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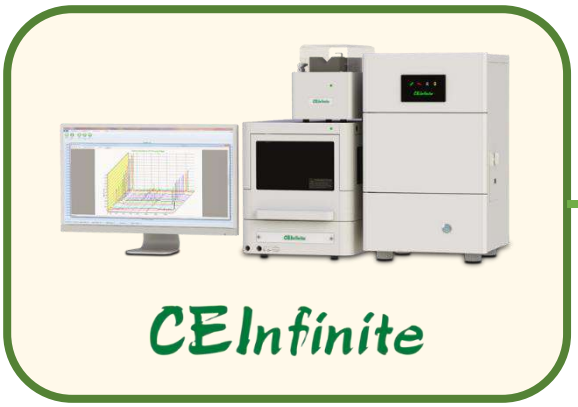
Application Note

Enjoy your iCIEF-HRMS

The best answer to the challenges of protein heterogeneity characterization in biopharma discovery: state of the art technology in CEInfinite iCIEF tandem Thermo Fisher quadrupole-orbitrap mass spectrometer



A Comprehensive Solution for Biological Product Development

Introduction

Recombinant monoclonal antibodies (mAbs) across the pharmaceutical industry have been spurring rapid growth in the commercial and clinical products of biotherapeutics. Recently, complex proteins including antibody-drug conjugates (ADC), bi-specific Abs and fusion proteins have further attracted great attentions to biopharma industry. The charged heterogeneity of protein drugs (resulting from several mechanisms including chemical degradation, cellular processes, and production conditions during the manufacturing process) requires in-depth structural characterization for critical quality attribute (CQA) assessment to ensure safety, efficacy and potency [1-2]. Charge heterogeneity of protein results from diverse mechanisms including cellular processes, chemical degradation and production conditions during the manufacturing process [3-4]. Post translational modifications (PTMs) including C-terminal lysine truncation, pyroglutamate formation, deamidation, sialylation and glycation occurred could result in the formation of charge variants [5-6]. Imaged capillary isoelectric focusing (iCIEF) based on pI differentiation is robust for high-efficiently analyzing diverse PTMs and degradation products [7-8] and high-resolution mass spectrometry (HRMS) is critical for molecular mass identification of protein variants [9-10].

Since the first commercial instrument developed, iCIEF (Imaging capillary isoelectric focusing) technology has been becoming the gold criteria of the quality and manufactory process control in the biopharmaceutical industry due to its high-resolution characterization of protein drugs with high-throughput. However, with the rapid development of biopharma discovery, the critical three bottlenecks as below in term to iCIEF coupled to HRMS for protein charged variants have been frustrating the scientists for a long time. Without promising answers, the width and depth in biopharmaceutical discovery will be greatly impeded.

1. Can iCIEF realize the industry-level tandem HRMS platform for characterizing protein-charged variants with “SOP” guide, easy-going control and ultra-high repeatability, to avoid the trail-and-error operation currently coupled strategies reported are facing?

2. Can iCIEF be developed for being the most compatible with HRMS identification including coated separation channel, critical reagents and seamless interface to achieve the accurate structural elucidation of diverse proteins, to avoid the resolution and sensitivity sacrifice during HRMS identification.
3. Can a routine-iCIEF be evolved to triple-iCIEF realizing the highly-auto QC analysis, fractionation and direct connection to HRMS of protein charged variants, to avoid “Low Return of Investment”?

“Innovations Make Breakthrough Solutions” has been accelerating the “answers” to above “challenges” from Advanced Electrophoresis Solutions (AES) [11-15]. In terms to protein heterogeneity characterization, the first hybrid-commercial iCIEF instrument has been introduced in 2019 for achieving one-button analysis in quality control, full-auto fractionation and HRMS direct connection.

After 6-years development, AES has achieved a further successful jump in iCIEF-HRMS based on the collaboration with key customers in biopharma industry as shown in Figure 1 [16-19], to gain the rapid iCIEF separation and reliable HRMS identification of protein charged variants. The whole iCIEF-HRMS analysis based on seamless MS interface can be solved within 30min and the complete compatibility with HRMS is achieved due to free urine and polymers such as carboxymethylcellulose (MC) in iCIEF separation resulted from customized coating capillary and ampholytes.

