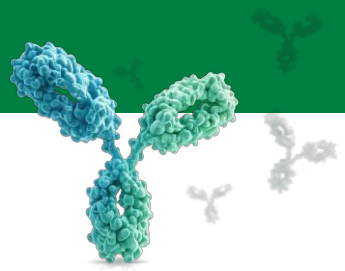


Technical Note

Rapid Platform icIEF Methods Development with AESlyte® HR 5–10.5 for Diverse mAbs

1.Introduction

Charge variant profiling of therapeutic monoclonal antibodies (mAbs), particularly the resolution of charge variants such as C-terminal lysine variants, succinimide intermediates, and N-terminal pyroglutamate species, requires exceptional pH gradient linearity and local buffering capacity in imaged capillary isoelectric focusing (icIEF). Historically, icIEF methods have relied on empirically optimized blends of broad-range and narrow-range carrier ampholytes (CAs), such as pH 3–10 and pH 8–10.5 ampholytes, to improve local resolution in the basic isoelectric points (pI) region.

Although effective in certain applications, blended ampholyte systems introduce additional sample preparation complexity, increase operator-dependent variability, and frequently require molecule-specific optimization for different mAbs. Such variability is undesirable in regulated quality control (QC) environments, where robustness, reproducibility, and long-term consistency are essential. These challenges are further exacerbated when broad-range and narrow-range ampholytes are sourced from different vendors or are subject to lot-to-lot quality variability, potentially increasing operational risk and costs for biopharmaceutical manufacturers.

From a physicochemical perspective, physically blended ampholyte systems may also produce spatially heterogeneous pH gradients due to differential electrokinetic migration of structurally distinct ampholyte populations under high electric fields. Consequently, localized gradient compression may occur, reducing the effective peak capacity in critical basic regions of the separation profile [1-2].

To address these limitations, AESlyte® HR 5–10.5 was developed as a single-component carrier ampholyte system spanning pH 5.0–10.5, thereby eliminating the need for offline ampholyte blending while enabling standardized and reproducible icIEF methods across diverse mAbs. In addition, AESlyte® HR 5–10.5 is manufactured under tightly controlled and fully traceable processes encompassing raw material qualification, chemical synthesis, and quality control, thereby supporting long-term lot-to-lot consistency and supply reliability for QC applications.

In this work, 37 representative mAbs spanning a broad range of isoelectric points (pI) were analyzed using a single standardized icIEF workflow without molecule-specific method optimization. All samples were prepared using an identical formulation containing 4% AESlyte® HR 5–10.5, 1% peptide pI marker 5.72, 1% peptide pI marker 9.71, 3 M urea, and 0.35% methylcellulose. The peptide pI markers are compatible with both UV absorbance and fluorescence detection, further

extending the versatility of the standardized workflow. Samples were analyzed under identical focusing conditions consisting of 1 min at 1500 V followed by 10 min at 3000 V.

This study illustrates the broad applicability of AESlyte® HR 5–10.5 for charge variant analysis across diverse mAbs, highlighting its potential to eliminate the need for molecule-specific method optimization and simplify method standardization for QC implementation.

2. Instrument and sample preparation

	Name	Part Number
Instrument	CEInfinite Analytical icIEF System	CE10002
Cartridge	FC coated,100 um internal diameter	CP00201
Carrier ampholytes	AESlyte® HR 5–10.5	101088
Sample concentration	0.25 mg/mL	n/a
Low pI marker	pI Marker 5.72 Peptide	100250
High pI marker	pI Marker 9.71 Peptide	100253
Additives	0.35% MC 3M urea	101099 n/a

Table 1. Instrument and Reagent Specifications

3. Discussion

As illustrated by the representative icIEF electropherograms in Figure 1, the standardized icIEF method based on AESlyte® HR 5–10.5 generated highly resolved charge variant profiles for 37 representative mAbs spanning a broad range of pI values, demonstrating its broad applicability without the need for molecule-specific method optimization. A single pair of peptide pI markers (pI 5.72 and 9.71) was used for all samples, providing a standardized calibration strategy applicable across diverse mAbs.

The excellent pH linearity of AESlyte® HR 5–10.5 over the pH 5.0–10.5 range enabled accurate pI determination without the need to select sample-specific pI markers. Furthermore, the electropherograms exhibited stable baselines throughout the entire separation window, indicating minimal interference from artifact peaks and baseline fluctuations that are often associated with physically blended ampholyte systems.

