White paper: Critical reagents for successful iCIEF

“Innovations Make Breakthrough Solutions”

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ELECTROPHORESIS SOLUTIONS LTD.

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1. Introduction

Capillary isoelectric focusing (CIEF) is a fundamental technology for measuring charge heterogeneity in protein samples as illustrated in Figure 1. In CIEF, iCIEF (whole column detection image CIEF) and traditional IEF, proteins are sorted by their isoelectric point (pI) along a pH gradient created by applying an electric field to a diverse mixture of carrier ampholytes (CAs).

The quality of an iCIEF separation is highly dependent on several attributes of the CAs used including baseline signal, linearity of the pH gradient, and consistency between manufactured lots. Recently, commercial brands of ampholytes have been frustrating the biopharmaceutical industry. The use of these ampholytes can cause spectral shifts which have extremely negative impacts on PI measurement and quantitation of protein charge. These artifacts include a "dip" of the baseline and "shift" of both peak and pI during iCIEF separation. A "dip" is commonly observed in the acidic range when using pH 3-10 and mixed ampholytes 5-8 and 8-10.5 provided by routine commercial brands, and occurs because of variable lots and sample excipients. Similarly, a "shift" of both peak and pI results from instable lots of ampholytes and is disastrous in quality control and product release. Moreover, method optimization usually cannot overcome the above troubles with the use of routine commercial ampholytes.

AESlytes, created by Advanced Electrophoresis Solutions Ltd. (AES), demonstrate a reduction in baseline noise and distinguishably increased consistency between lots when compared to other CAs brands of the same pH range. Unparallel lot-to-lot stability of AESlyte CAs mitigates "Dip" occurrence and "shift" of pI and peak in iCIEF separation, which guarantees consistency in QC method development. AESlytes also have a greater proportion of "working" CA molecules so that sufficient resolution is maintained at lower concentrations. Finally, AESlytes with ranges as narrow as one pH unit were created for further increased resolution.

AES provides the total solutions of critical reagents for iCIEF platform:

- **World leader in critical consumables such as CAs, coated separation columns, pI markers;**
- **Technology leader in prep iCIEF, iCIEF-MS, and carrier ampholytes manufacturing;**
- **World only solution provider for life cycle iCIEF method CMC.**

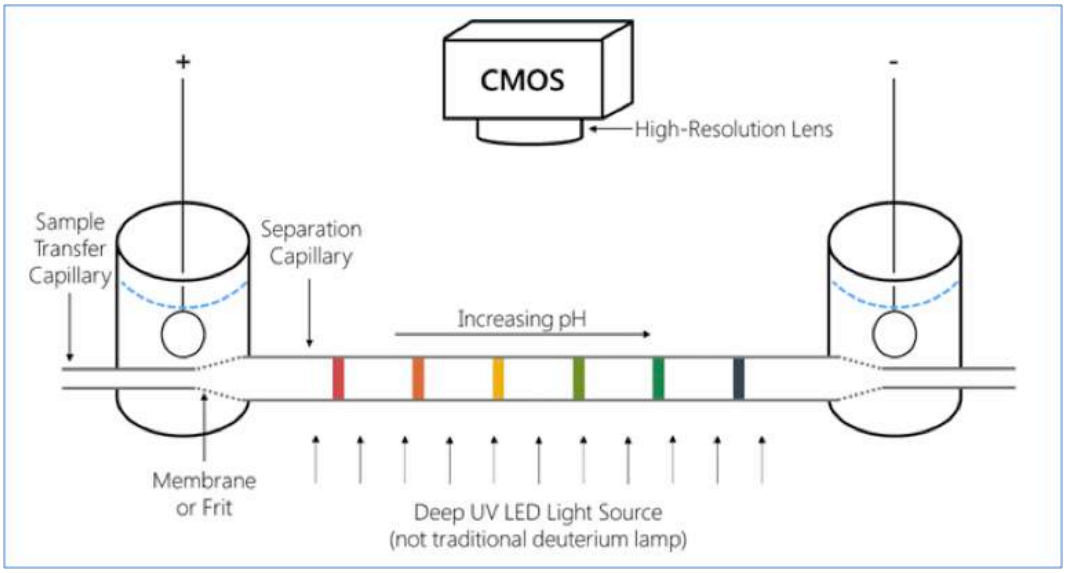


Figure 1. Schematic of iCIEF. UV-light source allows whole-column detection of separating proteins

2. Why AESlytes in iCIEF

2.1. Comprehensive AESlytes

AES is providing the most comprehensive collection of critical reagents for iCIEF including ampholytes and pI markers with the widest pH range and the highest pI resolutions in the industry, as shown in Figure 2 and 3. This diverse set of reagents can flexibly satisfy any urgent request from biopharmaceutical industry.

Especially facing challenges such as protein drugs with high pI, the extremely high pH CAs and pI markers from AElytes can effectively provide solutions as indicated in Figure 4.

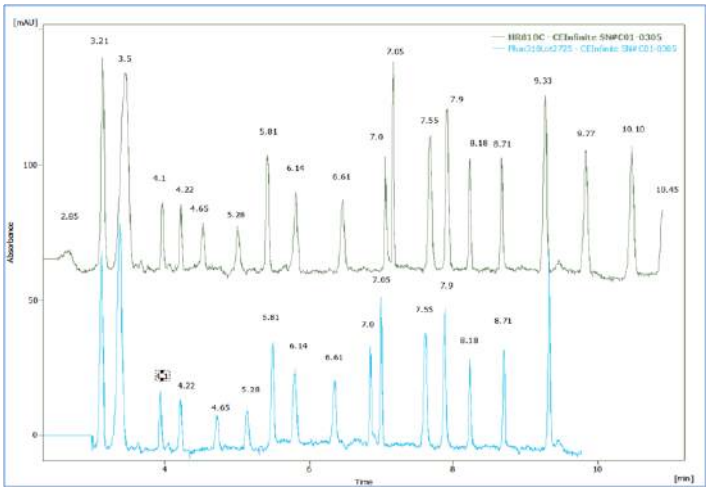


Figure 3. Comprehensive catalog of AES pI markers (black) with wider pH capacity than routine commercial brands (blue).

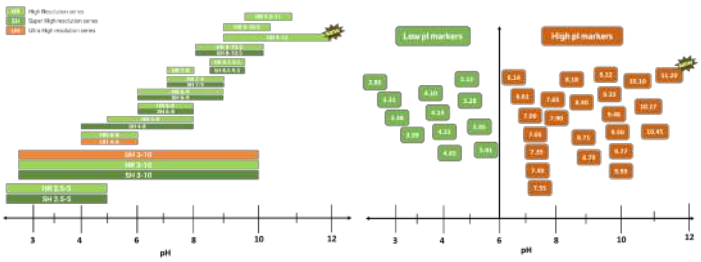


Figure 2. Full list of AESlyte series.

