

# **Method Development Using Imaged Capillary Isoelectric Focusing (iCIEF) Techniques for Therapeutic Biologics**

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# Abstract

Analysis and characterization of therapeutic proteins in the biopharmaceutical industry is one of the most challenging tasks in the development of biological drugs. Historically, conventional electrophoretic technologies such as SDS-PAGE, Western blot, and gel IEF have played important roles in the analysis of biomolecules. However, new technologies are emerging to complement traditional analytical methods and are recommended by health authorities to provide orthogonal analytical abilities. Over the past several years, electrophoretic based microseparation techniques such as capillary electrophoresis (CE) have been applied for the separation and analysis of a wide variety of compounds in the areas of biochemical and biological research. Most recently, imaged capillary isoelectric focusing (iCIEF) has demonstrated great potential in the separation of charge variants, offering unique application and analysis abilities. This presentation will demonstrate the utility of this new technology for the separation and analysis of biomolecules in biopharmaceutical method development processes, and its potential application for protein characterization, in-process, and GMP testing.

# **Gel IEF, Capillary IEF (CIEF), and Ion Exchange Chromatography**

- **Gel IEF**
  - High resolving power (pI differences of 0.001 pH units)
  - Traditionally performed in polyacrylamide slab gels
  - Slow, labor-intensive (staining/destaining), generally not quantitative
- **CIEF**
  - Performed in capillary format
  - Advantages over gel IEF, automation, speed, quantitation, could be realized
  - Single-point, on-column detection at one end of the capillary
  - Requires two steps (> 30 min/run):
    - Focusing (separation), based on pI
    - Mobilization, separated protein zones pass the detection window
  - Introduce uneven separation resolution, poor reproducibility
- **Ion Exchange Chromatography**
  - Lower resolving power
  - Increased interaction with the capillary/column
  - Slow analysis time, method development
  - Large sample consumption

# Imaged CIEF (iCIEF) Technology

## iCIEF

- **Whole-column UV image detection (captured by a camera with imaging lenses and CCD sensor) for rapid analysis**
- **Quick and easy method development**
  - **Fast sample analysis time (up to 6 runs/hour)**
    - **Different conditions can be tested quickly**
  - **Online monitoring IEF process**
    - **Quick optimization of key IEF parameters: focusing time and voltage**
    - **Sample aggregation and precipitation during IEF can be identified (in “real-time”)**
    - **Additives can be selected according to the identified problems**
  - **Electrolytes and instrument parameters are not affected by switching carrier ampholytes**
- **Optimal focusing time**
  - **Normally 5 – 10 minutes for most proteins**
    - **Certain proteins need longer than usual focusing time**
    - **Easy and quick optimization**
- **Reproducible peak pattern and high resolution**
  - **Optimal focusing time, without mobilization**