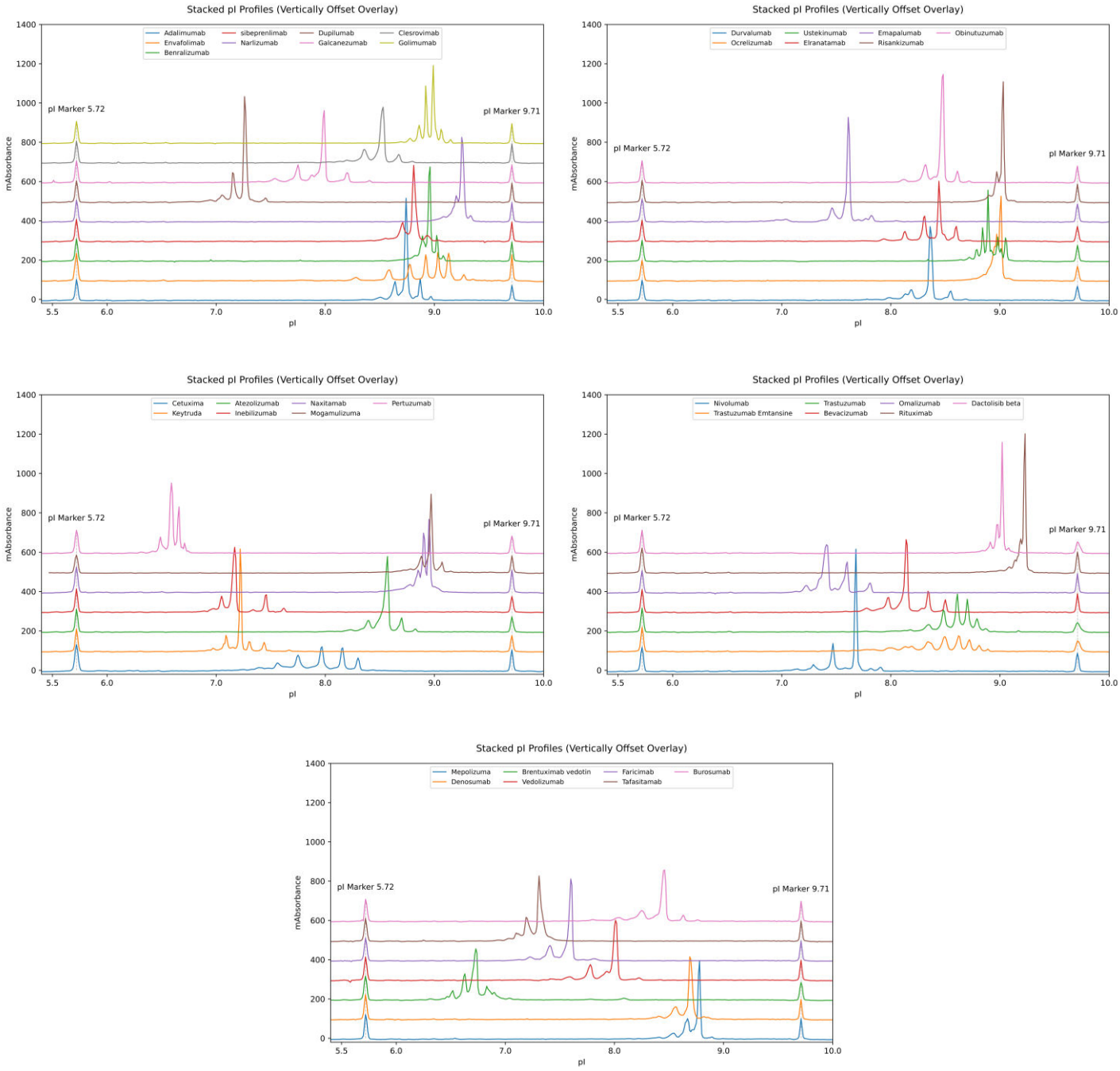


Figure 1. Replicate icIEF Electropherograms of 37 mAbs using AESlyte® HR 5–10.5 and pI marker pair (5.72 and 9.71).

3.1 Repeatability of standardized icIEF method for the studied mAbs

The repeatability of the standardized icIEF workflow was evaluated for each mAb tested. Across all samples, excellent reproducibility was achieved for both pI determination and relative peak area quantification. The relative standard deviation (RSD) values of the measured pI for acidic, main, and basic charge variants were all below 0.2%, demonstrating highly reproducible pI assignment independent of charge heterogeneity.