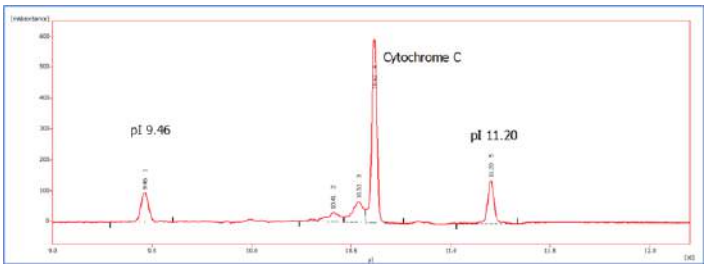


Figure 4. Extreme high pH range and pI marker: AESlyte HR 9-12 and pI marker 11.20.

2.2. Reduced baseline noise

iCIEF uses ultraviolet (UV) light at 280 nm to detect proteins but the CAs themselves can absorb UV light as well. Figure 5 shows a narrower and more uniform baseline for pH 3-10 HR AESlytes compared to the commercial brands most notably between pH 6 and 9.

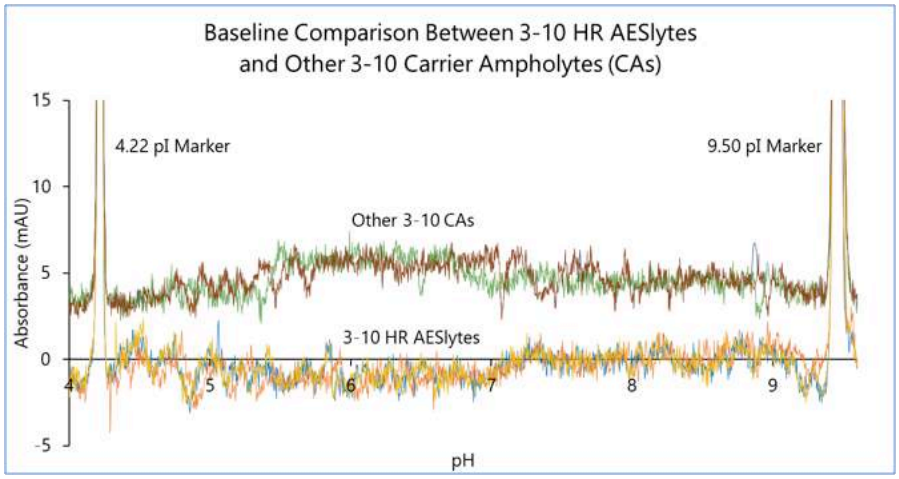
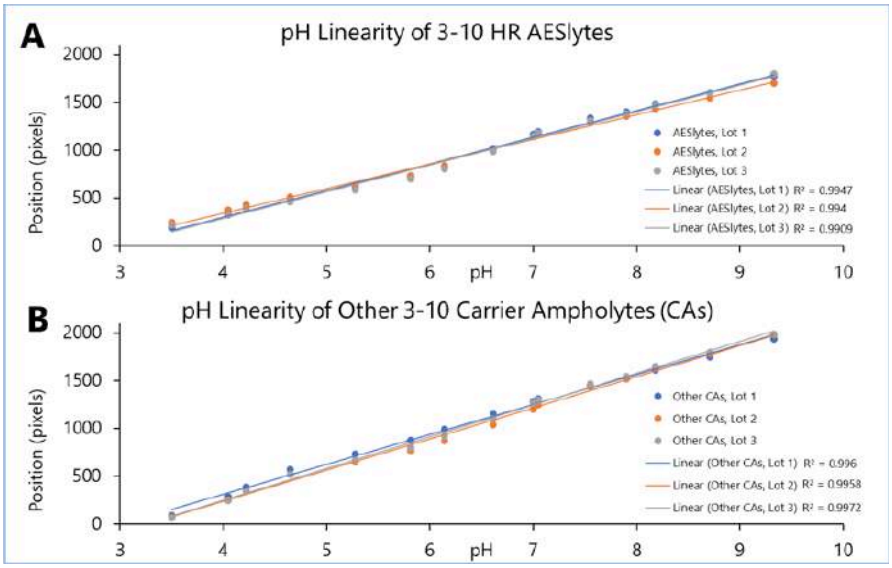


Figure 5. iCIEF Baseline signals of three Lots of 3-10 HR AESlytes and of other commercial brands 3-10 carrier ampholytes.

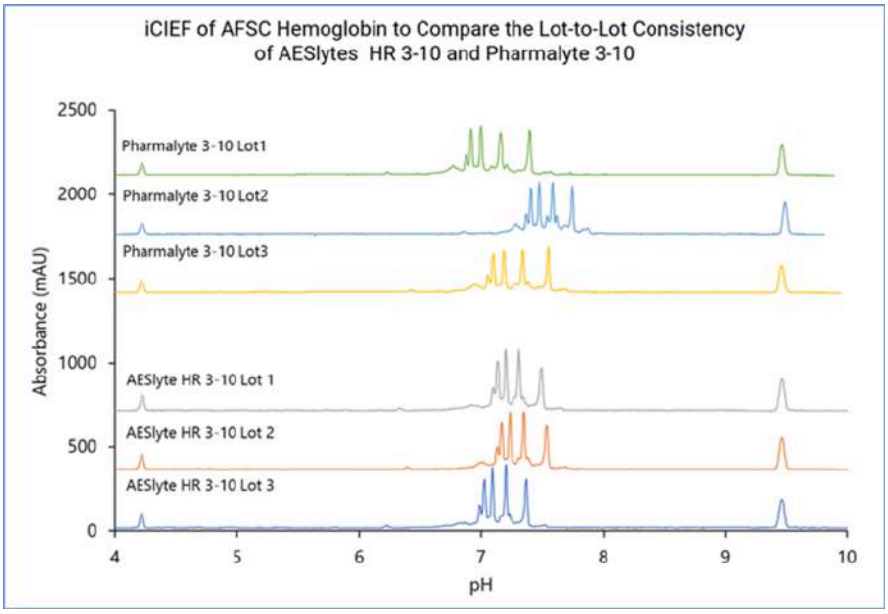
2.3. pH gradient linearity



To calculate the linearity of the pH gradient, 15 pI markers between pH 3 and 10 were focused. The AESlytes (Figure 6A) showed comparable linearity to the other 3-10 carrier ampholytes (Figure 6B).

