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A Robust cIEF Method: Intermediate Precision for the pH 5-7 Range

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Introduction

Optimization of the numerous variables associated with Capillary Isoelectric Focusing (cIEF) allows for the ability to obtain highly resolved, reproducible separations (Beckman Coulter Application Information Bulletin[1], p/n A-11634A). Taking key assay elements into consideration, a cIEF separation method was developed using mouse IgG1κ as model protein. Narrow-range ampholytes 5-8 were used for maximum resolution and urea was used as a solubilizer to prevent protein precipitation and aggregation during focusing. The high resolution obtained by the cIEF separation method was able to differentiate the distribution of charge heterogeneity between different commercial batches of IgG1κ. This separation was tested for reproducibility and robustness by performing an intermediate precision study with five instruments, three operators and different sets of reagents. Results showed a high level of reproducibility for cIEF following careful optimization of key experimental parameters including sample preparation, separation temperature and control sample solubility.

Experimental

Instrumentation and Conditions. All experiments were performed using the PA 800 Protein Characterization System (Beckman Coulter, Inc., Fullerton, CA) equipped with a UV detector and a 280 nm filter. All separations were carried out using a Neutral Capillary (Beckman Coulter, p/n 477441), which was 30.2 cm long and 20 cm from inlet to detector. An aperture of 200 μm was used in the capillary cartridge. The capillary temperature was maintained at 20°C in all separations, unless otherwise specified. Chemical mobilization was used in all cIEF experiments and voltage was applied in normal polarity.

Chemical Reagents. DDI water was obtained from an E-pure* deionizer (Barnstead Thermolyne, Dubuque, IA). Pharmalyte* 5-8 carrier ampholytes (p/n 17-0453-01) were purchased from GE-Healthcare Bio-Sciences AB (Uppsala, Sweden). Synthetic peptides were manufactured and used as peptide pI markers[2] (Table 1).

Each peptide solution was 1.25 mM in DDI water. Peptide solutions were aliquoted in 0.5 mL volumes and stored at -20°C until needed. During use, the peptide solutions can be stored at 2-8°C for up to 4 months.

The following solutions were prepared and used in all cIEF experiments. These solutions were stored at room temperature, unless indicated otherwise, and discarded 30 days after their preparation date.

Peptide	pI Value
Trp-Glu-His-Arg	7.0
Trp-Glu-His-His	6.7
Trp-Glu-His	5.5

Table 1: List of synthetic peptide pI markers and their pI values.

Anolyte: The anolyte consisted of a 200 mM phosphoric acid solution. It was prepared by diluting 685 µL of 85% phosphoric acid (Sigma-Aldrich, St. Louis, MO, p/n 343245) to a final volume of 50.0 mL with DDI water.

Catholyte: The catholyte consisted of a 300 mM sodium hydroxide solution. It was prepared by dissolving 0.60 g of sodium hydroxide (J.T.Baker, Phillipsburg, NJ, p/n 3722-11) in DDI water to a final volume of 50.0 mL.

Chemical Mobilizing Solution: This solution consisted of 350 mM acetic acid. It was prepared by diluting 1.0 mL of glacial acetic acid (Sigma, p/n A6283) with DDI water to a final volume of 50.0 mL.

Cathodic Stabilizer: The cathodic stabilizer consists of a 0.5 M arginine solution. It was prepared by dissolving 0.87 g of arginine (Sigma, p/n A5006) in 10.0 mL of DDI water.

Anodic Stabilizer: The anodic stabilizer consisted of a 0.2 M iminodiacetic acid (IDA, Sigma, p/n 220,000) solution. It was prepared by dissolving 0.27 g of IDA in 10.0 mL of DDI water.

Capillary Cleaning Solution: This solution consisted of 4.3 M urea in DDI water. It was prepared by mixing 10.8 g of urea (Sigma, p/n U0631) in 30.0 mL of DDI water. The solid material was dissolved by vortexing it for at least 15 min. This solution was stored at 2-8°C to minimize urea degradation.

Urea-cIEF Solution: Solutions with known molar concentrations of urea were prepared by dissolving the required amount of urea (Sigma, p/n U0631) to a final volume of 10.0 mL using cIEF gel (Beckman Coulter, p/n 477497). The urea-cIEF gel solutions were stored at 2-8°C to minimize urea degradation.