Similarly, relative peak areas exhibited excellent repeatability despite the varying complexity of charge variant profiles among the eight mAbs. The RSD values ranged from 0.83% to 2.19% for acidic variants, 0.24% to 1.67% for main variants, and 1.34% to 6.05% for basic variants. Figure 2 shows the representative Pertuzumab egram, and repeatability data are summarized in Tables 2 and 3.

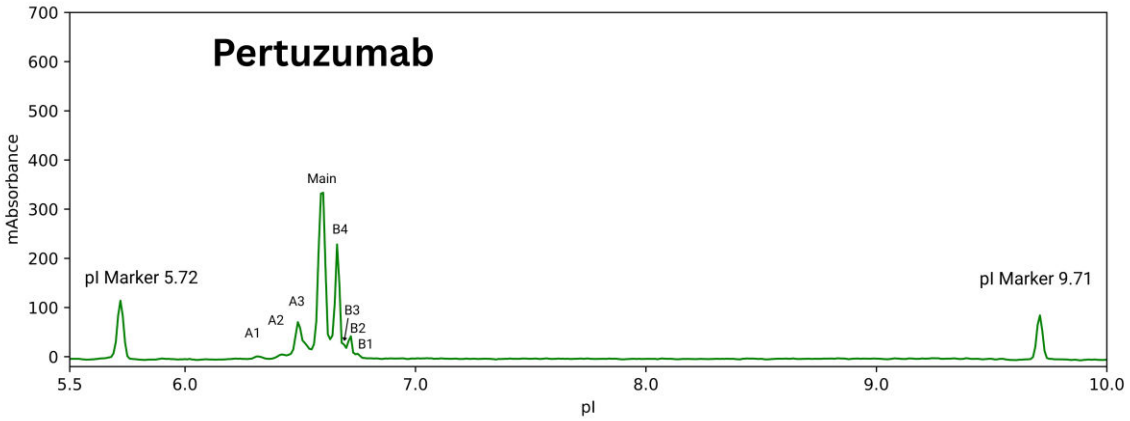


Figure 2. Electropherograms of Pertuzumab with AESlyte® HR 5–10.5 and pI marker 5.72 and 9.71.

pI Value								
	Acidic			Main	Basic			
	A1	A2	A3	Main	B4	B3	B2	B1
RUN 1	6.329	6.427	6.502	6.605	6.673	6.7	6.725	6.77
RUN 2	6.314	6.422	6.497	6.597	6.668	6.695	6.718	6.753
RUN 3	6.317	6.42	6.495	6.595	6.665	6.693	6.718	6.753
RUN 4	6.31	6.418	6.493	6.593	6.661	6.688	6.714	6.746
RUN 5	6.311	6.417	6.489	6.587	6.658	6.685	6.71	6.74
RUN 6	6.301	6.412	6.484	6.59	6.655	6.68	6.705	6.735
AVERAGE	**6.314**	**6.419**	**6.493**	**6.595**	**6.663**	**6.690**	**6.715**	**6.750**
RSD%	**0.15%**	**0.08%**	**0.10%**	**0.09%**	**0.10%**	**0.11%**	**0.10%**	**0.18%**

Table 2. Reproducibility of pI measurements for Pertuzumab (n=6)

	Acidic Area%			Main Area%	Basic Area%			
	A1	**A2**	**A3**	**Main**	**B4**	**B3**	**B2**	**B1**
RUN 1	1.6	1.7	13.9	50.3	24.6	2.5	3.8	1.5
RUN 1-SUM	17.2			n/a	24.6			
RUN 2	1.9	1.9	13.6	50.3	25	1.9	4.1	1.3
RUN 2-SUM	17.4			n/a	25			
RUN 3	1.6	1.5	14.2	50.6	24.7	2.2	4	1.2
RUN 3-SUM	17.3			n/a	24.7			
RUN 4	1.6	1.8	14.1	50.2	24.5	2.3	4.2	1.3
RUN 4-SUM	17.5			n/a	24.5			
RUN 5	1.5	1.6	14	50.4	24.8	2.2	4.1	1.3
RUN 5-SUM	17.1			n/a	24.8			
RUN 6	1.2	1.7	14.2	51.5	24	2.1	4.1	1.2
RUN 6-SUM	17.1			n/a	24			
AVERAGE	**17.267**			**50.550**	**24.600**			
RSD%	**0.95%**			**0.96%**	**1.38%**			

