Achieving Robust cIEF Analysis of Monoclonal Antibodies While Increasing Capillary Run-Life and Maintaining High Resolution and Reproducibility

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Overview

Capillary isoelectric focusing (cIEF) is widely used for characterization of therapeutic monoclonal antibodies (mAbs). By determining the isoelectric points (pI) of mAb charge isoforms, cIEF provides information crucial for establishing identity, purity, post-translational modifications, and stability. Achieving optimum cIEF resolution, repeatability, and reproducibility, however, can be challenging. Doing so while also achieving long capillary run-life can be even more difficult. Method- and instrument-related issues such as capillary variability, cross-contamination, and capillary coating degradation can negatively impact analytical cIEF performance. External factors such as sample purity can do the same.

This note describes an analytical cIEF method carefully designed to extend capillary run-life and, at the same time, maximize pI resolution and experimental repeatability and reproducibility for the analysis of mAbs. The method was developed on and for the SCIEX PA 800 Plus Pharmaceutical Analysis System, which was then used to test batches of neutral capillaries. The neutral-coated capillaries used in this work were produced through an improved manufacturing process designed to increase quality and reduce variation. The result was excellent cIEF resolution and reproducibility with improved capillary run-life.

Key Challenges:

- Salts and detergents in samples can interact with cIEF reagents, degrading analytical performance and reducing capillary run-life[1]
- Manufacturing processes can affect capillary run-life and result in inter-capillary variations, impacting reproducibility
- Inadequate capillary cleaning between runs can lead to cross-contamination, which is detrimental to repeatability and reproducibility

Key Features:

- Improved manufacturing processes with extensive quality monitoring of coating reagents produce neutral-coated capillaries with increased run-life
- cIEF method is optimized to eliminate cross-contamination and establish stable and reproducible pH gradients
- Use of a single master mix formulation for monoclonal antibodies with pI values of 7.0–9.5 enhances reproducibility and ease of operation
- Capillary cleaning and rest method aids capillary coating recovery and prolongs the life of the coating
- New neutral-coated capillaries and optimized cIEF method enabled over 100 analyses of United States Pharmacopeia (USP) immunoglobulin G (IgG) with highly reproducible results over multiple days and instruments
- Resolution of USP IgG peaks with pI differences of only 0.03 was achieved

Experimental

Sample Preparation

The samples used for this work were USP IgG and National Institute of Standards and Technology (NIST) IgG reference standards in a master mix that included urea in cIEF gel, iminodiacetic acid (IDA), Pharmalyte 3-10, arginine, and peptide pI markers. The final IgG concentration was 0.24% m/v. The master mix used was suitable for reproducible separation of monoclonal antibodies with pI values from 7.0 to 9.5.

Concentrations of anodic and cathodic blockers and urea were optimized during method development.

Sample pre-treatment is important to remove contaminants such as salts and detergents that can interact with cIEF reagents. Details of sample preparation are described in the cIEF manual. USP and NIST IgG were reconstituted in DI water to obtain concentration of 5 mg/mL solutions. No sample pre-treatment needed.

Figure 1 diagrams the sample preparation.