Desalting the IgG Sample. Three different commercial lots of mouse IgG1κ (Sigma, p/n M9269) were buffer exchanged with 20 mM Tris, pH 8.0 buffer before cIEF. A 20 mM Tris, pH 8.0 buffer was prepared by diluting 4.0 mL of 50 mM Tris buffer, pH 8.0 (Beckman Coulter, p/n 477427) to a final volume of 10.0 mL with DDI water. The IgG1κ sample (each vial contains 1 mL at a concentration of 1.1 mg/mL) was thawed at room temperature. Next 500 µL of IgG sample was placed into a Microcon* Centrifugal Filter Device YM-30 (p/n 42410, Millipore, Bedford, MA). Immediately the samples were centrifuged at 12,000 rcf for 5 min in a Microfuge® 18 (Beckman Coulter). The flow-through was discarded when necessary. Next, 250 µL of Tris buffer were added into each Microcon device and the samples were again centrifuged at 12,000 rcf for 10 min. Again, 250 µL of Tris buffer were added into each Microcon device and the samples were centrifuged at 12,000 rcf for 3 min. Finally, 200 µL of Tris buffer were added into each Microcon device and centrifuged for 5 min at 12,000 rcf. The volume inside each Microcon filter was collected by placing the inverted Microcon filters into new centrifuge tubes and centrifuging at 2,000 rcf for 3 min. The volume collected was measured with a pipet and used to calculate the IgG concentration in the desalted sample, which was adjusted to 5.0 mg/mL. Finally, the IgG sample was aliquoted in 5.0 µL volumes and stored at -20°C until use.

cIEF Methods. The following three 32 Karat™ Software methods were used:

- Capillary Conditioning – conditions the Neutral Capillary at the start of each working day.
- cIEF 5-8 Separation – performs the cIEF separation of IgG1κ in the pH 5-8 range.
- Shutdown – rinses the capillary before storage and turns off the UV lamp.

All three methods use the same Initial Conditions (Fig. 1) and UV Detector Settings (Fig. 2). In summary, the detector was set to direct absorbance at 280 nm with a collection rate of 2 Hz. The electronic filter of the detector was set to normal with a peak width of 16-25 points. All separations were performed at a capillary temperature of 20°C, unless indicated otherwise. The system autosampler was set to a temperature of 10°C. The trigger settings for the cartridge coolant and the sample storage were checked in to ensure the system is in equilibrium before use. The maximum electrical current was set to 20 µA. The auxiliary data channel corresponding to the electrical current was checked in, in order to save its data with its corresponding UV trace.

Each method has a different time program, as explained next.

Capillary Conditioning Method. The Neutral Capillary was conditioned with the following 50 psi rinses: DDI water for 2 min, chemical mobilizing

Figure 1: Initial conditions in Capillary Conditioning, cIEF 5-8 Separation and Shutdown methods.

Figure 2: UV detector settings in Capillary Conditioning, cIEF 5-8 Separation and Shutdown methods.

solution for 2 min and cIEF gel for 5 min. The rinses were performed in the forward direction and collected in an empty vial used as waste. The method ended by submerging both capillary ends in DDI water.

cIEF 5-8 Separation Method. The cIEF separation method started by performing two 50 psi rinses in the forward direction: capillary cleaning solution for 3 min and then DDI water for 2 min. The mixture of sample, ampholytes and pI markers was introduced into the capillary by performing a 99.9 sec injection at 25 psi. Immediately, both capillary ends were cleaned by submerging both capillary ends in DDI water for a few seconds. Focusing was performed at 25 kV for 5 min under normal polarity with the inlet side of the capillary submerged in anolyte and the outlet side submerged in catholyte. Chemical mobilization was carried out at 30 kV for 30 min under normal polarity, with the inlet side of the capillary submerged in anolyte and the outlet side in chemical mobilizer. The voltage ramp of 0.17 min was used for both focusing and mobilization steps. At the end of the mobilization step, the data collection was stopped, the capillary was rinsed with DDI water for 2 min at 50 psi, and then both capillary ends were submerged in DDI water. The vials used in the separation method were incremented every three consecutive runs to minimize carry over.

Shutdown Method. The shutdown method consisted of a 2 min rinse with DDI water followed by a 10 min rinse with cIEF gel. Both rinses were carried out at 50 psi in the forward direction. The UV lamp was turned off at the end of the final rinse.

The configuration of the buffer trays is shown in Fig. 3. The glass vials (Beckman Coulter, p/n 144980) were filled with 1.6 mL of the indicated cIEF reagent and placed at the specified location on the buffer trays. An empty glass vial was used for waste and all vials were capped using red caps (Beckman Coulter, p/n 144648). Arrows in the buffer tray configuration indicate vials that are incremented every three consecutive runs as programmed in the cIEF 5-8 Separation method. All cIEF separations were repeated at least three times to determine reproducibility of the results.

Preparation of cIEF Samples. Samples were prepared by mixing the following reagents at the volume ratios indicated in Table 2: urea-cIEF gel solution, Pharmalyte 5-8, cathodic stabilizer, anodic stabilizer, peptide pI markers (Table 1), and desalted mouse IgG1κ. Samples were mixed by vortexing. A 200 μL volume of cIEF sample mixture was transferred to a PCR vial (Beckman Coulter, p/n 144709), which was placed in a PCR vial holder (Beckman Coulter, p/n 144657) equipped with a micro vial spring (Beckman Coulter, p/n 358821) and then capped with a gray cap (Beckman Coulter, p/n 144656). The cIEF samples were placed at the inlet sample tray and analyzed the same day.