Figure 6. Linearity of pH gradient generated with 3-10 HR AESlytes (A) and other 3-10 carrier ampholytes (B).

2.4. Lot-to-lot consistency



An ASFC hemoglobin isomer mixture was focused with three different lots of 3-10 HR AESlytes as well as three lots of other 3-10 CAs, as the pI of hemoglobin A is standard for ampholyte manufacturing. Figure 7 shows than in comparison to the three lots of AESlytes, the other commercial brands show an increase in hemoglobin isomer variability.

Figure 7. iCIEF separation of ASFC Hb to demonstration pH gradient consistency across three Lots of 3-10 HR AESlytes (bottom three) and other commercial brands 3-10 carrier ampholytes (top three).

The pI reproducibility of the four main isomers was calculated for both types of CAs. Table 1 shows the reduction in variability by at least a factor of two when comparing the other brands CAs to the AESlytes.

Charge variants	pI Reproducibility (% RSD)			
	A	S	F	C
3-10 HR AESlytes	1.0%	1.0%	1.1%	1.2%
Other 3-10 CAs	3.5%	3.3%	2.9%	2.3%

Table 1. Relative Standard Deviation (%RSD) of Main Hemoglobin Isomers Separated with AESlytes or Other CAs.

2.5. Resolution at lower concentrations

For direct coupling of iCIEF to a mass spectrometer (MS), a lower concentration of CAs entering the MS with the sample will result in a stronger sample signal for deconvolution. AESlytes have an increased proportion of "working" CAs such that they sufficiently separate samples at lower concentrations than recommended for other CAs.

Figure 8 shows the maintained resolution of iCIEF separation of the NISTmAb reference material with AESlyte concentrations decreasing from 4% to 1%.

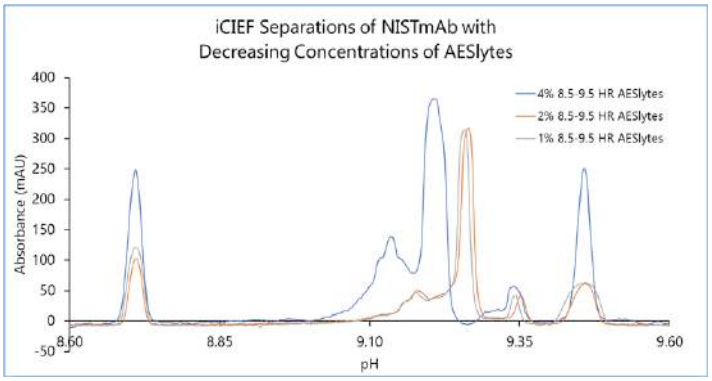


Figure 8. iCIEF Separation of the NISTmAb Reference Material at Decreasing Concentrations of 9.5-9.5 HR AESlytes.

2.6. Increased separation resolution

Increased CA resolving power is desired, especially for more basic samples (pH 7-11). Narrow range AESlytes, with pH ranges as small as 1 pH unit used in combination with CEInfinite Preparative iCIEF facilitate isolation of narrower charged isomer peaks.

Figure 9 illustrates the increase in resolving power of 8.5-9.5 HR AESlytes compared to a mixture of 3-10 and 8-10.5 HR AESlytes when focusing the NISTmAb reference material.

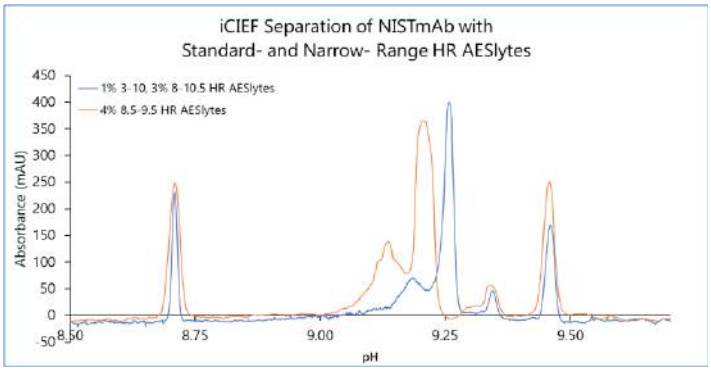


Figure 9. Comparison of NISTmAb separations with standard range AESlytes and new narrow range 8.5-9.5 HR AESlytes

2.7. Service: customized AESlytes

Since its inception, iCIEF has quickly become the quintessential technique for analyzing protein charge heterogeneity. Carrier ampholyte quality dictates the reproducibility of the separation itself as well as the potential for downstream assays through fractionation or iCIEF-MS coupling.

A lack of consistency between CA lots from a single manufacturer can introduce significant problems in the application of CIEF as a reproducible tool in the bio-pharmaceutical industry, particularly in quality control. AESlytes are consistent in their reduced baseline signal, pH linearity, and pH gradient between lots. AESlytes can also be used at a lower concentration than other CAs for the same resolution, opening the door to direct coupling to MS. New narrow range AESlytes increase separation resolution of samples in the pH 7-11 range which is crucial for analyzing biologics. CEInfinite's high performance AESlytes were developed in response to a need from all stages of the drug development lifecycle for more reproducible and dependable CAs (Figure 10). Based on our expertise, we are providing quality "SERVICE" as to help our customer with any challenges they come across in drug discovery. These services include:

- Carrier ampholyte customizations
- QC using customers' criteria
- Existing iCIEF profile matching
- Method development support for fusion protein or other complex molecules
- Guaranteed lot to lot consistency
- Re-supply service
- Help to succeed regulation filing