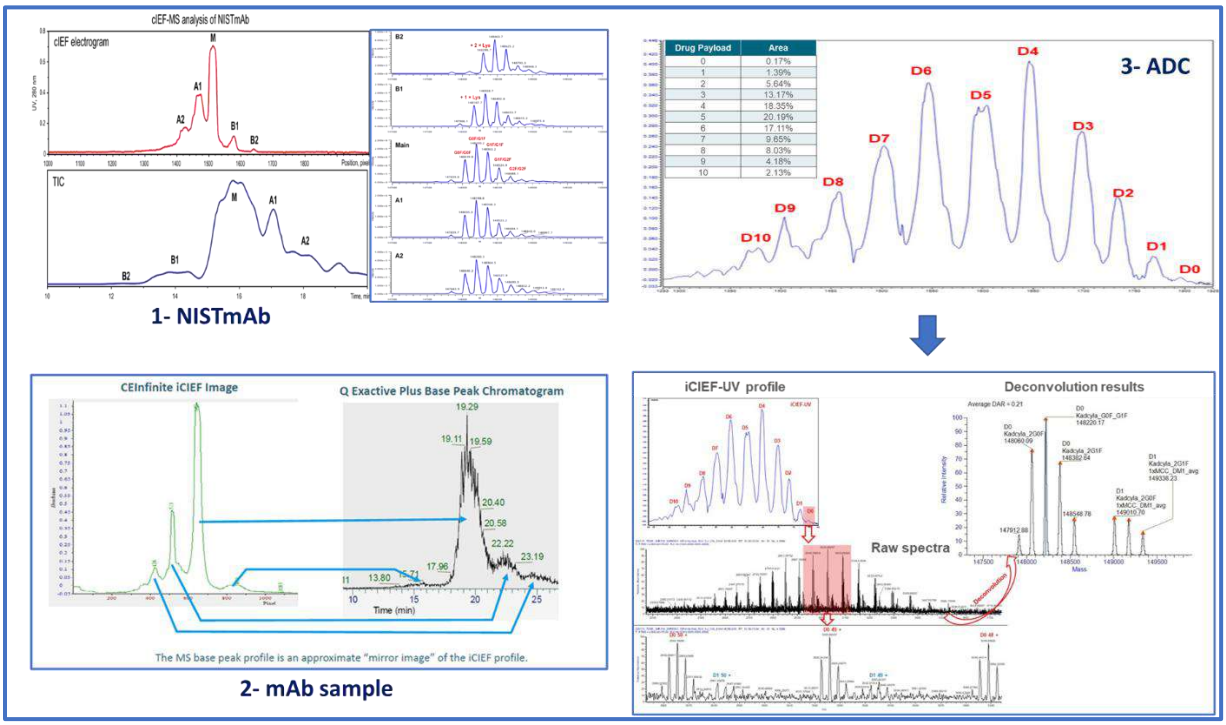


Figure 1. Achieved iCIEF-HRMS characterization of protein charged heterogeneity based on key collaborations with biopharma partners.

In this technique note, employing bevacizumab as targeted protein drug, the "SOP" based on CEInfite iCIEF, Thermo Fisher quadrupole-orbitrap (QE) mass spectrometer and Biopharma Finder Software 5.0 is demonstrated for protein charged variant characterization, including step-by-step workflow, complete iCIEF & HRMS experiment conditions, sample preparation, data process and operation details keeping reliable and repeatable analysis. This can be a guide to the operators who can follow up to achieve the easy-going and accurate iCIEF-HRMS standard operation.

Materials and methods

The CEInfinite system and patented iCIEF-MS cartridges eliminate the need for chemical migration when coupled to online mass spectrometry as shown in Figure 1, while only using proprietary capillary-coated cartridges and separation solvents during iCIEF separations greatly reduce the need for polymers and urea. It enables the isolated protein charge heterogeneity to be directly used for high-sensitivity MS characterization, thus retaining the excellent separation resolution of iCIEF for mass spectrometry analysis. The CEInfinite system requires no special modifications to the ionization source and can be directly connected to the mass spectrometer ionization source from the different mass spectrometry brands.

After proteins' focusing is completed along the separation capillary, water containing 0.1% (v/v) formic acid as mobilization solvent from syringe pump drives the focused protein bands out of the separation capillary towards MS ion source (ESI) at 50-200nL/min flowing rate depending on selected separation capillary cartridge I.D. (typically 50nL/min for 200µm I.D. cartridge; and 160nL/min for 320µm I.D. cartridge). Sheath liquid (water: acetonitrile =1:1 v/v, containing 1% formic acid v/v) helps the effluents direct into ESI though a seamless interface. The whole process is automatic and friendly even for the new operator.

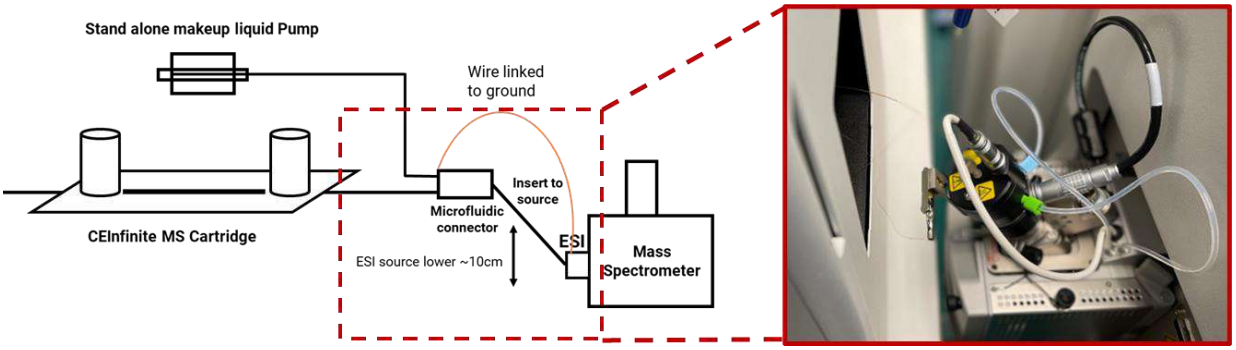


Figure 2. iCIEF-HRMS schematics

Workflow of iCIEF-HRMS

The workflow established by iCIEF-HRMS is straight forward, concise and full-automatic as illustrated in Figure 2, including sample preparation, iCIEF separation, MS identification and data processing. Based on the workflow, the intact protein elucidation and peptide mapping analysis can be easily and reliably achieved. In addition, customized ampholytes and capillary coatings allow to be the most compatible for MS connection, which can simplify the operation steps and prevent the ESI from contamination.

