Rediscover the Strengths of Your PA 800 Plus - A Multi-Attribute Protein Analyzer

Marcia R Santos
SCIEX Separations, Brea, CA

Introduction

Capillary Electrophoresis is a well-accepted analytical separation technology in many different environments within the biologics domains. From screening glycans in the upstream bioprocessing during cell culture optimization and clone selection, through analytical development for the characterization of a monoclonal antibody drug product, to lot release in final quality control. The SCIEX PA 800 Plus has been in the forefront of the lifecycle in the biologic industry as the leading Capillary Electrophoresis platform.

This work illustrates one reason the PA 800 Plus is the preferred platform for CE-SDS IgG Purity and Heterogeneity Assay quantitation in biologics analysis and characterizations. Here its quantitative capabilities are highlighted from a broad linear dynamic range attributed to robust chemistry, flexible modular detectors that could be easily be interchangeable, to superior reproducibility and repeatability. In addition, the system provides superior analytical assay performance due to patented temperature control system for both the sample and the capillary.

The combination of an award-winning chemistry, automation friendly sample preparation, fast separation, and streamline glycan identification software capabilities; the Fast Glycan Labeling and Analysis kit enables users to quickly identify and quantity the released N-linked glycans of a monoclonal antibody from sample to answer.

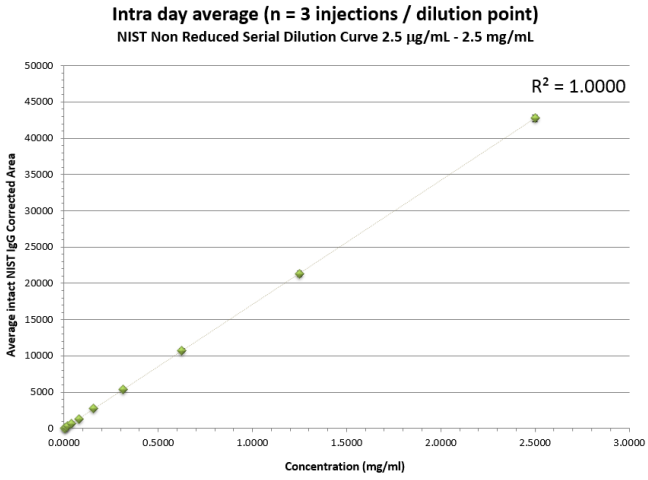

Figure 1. Linear Dynamic Range for NIST Non-Reduced IgG. The LDR for this protein over 3 logs was recorded in 1 Instrument, 1 cartridge and 3 Separation gel lots.

Key Features

- Highlight sample preparation workflow for both TAMRA and FQ
- Data comparison between traditional UV based detection and and LIF based.

Materials and Methods:

Instrument and Software Set up for the IgG Assay: The capillary electrophoresis instrument used was a PA 800 plus equipped with either LIF detection with solid state laser with excitation wavelength at 488 nm and the emission filters were 560 nm for TAMRA (SCIEX P/N 149068) or PDA detector set at 220 nm wavelength with 10 nm bandwidth. CE-EZ Cartridge (Figure 2) was used for the separation of NIST antibody in both assays. Separations were performed at 500 V/cm and injection was electrokinetic at 20s/-5kV. The separation gel used was from the IgG Heterogeneity and Purity Assay (Figure 3). Data acquisition and analysis was performed using 32Karat software V10.2. The IgG instrument methods used were: IgG HS Conditioning – PA 800 plus, IgG HS Separation – PA 800 plus and IgG HS Shutdown – PA 800 plus.[2]

Instrument Set up for the Fast Glycan Assay: PA 800 plus Pharmaceutical Analysis System was equipped with a 488 nm solid state laser. The LIF detector was equipped with a 488 nm excitation and 520 nm emission filters. In this study, the methods used were: Fast Glycan conditioning, separation and shutdown.[3]

Figure 2. The EZ-CE Cartridge. SCIEX P/N A55625. This cartridge was used for both the separation of NIST antibody and for the analyses of released N-linked glycans of NIST antibody.

EZCartridge was also used for the analyses of released N-linked glycans of NIST antibody (Figure 1).