## Methods

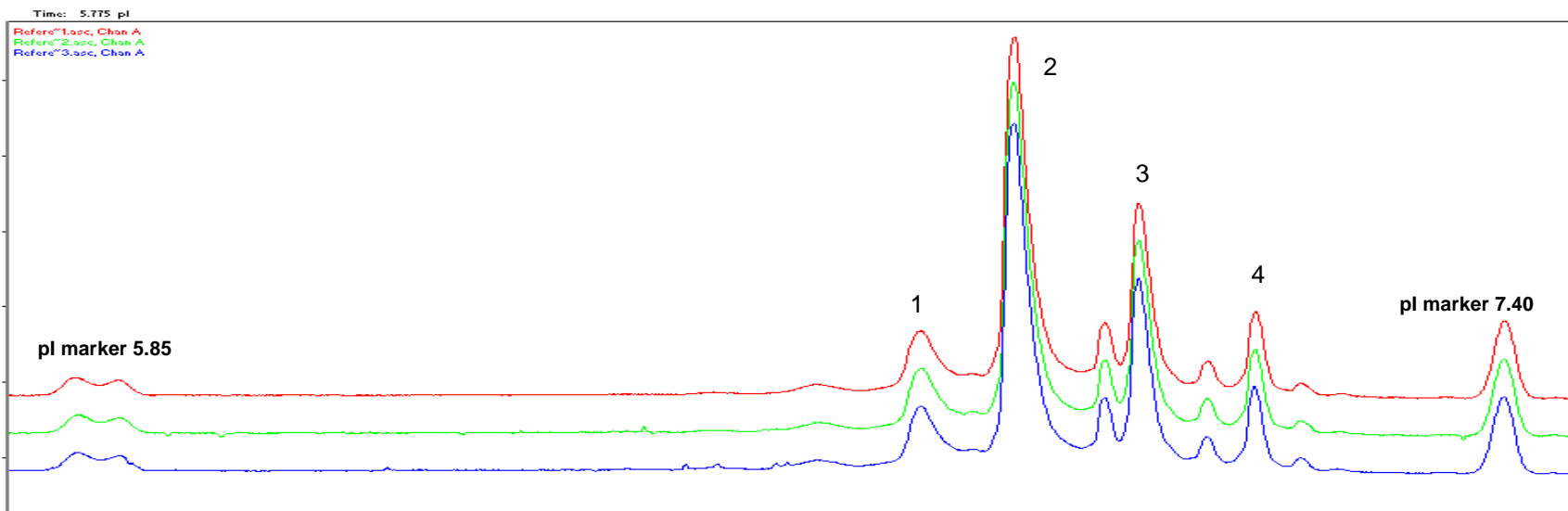
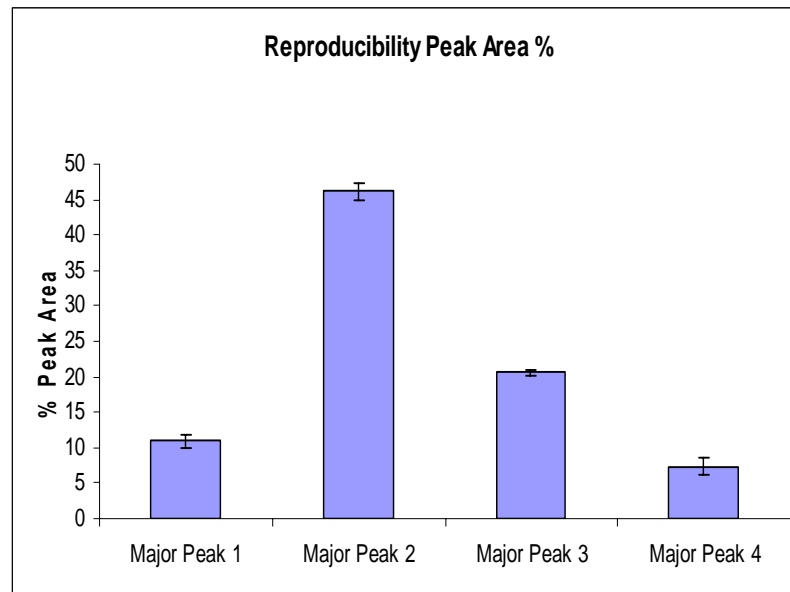
- **iCIEF Analysis:** BMS-Mab1 reference material, drug substance, and drug product isoforms are resolved in Pharmalyte (pH 5-8) using the Convergent Biosciences iCE280 imaged capillary electrophoresis system. BMS-Mab1 reference material and test articles are diluted to 1mg/mL in Milli-Q water, 8% Pharmalyte, 2M Urea, and 0.35% Methyl Cellulose (MC), with pI markers of 5.85 and 7.40. 35 $\mu$ L of each test article are injected by an Alcott 719AL autosampler into a fluorocarbon (FC) coated capillary cartridge in the iCE280. An anolyte solution of 80mM phosphoric acid and a catholyte solution of 100mM sodium hydroxide are utilized to establish a pH gradient across the capillary chamber. The sample is prefocused for 1 minute at 300V/cm and then focused for 9 minutes at 600V/cm. After focusing, sample migration is captured by a CCD camera for quantitative analysis.
- **Gel IEF Analysis:** BMS-Mab1 drug substance and drug product isoforms are resolved in an Ampholine PAGplate (pH 3.5-9.5) using the Multiphor II horizontal electrophoresis system. BMS-Mab1 reference material and test articles are diluted to 2mg/mL in Milli-Q water and manually loaded onto sample application pieces on the gel. An anolyte solution pH 3 and a catholyte solution pH 10 are utilized to establish a pH gradient across the gel. The samples are focused at 3000V, 50mA, 30W for 2.5 hours. Gels are processed by Coomassie blue staining and laser densitometry is employed for analysis.