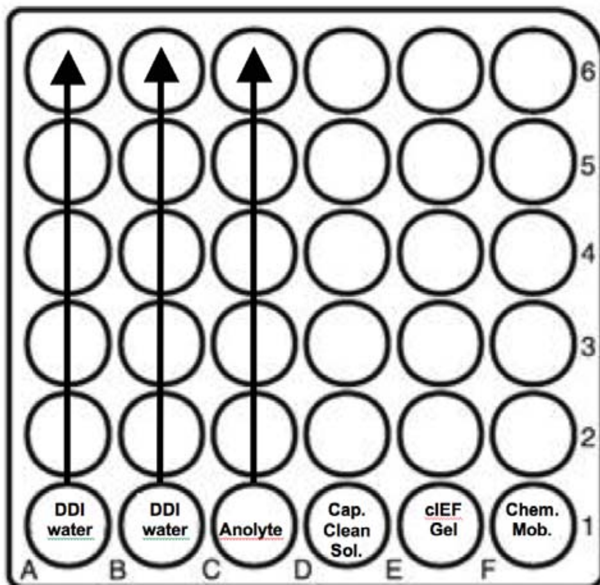
Results and Discussion

Accurate Pipetting Increases Robustness. The cIEF step most susceptible to errors is sample preparation because it requires mixing microliter volumes of multiple reagents (cIEF gel, ampholytes, sample, pI markers and stabilizers). Even minor errors in pipetting can lead to significant differences in both qualitative and quantitative aspects of the cIEF separation and can be exacerbated especially when measuring small volumes, such as 2.0 μL. The preparation of large-volume mixtures, referred to as a master mix, can help in prevention of this error (Table 2).

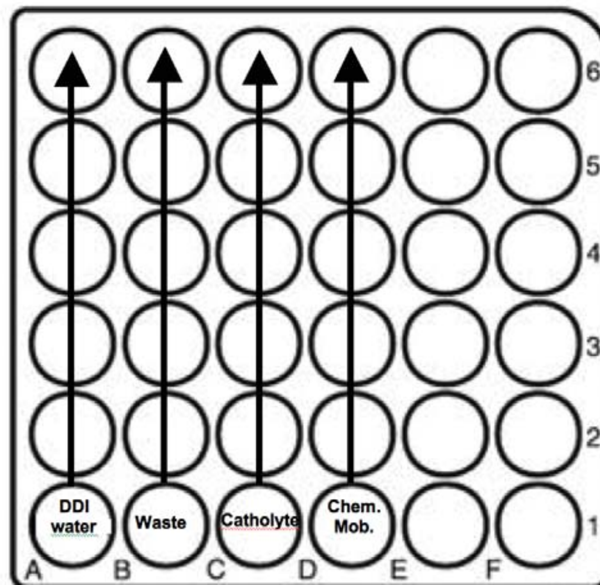
Mix the master mix completely by repeated aspiration using a pipet. It is critical that all reagents are mixed evenly throughout the sample to ensure maximum reproducibility. Vortexing may not be sufficient to overcome differences in density of the various reagents.

Differences Between Commercial Lots of IgG1κ. Three commercial lots of mouse IgG1κ were separated by cIEF under identical experimental conditions using 3.0 M urea in the sample and a capillary separation temperature of 20°C (Fig. 4). Each commercial lot yielded a unique cIEF IgG profile due to its charge heterogeneity. The effects of urea concentration and separation temperature in the cIEF separation were examined using IgG commercial lots #1 and #2, respectively. IgG commercial lot #3 was chosen to study the reproducibility of the cIEF method due to the simplicity of its profile.

Optimization of the Urea Concentration in the cIEF Sample. Urea retains solubility and helps prevent aggregation of proteins as they concentrate at their pI during focusing. Because of the intrinsic variability of proteins, optimal concentration of urea in the sample ultimately depends on the solubility of the protein to be analyzed. The cIEF profile of mouse IgG1κ lot #1 was evaluated using different urea concentrations (Fig. 5). In an environment lacking urea, the separation profile for IgG1κ is poorly defined, presumably due to its low solubility. In contrast, the



BI (Inlet Buffer Tray)



BO (Outlet Buffer Tray)

Figure 3: Buffer tray configuration for Capillary Conditioning, cIEF 5-8 Separation and Shutdown methods. "Cap. Clean Sol." stands for capillary cleaning solution, and "Chem. Mob." for chemical mobilizing solution.

