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Discover new icIEF solutions.

A guide for those open to rethinking their current icIEF approach...

An Update on the Recent and Rapid Progression of icIEF

Advanced Electrophoresis Solutions Ltd.

SUMMARY: Imaged capillary isoelectric focusing (icIEF) has experienced rapid change in recent years, and provided the biopharma industry with a variety of new tools for charge variant characterization. Advanced Electrophoresis Solutions Ltd. (AES) has been leading the way with high-resolution carrier ampholytes and preparative icIEF capabilities. The purpose of this report is to provide a brief update on the field of icIEF, with an emphasis on the AES technologies that are redefining analytical development.

Introduction

icIEF is a powerful tool for characterizing protein charge heterogeneity, and in particular, has become essential for monitoring the quality of monoclonal antibodies (mAbs). However, the icIEF landscape is rapidly changing:

a) There is a growing need to understand, in great detail, the modifications underlying each charge variant, as this information can help avoid costly delays during the late stages of development and manufacturing.

b) Emerging modalities like ADCs, fusion proteins, and AAVs have demonstrated great potential, but their complexity challenges the limits of existing charge variant characterization methods.

c) A heightened awareness of instrument discontinuation and critical reagent shortages have highlighted the need for a greater selection of icIEF vendors.

In response to these industry demands, many companies have been reconsidering their current approach and evaluating more advanced icIEF tools. Throughout this transition, AES has been actively supporting customers with various products and services:

a) CEInfinite. Run analytical and preparative icIEF with confidence, and begin to identify the modifications hidden beneath each charge variant peak (Fig 1).

b) Critical Reagents. Simplify the analysis of complex samples by leveraging the next generation of carrier ampholytes, pI markers, and capillary coatings (Fig 2).

Fig 1. The CEInfinite can flexibly switch between analytical icIEF, icIEF fractionation, and icIEF-MS.

c) Advanced Solutions. AES is much more than an icIEF vendor. We are a reliable partner that is committed to keeping all areas of your icIEF operations running smoothly and consistently. Furthermore, we have the expertise and resources to help you address your most urgent challenges. As biopharma becomes increasingly reliant on icIEF, and the technical demands become more complex, the benefits of working with a partner like AES will become more clear.

The remainder of this document will expand on the end-to-end icIEF solutions offered by AES, and hopefully stimulate an in-depth discussion based on your current needs.

HR AESlyte®

2.5 → 5	7 → 8
3 → 7	7 → 9
3 → 10	7.5 → 8.5
4 → 6	8 → 10.5
5 → 8	8.5 → 9.5
6 → 8	8.8 → 9.5
6 → 9	9 → 10.5
6.5 → 7.5	9.5 → 11

SH AESlyte®

2.5 → 5	7 → 8
3 → 10	7 → 9
4 → 6	8 → 10.5
4 → 8	8 → 9
6 → 8	8.5 → 9.5
6 → 9	

UH AESlyte®

2.5 → 5	6 → 9
3 → 10	7 → 8
4 → 8	7 → 9
4 → 10	8 → 10.5
4 → 5	8.5 → 9.5

pI Markers

2.85*	5.91*	8.71
3.21*	6.14	8.79
3.38	6.61	9.22
3.59*	7.00	9.33
4.05	7.03	9.46
4.10	7.05*	9.50
4.14*	7.35	9.77
4.22*	7.40	9.86
4.50	7.46*	9.99
4.65	7.55	10.00
5.12	7.65	10.10
5.28*	7.90	10.17
5.50*	8.18	10.45
5.85*	8.40	11.20

Fig 2. There is a wide selection of both broad and narrow range carrier ampholytes. The HR, SH, and UH AESlytes® each have different molecular properties that can be leveraged to optimize icIEF resolution for specific samples. There is also a wide selection of pI markers, with the ones highlighted in green representing fluorescent molecules, and the ones marked with an asterisk (*) representing those that are resistant to digestion by the carboxypeptidase B (CpB) enzyme.

icIEF Critical Reagents

Critical reagents influence the resolution and reliability of icIEF, and their availability and quality are essential to the success of biopharma operations.

The Industry Challenge. icIEF is known for its simple method development. The standard master mix can be finely tuned in a day, and trusted to separate most monoclonal antibodies.

However, with the emergence of more complex modalities like bispecific antibodies, fusion proteins, antibody/drug conjugates (ADCs), adeno-associated viruses (AAVs), and lipid nanoparticles, many of the technical difficulties affecting performance have been magnified, including solubility, pH gradient resolution, capillary wall adsorption, background interference, lot-to-lot consistency, and more.

If the charge heterogeneity of these complex samples cannot be adequately resolved, or if reproducibility issues arise during quality control, this will undoubtedly delay their release. Consequently, there is an urgency to develop a wider selection of critical reagents for a more tailored approach to method development. In general, more choice would also help ease concerns related to supply chain disruptions after their vulnerability was exposed during the recent pandemic.

