 8671, Lot 14HB-D-002) is a representative test molecule for the evaluation of therapeutic protein characterization technologies². NIST mAb RM 8671 comes as an aqueous 10-mg/mL solution and was prepared as described in the SDS-PAGE and Maurice CE-SDS sample preparation and running sections.

REAGENTS

REAGENT	VENDOR	PRODUCT NUMBER
Maurice CE-SDS Application Kit	ProteinSimple	PS-MAK02-S
β -Mercaptoethanol (β -ME, >98%)	Sigma-Aldrich	A3221
Iodoacetamide (IAM)	Sigma-Aldrich	A3221
2X Laemmli Sample Buffer	Bio-Rad	161-0737
Precision Plus Protein™ Dual Color Standard	Bio-Rad	161-0374
4–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gels	Bio-Rad	456-8085
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	Bio-Rad	165-8004

TABLE 1. Reagents used in this application note.

SDS-PAGE

Non-Reduced Sample Preparation

Each sample was first diluted to 1 mg/mL in Maurice Sample Buffer, then further diluted to 0.5 mg/mL with 2X Laemmli Sample Buffer at a ratio of 1:1. All samples were heat denatured at 70 °C for 10 minutes.

Reduced Sample Preparation

Each sample was first diluted to 1 mg/mL in Maurice Sample Buffer. Next, 3 μ L of 14.2-M β -ME was added per 57 μ L of 2X Laemmli Sample Buffer and combined with 60 μ L of the 1-mg/mL sample to make a 1:1 mixture with a final concentration of 0.5 mg/mL. Each sample was heat denatured at 70 °C for 10 minutes.

Sample Running and Data Visualization

The 4%–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gels from Bio-Rad Laboratories (PN 4568086) were used for all SDS-PAGE experiments performed. 12.5 μ g (20 μ L) of each sample was loaded in replicates of five according to the manufacturer's instructions. We used 10 μ L of Precision Plus Protein™ Dual Color Standard (Bio-Rad, PN 1610374) for molecular weight determination.

Protein samples were separated at 200 V for 45 minutes in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for mini precast gels (Bio-Rad, PN 1658004). Gel images were acquired using ProteinSimple's FluorChem M imaging system (PN 92-15312-00) according to the application note, "Total Protein Normalization with FluorChem Imagers."

Briefly, gels were exposed to TransUV excitation and a green filter for emission (542 nm) for 5 minutes. A second exposure was performed, this time with the exposure settings set to Auto. The highest quality image from the Auto exposure cycle was selected for presentation.

MAURICE CE-SDS

The Maurice CE-SDS Application Kit includes all batch reagents required for a run except β -ME and IAM.

Non-Reduced Sample Preparation

Before sample loading, 2.5 μ L of a 250-mM stock solution of the freshly prepared alkylating agent, IAM, was added to each 50- μ L sample to block disulfide scrambling or exchange. All samples were diluted in Maurice 1X SDS Sample Buffer to a final concentration of 1 mg/mL, denatured at 70 °C for 10 minutes, cooled on ice for 5 minutes, and mixed by vortex. A 50- μ L aliquot of each sample was transferred to a 96-well plate and spun down in a centrifuge for 10 minutes at 1000 x g. To mimic stressed conditions, native samples were incubated at 37 °C for 72 hours prior to preparation for analysis.

Reduced Sample Preparation

We added 2.5 μ L of 14.2-M β -ME to 50 μ L of sample, followed by 2 μ L of CE-SDS 25X Internal Standard previously reconstituted in 240 μ L of Maurice CE-SDS 1X Sample Buffer. All samples were diluted in 1X Sample Buffer to a final concentration of 1 mg/mL, denatured at 70 °C for 10 minutes, cooled on ice for 5 minutes, and mixed by vortex. A 50- μ L aliquot of each sample was then transferred to a 96-well plate and spun down in a centrifuge for 10 minutes at 1000 x g. To mimic stressed conditions, native samples were incubated at 37 °C for 72 hours.

Sample Running

As an added precaution, non-reduced and reduced samples were allocated to opposite sides of a 96-well plate to prevent contamination of non-reduced samples by β -ME. All samples were run in triplicate and electrokinetically injected into the cartridge capillary by applying voltage for 20 seconds at 4600 V before separation by electrophoresis at 5750 V. Reduced samples were separated for 25 minutes, and non-reduced samples were separated for 35 minutes.