Reagent	Volume per sample (µL)	Number of Samples	Total volume to be measured (µL)
cIEF gel	200	X 5 =	1,000
Pharmalyte 5-8	6.0	X 5 =	30.0
Cathodic Stabilizer	9.0	X 5 =	45.0
Anodic Stabilizer	5.0	X 5 =	25.0
pI 7.0 marker	2.0	X 5 =	10.0
pI 6.7 marker	2.0	X 5 =	10.0
pI 5.5 marker	2.0	X 5 =	10.0

Table 2: Example of the preparation of a cIEF master mix for four samples of mouse IgG1κ using Pharmalyte 5-8. Enter the number of required samples plus one (no less than 3) and multiply each reagent volume by it. In this example, the number of samples is four plus one, which equals to 5. Next, prepare each IgG sample by mixing 226 µL of cIEF master mix with 20 µL of desalted IgG1κ (5 mg/mL). The master mix can be stored at 2-8°C and is usually discarded at the end of the working day.

IgG isoforms are clearly defined when using 1.5 and 3.0 M urea. At 4.5 and 6.0 M urea, the IgG profile degrades, possibly due to protein denaturation caused by high urea concentration. Further analysis indicates that the pI values of the IgG sample were unaffected by urea concentration (Table 3).

Multiple separations were carried out using 1.5 and 3.0 M urea to determine which concentration yielded the best reproducibility for the IgG profile. The optimum concentration of urea for IgG1κ was determined to be 3.0 M because it yielded the lowest variability of IgG peak area composition[3]. Since the ionic products associated with the thermal degradation of urea are known to be detrimental to the cIEF separation[4], the samples were stored at 10°C in the PA 800 instrument to minimize urea degradation.

Effect of Temperature on cIEF. Separation temperature has a significant effect on the cIEF resolution of IgG1κ (Fig. 6). The IgG peaks D, E and F were less resolved when the temperature was 25°C and above. However, IgG peak A was more defined at temperatures above 15°C. Overall detection time decreased with increasing temperature, thus

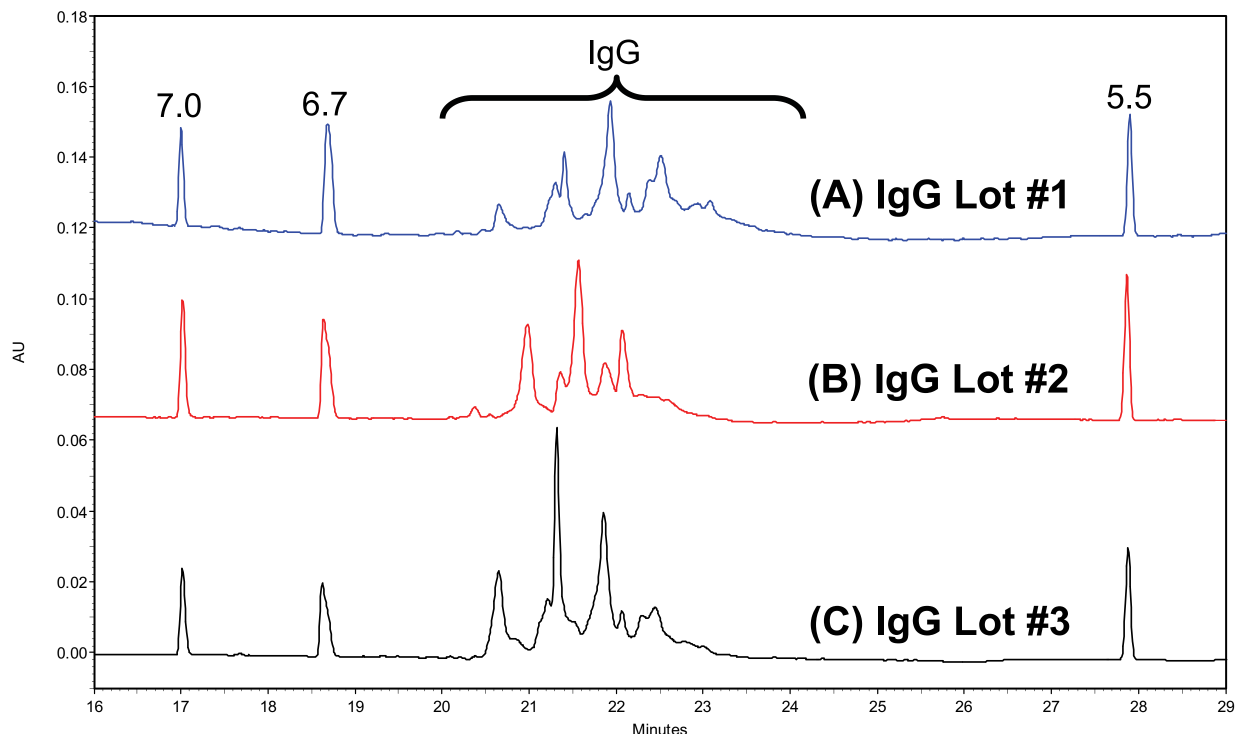


Figure 4: The cIEF separation of different commercial lots of mouse IgG1κ under identical separation conditions: (a) lot #1, (b) lot #2, and (c) lot #3. Data was aligned and stretched with respect to the detection times of the pI 7.0 and 5.5 markers for easy comparison.