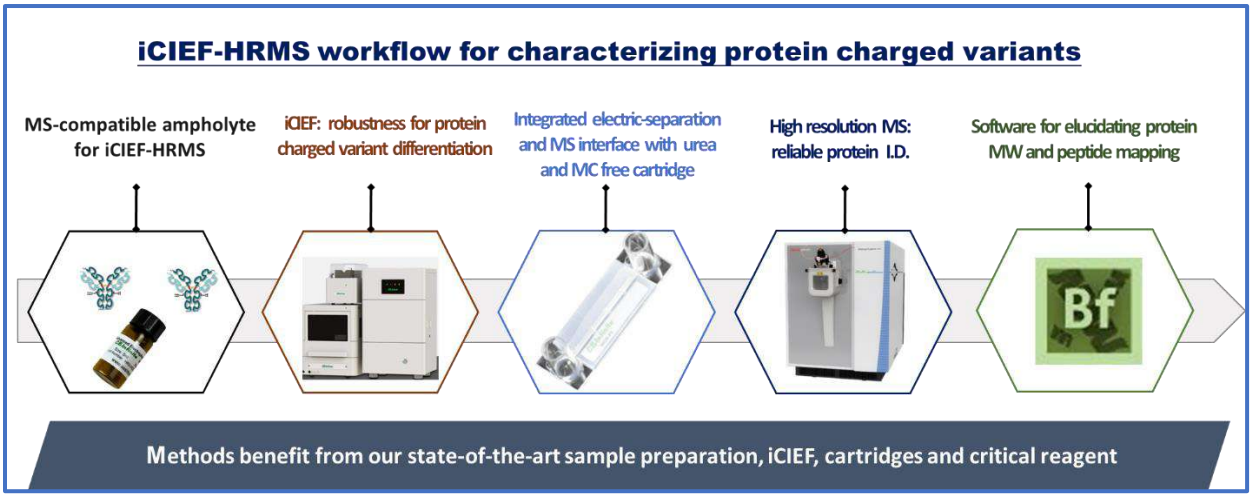


Figure 3. iCIEF-HRMS workflow for characterizing protein charged heterogeneity.

iCIEF and HRMS conditions

The CEInfinite iCIEF and Thermo Fisher QE plus MS were employed and the most optimal conditions are as follows.

iCIEF Conditions (CEInfinite)	
Sample final concentration	1mg/ml
Ampholyte	2% v/v AESlyte HR 7-9
Additive	10% v/v formamide
iCIEF focusing	1 min at 1,000 V, 1 min at 2,000 V, 10min at 3,000 V
iCIEF mobilization	0.1% formic acid, 0.05ul/min, 30mins
Cartridge	CEinfinite iCIEF-MS cartridge I.D. 200µm ; AD coating
Anolyte	80mM H3PO4 solution
Catholyte	100mM NaOH solution

HRMS parameters (QE+ or Exploris series)	
ESI mode	Positive
Spray voltage (kV)	3.6
Capillary temp. (℃)	275
Spray temp. (℃)	275
Sheath gas flow rate (L/min)	20
Aux gas flow rate (L/min)	5L
S-lens RF (eV)	70
Resolution	35,000@m/z 200
Scan range of precursor ion	m/z 2000-8000
AGC target	$3e^6$
Maximum injection time	200 ms
microscan	10
Insource CID	75
SID (V)	110
Data processing	Biopharma Finder 5.0
Spray needle OPTON-53011	**75μm I.D. 34 Gauge**

Experimental steps :

1. AD coating cartridge is rinsed into waste vial with condition solution containing 0.35%MC and followed with water.
2. Prepare “Master Solution” and mix with bevacizumab sample to have 1mg/ml final concentration and 10% v/v formamide, 2% ampholyte (AESlyte HR 7-9);
3. Fill mobilization syringe (250ul) with 0.1% formic acid and install on pump;
4. Fill sheath syringe (5ml, independent pump) with 0.5% formic acid, 50% acetonitrile in water and install on the independent pump;
5. Follow CEInfinite cartridge installation and startup procedure;
6. Turn sheath syringe pump on with 5ul/min flowrate and keep on;
7. Run iCIEF and MS.

Results and discussion

Figure.4 demonstrates the iCIEF electropherogram and MS total ion chromatogram (TIC) for the identification of bevacizumab’ acidic (A1 and A2) and basic (B1 and B2) charged variants.