Sample Preparation for UV Detection (Reduced and Non-Reduced): NIST Monoclonal Antibody (Reference Material 8671) 8mg/mL stock solution was diluted to 5 mg/ml in the CE-SDS sample buffer. A 2.5 mg/mL NIST sample was reduced and denatured by mixing beta-mercapto ethanol and 10 kDa marker (used as mobility marker) and heating the mixture to 60ºC for 10 minutes. Similarly, a 2.5 mg/mL NIST sample non-reduced sample was prepared by mixing a iodoacetamide solution and 10 kDa marker (used as mobility marker) and heating the mixture to 60ºC for 10 minutes.

Serial Dilution: A serial dilution of both reduced and non-reduced NIST sample was prepared by diluting the 2.5 mg/mL solution directly into the CE-SDS sample buffer.

Sample Preparation for LIF detection: Reagents used for 5-TAMRA.SE labeling and clean up for antibody analysis using LIF detection: 5-TAMRA.SE (5-Carboxytetramethylrhodamine; ThermoFisher Scientific; Grand Island – NY. Dimethyl sulfoxide; Citric acid anhydrous; Sodium Phosphate, Dibasic; Sodium Bicarbonate; Sodium dodecyl sulfate, Iodoacetamide and dithiothreitol were purchased from Sigma- Aldrich; St. Louis – MO; NAP 5 columns, GE-Healthcare, Milwaukee – WI.

Labeling of NIST Antibody with 5-TAMRA.SE[1] (briefly): 500 µg of starting NIST material was buffer exchanged into 0.1 M sodium bicarbonate solution pH 8.3 using previously equilibrated NAP-5 column. A portion of the flow through was incubated for 30 min at 30oC with a solution of 5-TAMRA-SE solution in DMSO. The excess dye was removed by buffer exchanged the antibody-dye reaction mix in citrate-phosphate buffer pH 6.6 using NAP 5 column. Samples are ready for reduction and alkylation.

Figure 3. The IgG Heterogeneity and Purity Assay Kit. P/N A10663.

Fast Glycan Sample Preparation Workflow[3]: A human Serum antibody (Sigma PN I4506) was reconstituted to a 10 mg/mL solution in 150 mM NaCl.

100 mg of Human Serum IgG sample was mixed with magnetic beads. The mix was denatured and reduced for 8 min at 60ºC followed by PNGase F digestion for 20 min at 60ºC. Acetonitrile was added to the enzymatic reaction mix to promote the capture of the released N-glycan by the beads. Next, APTS dye was added to the bead mixture and the sample was incubated for 20 min at 60ºC. The excess dye was removed by a series of bead washes using 90-80% acetonitrile solution. The final N-liked labeled glycan was eluted from the beads using ddi water. Figure 4 illustrates the sample preparation workflow.

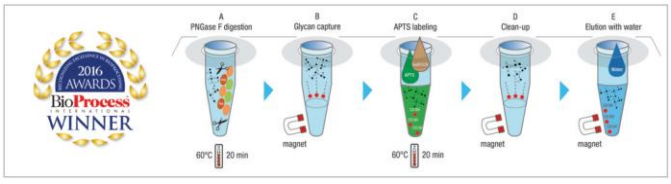

Figure 4. The Fast Glycan Workflow. (Top) Labeling and analysis kit – P/N B94499PTO. (Bottom) Fast Glycan Sample Preparation Workflow.

IgG Purity and Heterogeneity Assay Demonstrates a Linear Dynamic Range over 3 Logs of Concentration

Figures 5 and 1 (front page) show the linearity over 3 logs of dynamic range as well as intermediate precision. Figure 5 shows intermediate precision over 3 instruments and 3 cartridges and Figure 1 shows the intermediate precision of one instrument, one cartridge over 3 lots of separation gel.

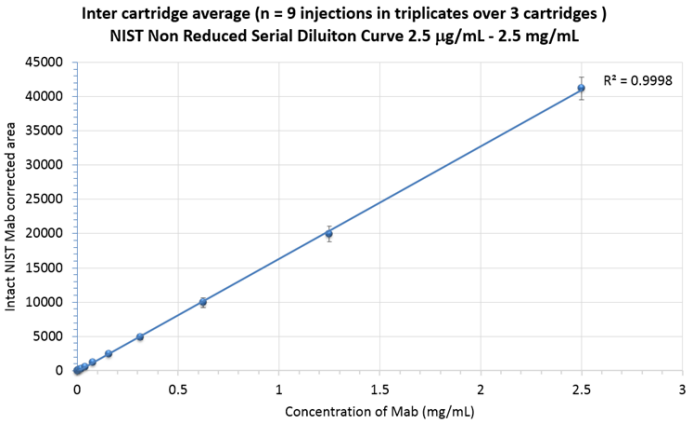

Figure 5. Linear Dynamic Range for NIST Non-Reduced IgG. The LDR for this protein over 3 logs recorded in 3 instruments and 3 cartridges is shown.