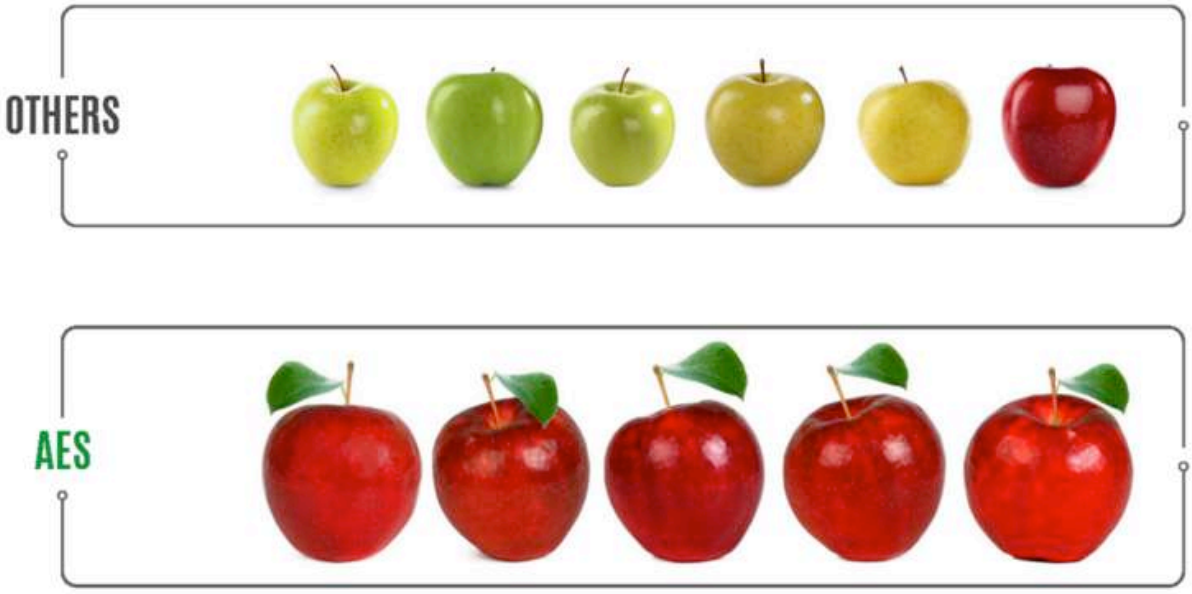


Figure 10. Reproducible "service" guarantee from AES warranty customers' success in their drug discovery.

3. AESlytes changed iCIEF' world for complex proteins

Complex protein drugs including bi-specific Ab, ADC (antibody-drug-conjugate) and fusion proteins are popular in biopharmaceutical industry due to their targeted drug delivery and selective therapeutic effects. However, their complex charge heterogeneity makes iCIEF characterization rather difficult and routine commercial CAs cannot address such a challenge. AESlytes provide unparalleled resolution capacity to ultra-highly characterize the charge variants of difficult protein drugs.

3.1. AESlytes for fusion proteins

In Figure 11 and 12, AESlytes demonstrate much better separation resolution of protein isomers than routine commercial brand CAs. In addition, the repeatability by AESlytes was excellent in terms to pI and peak area. AESlytes were employed to characterize a diverse of fusion proteins with unparallel resolutions as demonstrated in Figure 13.

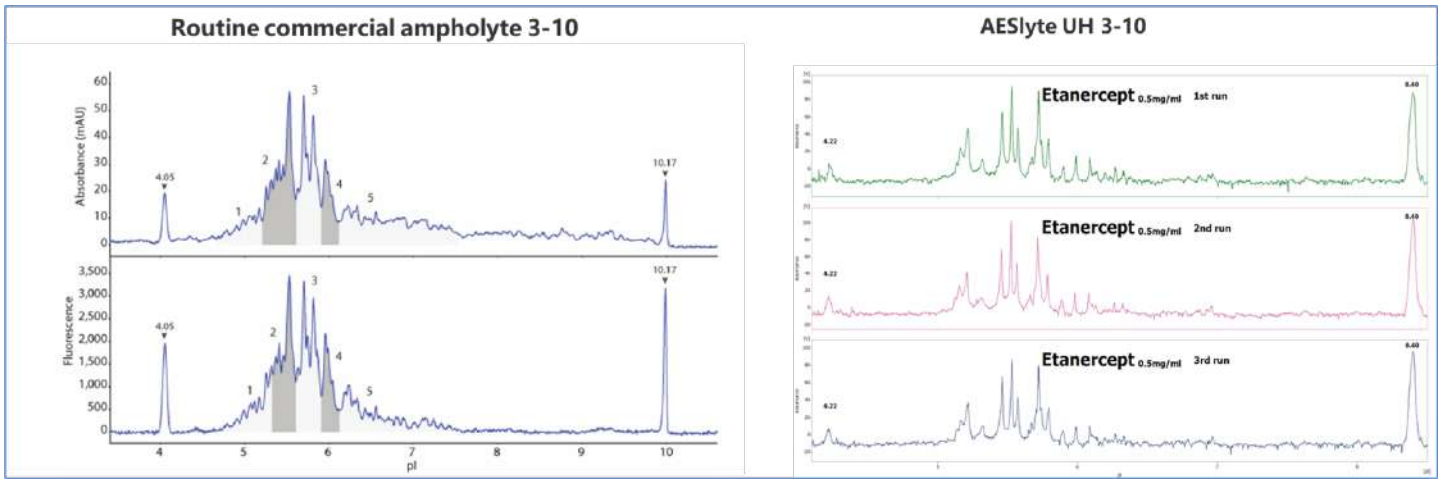


Figure 11. iCIEF comparison of etanercept (fusion protein) between AESlytes 3-10 and routine commercial ampholyte 3-10.

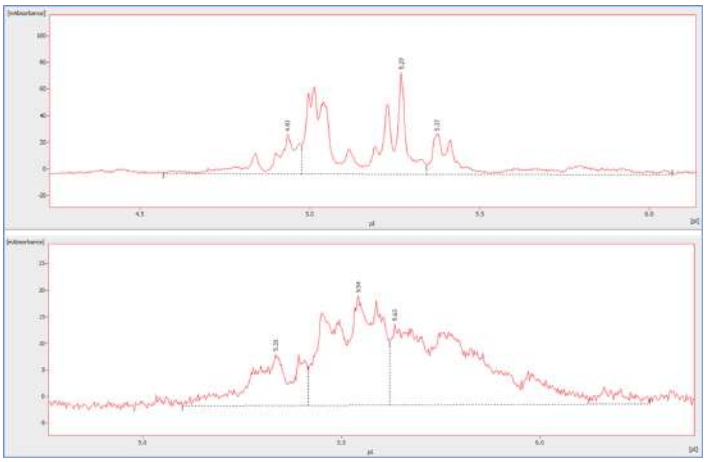


Figure 12. iCIEF comparison of fusion protein (FP-AT-1) between AESlytes UH3-10 and routine commercial ampholyte 2-9.