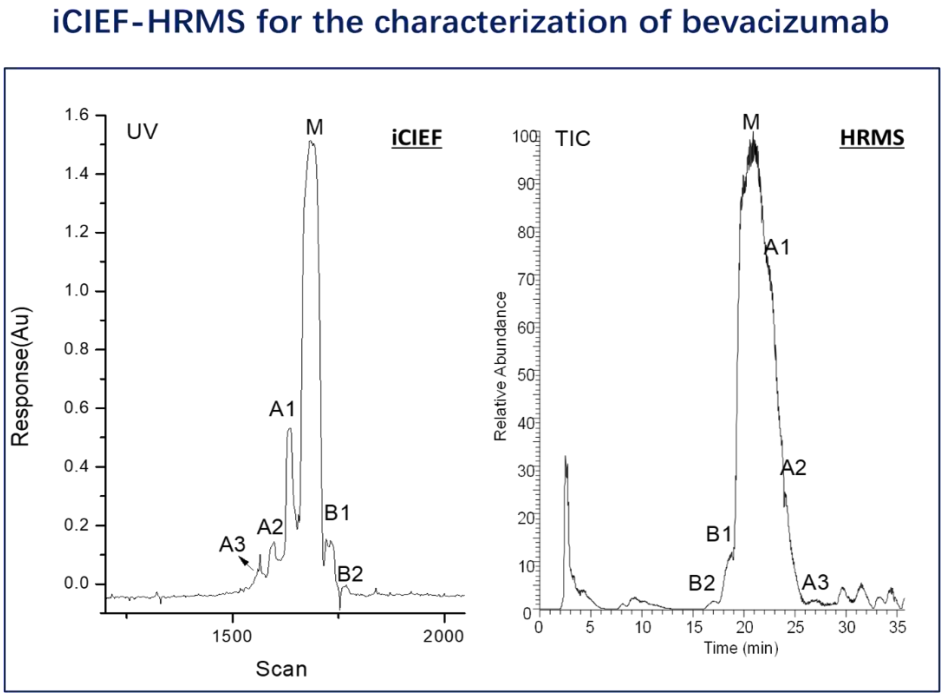


Figure 4. Electropherogram and total ion chromatogram (TIC) in iCIEF-HRMS analysis.

As illustrated workflow of data processing in Figure 5, Thermo Fisher Biopharma Finder 5.0 utilizes the accuracy of high-resolution data for confident intact mass analyses. For absolute confidence in deconvoluted molecular weights in basic, acidic and native conditions, advanced algorithms make the most of high-quality high resolution mass analyzer data. Xtract deconvolution using sequence-specific isotope tables provides accurate results for deconvolution of complex biotherapeutics. Confidently discover, identify, quantify and monitor product quality attributes with easy-to-understand data visualization within a single software platform. Figure 6 displays the results of protein identification based on Biopharma Finder.

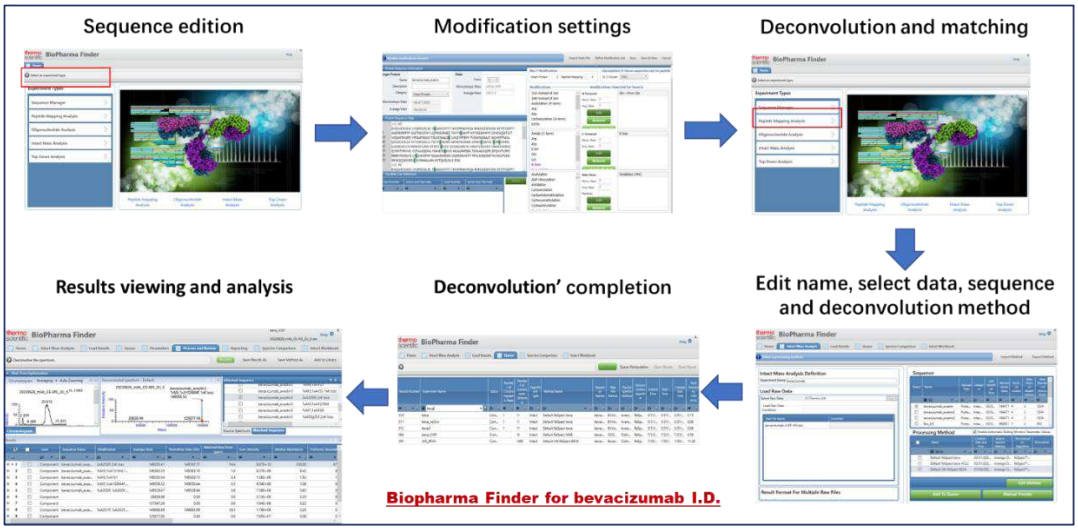


Figure 5. Workflow of Biopharma Finder 5.0 for the processing of data from iCIEF-HRMS.