To further increase the limit of detection, the use of laser induced fluorescence detection allows for the detection of low-level impurities. Figure 6 shows a comparison between a 1 mg/mL NIST on UV (black) vs 1 mg/mL of Tamra labeled NIST (blue).

Intermediate Precision: 10 KD marker – Mobility Marker

The intermediate precision for the 10 kDa marker used in the IgG Assay as mobility marker is illustrated in Figure 7. The remarkable reproducibility obtained over 558 runs, 3 instruments and under both reduced and non-reducing conditions combined shows not only the robustness of the assay but also highlights the flexibility of the temperature-controlled platform to allow the user to run both experimental conditions reliably.

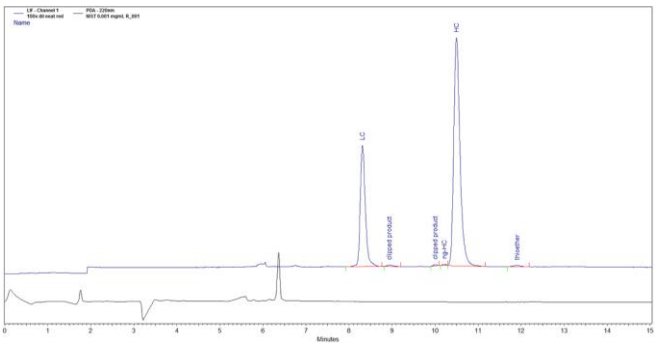

Figure 6. Comparison LIF and UV Runs of 1 µg /mL NIST Antibody. (Top blue trace) Tamra labeled NIST IgG measured by LIF. (Bottom black trace) UV analysis of same NIST IgG.

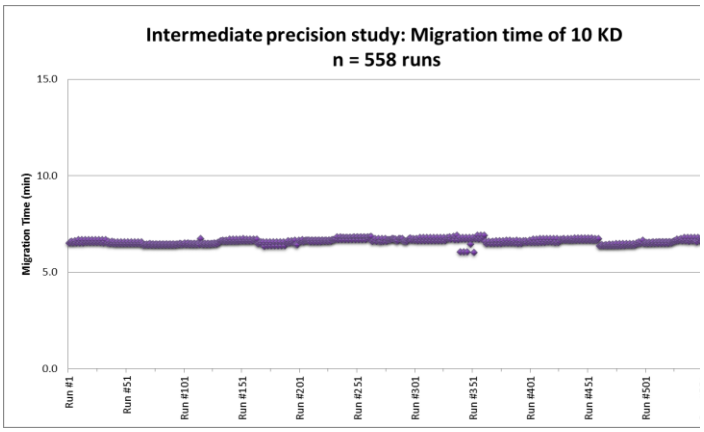

Figure 7. Intermediate Precision of 10 KD Marker.

Conditions:

- 3 PA 800 plus;
- Reduced and Non-Reduced;
- 3 lots of gels;
- 9 Pre-built cartridges
- 6 days

Results:

- Ave. MT: 6.57 min
- Std Dev.: 0.13
- %CV: 1.98 (n=558)

Purity of Monomer

Table 1 shows the intermediate precision for the purity of the monomer. The report of Investigation for NIST ref. material 8671 (lot no. 14HB-D-002)[4] indicates a size heterogeneity of 98.47%. The combined results reported here shows 98.63 % purity of monomer determined over 3 instruments, 3 lots of gel 9 cartridges. Figure 8 shows the e-gram of non-reduced NIST and its impurities.

Table 1. Purity of Monomer – NIST Intermediate Precision.

	LMW CA*	CA Intact IgG (Glyc + Non-Glycosylated)	Total CA	% LMW	% Purity of Monomer
Average	*251.49*	*18326.36*	*18577.85*	*1.37*	*98.63*
Std Dev	*15.22*	*1963.23*	*1968.46*	*0.14*	*0.14*
% CV	*6.1*	*10.7*	*10.6*	*10.0*	*0.1*

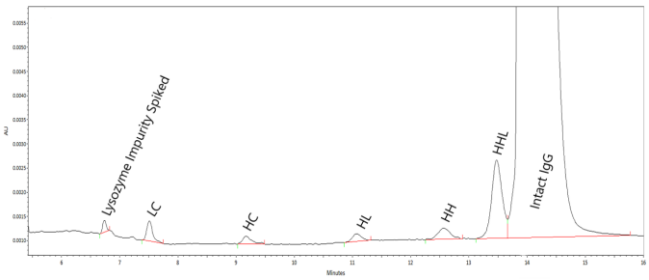

Figure 8. Representative Electropherogram of NIST Antibody. NIST under non-reducing conditions showing its low-level impurities, including the quantitation of 0.05 mg of lysozyme spiked into 1 mg of NIST antibody.