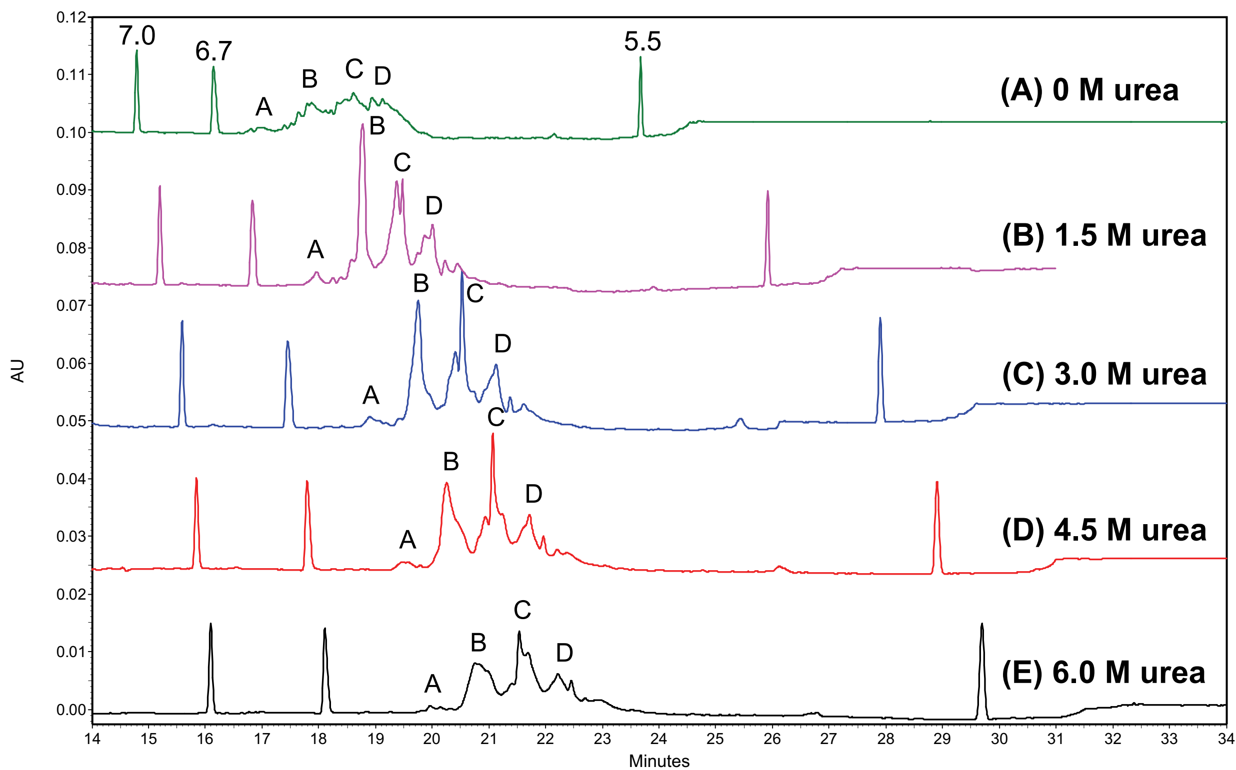


Figure 5: The cIEF separation of mouse IgG1κ from commercial lot #1 at different urea concentrations: (a) 0, (b) 1.5, (c) 3.0, (d) 4.5 and (e) 6.0 M urea in cIEF gel. Sample: cIEF master mix using 10.0 μL of mouse IgG1κ (5 mg/mL). IgG peaks are labeled A, B, C and D.

[Urea] (M)	pI value of IgG Peak A	pI value of IgG Peak B	pI value of IgG Peak C	pI value of IgG Peak D
0.0	6.61	6.45	6.33	6.28
1.5	6.58	6.47	6.38	6.30
3.0	6.53	6.47	6.37	6.30
4.5	6.54	6.47	6.38	6.30
6.0	6.54	6.46	6.38	6.30

Table 3: The pI values of the IgG1κ peaks (A, B, C and D) indicated on Fig. 8 at different urea concentrations. The pI values of the IgG isoforms were calculated using a linear relationship between detection time and the theoretical pI values of the peptide pI markers.

compressing the pH gradient and resulting in loss of resolution. These data illustrate the importance of temperature control in obtaining reproducible separation profiles.

Reproducibility of cIEF Method. The cIEF method was tested for reproducibility by performing seven sets of six consecutive runs (n=42) of the same desalted IgG1κ sample using five different instruments and three analysts utilizing different cIEF chemical reagents and different lots of Neutral Capillary over a one week period. Each set of cIEF runs provided IgG profiles of high reproducibility (Fig. 7). The IgG profile was divided into four major peaks (Fig. 8). Table 4 summarizes reproducibility of the cIEF separations with regard to peak migration time, pI value, and IgG peak area composition (peak area %). Minimal fluctuation in peak profile and migration time was observed within each set of runs (Fig. 7). The standard deviation in pI was less than 0.01 pH units. The standard deviation in IgG peak area composition was less than 1.5 %. In conclusion, these data demonstrate the robustness of this cIEF method and chemistry, yielding high reproducibility in terms of pI values and IgG composition.