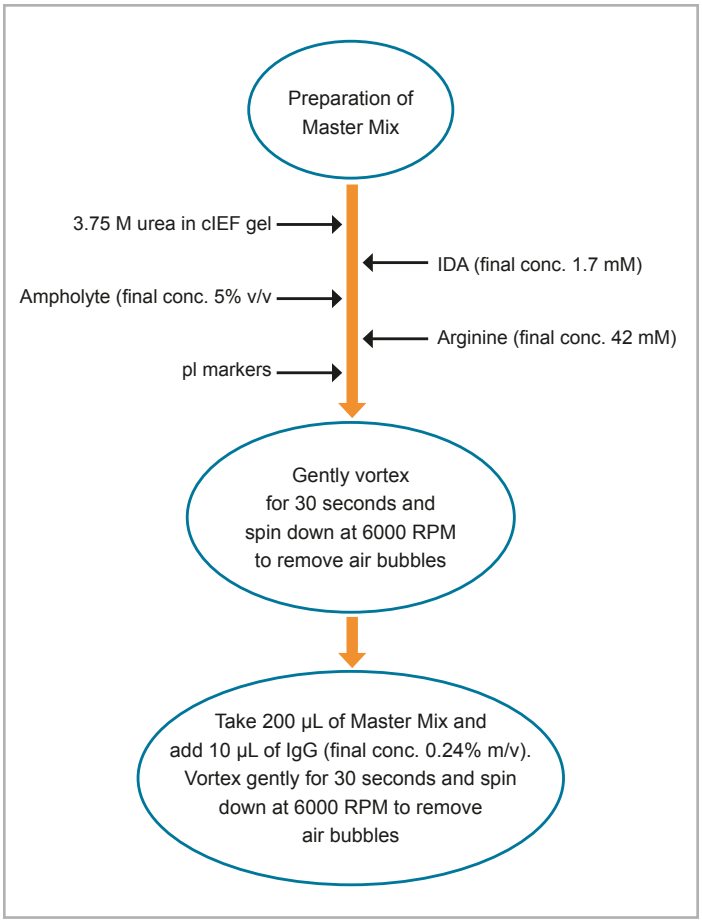


Figure 1. Sample preparation for cIEF analysis.

Separation and Analysis

Initial Capillary Conditioning

Initial capillary conditioning was carried out as shown in Figure 2. Sample loading solution (SLS, SCIEX PN 608082) was used instead of 4.3 M urea solution because SLS is a more efficient way to clean capillary inner surface.[2]

cIEF Separation

All cIEF separations were carried out on PA 800 Plus systems. 32 Karat software was used for data collection and analysis. All aspects of the cIEF separation method were optimized to eliminate cross-contamination and establish stable and reproducible pH gradients. The steps of the cIEF separation method are shown in Figure 3. Notes regarding some individual steps:

Step 1: Rinse the capillary with SLS at the beginning of each cIEF separation run to clean the capillary inner surface.

Step 4: Introduce the sample, in master mix, into the capillary at low pressure (15 psi) for 150 seconds to replace multiple capillary volumes.

Step 5: Dip the capillary ends in double-deionized (DDI) water before moving the capillary to anolyte/catholyte vials for the focusing step.

Step 8: Dip the capillary ends in DDI water before moving the capillary to anolyte/chemical mobilizer vials for the mobilization step.

Initial Conditions | UV Detector Initial Conditions | Time Program

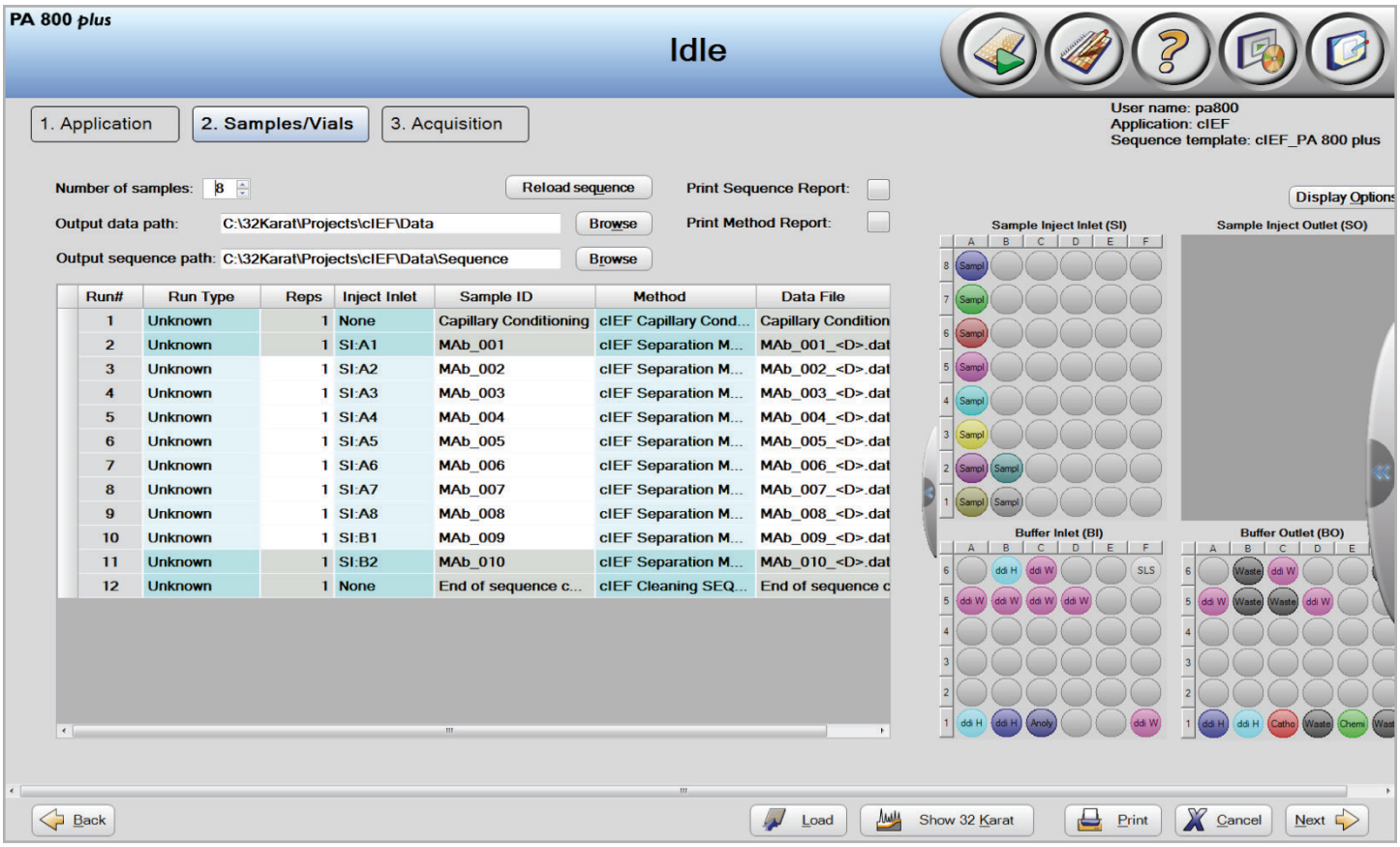
	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	5.00 min	BI:A1	BO:B1	forward	water
2		Rinse - Pressure	50.0 psi	5.00 min	BI:D1	BO:B4	forward	SLS
3		Rinse - Pressure	50.0 psi	3.00 min	BI:B1	BO:B3	forward	water
4		Rinse - Pressure	50.0 psi	3.00 min	BI:A1	BO:B1	forward	Water
5		Wait		0.00 min	BI:A1	BO:A1		Idle Position
6								