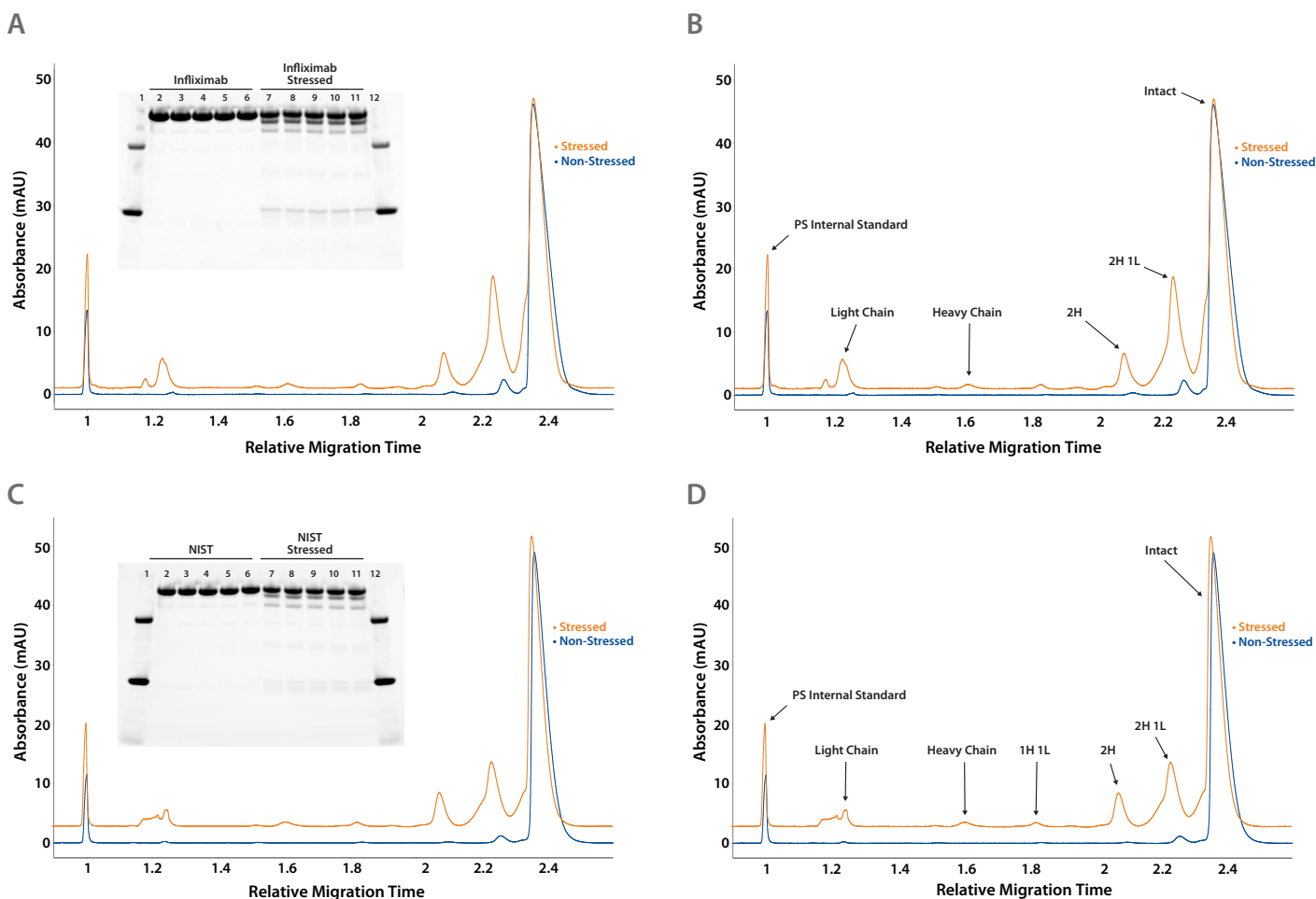
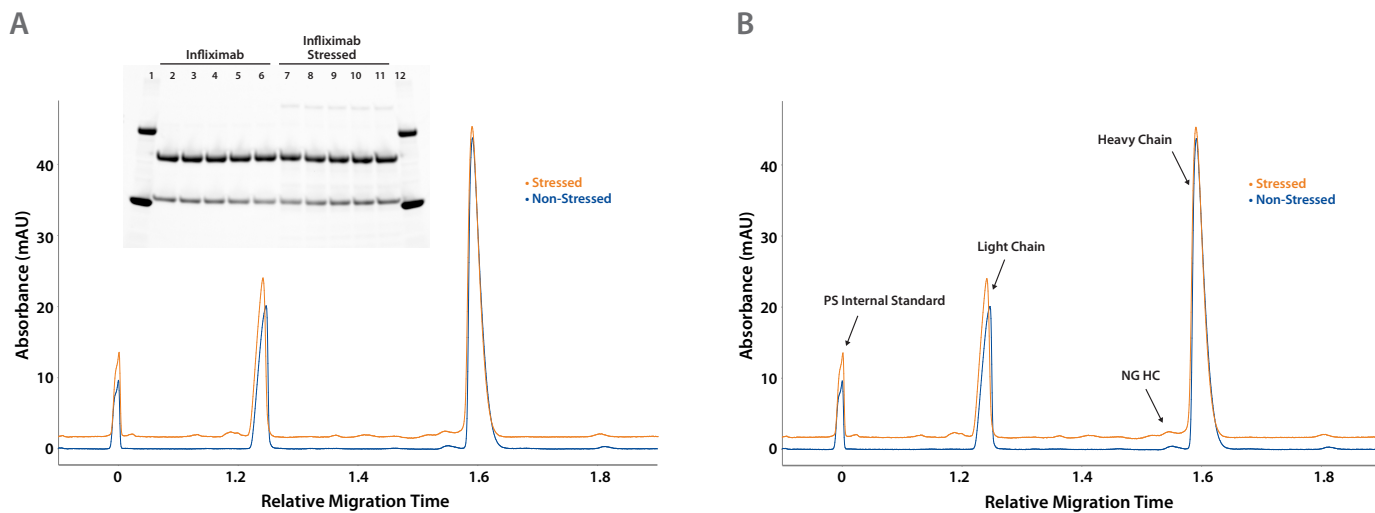