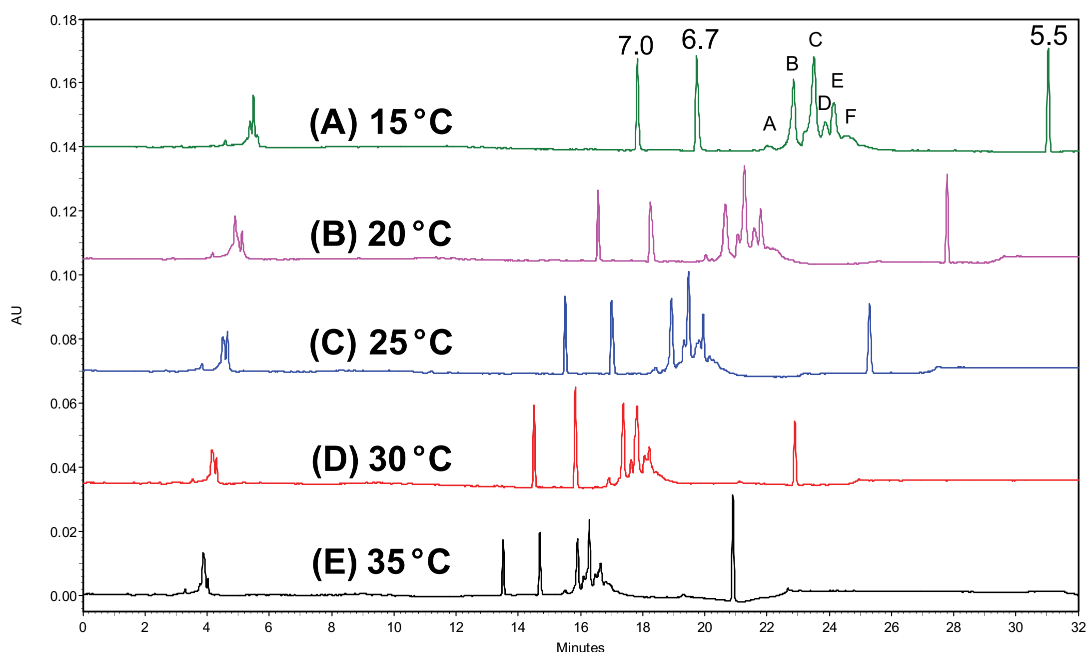
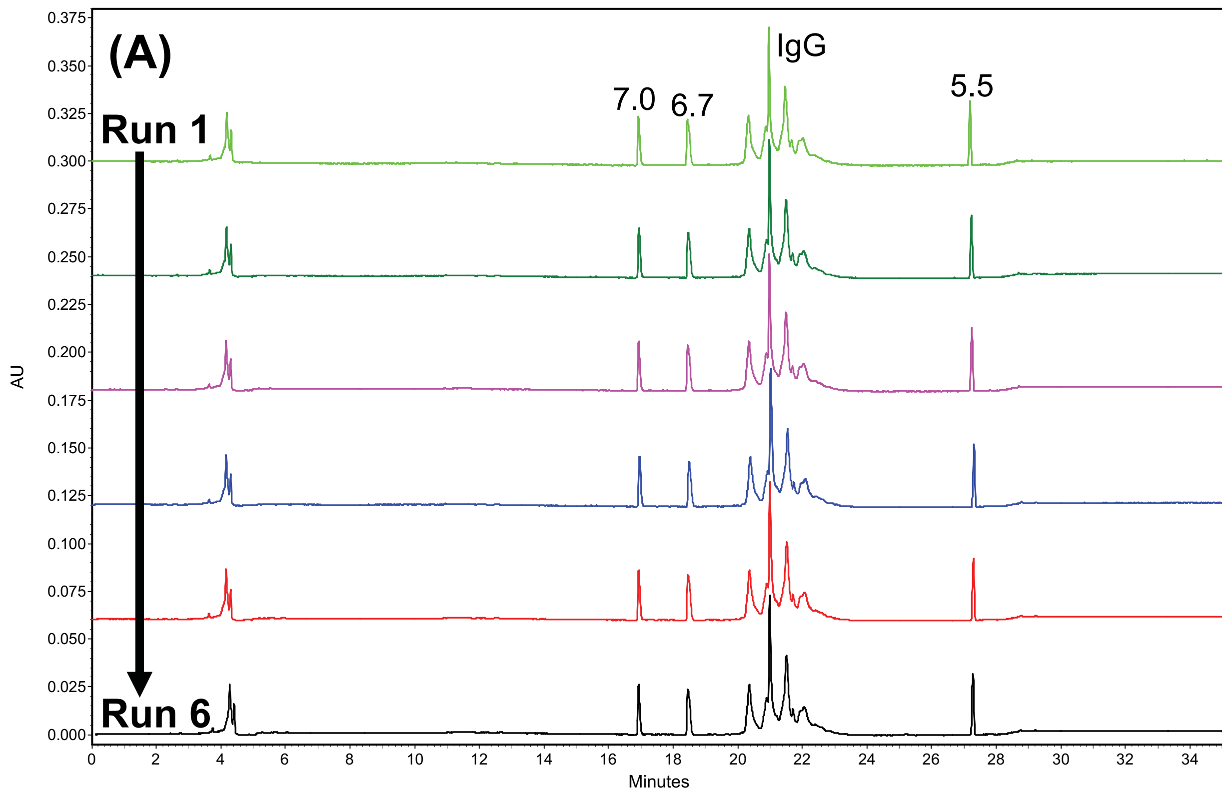