Figure 2. cIEF capillary conditioning method.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	1.00 min	BI:F6	BO:F6	forward	SLS
2		Rinse - Pressure	20.0 psi	3.00 min	BI:F1	BO:F1	forward, In vial inc 10	Water rinse 1
3		Rinse - Pressure	50.0 psi	2.00 min	BI:B1	BO:B1	forward, In / Out vial inc 10	Water rinse 2
4		Inject - Pressure	15.0 psi	150.0 sec	SI:A1	BO:B1	Override, forward	Sample fill
5		Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	Water Dip 1
6	0.00	Separate - Voltage	25.0 KV	15.00 min	BI:C1	BO:C1	0.17 Min ramp, normal polarity, In / Out vial inc 10	Focusing Step
7	1.00	Autozero						
8	15.10	Wait		0.00 min	BI:C1	BO:A1	In / Out vial inc 10	Water Dip 2
9	15.20	Separate - Voltage	30.0 KV	25.00 min	BI:C1	BO:E1	0.17 Min ramp, normal polarity, In / Out vial inc 10	Chemical Mobilization Step
10	40.20	Stop data						Stop cIEF separation
11	40.30	Rinse - Pressure	50.0 psi	3.00 min	BI:B1	BO:D1	forward, In / Out vial inc 10	Water rinse 3
12	43.40	Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	Water Dip 3
13	43.50	End						Method End

Figure 3. cIEF separation method.



Run#	Run Type	Reps	Inject Inlet	Sample ID	Method	Data File
1	Unknown	1	None	Capillary Conditioning	cIEF Capillary Cond...	Capillary Condition
2	Unknown	1	SI:A1	MAb_001	cIEF Separation M...	MAb_001_<D>.dat
3	Unknown	1	SI:A2	MAb_002	cIEF Separation M...	MAb_002_<D>.dat
4	Unknown	1	SI:A3	MAb_003	cIEF Separation M...	MAb_003_<D>.dat
5	Unknown	1	SI:A4	MAb_004	cIEF Separation M...	MAb_004_<D>.dat
6	Unknown	1	SI:A5	MAb_005	cIEF Separation M...	MAb_005_<D>.dat
7	Unknown	1	SI:A6	MAb_006	cIEF Separation M...	MAb_006_<D>.dat
8	Unknown	1	SI:A7	MAb_007	cIEF Separation M...	MAb_007_<D>.dat
9	Unknown	1	SI:A8	MAb_008	cIEF Separation M...	MAb_008_<D>.dat
10	Unknown	1	SI:B1	MAb_009	cIEF Separation M...	MAb_009_<D>.dat
11	Unknown	1	SI:B2	MAb_010	cIEF Separation M...	MAb_010_<D>.dat
12	Unknown	1	None	End of sequence c...	cIEF Cleaning SEQ...	End of sequence c

Figure 4. cIEF run sequence

Capillary cleaning and rest

To maintain optimum resolution, repeatability, and reproducibility, and extend capillary run-life, use of the capillary cleaning and rest method is recommended after no more than 20 runs. The capillary cleaning and rest method is shown in Figure 5. Notes regarding some individual steps:

Steps 1, 2, & 3: Rinse the capillary with DDI water, chemical mobilizer (350 mM acetic acid), and DDI water again.