FIGURE 1. Comparative results from SDS-PAGE (inset) and Maurice CE-SDS e-grams for non-reduced infliximab (A, B) and NIST mAb (C, D) under heat-stressed and non-stressed conditions (color overlays). The impurity peaks are assigned and shown in the duplicate CE-SDS e-grams for infliximab (B) and NIST mAb (D). The peak labels are not shown in parts (A) and (C) for simplicity. CE-SDS data were offset for ease of visual comparison between various samples and conditions analyzed.



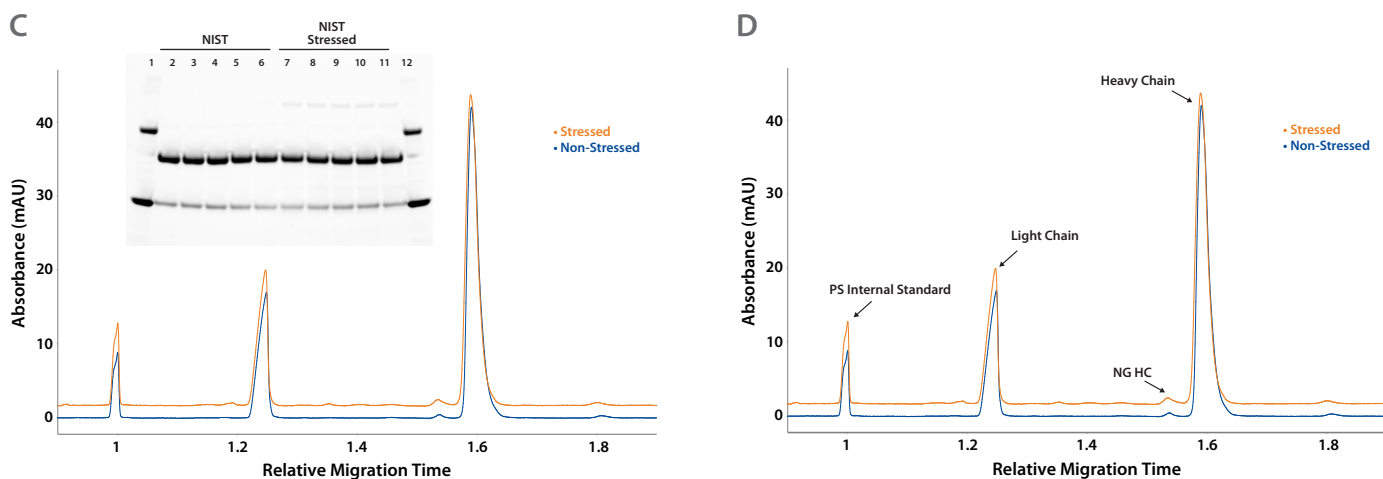


FIGURE 2. Comparative results from SDS-PAGE (inset) and Maurice CE-SDS e-grams for reduced infliximab (A, B) and NIST mAb (C, D) under heat-stressed and non-stressed conditions (overlays). The impurity peaks are assigned in the duplicate CE-SDS e-grams for infliximab (B) and NIST mAb (D) and were left out of parts (A) and (C) for simplicity. CE-SDS data were offset for ease of visual comparison between various samples and conditions analyzed.