The Advanced Electrophoresis Solution. AES has been meeting these industry demands by leveraging a deep understanding of icIEF and developing an innovative approach to chemical design and synthesis.

→ AESlyte®. There are 3 different carrier ampholyte series to choose from, and each includes a variety of narrow pH ranges for increased resolution (Fig 2). The HR series delivers results similar to that of Pharmalyte® while providing outstanding lot-to-lot consistency[15,16]. The SH and UH series can be used independently or mixed with the HR series. The UH series, in particular, consists of entirely new molecular structures with unmatched resolution (Fig 3).

→ Capillary Coatings. In addition to the hydrophobic fluorocarbon (FC) coating, there are durable hydrophilic coatings made from either acrylamide derivative (AD) or methylcellulose (MC). These coatings expand the diversity of samples that can be analyzed by icIEF while eliminating the need for MC in solution, thereby avoiding issues like pre-rinse, bubble formation, and capillary clogging.[9]

→ pI Markers. There are 40+ markers ranging from 2.85 to 11.2 (Fig 2). Many are fluorescent, and several are resistant to carboxypeptidase B (CpB) enzyme digestion.

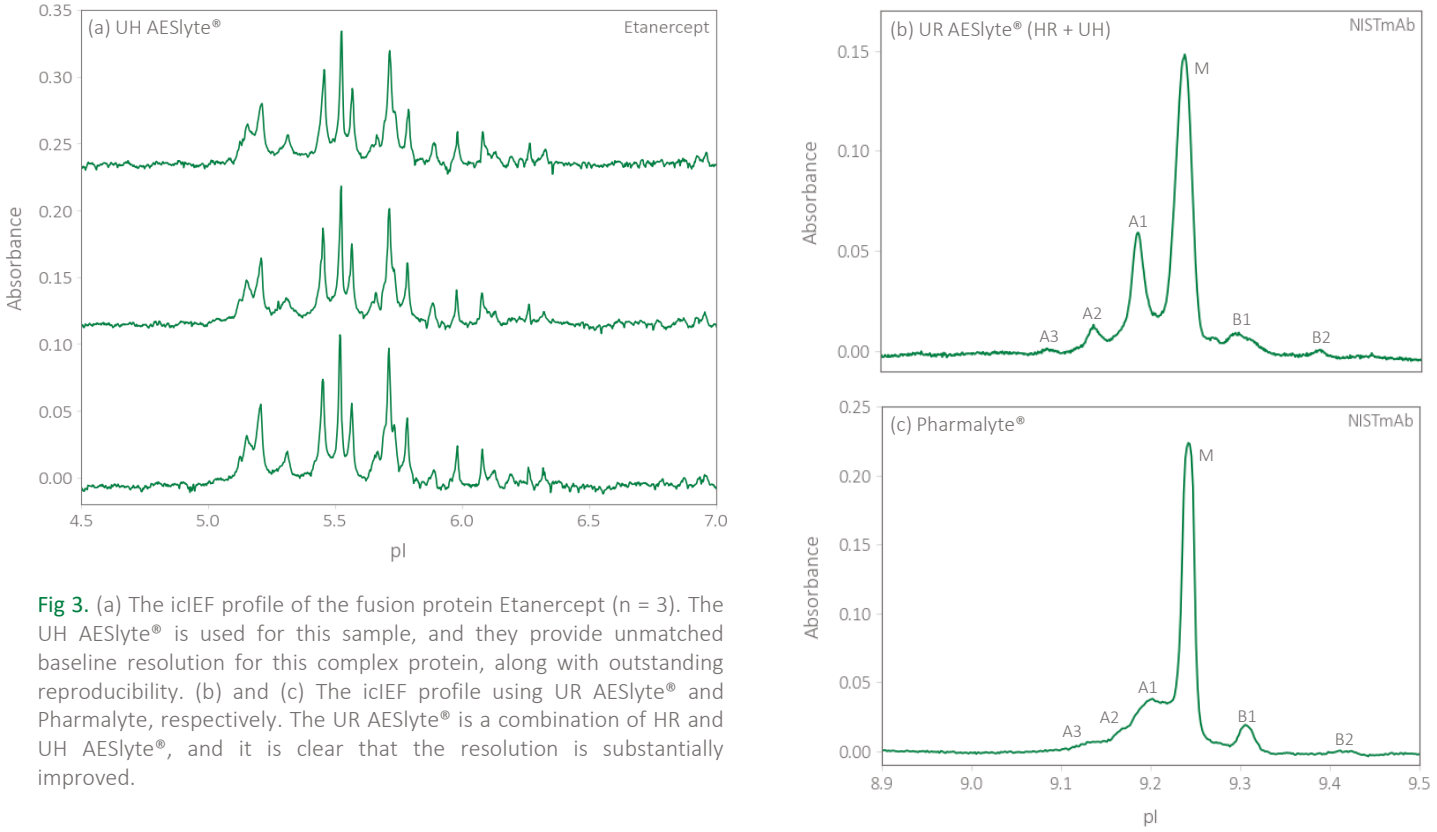


Fig 3. (a) The icIEF profile of the fusion protein Etanercept (n = 3). The UH AESlyte® is used for this sample, and they provide unmatched baseline resolution for this complex protein, along with outstanding reproducibility. (b) and (c) The icIEF profile using UR AESlyte® and Pharmalyte, respectively. The UR AESlyte® is a combination of HR and UH AESlyte®, and it is clear that the resolution is substantially improved.

icIEF Replacement

The iCE3 platform has become an essential tool for monitoring the quality of monoclonal antibodies. However, its upcoming discontinuation has companies searching for an alternative icIEF instrument.

The Industry Challenge. Finding an icIEF replacement is not straightforward, and there are numerous expenses to consider beyond the initial capital investment. For one, the time and resources required for method transfer, employee training, and revalidation can be significant. This is especially true if the new instrument has difficulty reproducing the original data.