Figure 6: The cIEF separation of mouse IgG1κ from commercial lot #2 at different separation temperatures: (a) 15, (b) 20, (c) 25, (d) 30 and (e) 35 °C. IgG peaks are labeled A - F.



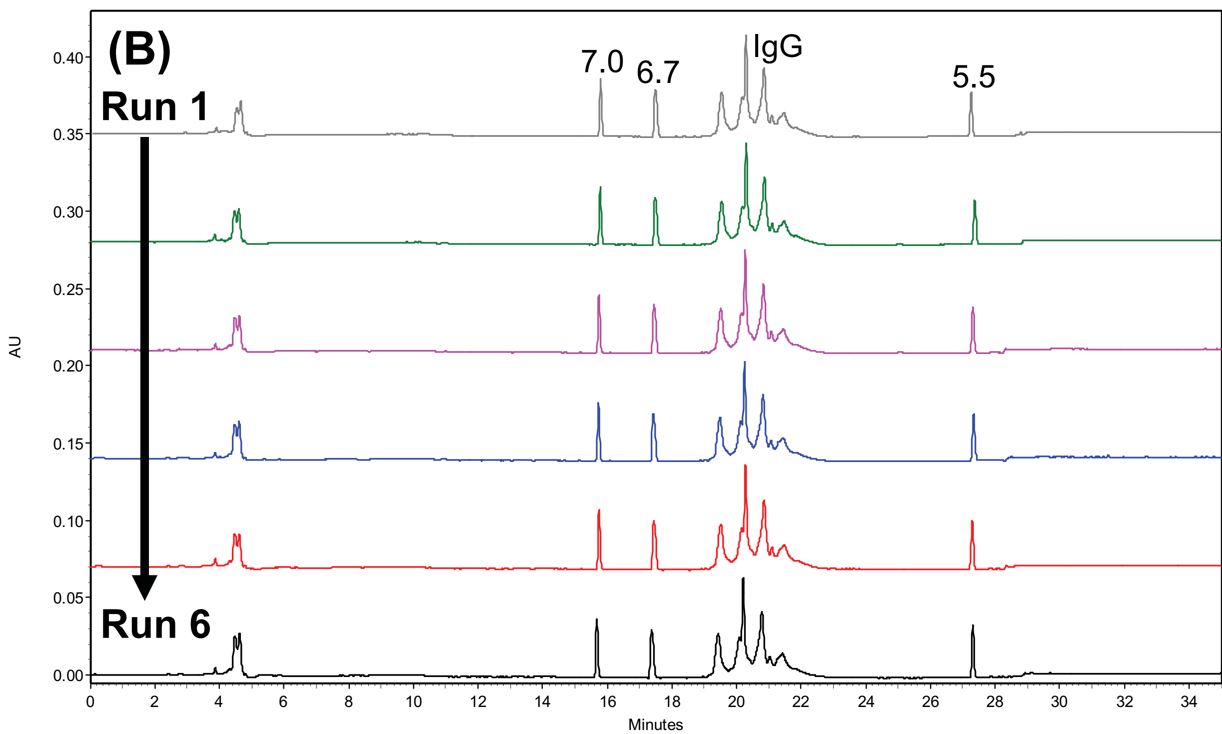


Figure 7: Sets (A) and (B), each with six consecutive cIEF separations of mouse IgG1κ using the same desalted IgG (commercial lot #3) sample and cIEF separation method. Each set was performed using a different PA 800 system, operator, reagent solutions and Neutral Capillary lot.