Comparative Analysis: Maurice Against Slab-Gels

When compared with Maurice CE-SDS for protein purity analyses, the SDS-PAGE slab-gel approach has significant disadvantages in terms of sample volume used, assay sensitivity, time to result and confidence in those results. This section describes just how well Maurice CE-SDS outperforms SDS-PAGE using the same samples in parallel.

Under non-reduced conditions, non-stressed infliximab and NIST mAb samples show a single high-molecular-weight band (**Figure 1 A and C**, inset, lanes 2–6). Heat-stressed samples show the same major high-molecular-weight band, but also show additional minor bands at lower molecular weights, which represent stress-induced fragmentation (**Figure 1 A and C**, inset, lanes 7–11), with comparable results between infliximab (**Figure 1A**, inset) and NIST mAb (**Figure 1C**, inset). Lanes 1 and 12 contain both high and low molecular-weight markings, according to the Precision Plus Protein Dual Color Standard used. Samples run by CE-SDS on Maurice exhibit greater resolution and signal-to-noise compared with SDS-PAGE results (**Figure 1 A and C**) due to the high-resolving power characteristic of this technique. This translates to accurate quantitation and assignment of fragmentation

peaks or impurities in the resulting e-grams of non-reduced stressed and non-stressed infliximab (**Figure 1B**, overlay) and NIST mAb (**Figure 1D**, overlay) samples using Compass for iCE software. For non-reduced, non-stressed infliximab (**Figure 1B**, blue line) and NIST mAb (**Figure 1D**, blue line) the impurities that are shown are a single light chain (LC), a mixture of two heavy chains and one light chain (2H 1L), and a minor amount of 2H. However, for non-reduced, heat-stressed samples (orange line), the major degradation products assigned are LC, two heavy chains (2H) and 2H 1L.

Under reduced conditions, samples are less affected by heat stress compared with their non-reduced counterparts. Non-stressed and heat-stressed infliximab and NIST mAb samples show similar single high-molecular-weight and low-molecular-weight bands, corresponding to the antibody heavy chain (HC) and LC, in both SDS-PAGE and Maurice CE-SDS (**Figure 2 A and C**, inset, lanes 2–11). The high molecular weight species detected in SDS-PAGE but not by Maurice is likely as a result of being too large for the separation time used. When run by CE-SDS on Maurice, the peaks are nevertheless clearly separated and assigned as the antibody LC and HC, in addition to the non-glycosylated heavy chain (NGHC) (**Figure 2 B and D**, overlays).

