Using Pharmalyte® in capillary isoelectric focusing (cIEF) for charge variant analysis of monoclonal antibodies

Pharmalyte is an excellent ampholyte option for capillary isoelectric focusing (cIEF) of proteins providing robust high resolution separations of charge variants

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There is an increased demand in the biopharmaceutical industry for improved resolution and an increase in robustness of standard capillary isoelectric focusing electrophoresis (cIEF) methods for charge variants separation in monoclonal antibodies (mAb) quality control. cIEF was designed as a substitute for gel isoelectric focusing, which is used for the separation of molecules by the difference in their isoelectric point (pI). It has been used in quality control of mAb produced by biopharmaceutical companies for over 10 years.[1]

cIEF works by mixing the protein sample with ampholytes, which are mixtures of charged molecules with a range of isoelectric points (pI) that encompass the pI range of the protein sample. The whole mixture is then injected into a capillary, an electric voltage is applied, and the migration of the ampholytes encourages the proteins to move along the pH gradient until they reach a point where they have no charge.[2] Ampholyte quality can have a big impact on results, so the source of these chemicals is important. Pharmalyte ampholytes are mixtures of 600-700 different amphoteric compounds and are synthesized by the co-polymerization of glycine, glycylglycine, various amines and epichlorhydrin. Because of its hydrophilic nature, Pharmalyte does not bind to proteins and is available in both wide and narrow pH ranges which makes it ideal for tailor made mixtures to produce high resolution and reproducible separation of protein charge variants.

Key benefits of using Pharmalyte for cIEF

- Reduction in background interferences speeding up analysis by improving peak integration
- Improves data quality by providing highly reproducible pI values for protein charge variants
- Highly adaptable providing different pI ranges to fine tune the separation for the target protein

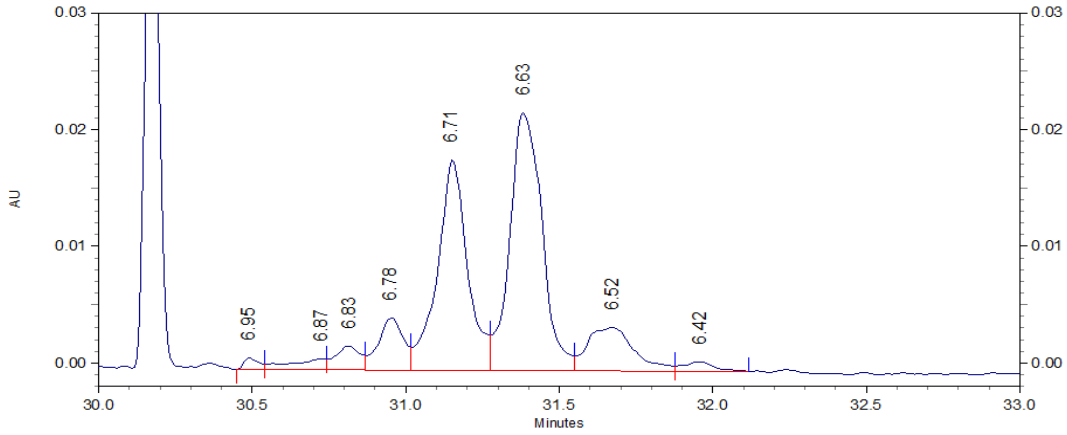


Figure 1. cIEF electropherogram for Vectibix highlighting a typical result obtained using Pharmalyte 3-10 and demonstrating a high-resolution separation of protein charge variants with the peaks labeled with the pI values for the charge variants.

Materials and methods

Materials:

L-arginine free base (PN A5006-100G), iminodiacetic acid, 98% (PN 220000-25G), urea BIOXTRA, pH 7.5-9.5 (PN U0631-500G), glacial acetic acid, >=99.85% (PN 695084-1L), phosphoric acid, 85 wt. % solution (PN 345245-100ML), sodium hydroxide, 1.0 N for HPCE (PN 72082-100ML) and Amicon Ultra-0.5 Centrifugal Filters with 10,000 NMWL were obtained from Merck (Darmstadt, Germany). Sample Loading Solution (PN 608082), Universal vials (PN A62251), Universal vial caps (PN A62250), PCR vials (PN 144709), blank cartridge (PN 144738) and Advanced cIEF Starter Kit (PN A809960, which included eCAP Neutral Capillary (PN 477441), cIEF Gel buffer 100 mL (PN 477497) and pI Peptide Marker Kit PN A58481)] were from SCIEX (Framingham, MA, U.S.A.). Pharmalyte 3-10 (PN 17045601) and Pharmalyte 5-8: (PN 17045301) Pharmalyte 3-10 were obtained from Cytiva (Uppsala, Sweden).