Furthermore, the ability to maintain and repair the new instrument must be taken seriously, as potential delays in manufacturing can result in substantial losses. Finally, the replacement itself may be discontinued at some point, causing this entire process to repeat itself.

The Advanced Electrophoresis Solution. There are several companies considering CEInfinite to replace iCE3. AES has been working closely with them to ensure a seamless transition and several benefits have been noted:

→ Method Transfer and Data Reproducibility. Both iCE3 and CEInfinite use similar methods, so the existing data can be reproduced while minimizing the cost of method transfer and revalidation.

→ Instrument Reliability. The mechanical and optical components have few moving parts, and this simplicity increases the likelihood of generating consistent data. This also reduces the risk of malfunction, and in any event, should allow for a timely repair.

→ Customer Support. AES offers lifetime support to eliminate the uncertainty of future discontinuations. Furthermore, we have the infrastructure to deliver prompt maintenance and consumables.

→ Preparative Capabilities. The CEInfinite can flexibly switch between the analytical mode, the fractionation mode, and the icIEF-MS mode without significant changes to the method. This technology is becoming essential to the investigation of icIEF peaks and is likely to help navigate regulatory compliance as the industry evolves.

→ Critical Reagents and icIEF Services. Choosing CEInfinite also creates additional opportunities to partner with AES and leverage several decades of experience. In addition to a steady supply of reagents manufactured in-house, AES can assist with method development, while also providing custom solutions for specific needs. As biotherapeutics become more complex, and the demand for preparative icIEF increases, the need for a dependable partner like AES will become more clear.

(a)	Apparent Main pI			Acidic Area			Main Area			Basic Area		
	Average	SD	%RSD	Average	SD	%RSD	Average	SD	%RSD	Average	SD	%RSD
NISTmAb	9.24	0.0016	0.018	31.1%	0.48	1.56	59.9%	0.72	1.21	9.1%	0.21	2.35
USP mAb001	9.20	0.007	0.076	36.5%	0.67	1.84	56.9%	0.63	1.11	6.6%	0.23	3.50
USP mAb002	7.94	0.012	0.145	24.4%	0.17	8.05	71.3%	0.33	0.47	4.3%	0.12	6.43
USP mAb003	7.87	0.015	0.192	17.6%	0.57	6.92	63.1%	0.56	0.89	19.4%	0.20	0.65