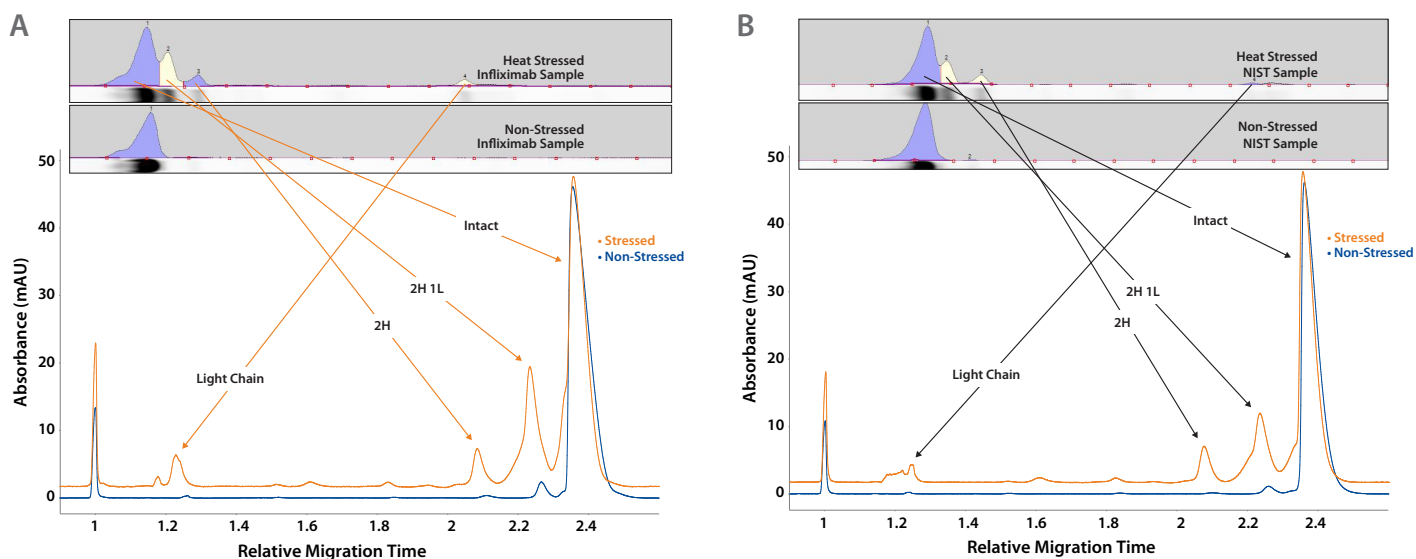


FIGURE 3. Assignment of impurity bands/peaks detected by SDS-PAGE and CE-SDS for non-reduced infliximab (A) and NIST mAb (B) under heat-stressed and nonstressed conditions. The inset SDS-PAGE gels were scanned using the FluorChem M imaging system and analyzed by accompanying AlphaView software. CE-SDS samples were separated using Maurice, and impurity peaks were assigned using Compass for iCE software. CE-SDS data were offset for ease of visual comparison between various samples and conditions analyzed.

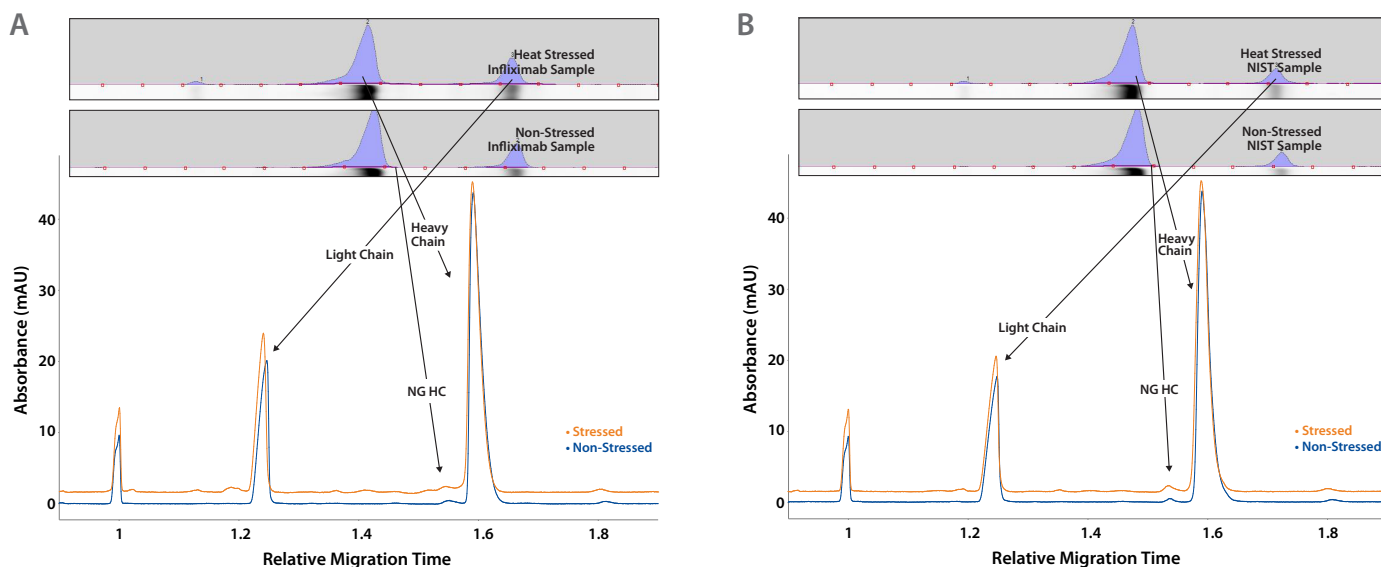


FIGURE 4. Assignment of impurity bands/peaks detected by SDS-PAGE and CE-SDS for reduced infliximab (A) and NIST mAb (B) under heat-stressed and nonstressed conditions. The inset SDS-PAGE gels were scanned using the FluorChem M imaging system and analyzed by accompanying AlphaView® software. CE-SDS samples were separated using Maurice, and impurity peaks were assigned using Compass for iCE software. CE-SDS data was offset for ease of visual comparison between various samples and conditions analyzed.