A PA 800 Plus Pharmaceutical Analysis System (SCIEX, Framingham, MA, U.S.A.) equipped with UV detector and 32 Karat™ Software were used for all the experiments. Data acquisition and processing were performed using 32 Karat Software.

Vectibix (20 mg/mL) was purchased from Distansapoteket, (Stockholm, Sweden) and, for analysis, was diluted 1 in 4 with water to obtain a final sample concentration of 5 mg/mL.

Sample preparation:

For broad range separations, a master mixture was prepared by combining cIEF gel containing 3.75M urea (200 µL, SCIEX PN 477497 prepared as described in SCIEX application guide) with Pharmalyte 3-10 (12 µL), cathodic stabilizer (20 µL, 500 mM L-arginine in water), anodic stabilizer (2 µL, 200 mM iminodiacetic acid), four pI markers 9.5, 7.0, 5.5. and 4.1 (each 2 µL, SCIEX PN A58481), water (4 µL) and diluted Vectibix sample (4 µL, 5mg/mL). Three independent samples were prepared.

Capillary electrophoresis:

All separations were performed using a neutral capillary (capillary length 30.2 cm, effective length 10 cm). Before the analysis started, the capillary was pretreated using the standard conditioning method involving water, sample loading solution and then two additional independent water rinse steps. Before each analysis the capillary was rinsed again with sample loading solution (1 min, 50 psi), water vial 1 (3 min, 20 psi), water vial 2 (2 min, 50 psi), and then the capillary was loaded with sample master mix (150 seconds, 15 psi). The capillary was cleaned using a dip step into a vial containing water on the outlet and inlet side. Proteins were focused by applying a separation voltage (25 kV positive mode, 15 minutes) at the inlet cathode capillary end, which was placed into 200mM phosphoric acid. The outlet anode end was in 300mM sodium hydroxide at the same time. Following separation, the outlet end was cleaned using a water dip step before placing in 300mM acetic acid and the peaks chemically mobilized to the detector by applying voltage (30kV, 25 minutes).

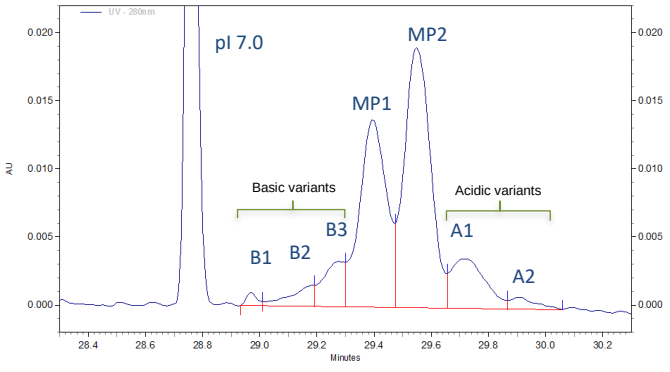
At the end of every batch a shutdown method was run which involved rinsing the capillary with water, sample loading solution and then two additional independent water steps.

Results and discussion

cIEF is designed for efficient separation of proteins by their isoelectric points (pI), using a stable and reproducible pI gradient that is generated by the ampholyte carrier stabilized by anodic and cathodic stabilizers. In cIEF, the resolution between peaks is affected by the type of ampholyte carrier (pI range) and gradient stretch (final ampholyte concentration and stabilizers) used. The quality of ampholytes impacts not only the goodness of fit of the generated pI gradient but also the stability of the linear gradient.