Impurity Quantitation

Following ICH Q2B guidelines to industry[5], % impurity can be determined by the relative weight of impurity to drug substance. Figure 9 shows a e-gram of 0.05 mg of lysozyme spiked to 1 mg of NIST antibody to demonstrate the *low-level impurity quantitation capability of PA 800 plus is 0.05%.*

% Glycan Site Occupancy (%SO) and Corrected Area % Composition

The presence of a glycan unit at the site of occupancy influences solubility, folding, secretion, modulate antigenicity, and increase in vivo half-life of the glycoprotein. Thus, reliable quantitation is of paramount importance. The % glycan site occupancy is determined based on the ratio between the corrected area of heavy chain and the sum corrected area of glycosylated non-glycosylated heavy chain multiplied by 100.

Table 2. Site Occupancy for NIST Antibody. On multiple PA 800 plus systems.

#1	% LC	% ng - HC	% HC	% Thioether	Site Occupancy
Average	*29.83*	*0.51*	*69.23*	*0.43*	*99.5*
Std Dev	*0.05*	*0.00*	*0.05*	*0.02*	*0.005*
% CV	*0.2*	*0.9*	*0.1*	*4.1*	*0.005*
#2					
Average	*30.65*	*0.47*	*68.49*	*0.39*	*99.4*
Std Dev	*0.02*	*0.003*	*0.03*	*0.01*	*0.03*
% CV	*0.1*	*0.7*	*0.04*	*1.9*	*0.03*
#3					
Average	*30.95*	*0.51*	*68.12*	*0.41*	*99.4*
Std Dev	*0.13*	*0.00*	*0.14*	*0.02*	*0.03*
% CV	*0.4*	*0.8*	*0.2*	*4.3*	*0.00*

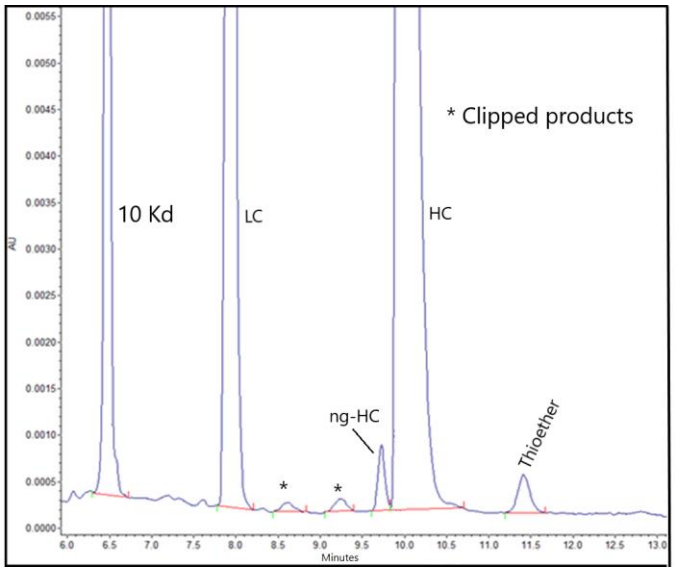

Figure 9. Electropherogram of Reduced NIST Antibody.

Figure 9 shows a representative electropherogram of the NIST under reducing conditions. Table 2 show the % SO determined for NIST antibody is in very good agreement between the 3 instruments used in this study.

Fast Glycan Labeling and Analysis

Glycosylation, an important quality attribute of monoclonal antibodies, play a significant role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of these therapeutic agents.

Typically, the analysis of such molecules relies on the enzymatic cleavage of the glycan molecule from the protein backbone, followed by a fluorescent tagging reaction which will impart charge and fluorescence so that these molecules can be separated detected and quantified. A laborious and lengthy process that can take overnight.