Step 4, 5, & 6: Clean the capillary inner surface with SLS, followed by DDI water, and fill the capillary with cIEF gel.

To extend capillary run-life, store the capillary at 2–8° C for 16–18 hours with the ends submerged in DDI water before the next use.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	5.00 min	BI:B6	BO:B6	forward	Water Rinse 1
2		Rinse - Pressure	50.0 psi	2.00 min	BI:A6	BO:A6	forward	Chemical mobilizer Rinse
3		Rinse - Pressure	50.0 psi	5.00 min	BI:B6	BO:B6	forward	Water rinse 2
4		Rinse - Pressure	50.0 psi	2.00 min	BI:F6	BO:F6	forward	SLS rinse
5		Rinse - Pressure	50.0 psi	5.00 min	BI:E6	BO:E6	forward	Water Rinse
6	0.00	Separate - Pressure	50.0 psi	3.00 min	BI:D6	BO:D6	forward	cIEF Gel Rinse
7	3.10	Lamp - Off						
8	3.20	Wait		0.00 min	BI:A1	BO:A1		Water

Figure 5. cIEF capillary cleaning and rest method.

Results and Discussion

The optimized cIEF method was used to analyze USP and NIST IgG samples on multiple batches of neutral-coated capillaries on multiple instruments to assess repeatability and reproducibility. Over 100 cIEF separations were conducted over 5 days. After each batch of runs (20 cIEF separations), capillary was cleaned and rested according to the capillary cleaning and rest method previously described. The neutral-coated capillary lots generated reproducible pI values and normalized peak areas.

Figure 6 shows a representative cIEF separation profile for USP IgG Reference Standard. Statistics for Acidic 1 and Acidic 2 peak resolutions are listed in Table 1.