In **Figures 3 and 4**, we use a molecular weight marker to determine the apparent molecular weight of the bands detected following separation of our non-reduced (**Figure 3**) and reduced (**Figure 4**) antibody samples by SDS-PAGE or Maurice CE-SDS. This effort demonstrates the large discrepancy in signal-to-noise between the two techniques. The power to detect an impurity signal over background is much lower by SDS-PAGE compared with Maurice CE-SDS. Autointegration of bands using the Maurice system is also far easier than the more manual process of analyzing SDS-PAGE results. This is especially

obvious when comparing heat-stressed versus non-stressed infliximab and NIST mAb under non-reduced conditions (**Figure 3**). Importantly, Maurice CE-SDS has the ability to resolve the NGHC from the HC, otherwise difficult to achieve by SDS-PAGE due to the low detection sensitivity associated with the method (**Figure 4**). Given that glycosylation status influences antibody effector function and stability, efficient separation of the NGHC from the HC is critical to the proper analysis of a biotherapeutic protein product.

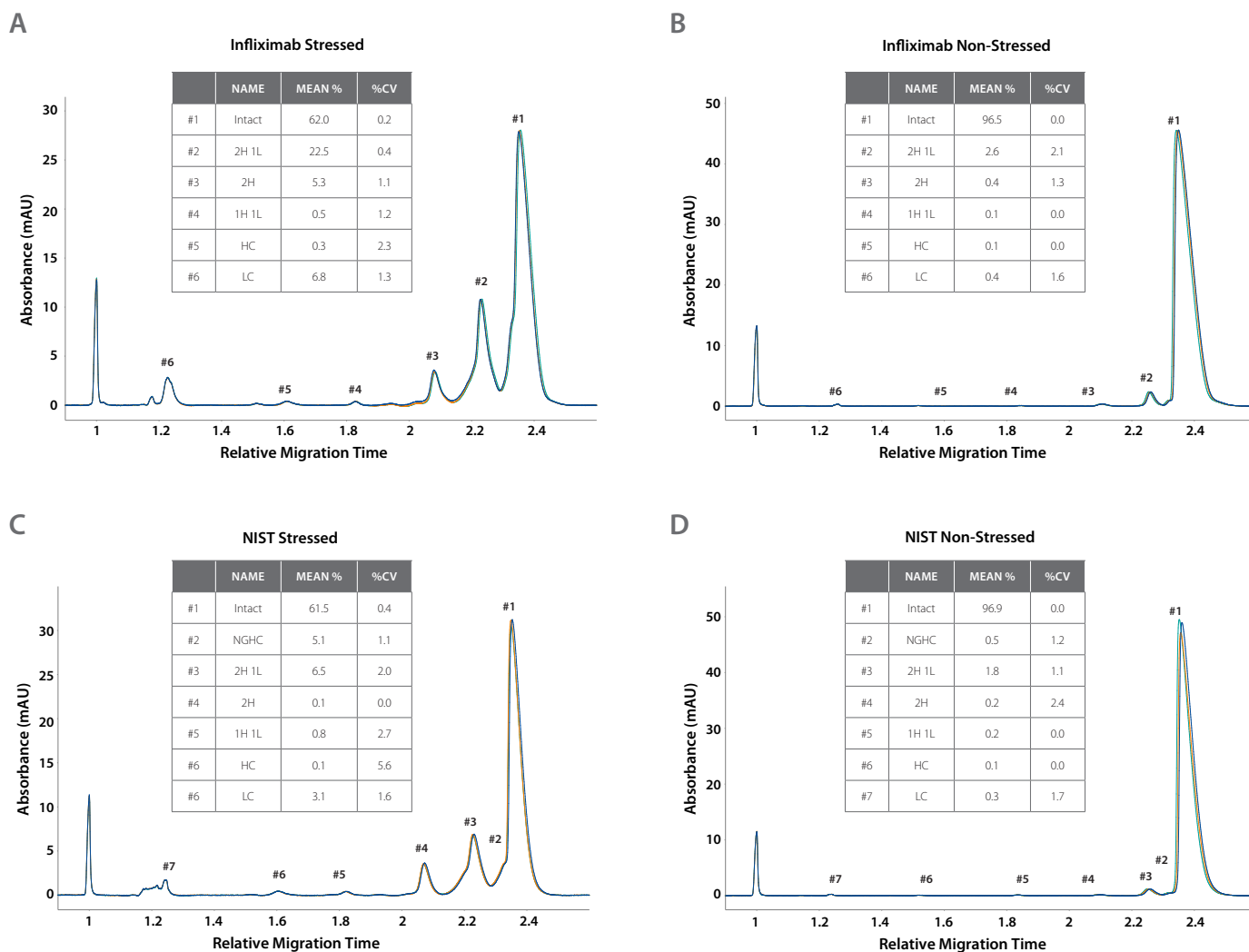


FIGURE 5. Triplicate injection e-gram overlays for nonreduced infliximab under heat-stressed (A) and non-stressed (B) conditions. Triplicate injection e-gram overlays for nonreduced NIST mAb under heat-stressed (C) and nonstressed (D) conditions. Mean values = percent peak area.