The Fast Glycan Labeling and Analysis kit was designed to reduce the glycan workflow from sample preparation to data analysis and reporting, thanks to built-in integration parameters and a search algorithm[6] to the separation method. The Fast Glycan application can integrate the data, assign GU (glucose unit) values and determine glycan ID's to 36 N-linked glycan species and print a report in just seconds.

Not only speed of the process that matters but reliability and robustness of the assay is also of utmost importance. Below the intermediate precision data obtained for the Fast Glycan Labeling and Analysis kit is shown.

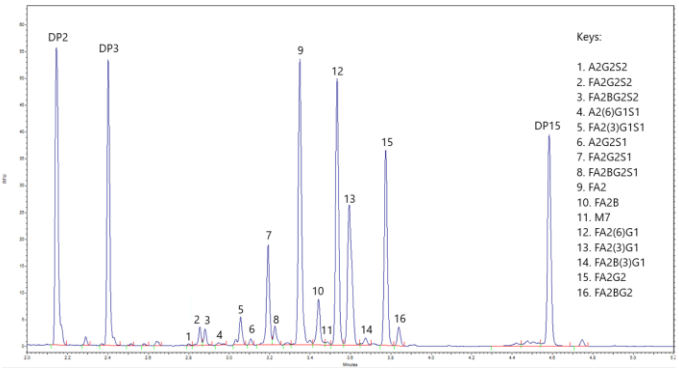

Figure 10. Representative Electropherogram of Released N-linked Glycans of Serum IgG sample.

Fast Glycan on PA 800 plus Intermediate Study

The experimental design space for this study involved two PA 800 plus instruments under OQ3 and 3 chemistry lots.

The separation of N-linked glycan from human Serum IgG is shown in Figure 10. This sample was chosen due to the variety of N-linked glycan peaks which help showcase the resolution power of this chemistry.

Table 3 summarizes the average migration time values for 4 major glycan peaks of FA2, FA2(6)G1, FA2(3)G1 and FA2G2 which have a target specification of ≤ 5 minutes at 25ºC. The target specification for run-to-run resolution between FA2(6)G1 and FA2(3)G1 is ≥ 1.0 and the migration time for FA2 peak, should be ≥ 3.0 and ≤ 4.0 minutes at 25ºC. The population size for this study was n=600.

Table 3: Average Migration Time for Major Glycans; Resolution between FA2(6)G1 and FA2(3)G1 and Migration Time of FA2

PA 800 plus #	chemistry lot #	Average Migration time of the major peaks of FA2, FA2(6)G1, FA2(3)G1 and FA2G2				Average Run-to-run resolution between FA2(6)G1 and FA2(3)G1	Average Migration time of the FA2 peak
		FA2	FA2(6)G1	FA2(3)G1	FA2G2		
1	1	3.399	3.587	3.649	3.832	1.750	3.399
1	2	3.417	3.606	3.667	3.852	1.705	3.417
1	3	3.402	3.589	3.650	3.834	1.772	3.402
2	1	3.355	3.539	3.600	3.782	1.767	3.355
2	2	3.371	3.560	3.617	3.800	1.776	3.371
2	3	3.331	3.515	3.577	3.755	1.786	3.331

In terms of instrument reproducibility, this study showed excellent %RSD for the parameters reported above. Table 4 summarizes the %RSD found for each instrument and chemistry lot studied.

Table 4: Reproducibility for Migration Time for Major Glycans; Resolution between FA2(6)G1 and FA2(3)G1 and Migration Time of FA2 (N=600)

PA 800 plus #	chemistry lot #	%RSD Migration time of the major peaks of FA2, FA2(6)G1, FA2(3)G1 and FA2G2				%RSD Run-to-run resolution between FA2(6)G1 and FA2(3)G1	%RSD Migration time of the FA2 peak
		FA2	FA2(6)G1	FA2(3)G1	FA2G2		
1	1	0.58	0.54	0.55	0.55	0.79	0.58
1	2	0.53	0.54	0.54	0.55	1.24	0.53
1	3	0.45	0.45	0.46	0.46	0.72	0.45
2	1	0.53	0.58	0.55	0.54	0.88	0.53
2	2	0.60	1.05	0.62	0.61	1.33	0.60
2	3	0.33	0.27	0.87	0.26	0.65	0.33

Conclusions

In this technical note, important figures of merit including the robustness and reliability of PA 800 plus instrument have been demonstrated, that combined with gold standard chemistry kits showcase unmatched performance in the characterization of monoclonal antibodies for the biopharma industry.

References

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Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

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