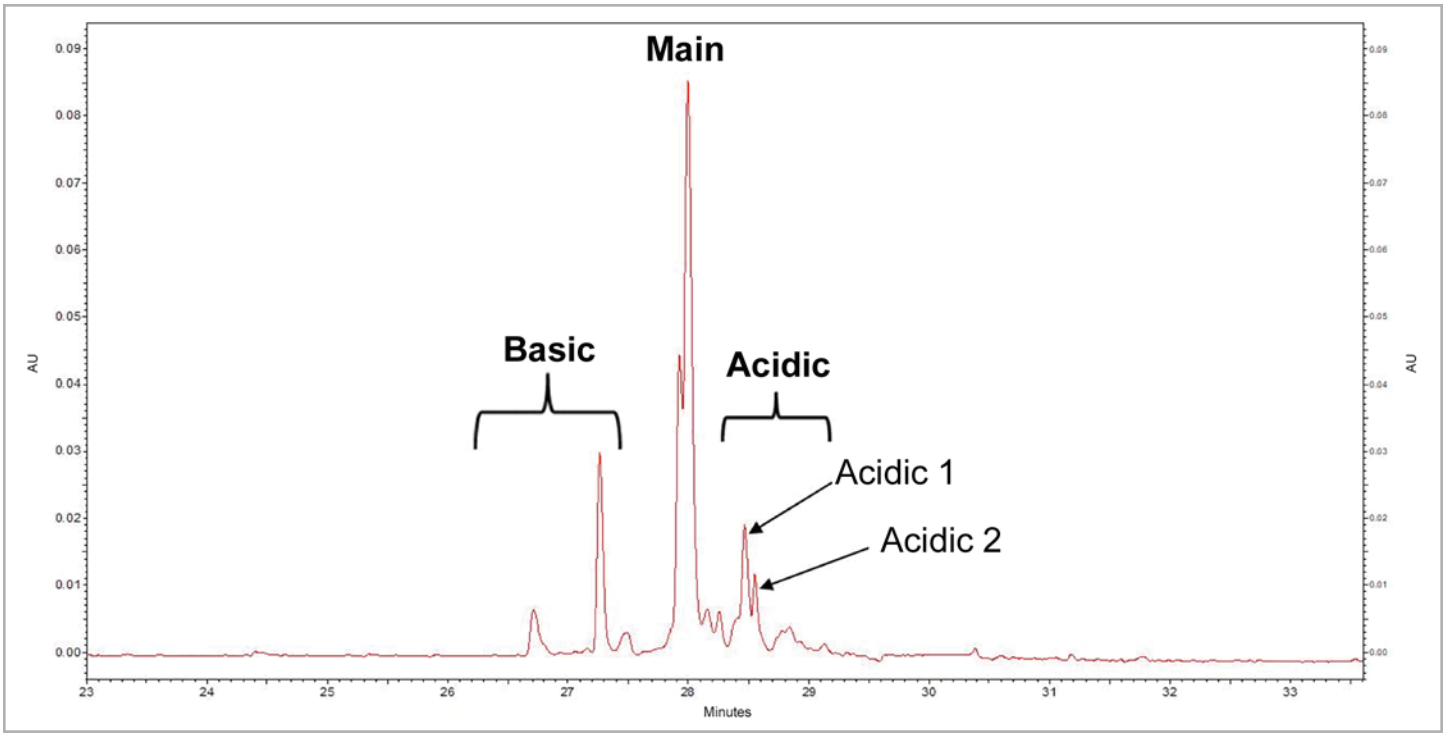


Figure 6. cIEF separation profile of USP IgG with 3-10 ampholytes. pI values of USP IgG were in the pH 7.0–7.6 range.

	Lot 2	Lot 3
AVE	0.80	0.65
STD	0.049	0.027
%RSD	6.16	4.18

Table 1: Calculated pI resolution of Acidic 1 & Acidic 2 peaks of USP IgG (N=20) in two manufacturing lots of neutral-coated capillaries.

Using the same master mix and method, the analyses generated highly reproducible results that clearly differentiated USP IgG from NIST IgG (Figure 7).