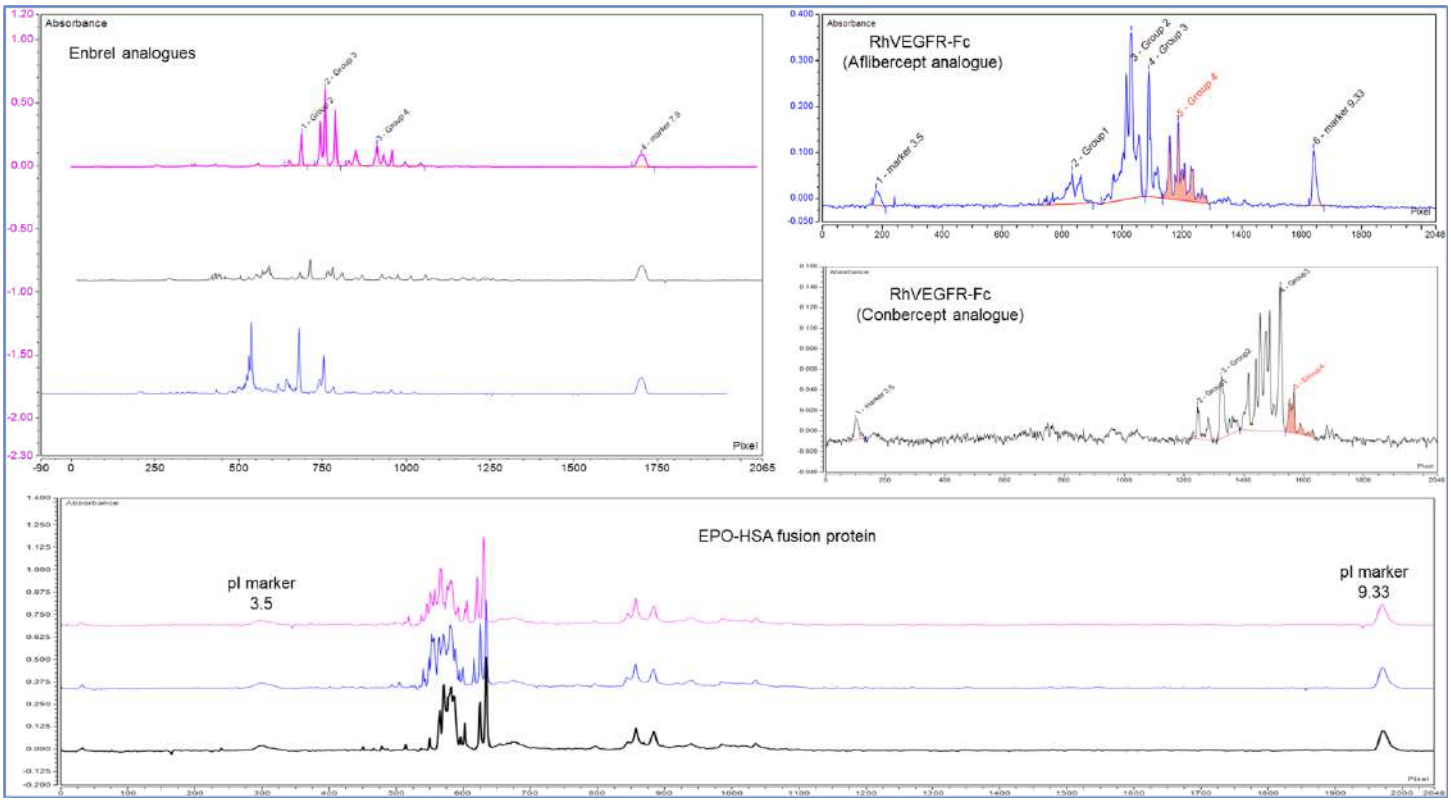
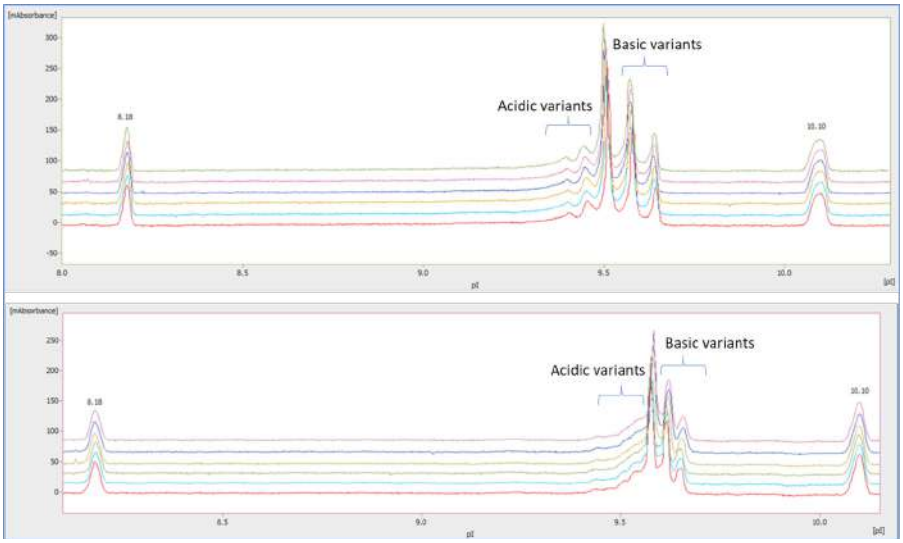


Figure 13. Fusion proteins with unparallel resolutions using AESlytes.

3.2. AESlytes for bi-specific antibody



As shown in Figure 14, the charge variants of bi-specific Ab were not well separated using routine commercial brand CAs but could be well solved by AESlytes, demonstrating the outstanding resolution of AESlytes for improving the separation of complex proteins.

Figure 14. AESlytes achieved outstanding resolution compared to other commercial brand CAs for bi-specific antibody (BSAb-AT-1).

3.3. AESlytes for ADCs

As shown in Figure 15, AESlytes HR and UH series were used for characterizing complex ADCs with excellent resolution of protein isomers with different binding ratios.