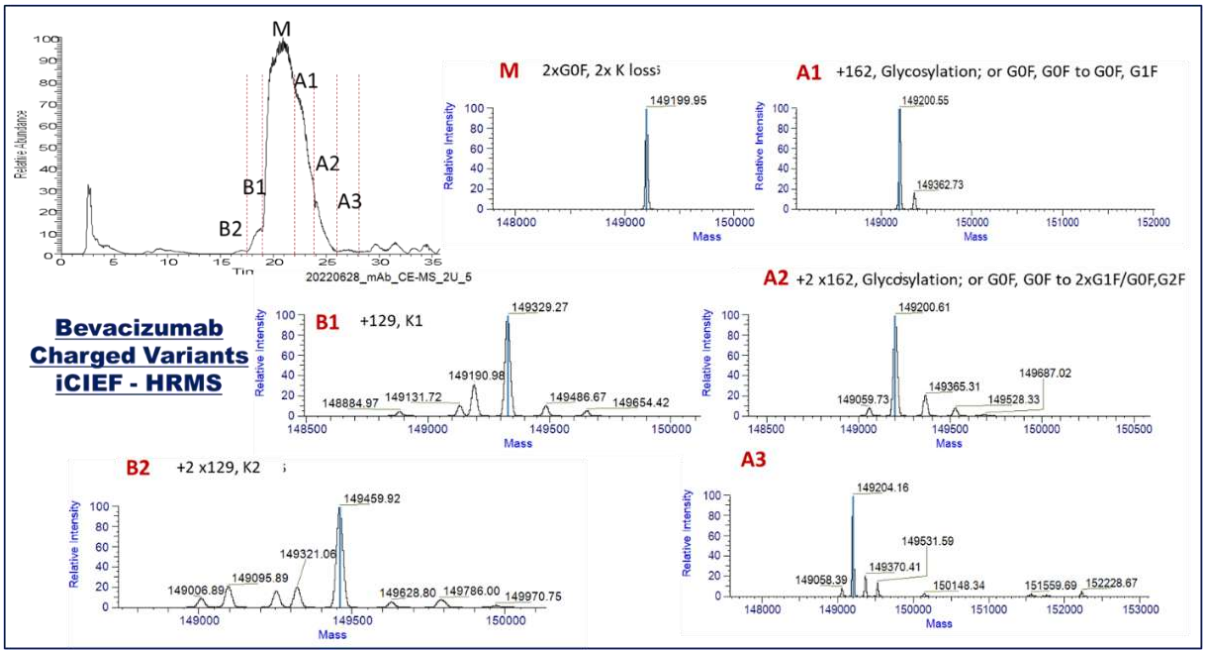


Figure 6. Structural elucidation of bevacizumab' charged variants by Biopharma Finder 5.0.

Method repeatability

Intact NISTmAb was utilized for the stability evaluation of methodology, to achieve excellent repeatability as shown in Figure 7. This warranties the applicability of iCIEF-MS established in QC stage.

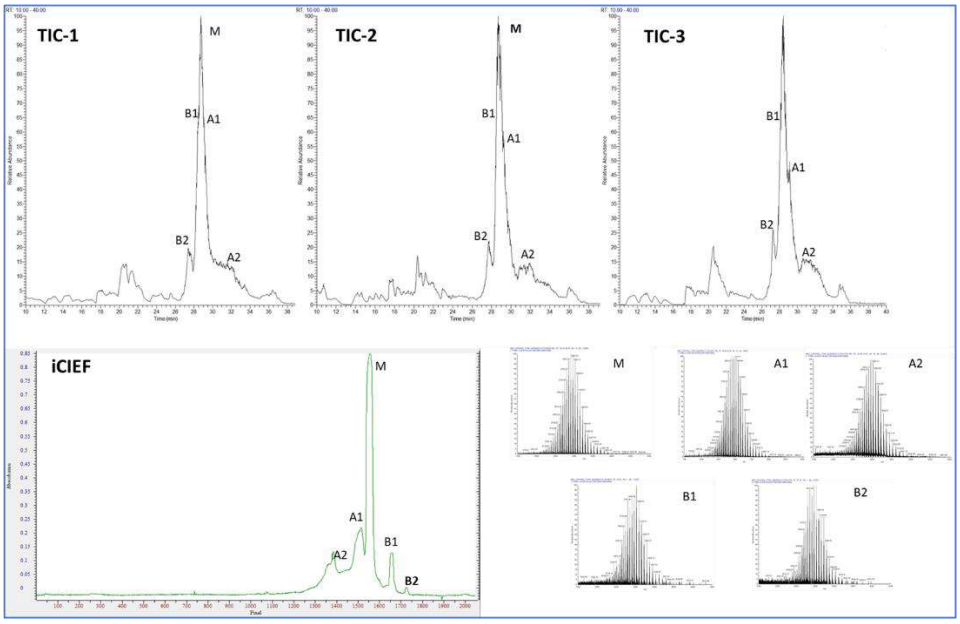


Figure 7. Method repeatability of iCIEF-HRMS for Intact NISTmAb (n=3)

How to install the CEInfinite System - MS-Coupling

Overview

The information in this technique note is designed to assist users wishing to install a CEInfinite system with MS-coupling. This section describes how the CEInfinite iCIEF system works with MS connection. Refer to Figure 8 & Figure 9 for a general illustration on how CEInfinite system is tandem MS source.