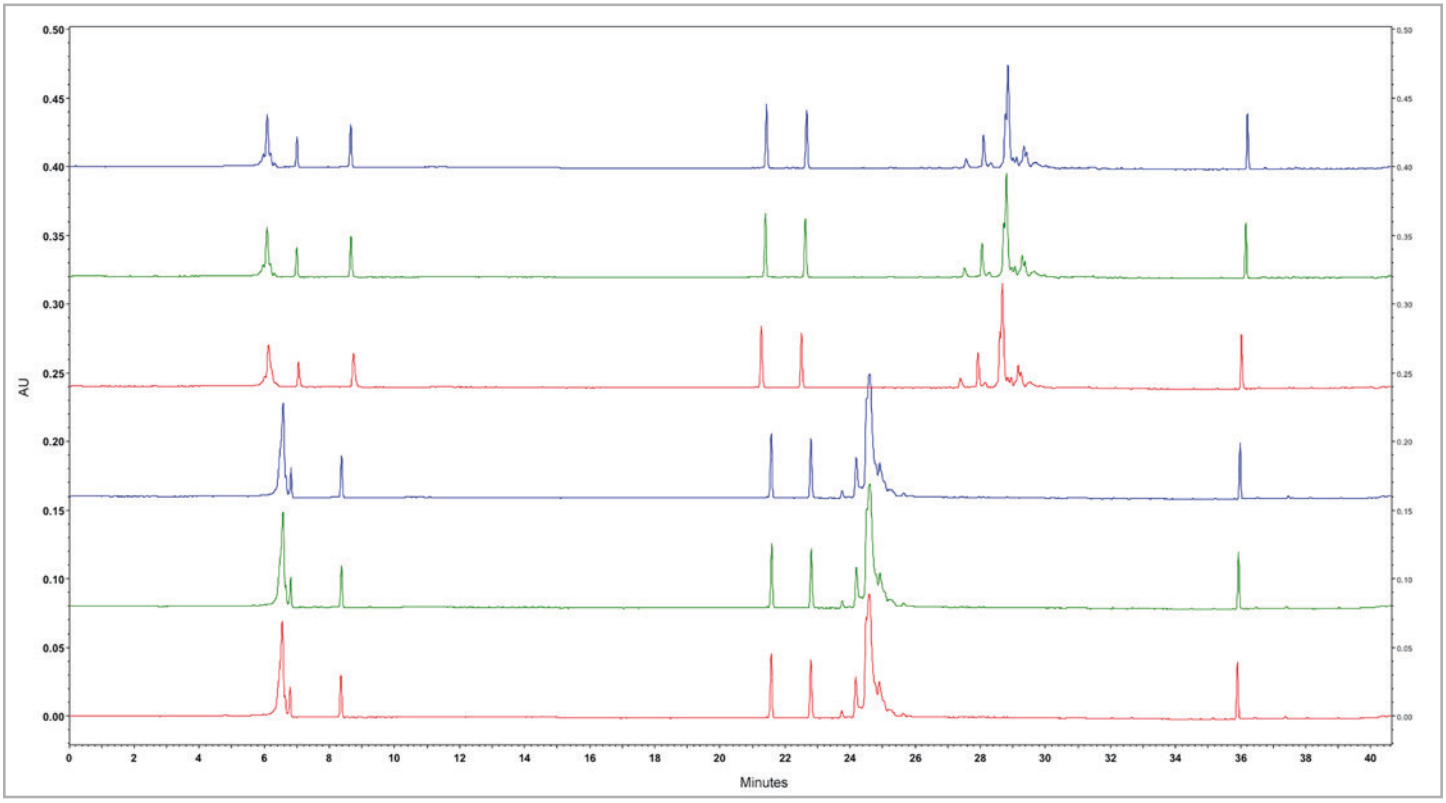


Figure 7. cIEF separation profiles of USP (top three traces) and NIST IgG (bottom three traces) using one master mix formulation of 3-10 ampholytes and one separation method. pI values of USP IgG are in the pH 7.0-7.6 range. pI values of NIST IgG are in the pH 8.7-9.5 range. Peptide pI markers 10.0, 9.5, and 5.5 were used in the master mix.

Over 100 runs of USP IgG were made over the course of 5 days on multiple PA 800 Plus systems with capillaries from multiple lots. Resolution and reproducibility, as displayed in Figures 8, 9, and 10, and calculated in Tables 2 and 3, were excellent.