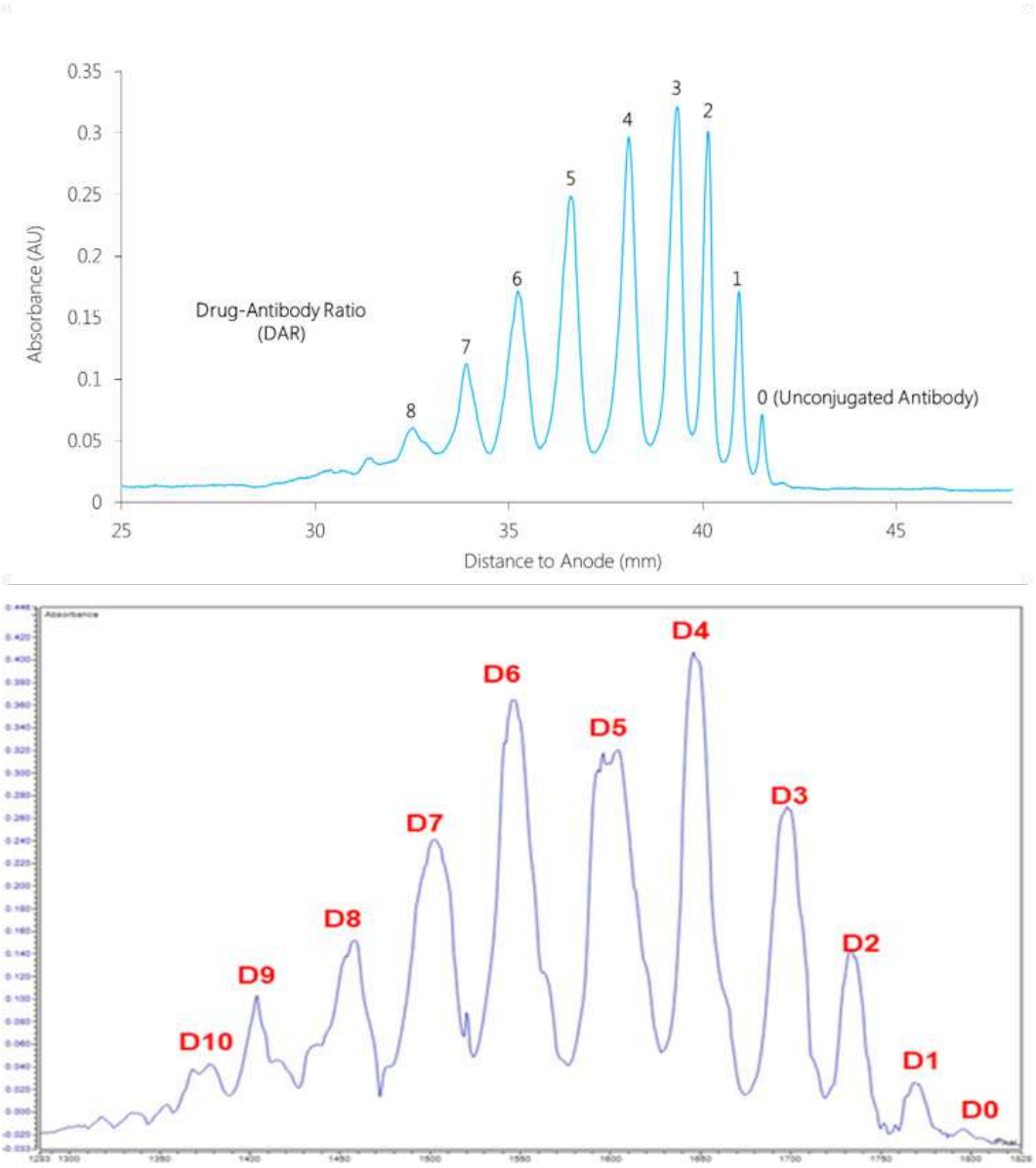


Figure 15. ADC iCIEF employing AESlytes HR and UH series

4. AESlytes in iCIEF-MS

Recently, a robust iCIEF-MS platform was developed to gain both rapid iCIEF separation and reliable high-resolution MS (HRMS) identification of protein charged variants simultaneously.
The established methodology is highly-sensitive and allows rapid heterogeneity characterization of monoclonal antibodies and other complex proteins including antibody-drug-conjugate (ADC) and bi-specific Abs. In addition, just by changing capillary separation cartridge, the developed iCIEF-MS configuration can flexibly switch to an iCIEF-based fraction collection model for isolating charged variants for subsequent peptide mapping by high performance liquid chromatography (HPLC) tandem high resolution mass spectrometry (HRMS). The whole workflow of iCIEF-MS for protein characterization, illustrated in Figure 16, is straight forward and accurate results can be obtained within 45 min.

As indicated in Figure 17, AESlytes exhibited much lower MS background interference than routine commercial brand CAs, meaning adequate MS sensitivity and selectivity could be obtained for trace protein characterization.