In this study, Vectibix (monoclonal antibody, panitumumab) was used to determine stability and reproducibility of migration time, corrected peak area distribution and the pI values across the same batch of wide range Pharmalyte 3-10. Three independent sample preparations were used, each injected 8 times in one single sequence of 24 runs. The pI values of the Vectibix peaks were determined by using bracketing pI markers of pI 7.0 and 5.5, with the gradient linearity calculated with four markers including additional pI markers at 9.5 and 4.1. Figure 1 shows a typical electropherogram for Vectibix.

Figure 2 Zoomed in section of cIEF electropherogram for Vectibix test sample obtained from using Pharmalyte 3-10.



Three different basic variants (B1 to B3), two well separated main peaks (MP1 and MP2) and two acidic isoforms (A1-A2) were identified for Vectibix (Figure 2).

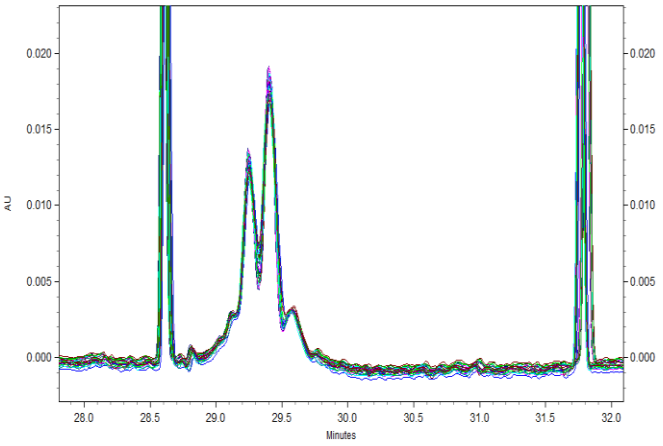


Figure 3. Overlay of all 24 runs of Vectibix samples with an x-axis offset to align the electropherograms around the pI markers.

In this analysis, peaks were clustered into one of three isoform groups: basic variants, main peaks and more acidic variants, because of very small differences in pI values observed for the peaks. This allows comparison of stability of corrected peak area distribution between selected three charge variants groups.

Method reproducibility

All 24 runs of eight replicate injections of three independent sample preparations, using the same sample and the same batch of wide range Pharmalyte 3-10, showed very good reproducibility. This is demonstrated in Figure 3 where a very small x axis offset was used to highlight the reproducible peak profiles for the obtained separations (minor peak shifting was expected due to buffer evaporation during this extended batch analysis). Table 1 highlights the reproducibility of this method with peak migration times showing % RSD of less than 0.5%.

pI value and linear gradient stability

Data analysis of pI value and gradient linearity stability shows extremely high reproducibility, which was independent of sample preparation (Table 2).

Sample	Migration Time						
	B1	B2	B3	Main peak 1	Main peak 2	A1	A2
Vbix768_1-Rep1	28.81	29.03	29.11	29.24	29.4	29.58	29.76
Vbix768_1-Rep2	28.88	29.09	29.18	29.32	29.48	29.65	29.83
Vbix768_1-Rep3	28.94	29.16	29.25	29.38	29.53	29.71	29.9
Vbix768_1-Rep4	28.9	29.12	29.21	29.33	29.49	29.68	29.85
Vbix768_1-Rep5	28.93	29.16	29.23	29.36	29.52	29.69	29.88
Vbix768_1-Rep6	28.94	29.18	29.25	29.38	29.53	29.71	29.9
Vbix768_1-Rep7	28.94	29.18	29.25	29.38	29.53	29.72	29.89
Vbix768_1-Rep8	28.99	29.21	29.3	29.43	29.59	29.76	29.95
Vbix768_2-Rep1	28.97	29.18	29.28	29.39	29.55	29.72	29.91
Vbix768_2-Rep2	28.98	29.2	29.28	29.41	29.57	29.74	29.92
Vbix768_2-Rep3	29	29.23	29.31	29.43	29.58	29.76	29.93
Vbix768_2-Rep4	29.03	29.25	29.33	29.45	29.61	29.78	29.97
Vbix768_2-Rep5	29.01	29.23	29.31	29.43	29.59	29.76	29.94
Vbix768_2-Rep6	28.96	29.18	29.28	29.39	29.54	29.72	29.9
Vbix768_2-Rep7	28.96	29.18	29.28	29.38	29.53	29.7	29.89
Vbix768_2-Rep8	28.94	29.15	29.24	29.37	29.52	29.69	29.87
Vbix768_3-Rep1	28.78	29	29.08	29.2	29.35	29.53	29.7
Vbix768_3-Rep2	28.74	28.98	29.06	29.17	29.33	29.5	29.67
Vbix768_3-Rep3	28.73	28.97	29.04	29.16	29.31	29.48	29.67
Vbix768_3-Rep4	28.73	28.95	29.03	29.14	29.3	29.46	29.66
Vbix768_3-Rep5	28.73	28.98	29.04	29.16	29.31	29.48	29.67
Vbix768_3-Rep6	28.71	28.92	29.03	29.13	29.28	29.43	29.7
Vbix768_3-Rep7	28.67	28.89	28.99	29.09	29.24	29.43	29.6
Vbix768_3-Rep8	28.64	28.87	28.95	29.07	29.22	29.38	29.58
Min:	28.64	28.87	28.95	29.07	29.22	29.38	29.58
Max:	29.03	29.25	29.33	29.45	29.61	29.78	29.97
Mean:	28.87	29.10	29.18	29.30	29.45	29.63	29.81
Std Dev:	0.12	0.12	0.12	0.12	0.13	0.13	0.12
%RSD:	0.42%	0.41%	0.41%	0.43%	0.43%	0.44%	0.41%