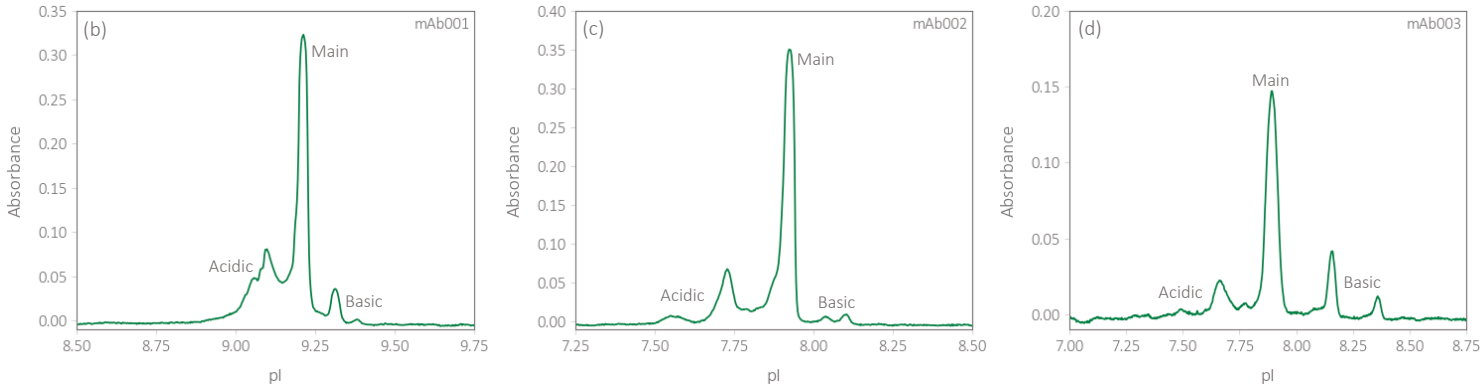


Fig 4. (a) A summary of icIEF data averaged over 6 consecutive runs with the CEInfinite for different monoclonal antibody reference standards. The methods were chosen according to similar evaluations by iCE3 and Maurice,[17-19] and the results are comparable. The corresponding icIEF profiles for the USP samples (a) mAb001, (b) mAb002, and (c) mAb003 are shown above, while the NISTmAb profile can be found in Fig 3c. Please contact AES for further information on the materials, methods, and data analysis.

icIEF Fractionation

icIEF peaks can now be collected in quantities suitable for peptide mapping, potency assays, and more.

The Industry Challenge. A comprehensive understanding of the modifications underlying each charge variant can help mitigate costly setbacks related to scale-up and regulatory compliance. And yet, this critical information often remains elusive during icIEF characterization.

The main issue is that it is difficult to mobilize the icIEF peaks for collection, as this process results in resolution loss and various instabilities affecting reproducibility. On top of that, the collected quantities are so minuscule that it is hard to do any kind of thorough analysis.

As a workaround, the charge variants are often analyzed with ion exchange chromatography or a related technique capable of fractionation. However, it can take months to develop a proper method on these platforms, and there is no guarantee that they can match the resolution of icIEF. With the rise of ADCs, fusion proteins, and more, this problem will only become more pronounced.

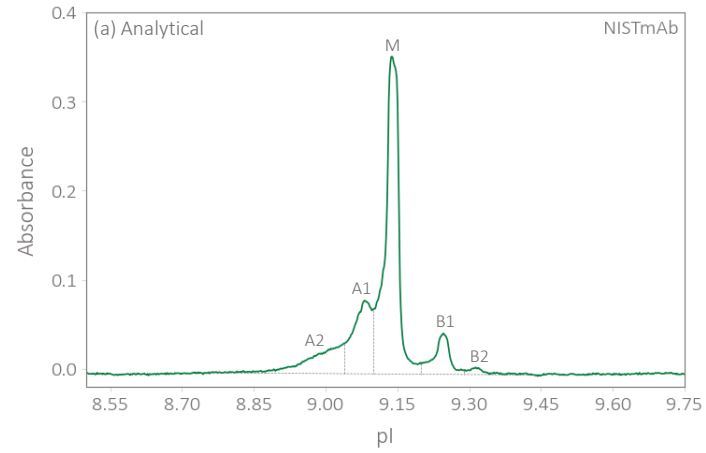
The Advanced Electrophoresis Solution. AES has addressed these challenges with several innovations (Fig 5).

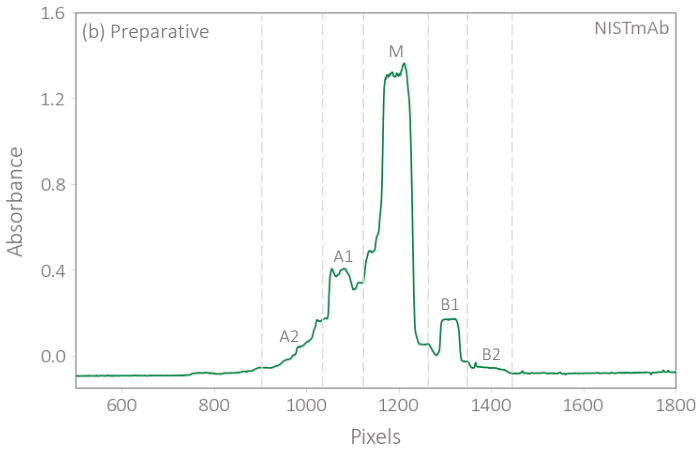
→ Speed and Reproducibility. Pressure mobilization is used instead of chemical mobilization to avoid instabilities related to Joule heating and pH gradient disruption. Each run is 45 minutes with excellent reliability.