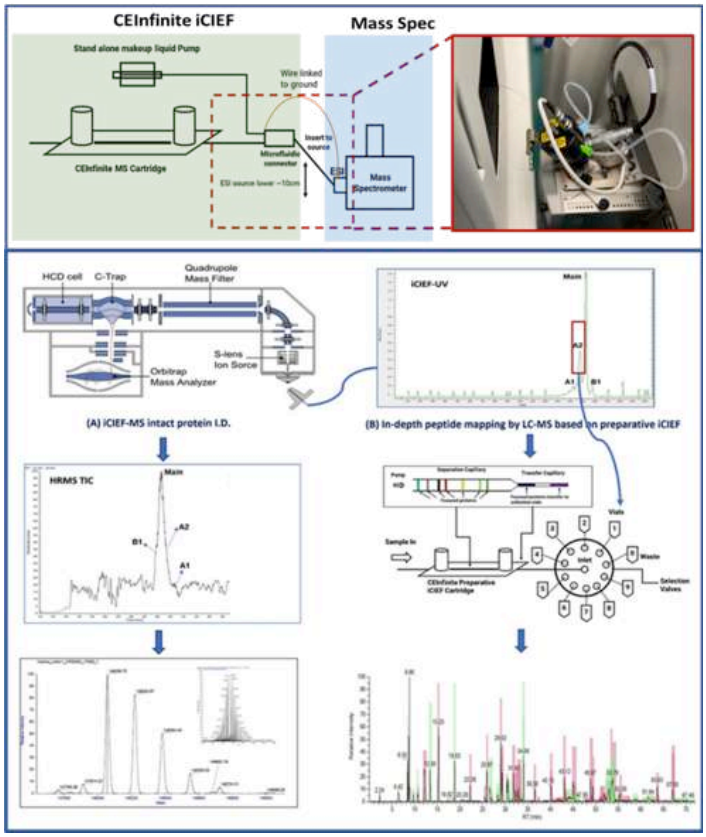


Figure 16. iCIEF-based mass spectrometry strategy for protein characterization

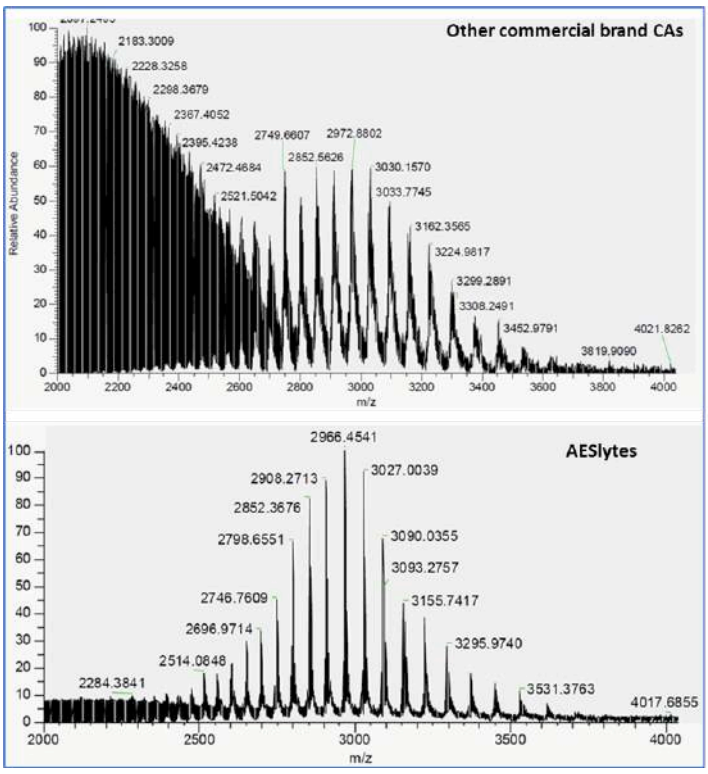


Figure 17. Much lower MS background interference from AESlytes series than routine commercial brand CAs.

4.1 Cutting edge MC coated capillary for high-throughput protein characterization

Recently, AES has developed MC coated capillary using state of the art two-layer polymerization technology (shown in Figure 18) to avoid the addition of MC as dynamic coatings and be free from pre-condition of MC solution before the iCIEF separation. Excellent long-term stability of MC coated capillary was achieved and the iCIEF behaviors of diverse protein drugs (mAb, ADC, bi-specific antibodies and fusion proteins) using routine FC coated and MC coatings were compared to ensure the consistency with the use of different types of coated capillaries. Furthermore, the MC coated capillary cartridge was then applied to iCIEF-MS for characterizing protein charge variants with reliable identification of MS after iCIEF separation. Routine FC coated capillaries usually needs a pre-rinse with MC solution before sample running. This new coating can greatly simplify the operation steps and prevent the contamination of ESI-MS that results from using routinely coated capillary. The developed method based on MC coatings together with employing AESlytes is robust, highly-efficient and high-throughput for iCIEF quality control and iCIEF-MS coupling.

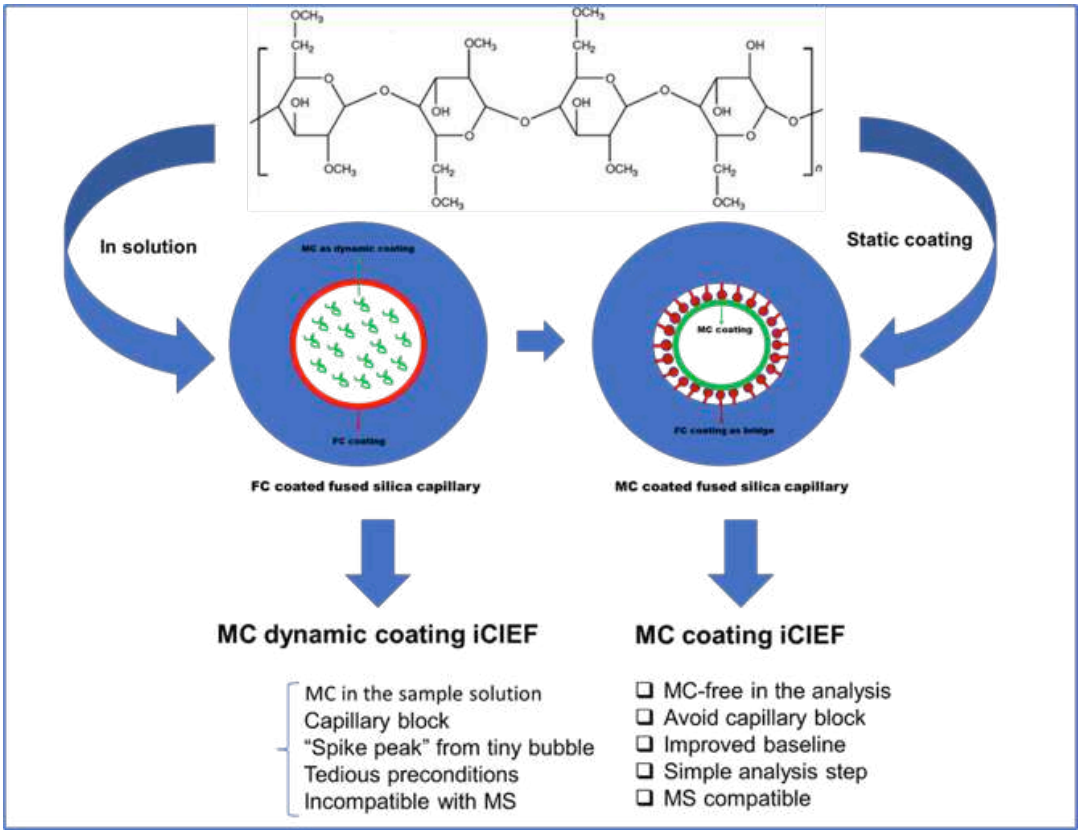


Fig. 18. Advantages of MC static coating in iCIEF as comparing to MC dynamic coating.

As illustrated in Figure 19, using very narrow pH range ampholyte (pH 7-8) and MC coated capillary, mAb-AT-1 with its four charge variants were profiled by iCIEF-MS with good sensitivity.