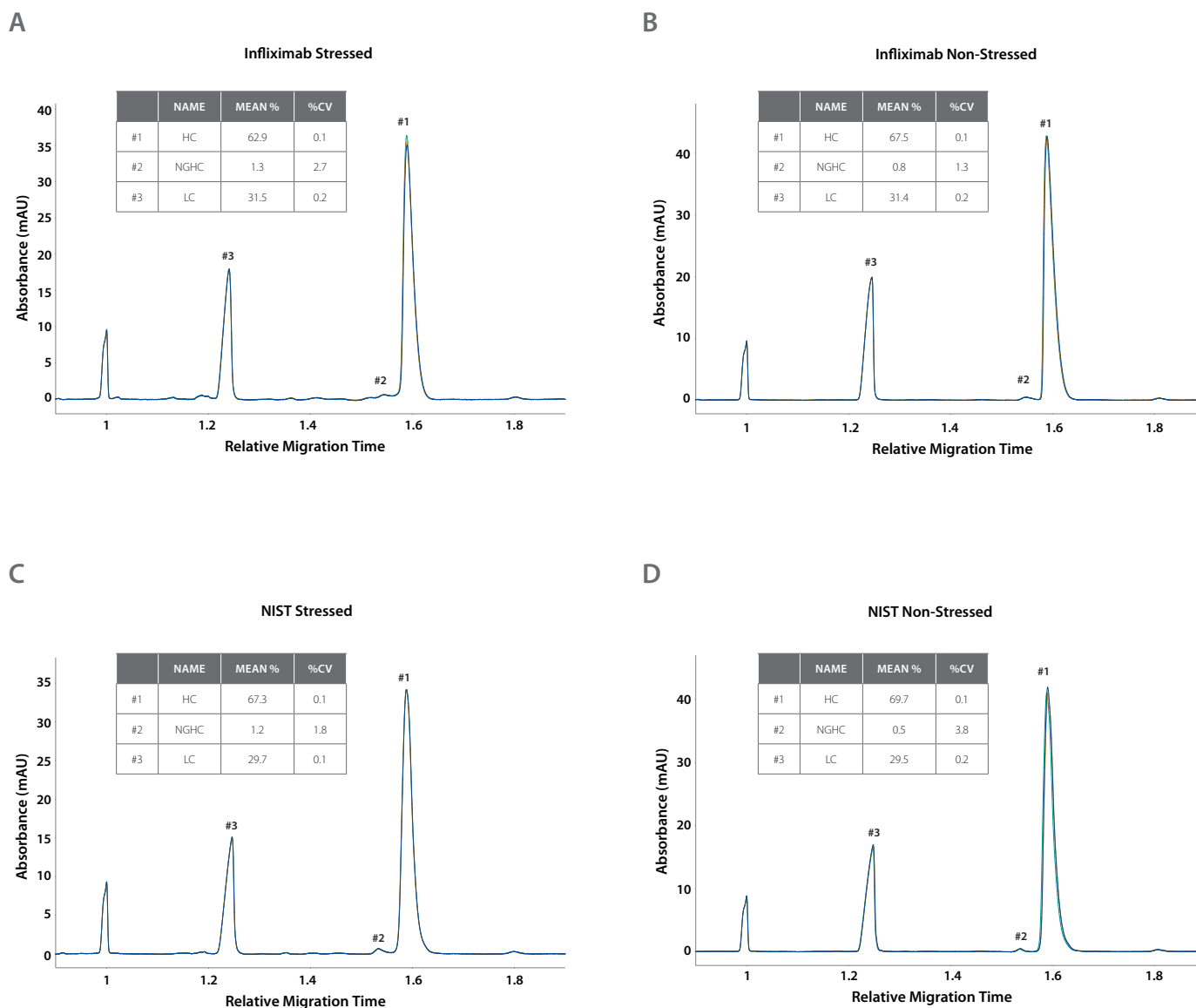


FIGURE 6. Triplicate injection e-gram overlays for reduced infliximab under heat-stressed (A) and non-stressed (B) conditions. Triplicate injection e-gram overlays for reduced NIST mAb under heat-stressed (C) and non-stressed (D) conditions.