Table 1. Migration time reproducibility for one single sequence of eight repetitions of three independent sample preparations.

The %RSD did not exceed the value of 0.001% for pI and was very low for gradient stability measured using the four synthetic peptide pI markers (Table 2). This highlighted the quality of the wide range of Pharmalyte 3-10 used in this general cIEF assay.

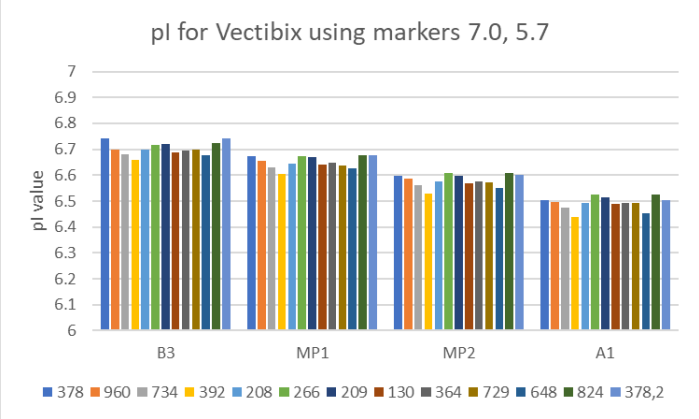


Figure 4. pI value variation for the same Vectibix test sample prepared from 12 different batches of Pharmalyte 3-10 (batch numbers are listed below graph).

To test Pharmalyte batch to batch reproducibility, the variation of the pI value was measured using samples prepared from different batches of Pharmalyte 3-10. In this test, pI values were determined using the two bracketing markers whose pI values were calculated at 7.0 and 5.7. The results in Figure 4 highlight the very good reproducibility of results obtained when using different batches of the same type of Pharmalyte.

Corrected peak area distribution

Corrected peak area distribution was also assessed in this study. Due to relatively small peak areas for both the acidic and basic peaks, we combined the acidic variants into one total area and the basic variants into another total area. We then compared the overall % areas with those of the two main variants present in the sample. Using this approach, which helped to normalize these small peaks, the results obtained showed very good reproducibility within the same sample, as well as within the runs in one single sequence of repeat analysis of 3 separate samples (Table 3).