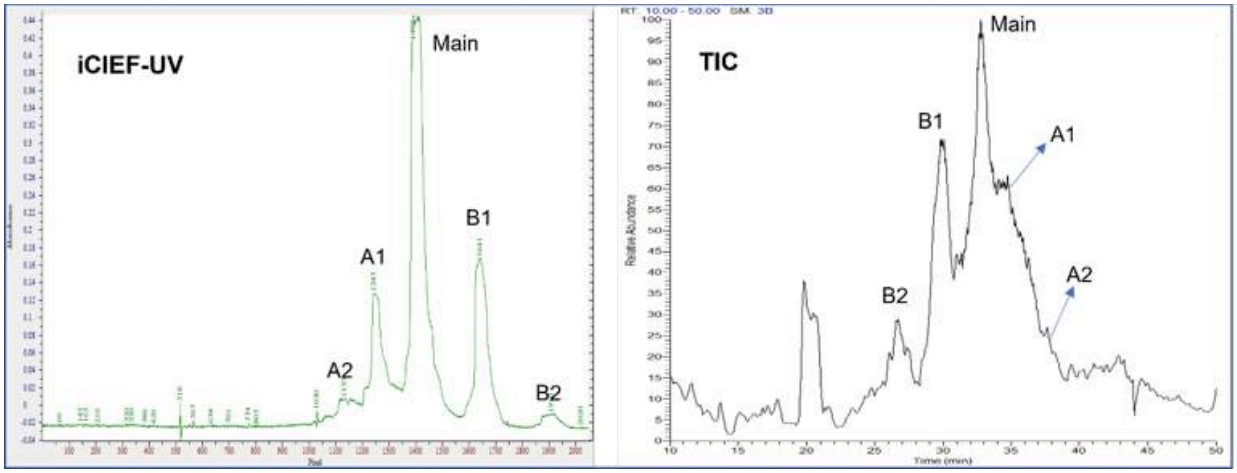


Fig. 19. iCIEF-HRMS of mAb-AT-1 with the use of MC coated capillary.

An integrated iCIEF-MS platform equipped with MC coated capillary and AESlytes was employed to characterize the charge variant of a bi-specific antibody (Figure 20). This study quickly demonstrated an accurate fingerprint of protein heterogeneity including a main component as well as acidic variants and basic variants.

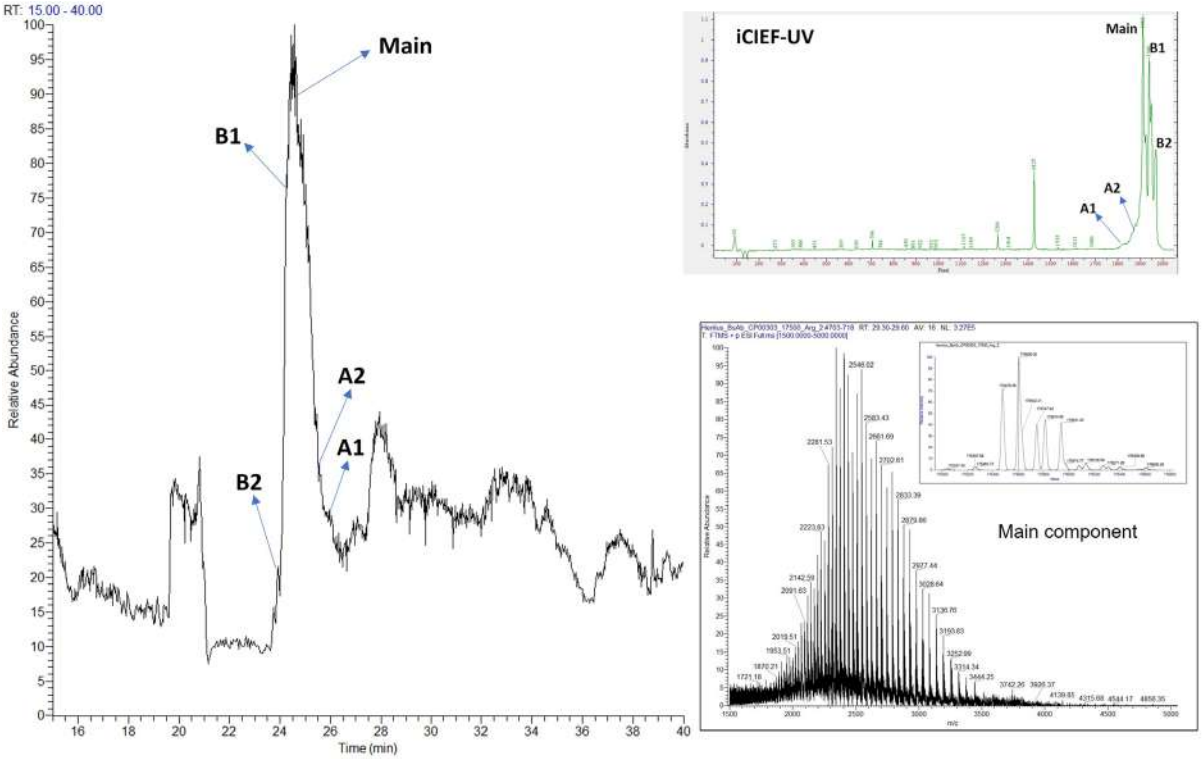


Figure 20. iCIEF-MS of bi-specific antibody-AT-1 with the use of MC coated capillary.

5. AESlytes (PN#)

HR series		SH series		UH series	
AESlyte HR 2.5-5	101013	AESlyte SH 5-8	101022	AESlyte UH 5-8	101062
AESlyte HR 5-8	101014	AESlyte SH 2.5-5	101023	AESlyte UH 2.5-5	101063
AESlyte HR 8-10.5	101015	AESlyte SH 4-8	101024	AESlyte UH 4-5	101064
AESlyte HR 3-10	101016	AESlyte SH 8-10.5	101025	AESlyte UH 8-10.5	101065
AESlyte HR 6-9	101017	AESlyte SH 3-10	101026	AESlyte UH 3-10	101066
AESlyte HR 7-8	101019	AESlyte SH 6-9	101027	AESlyte UH 6-9	101067
AESlyte HR 7-9	101011	AESlyte SH 7-8	101029	AESlyte UH 7-8	101069
AESlyte HR 8.5-9.5	101018	AESlyte SH 7-9	101021	AESlyte UH 7-9	101071
AESlyte HR 4.2-4.9	101020	AESlyte SH 8.5-9.5	101028	AESlyte UH 8.5-9.5	101068
AESlyte HR 4-6	101040	AESlyte SH 4-6	101030	AESlyte UH 4-6	101070
AESlyte HR 6-8	101041	AESlyte SH 6-8	101031	AESlyte UH 7-10	101060
AESlyte HR 8.8-9.5	101042	AESlyte SH 8-9	101032		
AESlyte HR 9-10.5	101043				
AESlyte HR 9.5-11	101044				
AESlyte HR 8-9	101045				
AESlyte HR 3-7	101046				
AESlyte HR 9-12	101047				
AESlyte HR 6.5-7.5	101048				

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AES Advanced Electrophoresis Solutions Ltd (AES)is a total solution provider in iCIEF and related cutting-edge technologies. AES has been collaborating with leading biopharmaceutical companies answering their diversely urgent needs. AES stays with customers to give supports for their CMC strategy and entire method development cycle.

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