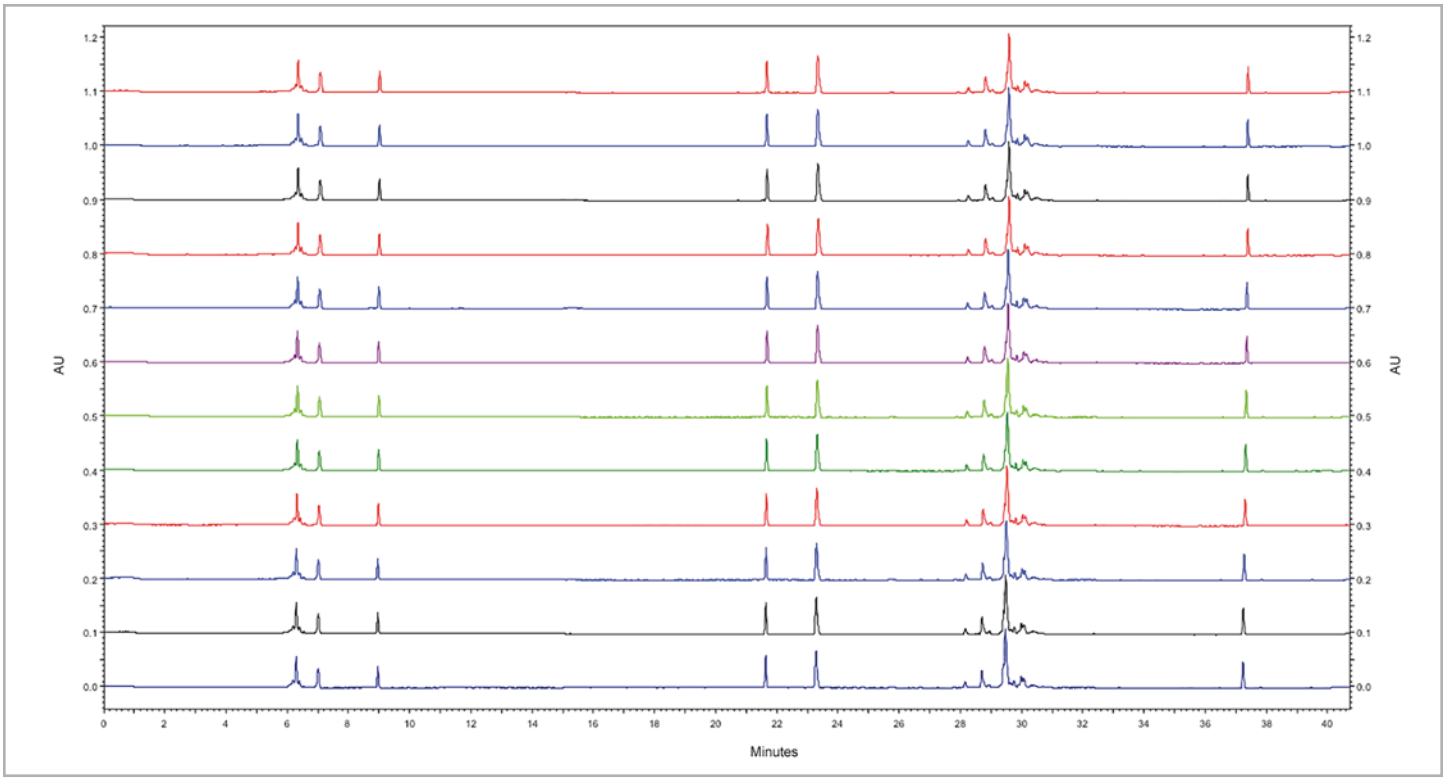


Figure 8. USP IgG, 12 consecutive cIEF separations. Run # 13–24 pI markers are 10.0, 9.5, and 5.5.

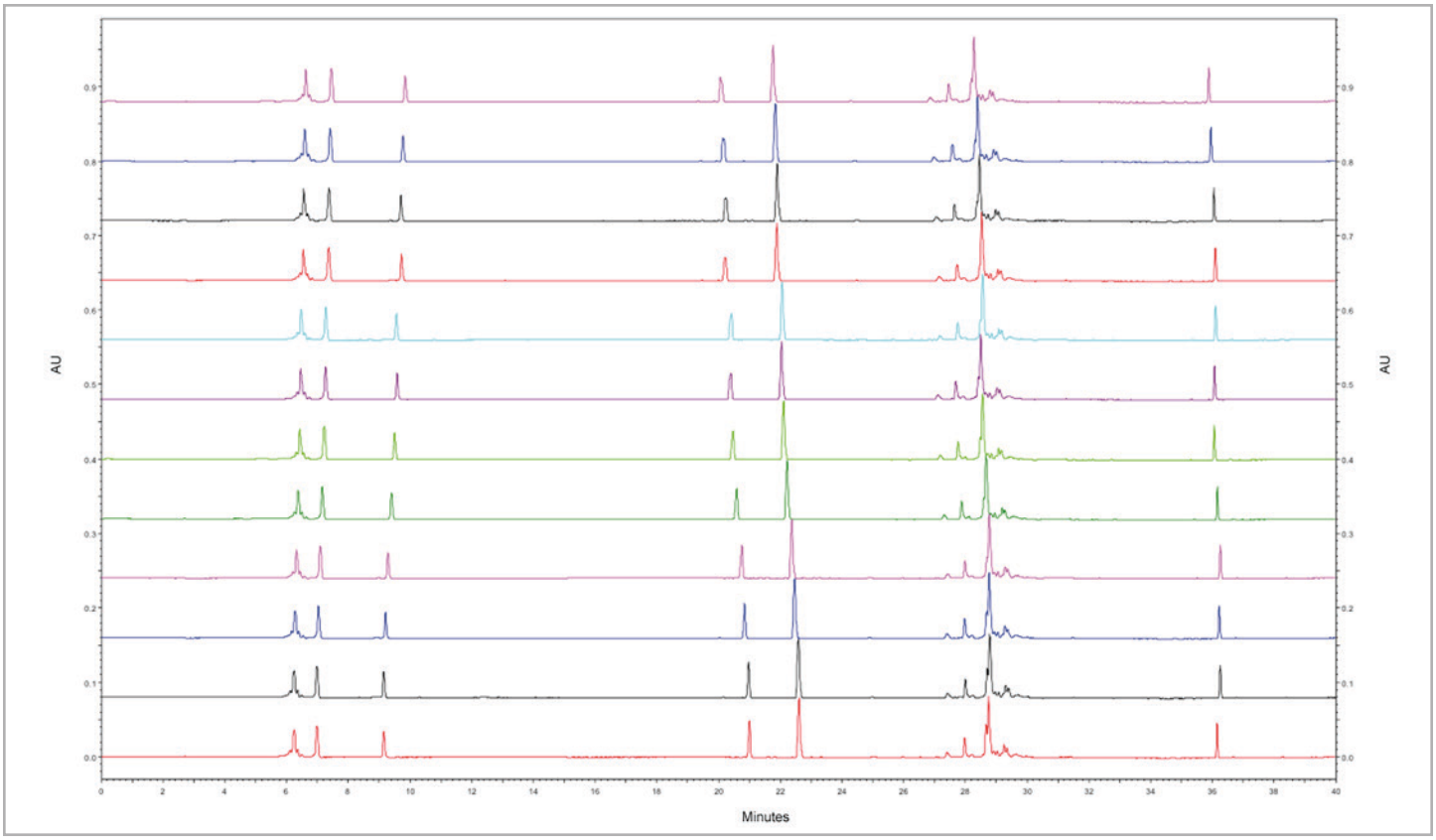


Figure 9. USP IgG, 12 consecutive cIEF separations. Run # 97–108 pI markers are 10.0, 9.5, and 5.5.