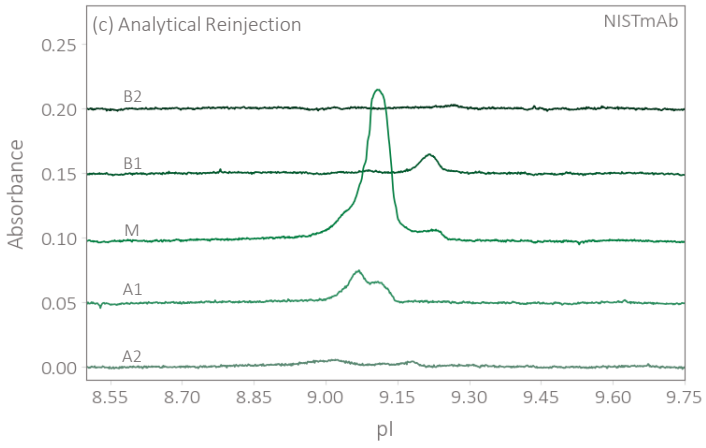
→ High Resolution. A transfer capillary with a reduced diameter and specialized coatings helps minimize dispersion during transit (Fig 9). A reversed polarity is also possible to improve the purity of the acidic fractions.

→ Automation and Throughput. The target regions for fractionation are manually selected during the first run. Each subsequent run is then automated based on this condition, thereby enabling more than 25 reproducible runs per day with minimal user intervention.

→ Orthogonal Characterization. icIEF can now be used in combination with other analytical techniques, such as SEC-MS, to obtain a more in-depth and unique fingerprint.







(d)	Initial Area	Est. Fraction Purity	Est. Fraction Recovery	Est. Amount per 25 runs
B2	1.43%	87.4%	65.4%	2.4 µg
B1	8.50%	89.2%	63.5%	13.5 µg
M	58.86%	86.3%	87.3%	129.2 µg
A1	16.97%	46.5%	58.4%	25.0 µg
A2	14.24%	63.0%	61.0%	21.8 µg

Fig 5. (a) The NISTmAb icIEF profile using the analytical capillary cartridge (100 µm diameter). (b) The NISTmAb icIEF profile using the preparative capillary cartridge (320 µm diameter). This is before pressure mobilization, with the different regions selected for fractionation. (c) The icIEF profile of the collected fractions reinjected into the analytical cartridge. (d) An estimation of the fraction purity, recovery, and quantity calculated based on the a starting concentration of 2.5 mg/mL in a 4.02 µL capillary. The recovery estimation assumes no sample loss during transfer to the collection vials.

icIEF-MS Coupling

icIEF-MS is a convenient alternative to icIEF fractionation. Although not as comprehensive, its ability to rapidly identify intact charge variants immediately following icIEF separation makes it a powerful tool throughout all stages of biotherapeutic development and manufacturing.

The Industry Challenge. There are several technical issues to overcome when directly coupling icIEF to MS. For one, there is the problem of peak mobilization, which was noted for fractionation. Second, critical reagents, such as carrier ampholytes, urea, and MC, can interfere with the electrospray ionization process. Third, it is difficult to design a reliable interface due to factors like backpressure, sensitivity, electrical grounding, and capillary clogging.

On top of that, the interface may not be compatible with your preferred MS instrument. Furthermore, if the method transfer from the analytical mode to icIEF-MS is not straightforward, the benefit of convenience may be lost. Worse, the icIEF profiles may differ significantly, and this would cast uncertainty onto the final results.

The Advanced Electrophoresis Solution. AES has also introduced several innovations to couple directly to MS.

→ Resolution and Reliability. The pressure mobilization and transfer capillary, along with a carefully designed icIEF-MS interface, have enabled robust performance.

→ Critical Reagents. AES has leveraged its expertise in chemical synthesis to develop carrier ampholytes and permanent MC coatings for better electrospray ionization.

→ MS Instrument Compatibility. The platform works with various MS brands, including Thermo Fisher, Waters, and Agilent. That said, it performs best when used with the Thermo Scientific Q Exactive Plus and a nanospray emitter.

→ Seamless Method Transfer. Switch between analytical icIEF, fractionation, and icIEF-MS with limited changes to the cartridge and method. As a result, this instrument provides a highly comprehensive and flexible charge variant characterization strategy.