Table 3. Reproducibility of relative charge variant peak area measurements for Pertuzumab (n=6)

3.2 Linearity of AESlyte® HR 5-10.5 and lot-to-lot consistency

The pH linearity and lot-to-lot consistency of carrier ampholytes are critical for establishing standardized and reliable icIEF methods for QC and manufacturing applications. Accordingly, both attributes were systematically evaluated for AESlyte® HR 5–10.5. As shown in Figures 2 and 3, calibration using 15 peptide pI markers demonstrated excellent pH linearity across the entire pH 5.0–10.5 range, with correlation coefficients (R) exceeding 0.99 for all three production lots. Moreover, the three independent lots exhibited nearly superimposable calibration curves, indicating outstanding lot-to-lot reproducibility. These results reflect the stringent control implemented throughout raw material qualification, manufacturing, process traceability, and final quality control, resulting in highly consistent ampholyte performance across production lots. Such consistency is essential for maintaining the long-term robustness of validated icIEF methods in QC and manufacturing environments by minimizing analytical risk, reducing the need for revalidation requirements, and lowering operational costs.

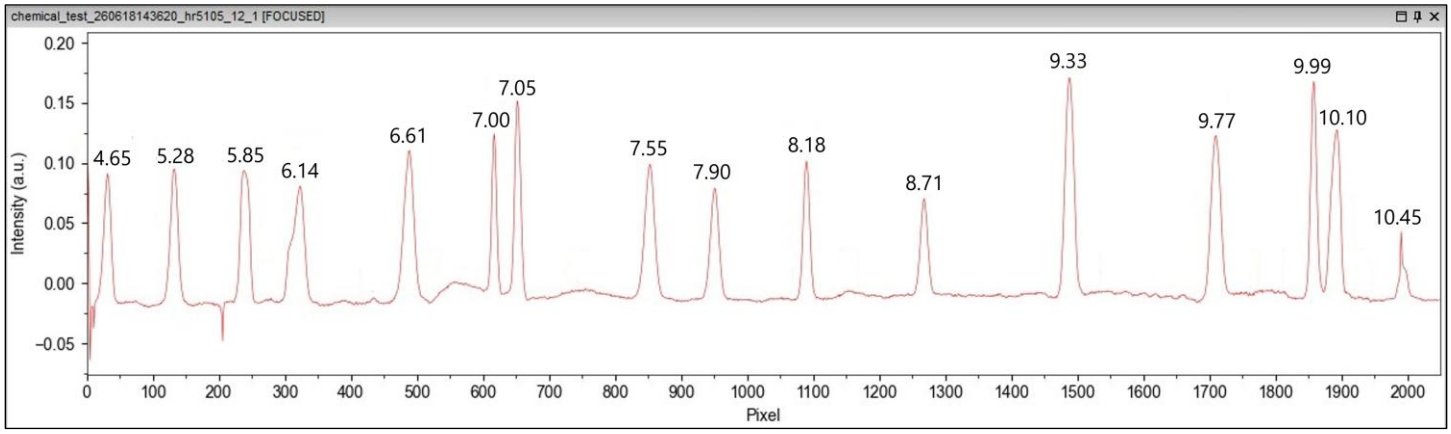
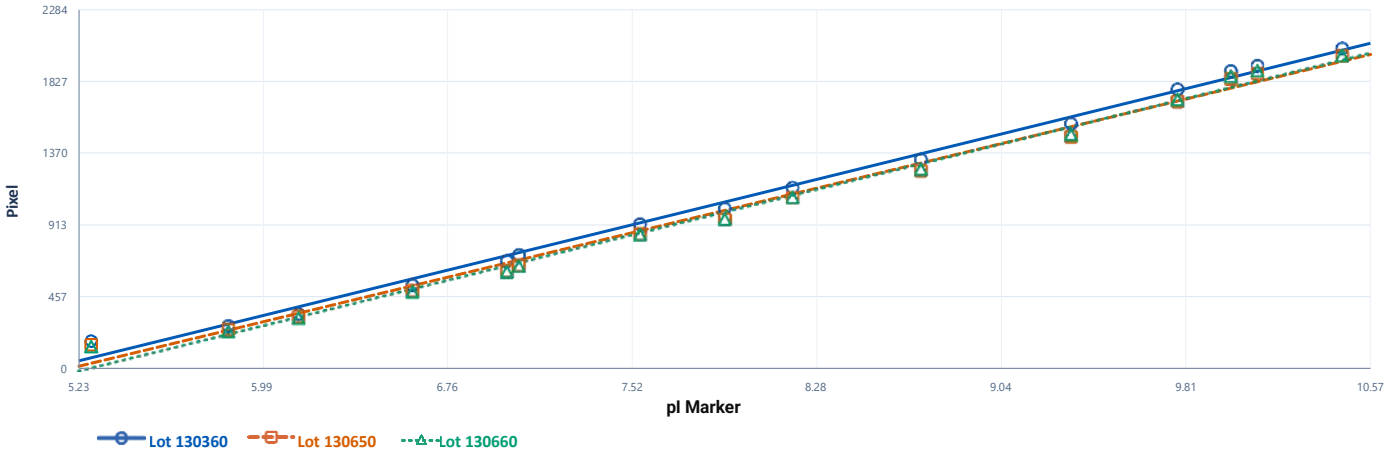