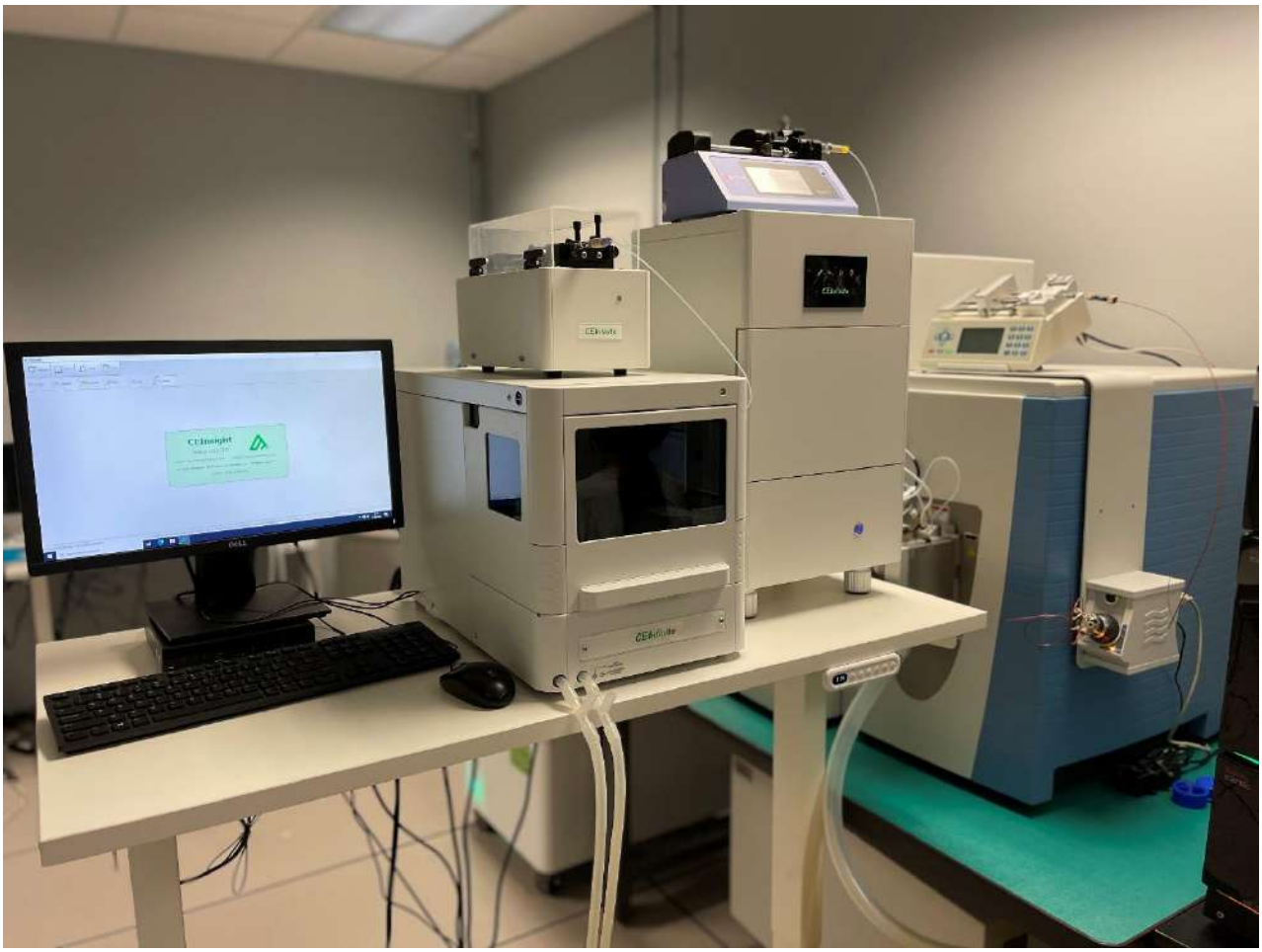


Figure 8. CEInfinite iCIEF system - MS coupling overview.

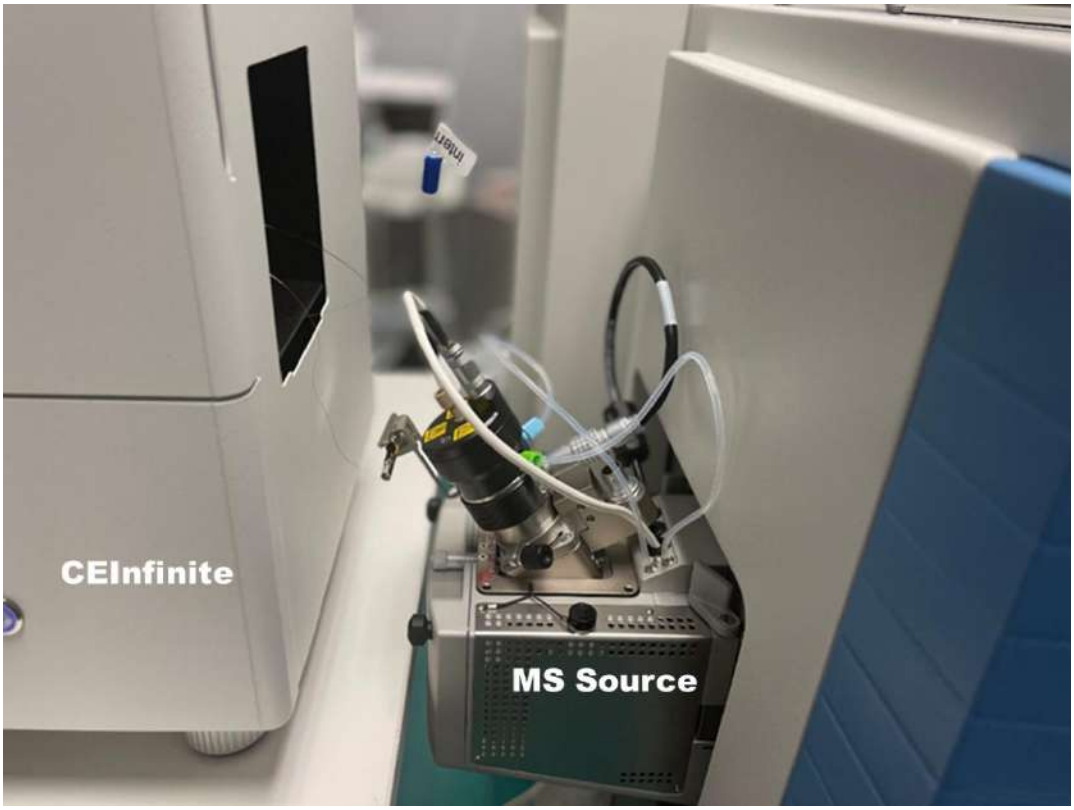
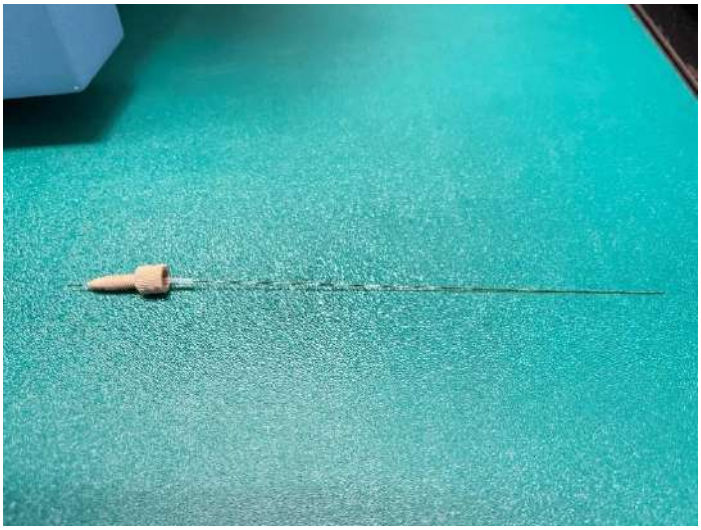