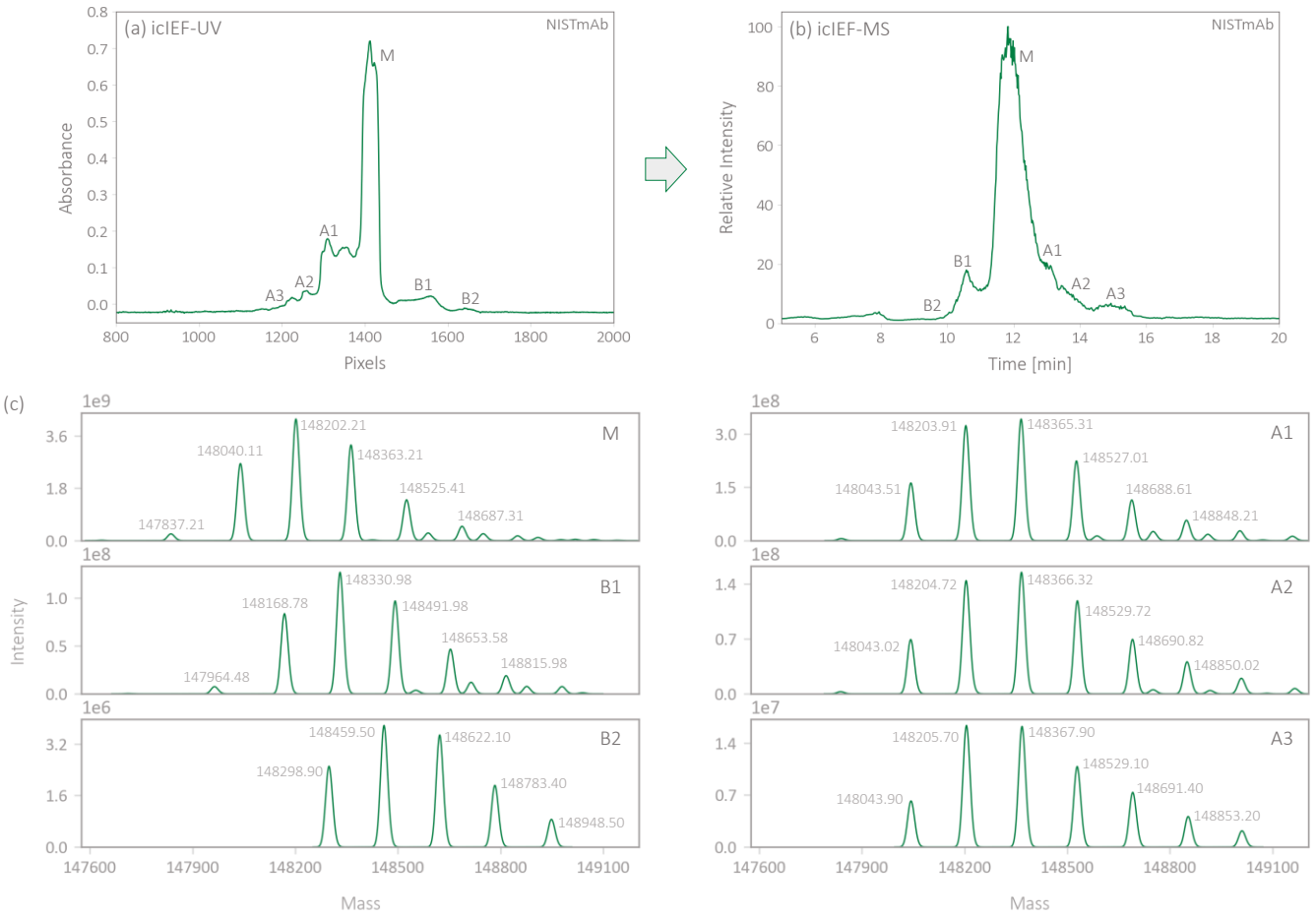


Fig 6. (a) The NISTmAb icIEF profile icIEF-MS capillary cartridge (200 µm diameter). The peaks are then mobilized toward the directly coupled MS instrument to produce the (b) total ion chromatogram (TIC) capable of detecting all relevant icIEF peaks. (c) Each of the different charge variant regions can then be highlighted and deconvoluted to obtain the mass spectrum. There is a clear shift in the basic variants likely corresponding to lysine clipping, as well as a more subtle shift in the acidic variants likely corresponding to deamidation.

icIEF Services

AES has built a state-of-the-art icIEF facility and developed a team of experienced scientists and engineers (Fig 7).

The Industry Challenge. The next generation of icIEF is upon us, and many are looking to access the most advanced tools. This is especially true as the industry places more of a premium on in-depth information, and becomes more ambitious in the development of therapeutics with complex charge profiles.

Maximizing the potential of these technologies can require a lot of time and resources, and everyone has a unique set of timelines when deciding to commit to such an investment.

The Advanced Electrophoresis Solution. AES services are helping companies adapt to the next generation of icIEF, regardless of their current timelines. For example, this is a great option for high-priority projects that simply cannot wait. Alternatively, we can assist you with smaller projects as you look to become more familiar with our technology.

→ Extensive Experience. Our leadership has been heavily involved in the development of icIEF for several decades. Furthermore, we have a team of capable scientists and engineers who have been working through some of the industry's most difficult icIEF problems to date.

→ Method Development. We routinely support customers developing icIEF methods for complex samples. For example, we can recommend different carrier ampholytes, capillary coatings, and system parameters.