Figure 2. icIEF electropherograms calibrated using 15 pI markers with AESlyte® HR 5-10.5

Linearity Report

R and R^2 are calculated from valid marker/pixel pairs.



1. Lot 130360 | HR 5-10.5 | Slope = 379.3353 px/pH | Intercept = -1938.54 | R = 0.9980 | R^2 = 0.9960 | n = 15

2. Lot 130650 | HR 5-10.5 | Slope = 371.9227 px/pH | Intercept = -1932.48 | R = 0.9968 | R^2 = 0.9936 | n = 15

3. Lot 130660 | HR 5-10.5 | Slope = 378.1197 px/pH | Intercept = -1985.82 | R = 0.9969 | R^2 = 0.9939 | n = 15

Figure 3. Lot-to-lot consistency of AESlyte® HR 5-10.5.

4. Conclusions

This work demonstrates that the single-component carrier ampholyte system, AESlyte® HR 5–10.5, provides a practical platform for establishing standardized and broadly applicable icIEF methods for charge variant analysis of diverse mAbs. Unlike conventional empirically blended broad- and narrow-range ampholyte systems, AESlyte® HR 5–10.5 generates a continuous pH gradient spanning pH 5.0–10.5, covering the pI range of most therapeutic mAbs reported in the literature [3]. Consequently, diverse mAbs can be analyzed using a single standardized workflow without product-specific ampholyte blending or method optimization. Using 37 representative mAbs spanning a broad pI range, the standardized method

demonstrated excellent separation performance, pH gradient linearity, and analytical reproducibility. Calibration with the peptide pI markers produced excellent linearity across the entire pH range, while three independent production lots exhibited highly consistent calibration curves, confirming outstanding lot-to-lot reproducibility. In addition, the standardized workflow achieved excellent repeatability in both pI determination and relative charge variant quantification across all tested mAbs. By eliminating offline ampholyte blending, AESlyte® HR 5–10.5 simplifies sample preparation, minimizes operator-dependent variability, and improves method standardization across laboratories. Its tightly controlled manufacturing process further supports the long-term reliability required for validated QC methods and manufacturing-release testing.

To date, the standardized icIEF workflow described here has been successfully applied to the charge heterogeneity characterization of more than 100 protein modalities from mAbs to bispecific Abs and ADCs, further demonstrating its robustness, broad applicability, and suitability as a universal platform method for both research and quality control environments.

Reference:

[1] Belfiore, M., Ascione, A., Ghizzani, V., Di Meo, S., & Luciani, F. (2024). A new paradigm for optimized experimental design in cIEF platforms aimed at an accurate robust and reliable mAbs charges variant assessment. Scientific Reports, 14(1), 28087.

[2] Barnthouse, K., Ganguly, S., Groeneveld, M., Lopez, Jr., M. A., Nedved, M., & Smith, K. D. (2020). Methods for producing anti-TNF antibody compositions. International Publication No. WO 2020/183270 A1. Geneva, Switzerland: World Intellectual Property Organization.

[3] Goyon, A., Excoffier, M., Janin-Bussat, M. C., Chevalier, M., Guillarme, D., & Beck, A. (2017). Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies. Journal of Chromatography B, 1065-1066, 119-128.

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