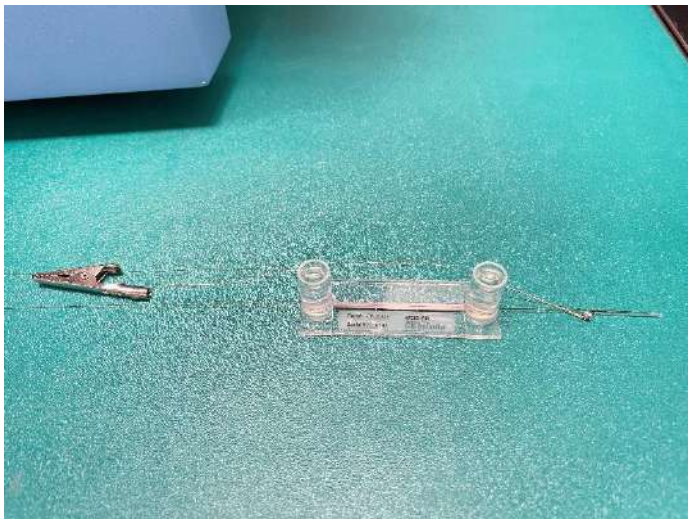
Figure 9. CEInfinite iCIEF system – MS Coupling details.

Configurations

1. A transfer capillary with 15cm or shorter length 100um I.D. included in the iCIEF-MS kit is used to connect between the cartridge outlet to the MS ion source. Cut the transfer capillary as short as possible to reduce the sample mobilization time and back-pressure.

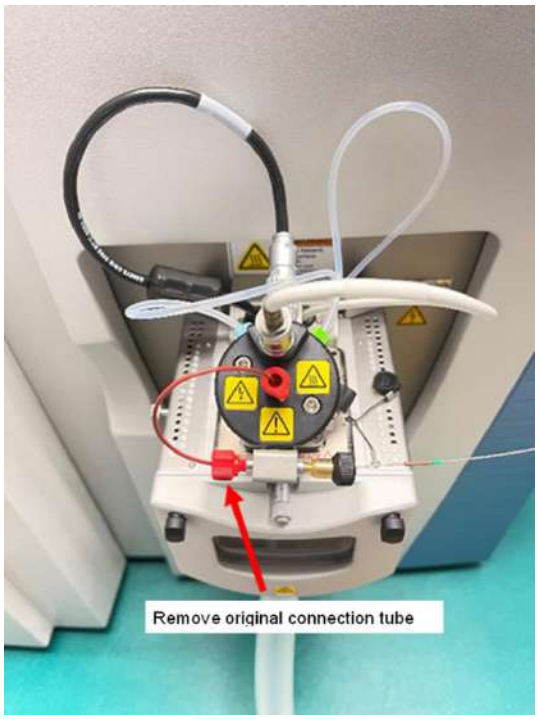


2. Cartridge with I.D. 200µm (CP00303M) as shown below allows users to direct coupled from CEInfinite system to Mass spectrometry.



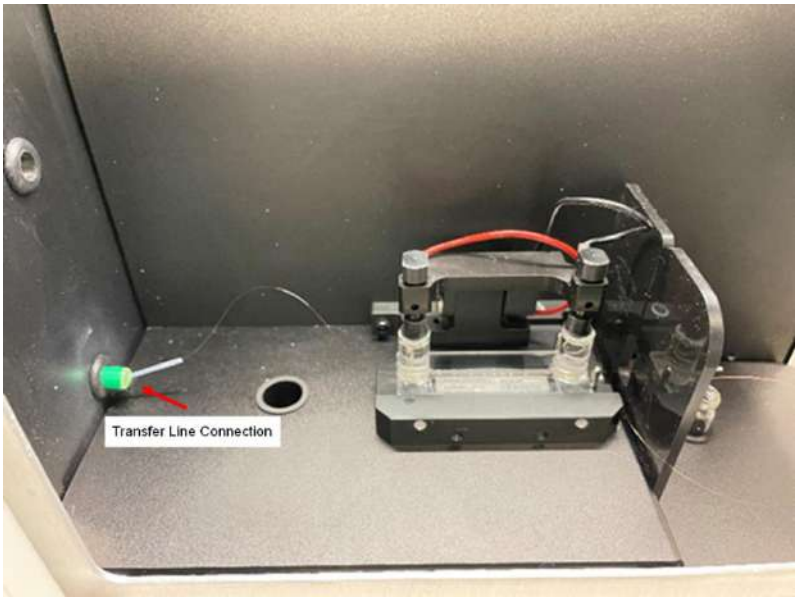
Operation procedure:

1) Remove mass spectrometry original connection tubes illustrated as follows.