→ High-Throughput Fractionation. Several CEInfinite units are available at our facility, allowing us to rapidly collect more than 50 µg of each icIEF peak. We can then run buffer exchange on the fractions before freezing and returning them for further analysis.

→ icIEF-MS and Peptide Mapping. Our facility includes a Thermo Scientific Q Exactive Plus mass spectrometer to run icIEF-MS with excellent resolution and sensitivity (Fig 8). We also have a Thermo Scientific Vanquish UHPLC system for in-depth peptide mapping after fractionation.

→ Custom Carrier Ampholytes. Thanks to our unique understanding of their design and synthesis, we can help alleviate many of the concerns associated with carrier ampholytes: a) custom formulations for optimal stability and resolution, b) profile matching of existing icIEF curves, c) guaranteed lot-to-lot consistency, d) resupply services, and e) regulatory filing support.

→ Instrument Evaluation. We are happy to accept samples from those who want to test the CEInfinite.

Fig 7. Partner with AES to leverage our expertise and cutting-edge icIEF technologies and services.

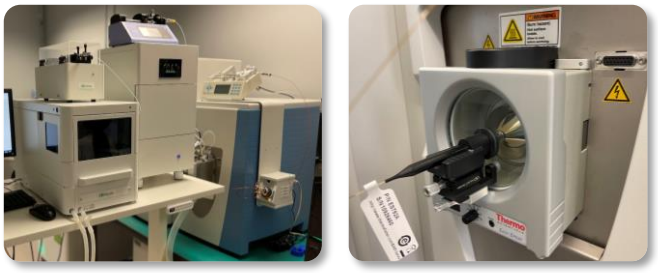


Fig 8. The CEInfinite in icIEF-MS mode, directly coupled to Thermo Scientific Q Exactive Plus, with a close-up of the nanospray emitter.

Software Integration

CEInsight software can be used to control the CEInfinite system while complying with FDA Title 21 CFR Part 11 requirements. The friendly user interface can facilitate the reliable operation of analytical icIEF, icIEF fractionation, and icIEF-MS. The data can then be exported and quantified using standard analysis software such as Chromeleon, Empower, Chemstation, and Clarity.

Recently, we announced our integration with Chromeleon software to control the operation and analysis of the analytical and icIEF-MS modes. This is a significant milestone that allows many of our customers to use CEInfinite with the software they are most familiar with. Moreover, our software team is actively working to develop a similar integration for Empower.

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System Overview

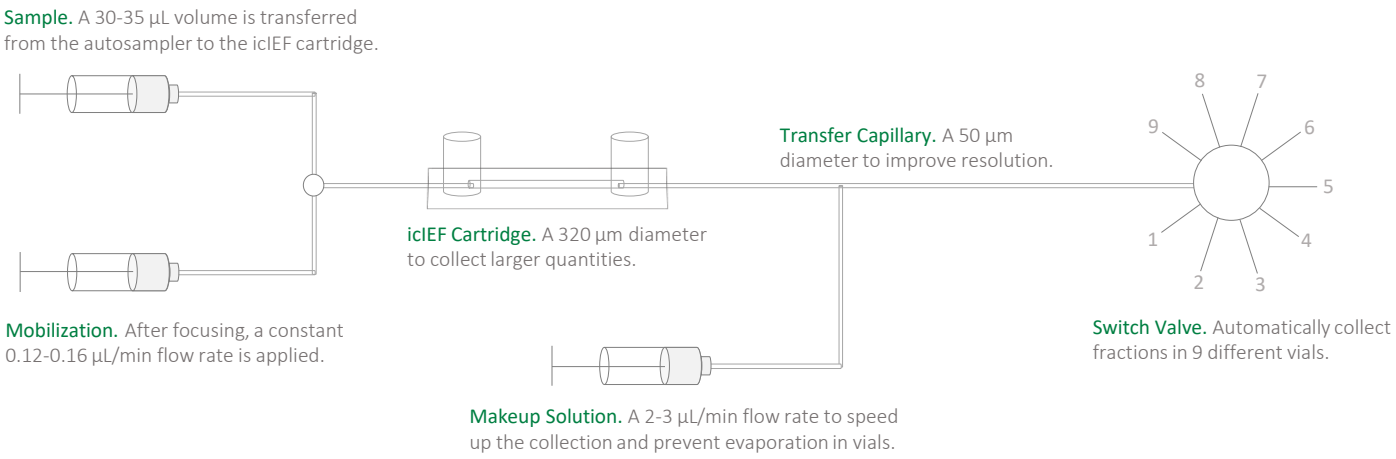

Fig 9. (a) An overview of the CEInfinite fractionation system. The sample is loaded in the autosampler before being transferred to the icIEF cartridge. The capillary diameter is increased from 100 µm to 320 µm to increase the amount of sample collected. The separation takes place for 10-20 minutes before the mobilization is activated. At this point, the charge variant peaks move toward the transfer capillary while the specific regions are defined for collection. The software calculates the time it takes for each region to reach the switch valve, which then adjusts automatically to direct the fractions into their respective vials. (b) The icIEF-MS system is a similar setup with the following exceptions. The icIEF cartridge has a 200 µm diameter. The make-up solution includes acetonitrile and formic acid for better electrospray ionization efficiency. Finally, the switch valve is replaced with direct coupling to a mass spectrometer.