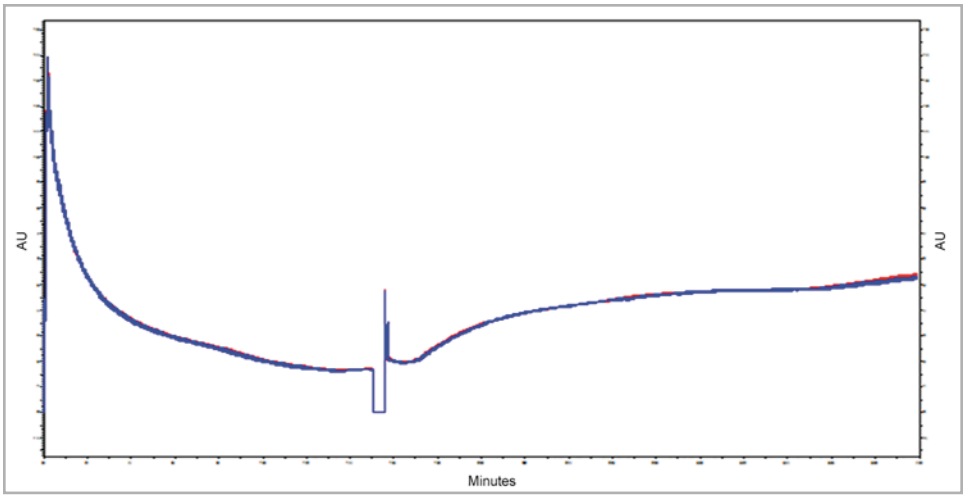


Figure 10. Overlaid CE electrical current traces of 24 consecutive cIEF separations.

	Neutral Capillary Lot 1			Neutral Capillary Lot 2			Neutral Capillary Lot 3		
	Basic	*Main*	*Acidic*	*Basic*	*Main*	*Acidic*	*Basic*	*Main*	*Acidic*
AVE	18.23	59.38	22.40	18.10	60.46	21.44	18.87	59.28	21.85
STD	0.306	0.567	0.397	0.661	0.709	0.674	0.770	0.787	0.913
%RSD	1.68	0.95	1.77	3.65	1.17	3.14	4.08	1.33	4.18

Table 2: Three different neutral capillary lots tested using multiple PA800 Plus instruments on five different days for each lot. Normalized peak areas of USP IgG are reported (N=100).

	Day 1 (Runs 1–24)		Day 2 (Runs 25–48)		Day 3 (Runs 49–72)		Day 4 (Runs 73–96)	
	Acidic 1	*Acidic 2*	*Acidic 1*	*Acidic 2*	*Acidic 1*	*Acidic 2*	*Acidic 1*	*Acidic 2*
AVE	7.53	7.50	7.55	7.52	7.56	7.53	7.53	7.50
STD	0.00	0.00	0.01	0.01	0.01	0.01	0.02	0.02
%RSD	0.03	0.02	0.1	0.11	0.07	0.08	0.22	0.30

Table 3: Calculated pI of Acidic 1 & Acidic 2 peaks of USP IgG (N=19).

Conclusions

Capillary isoelectric focusing is an important tool for the characterization of therapeutic monoclonal antibodies. However, a variety of internal (instrumentation- and method-related) and external (sample-related) factors can make it difficult to achieve the needed resolution, repeatability, and reproducibility over large numbers of runs. This work demonstrated that with improved capillary manufacturing processes, an optimized method, and excellent instrumentation, over 100 reproducible analyses of immunoglobulin G can be achieved with pI resolution as good as 0.03.

Reference

1. Mack, S., Cruzado-Park, I., Chapman, J., Ratnayake,C., Vigh, G., *Electrophoresis* 2009, 30, 4049-4058.
2. Bonn, R., Rampal, S., Rae, T., Fishpaugh, J., *Electrophoresis* 2013, 34, 825-832.

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