How well a technique reproduces the same result is at the crux of comparative inter-lab and intra-lab quality control analyses. Variations in purity results using the same sample in the same laboratory may result in indeterminate conclusions or, worse, false positive/negative reporting. In addition to being frustrating for analysts, irreproducible results incur added costs to the development process and negatively impact overall operations. To this end, the power of Maurice CE-SDS in minimizing inconsistencies and maintaining reproducibility is easy to appreciate.

Figures 5 and 6 illustrate the results of three consecutive injections and analyses performed using the same nonreduced (**Figure 5**) or reduced (**Figure 6**) heat-stressed or nonstressed antibody sample. The e-grams show overall equal and highly reproducible peak profiles between injections for the various fragments, where the inset tables define each peak detected. Attesting to the reproducibility of the system are, on average, very low, single-digit coefficient of variations (%CV) throughout. These data affirm that Maurice CE-SDS is a reliable system

		NON-STRESSED		STRESSED			
		Intact	2H 1L	Intact	2H 1L	2H	LC
Infliximab	Mean %	99.34	0.66	67.42	21.57	5.10	5.92
	Std. Dev	0.09	0.09	1.33	0.77	1.18	0.82
	% RSD	0.09	13.83	1.97	3.58	23.24	13.90
NIST mAb	% Area Avg	99.15	0.85	72.58	15.75	7.07	4.60
	Std. Dev	0.22	0.22	1.35	0.92	0.32	0.52
	% RSD	0.22	25.20	1.87	5.87	4.49	11.36

LC, light chain; %CV, coefficient of variation; mAb, monoclonal antibody.

TABLE 2. Reproducibility of SDS-PAGE under non-reduced conditions, performed using five replicate samples.

		NON-STRESSED		STRESSED		
		HC	LC	NGHC	HC	LC
Infliximab	Mean %	69.98	30.02	3.00	68.12	28.89
	Std. Dev	2.98	2.98	0.15	2.05	2.16
	% RSD	4.26	9.92	5.15	3.01	7.48
NIST mAb	% Area Avg	80.62	19.38	2.99	77.69	19.33
	Std. Dev	2.02	2.02	0.15	1.01	0.98
	% RSD	2.51	10.44	5.03	1.30	5.09

HC, heavy chain; LC, light chain; NGHC, non-glycosylated heavy chain; %CV, coefficient of variation; mAb, monoclonal antibody.

TABLE 3. Reproducibility of SDS-PAGE under reduced conditions, performed using five replicate samples..

for minimizing variability between results, providing you with confidence in the conclusions you make.

As a measure of reproducibility for the results obtained by SDS-PAGE, %CV calculations for the percent peak area were performed corresponding to the separation and detection of the intact protein and the respective impurities under both non-reduced and reduced conditions. These data are summarized in **Table 2** and **Table 3**. For the separation and percent peak area quantitation of the non-reduced

intact antibody, SDS-PAGE proved reproducible, yielding low %CV values (<2.0%). However, the reliability of the slab-gel approach for the consistent detection of impurities for the antibody samples analyzed is far lower than that of CE-SDS, with %CV values for percent peak area reaching as high as 25%. Taken together, assessing each technique’s reproducibility points to the conclusion that SDS-PAGE may not be a feasible option for the quantitative analysis of complex protein mixtures.

Conclusion: Running Away from Gel to Maurice

To improve your protein purity characterization, analysis by way of Maurice CE-SDS offers significantly higher peak profile resolution and more accurate quantitation of impurities than what traditional SDS-PAGE slab-gel methods can offer. As demonstrated in the side-by-side comparison, Maurice CE-SDS—not SDS-PAGE—enabled definitive and reproducible separation of reduced and nonreduced samples and associated impurities, while also reliably differentiating between the glycosylated and nonglycosylated heavy chain.

Although still used in the industry, SDS-PAGE is challenged by reproducibility issues, making it a risky choice for quantitative protein purity analysis. Today, more and more biopharmaceutical companies have adopted CE-SDS over slab-gels in their development process. Maurice, a cutting-edge CE-SDS system, excels in providing not only reproducible, high-resolution data, but also a simple, robust workflow that reduces hands-on time and minimizes user error.

References

1. Infliximab, R Fatima and M Aziz, *StatPearls*, Available online at <https://www.ncbi.nlm.nih.gov/books/NBK500021/>. Accessed September 11, 2018. Updated April 24, 2018.
2. National Institute of Standards and Technology, NIST Monoclonal Antibody Reference Material 8671. Available online at <https://www.nist.gov/programs-projects/nist-monoclonal-antibody-reference-material-8671>. Accessed September 11, 2018. Updated February 9, 2018.



Toll-free: (888) 607-9692
Tel: (408) 510-5500
info@proteinsimple.com
proteinsimple.com

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