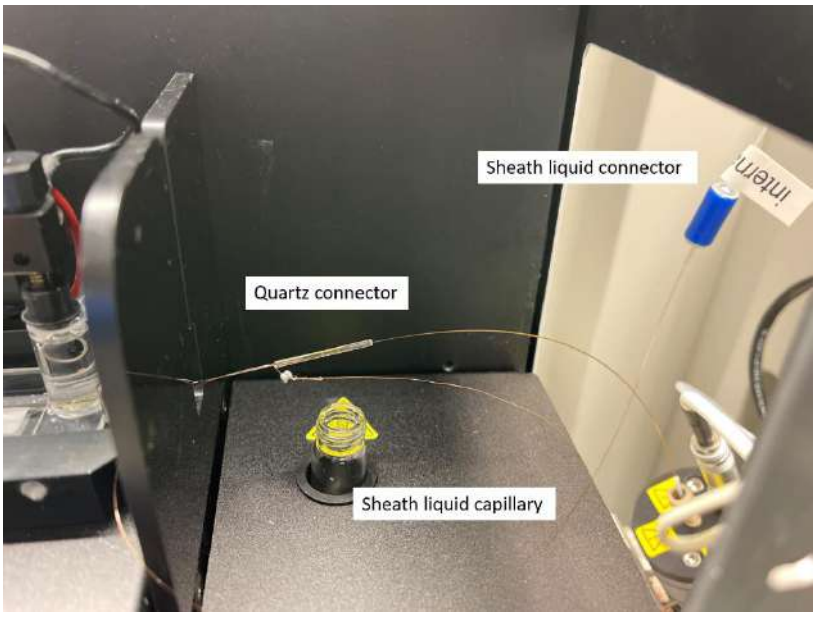


2) Install long transfer capillary into injection port.

3) Connect transfer line with left side of cartridge's capillary.

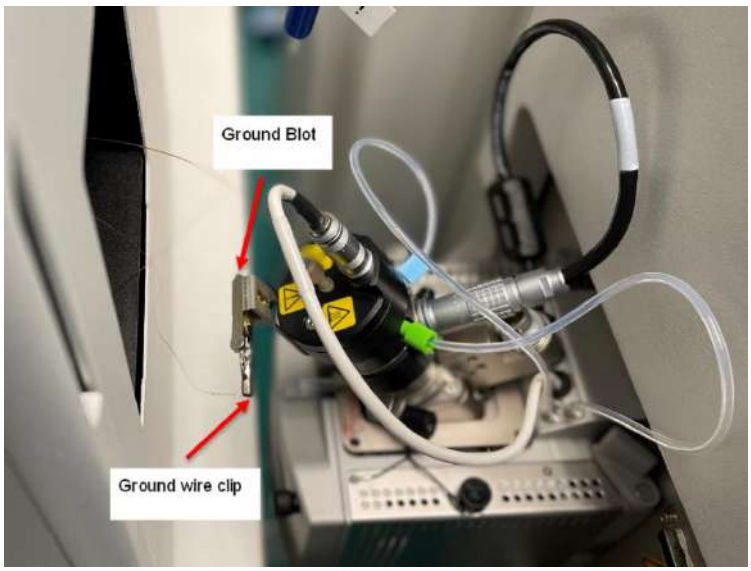


4) Adjust CEInfinite platform height and move as close as possible to the mass spec source. The customized iCIEF-MS adjustable bench is optional. The outlet should be ~10cm higher than the source. Connect sheath Liquid tube with sheath capillary line and then connect long transfer capillary with quartz connector.



5) Connect Ground wire to Ground blot.

Insert the long transfer capillary into the source directly, and connect the grounding wire with the ground blot with the clip (included in the kit).



Conclusion

Rapid and accurate characterization of protein charged heterogeneity is a critical need for therapeutics to support the rapid growth in biopharma industry, but for a long term there has not been a robust analytical platform that could simultaneously provide rapid and high-efficient charge variant separation along with the molecular mass identification of peaks. iCIEF-HRMS can provide a promising strategy for differentiation and identification of protein charged variants. The CEInfinite iCIEF-HRMS developed has overcome the demerits of reported CE-MS products which are frustrated by poor repeatability and low sensitivity, to realize the QC-level platform and promising workflow based on iCIEF-HRMS. In addition, iCIEF-MS configuration can flexibly and seamlessly switch to iCIEF preparative model for the μg level fraction collection of charged variants which can further be LC-MS analyzed at peptide mapping level. The total solutions allow to achieve the QA analysis, MS direct connection and fraction collection of protein charged variants on a “CEInfinite iCIEF".

CEInfinite iCIEF-MS kit

Part	Product number
0.35% MC solution	101099
80mM H3PO4 solution as anolyte	101007
100mM NaOH solution as catholyte	101008
WCID AD Cartridge for MS	CP00303M
Adjustable bench (optional)	AB00601
Syringe pump for sheath liquid delivery(optional)	SP00103
AESlyte HR, SR and UH series as ampholytes	Refer to “Consumable Brochure”

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System spec

iCIEF instrument

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

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