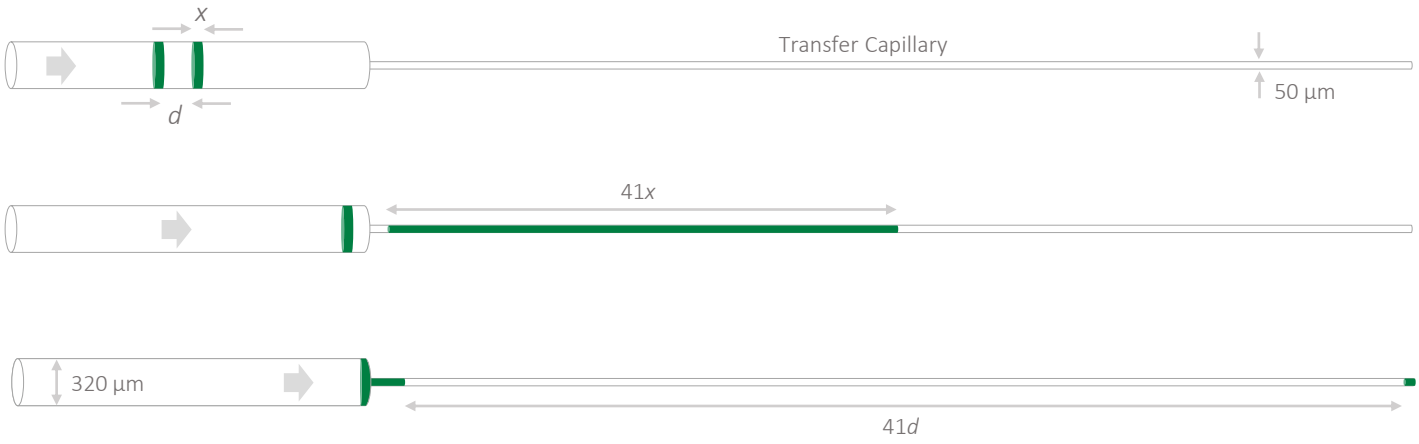

Fig 10. As the charge variant zones go from the 320 µm diameter icIEF capillary to the 50 µm diameter transfer capillary, the separation distance will increase by approximately 41 times, and therefore reduce the cross-contamination caused by molecular diffusion and the parabolic velocity flow profile. The pressure mobilization enables excellent consistency and speed while avoiding the chemical mobilization additives that can cause instabilities related to Joule heating and pH gradient disruption. The last peaks to elute will have the worst purity due to the extra-column effects influencing the preceding samples. Fortunately, pressure mobilization also supports reversed polarity to allow the acidic variants to elute first for a higher purity.

System Overview

CEInfinite

Detection Mode	Whole column, sCMOS imaging technology
Height x Width x Depth	54 cm x 33 cm x 30 cm
Weight	30 pounds (14 kg)
Detection Dynamic Range	250 (0.004 – 1.0 AU, 280 nm)
High Voltage Range	0 – 3000 Volts (Continuously Adjustable)
Sample Throughput	Up to 12 injections per hour
pI CV	<1%
Working Temperature	15 - 35°C
Humidity	20 - 80% RH
Electrical Requirement	100/240 VAC, 50 - 60 Hz
Exposure Time	0.02 – 99.9 ms
ADC Maximum	16386 AU
Operation Mode	Manual or Automatic
Detector Noise	Less than 0.001 AU, 280 nm
Separation pH Range	2.1 – 12

Auto-Sampler

Model	840
Sample Capacity	84+3 vial tray, 96 well plate
Sample Tray Temperature	4 - 40°C
Typical Sample Volume	15 µL
Electrical Requirement	95 – 240 VAC, 50 – 60 Hz
Height x Width x Depth	36 cm x 30 cm x 57.5 cm
Weight	46 pounds (21 kg)
Working Temperature	10 - 40°C
Humidity	20 – 80% RH

Injection Pump

Syringe Size	250 µL/5mL
Electrical Requirement	100 – 240 VAC, 50 – 60 Hz
Height x Width x Depth	12.14 cm x 10.8 cm x 24.1 cm
Weight	2.72 pounds (1.23kg)
Working Temperature	5 – 40°C

Innovations Make Breakthrough Solutions

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