Sample	pI value determined between two bracketing markes 7.0 and 5.5							Goodnes of Fit
	B1	B2	B3	Main peak1	Main peak2	A1	A2	for wide range of pI
Vbix768_1-Rep1	6.9	6.8	6.76	6.7	6.62	6.54	6.46	0.9987
Vbix768_1-Rep2	6.9	6.8	6.76	6.7	6.62	6.54	6.46	0.9987
Vbix768_1-Rep3	6.9	6.8	6.75	6.7	6.62	6.54	6.46	0.9987
Vbix768_1-Rep4	6.9	6.8	6.76	6.7	6.63	6.54	6.45	0.9986
Vbix768_1-Rep5	6.9	6.79	6.75	6.7	6.62	6.54	6.46	0.9987
Vbix768_1-Rep6	6.9	6.79	6.75	6.7	6.62	6.54	6.45	0.9986
Vbix768_1-Rep7	6.9	6.79	6.75	6.7	6.62	6.54	6.46	0.9986
Vbix768_1-Rep8	6.9	6.79	6.76	6.69	6.62	6.54	6.45	0.9987
Vbix768_2-Rep1	6.9	6.8	6.75	6.7	6.62	6.54	6.45	0.9986
Vbix768_2-Rep2	6.9	6.8	6.76	6.7	6.62	6.54	6.46	0.9986
Vbix768_2-Rep3	6.9	6.79	6.75	6.69	6.62	6.54	6.46	0.9986
Vbix768_2-Rep4	6.9	6.79	6.75	6.7	6.62	6.54	6.45	0.9986
Vbix768_2-Rep5	6.9	6.79	6.76	6.7	6.62	6.54	6.46	0.9980
Vbix768_2-Rep6	6.9	6.8	6.75	6.69	6.62	6.54	6.45	0.9986
Vbix768_2-Rep7	6.9	6.79	6.75	6.69	6.62	6.54	6.45	0.9986
Vbix768_2-Rep8	6.9	6.8	6.75	6.69	6.62	6.54	6.46	0.9986
Vbix768_3-Rep1	6.9	6.79	6.75	6.7	6.62	6.54	6.46	0.9986
Vbix768_3-Rep2	6.9	6.79	6.75	6.7	6.62	6.54	6.46	0.9986
Vbix768_3-Rep3	6.9	6.79	6.75	6.7	6.62	6.54	6.46	0.9986
Vbix768_3-Rep4	6.9	6.79	6.75	6.7	6.62	6.54	6.45	0.9986
Vbix768_3-Rep5	6.9	6.78	6.75	6.7	6.62	6.54	6.46	0.9986
Vbix768_3-Rep6	6.89	6.79	6.74	6.69	6.62	6.55	6.42	0.9985
Vbix768_3-Rep7	6.9	6.79	6.75	6.7	6.63	6.54	6.46	0.9985
Vbix768_3-Rep8	6.9	6.79	6.75	6.7	6.63	6.55	6.46	0.9985
Min:	6.89	6.78	6.74	6.69	6.62	6.54	6.42	0.9980
Max:	6.90	6.80	6.76	6.70	6.63	6.55	6.46	0.9987
Mean:	6.90	6.79	6.75	6.70	6.62	6.54	6.46	0.9986
Std Dev:	0.002	0.006	0.005	0.004	0.003	0.003	0.009	0.000
%RSD:	0.000	0.001	0.001	0.001	0.001	0.000	0.001	0.000

Table 2. pI value reproducibility and linear gradient stability obtained for all 24 runs performed for one single sequence of eight repetitions of three independent sample preparations using the same batch of wide range Pharmalyte 3-10.

Sample	Corrected Peak Area %				
	Basic variants	Main peak1	Main peak2	Acidic variants	Total %
Vbix768_1-Rep1	11.56	32.51	43.18	12.74	100
Vbix768_1-Rep2	10.99	32.49	43.17	13.35	100
Vbix768_1-Rep3	10.09	33.00	43.33	13.58	100
Vbix768_1-Rep4	11.00	32.65	43.45	12.89	100
Vbix768_1-Rep5	10.59	33.11	44.09	12.22	100
Vbix768_1-Rep6	10.19	32.60	42.87	13.69	100
Vbix768_1-Rep7	9.97	32.65	43.49	13.88	100
Vbix768_1-Rep8	9.76	32.90	44.36	12.97	100
Vbix768_2-Rep1	10.40	32.98	44.12	12.49	100
Vbix768_2-Rep2	10.42	32.35	44.38	12.85	100
Vbix768_2-Rep3	9.99	32.68	44.44	12.89	100
Vbix768_2-Rep4	10.57	32.92	44.31	12.20	100
Vbix768_2-Rep5	9.74	32.37	45.65	12.24	100
Vbix768_2-Rep6	9.62	33.20	44.55	12.62	100
Vbix768_2-Rep7	9.53	33.01	44.56	12.90	100
Vbix768_2-Rep8	10.41	32.26	44.38	12.96	100
Vbix768_3-Rep1	9.56	31.09	44.77	14.58	100
Vbix768_3-Rep2	9.56	30.97	45.87	13.59	100
Vbix768_3-Rep3	9.11	31.00	45.89	14.01	100
Vbix768_3-Rep4	9.82	30.89	46.20	13.09	100
Vbix768_3-Rep5	9.75	31.32	46.32	12.61	100
Vbix768_3-Rep6	10.97	30.11	45.13	13.80	100
Vbix768_3-Rep7	11.12	29.46	44.02	15.38	100
Vbix768_3-Rep8	9.93	30.96	45.02	14.09	100
Min:	9.11	29.46	42.87	12.2	
Max:	11.56	33.2	46.32	15.38	
Mean:	10.19	32.06	44.48	13.23	
Std Dev:	0.62	1.05	0.99	0.79	
%RSD:	6.03%	3.28%	2.22%	5.95%	