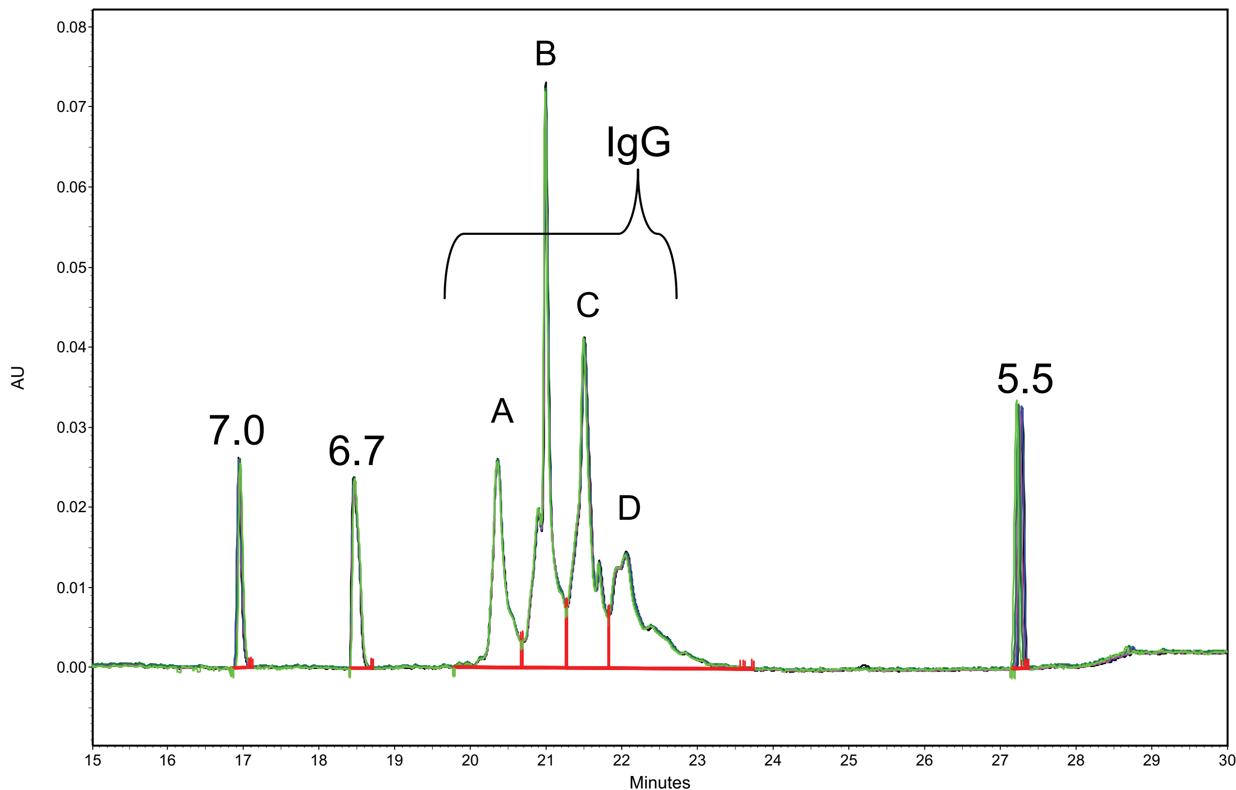


Figure 8: Overlay of six consecutive cIEF separations of mouse IgG1κ lot #3 from set (A), Fig. 10. Data was aligned with respect to the detection time of the pI 7.0 marker. IgG charge isoforms were grouped into four main peaks, which are labeled A, B, C and D.

	Time (min)	Time (min)	Time (min)
Peak	Mean	Std Dev	%RSD
pI 7.0	16.632	0.571	3.435
pI 6.7	18.239	0.513	2.815
IgG a	20.227	0.471	2.329
IgG b	20.916	0.437	2.090
IgG c	21.454	0.426	1.985
IgG d	22.021	0.396	1.800
pI 5.5	27.530	0.341	1.239
	pI exp	pI exp	pI exp
Peak	Mean	Std Dev	%RSD
pI 7.0	6.957	0.008	0.115
pI 6.7	6.741	0.007	0.103
IgG a	6.473	0.007	0.114
IgG b	6.381	0.007	0.115
IgG c	6.308	0.007	0.117
IgG d	6.232	0.009	0.152
pI 5.5	5.492	0.009	0.158
	%Area	%Area	%Area
Peak	Mean	Std Dev	%RSD
IgG a	16.34%	0.59%	3.61
IgG b	31.80%	1.10%	3.46
IgG c	29.58%	0.85%	2.89
IgG d	22.28%	1.25%	5.63

Table 4: Average detection times, pI values and peak area percent composition from intermediate precision study (n=42) of the cIEF separation of mouse IgG1κ lot #3 sample.

Conclusions

The results achieved in this study are a demonstration of the high level of robustness of this cIEF chemistry. The cIEF method developed for IgG1κ can be utilized for the separation of therapeutic IgG molecules in the pH 5-7 range. Critical factors to consider while pressing on for maximum reproducibility are concentration of urea within the sample, capillary temperature during the separation, and accurate pipetting and uniform mixing during sample preparation. A clear understanding of these variables is critical in the development and validation of new cIEF methods.

See References on next page.

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Notes

Notes

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