Table 3. Corrected peak area distribution obtained for all 24 runs performed for one single sequence of eight repetitions of three independent sample preparation using the same batch of wide range Pharmalyte 3-10.

From the results above, the use of Pharmalyte has been shown to obtain very reproducible results. In addition, Pharmalyte mixtures can also be fine-tuned to improve the resolution obtained. In the final experiment, we compare results from using the wide range Pharmalyte 3-10 with those from the narrow range Pharmalyte 5-8 (Figure 5). Figure 5 highlights how by narrowing the range, you can improve the peak shape and separation. This helps to improve the overall variant detection and peak area integration without dramatically altering the pI values obtained for the peaks present in the sample.

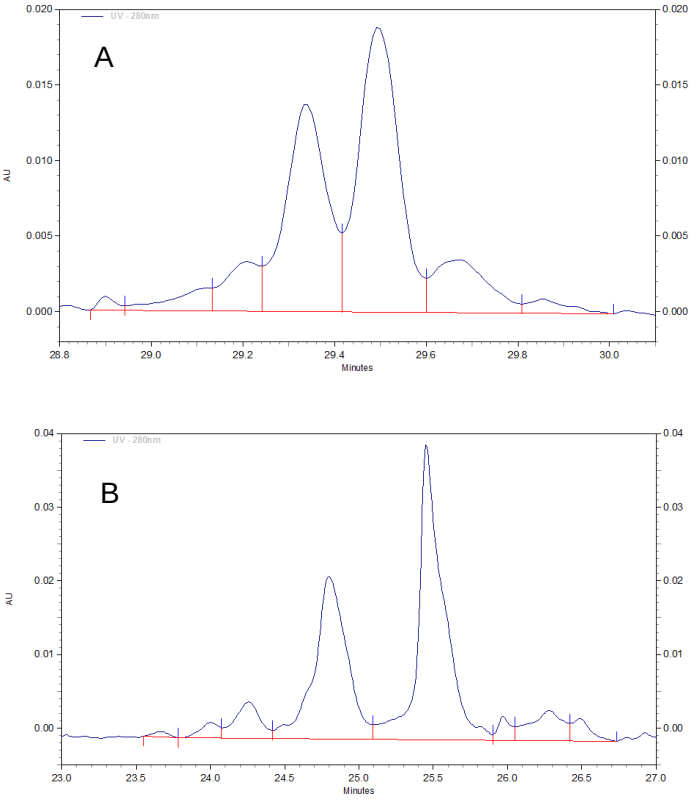


Figure 5. Comparison of Pharmalyte pI 3-10 versus pI 5-8 for mAb charge variant separation. (A) Separation of Vectibix using wide range Pharmalyte 3-10 (B); Separation of Vectibix using narrow range pI 5-8 Pharmalyte 5-8.

Conclusions

This technical note has demonstrated how the use of Pharmalyte together with the PA 800 Plus Pharmaceutical Analysis system can provide:

- Very reproducible pI values for charge variant analysis of monoclonal antibodies even across different batches of Pharmalyte
- Provides reproducible migration times and corrected peak areas results within a sample and between different samples
- Can be adjusted and fine-tuned by using different mixtures of Pharmalyte to optimize the separation for new protein samples

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