## Optimization Parameters

- **Urea Concentration:** 2M to 8M
- **Pharmalyte (5-8) Percentage:** 2% to 16%
- **Sample Concentration:** 0.5mg/mL to 3mg/mL
- **Autosampler Temperature:** 4C to 25C.
- **Focusing Duration:** Prefocus 0 to 1 Minute at 300V/cm  
Focus 5 to 15 Minutes at 600V/cm

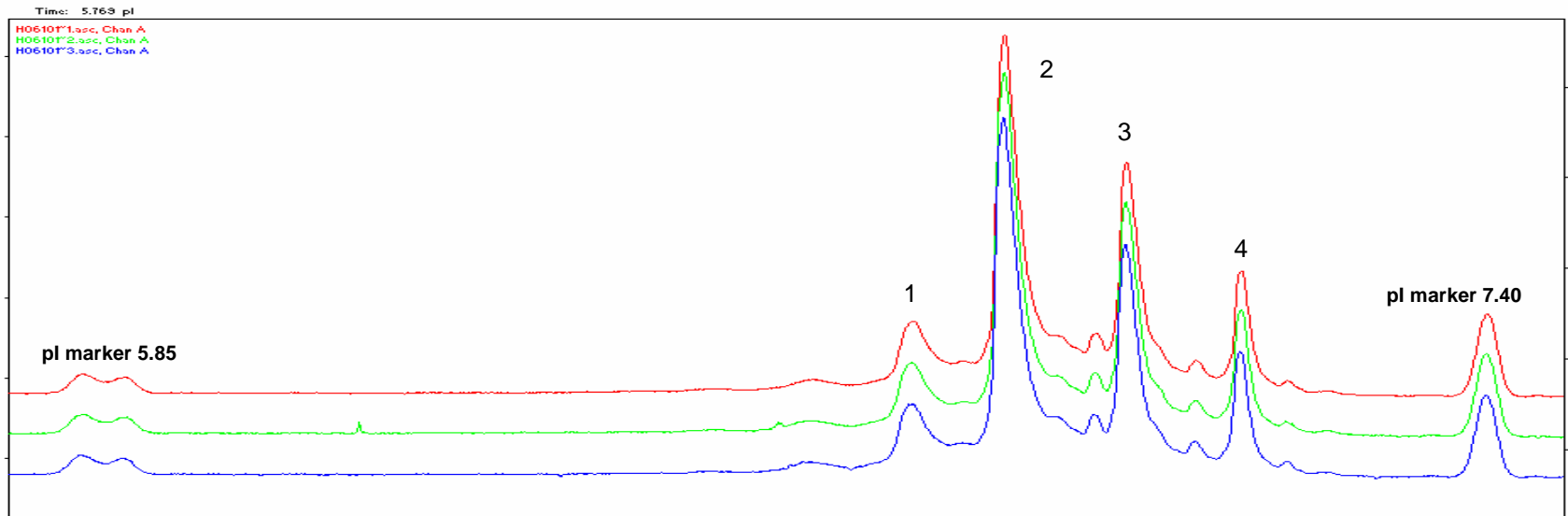
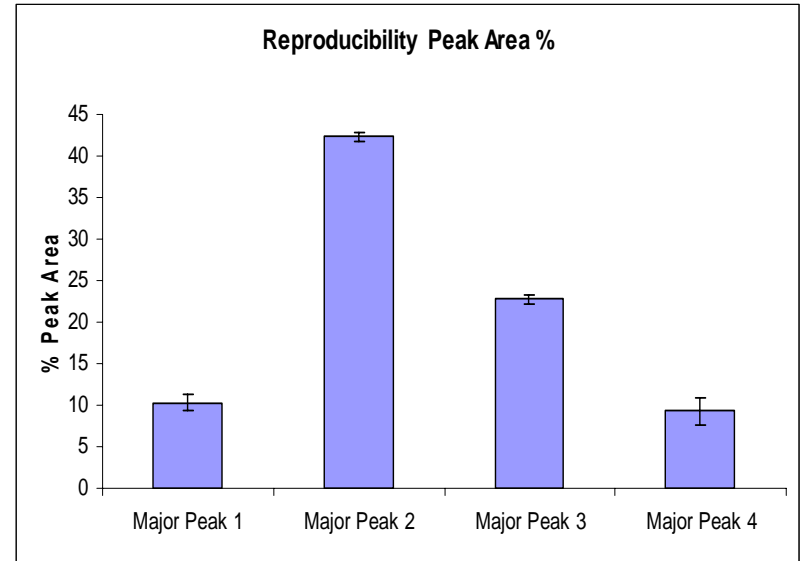
# BMS Mab1 Reference Material

Peak pl	Major Peak 1	Major Peak 2	Major Peak 3	Major Peak 4
Replicate 1	6.766	6.869	7.003	7.13
Replicate 2	6.766	6.868	7.003	7.129
Replicate 3	6.766	6.867	7.002	7.129
Mean	6.77	6.87	7.00	7.13
STD	0.00	0.00	0.00	0.00
%RSD	0.00	0.01	0.01	0.01



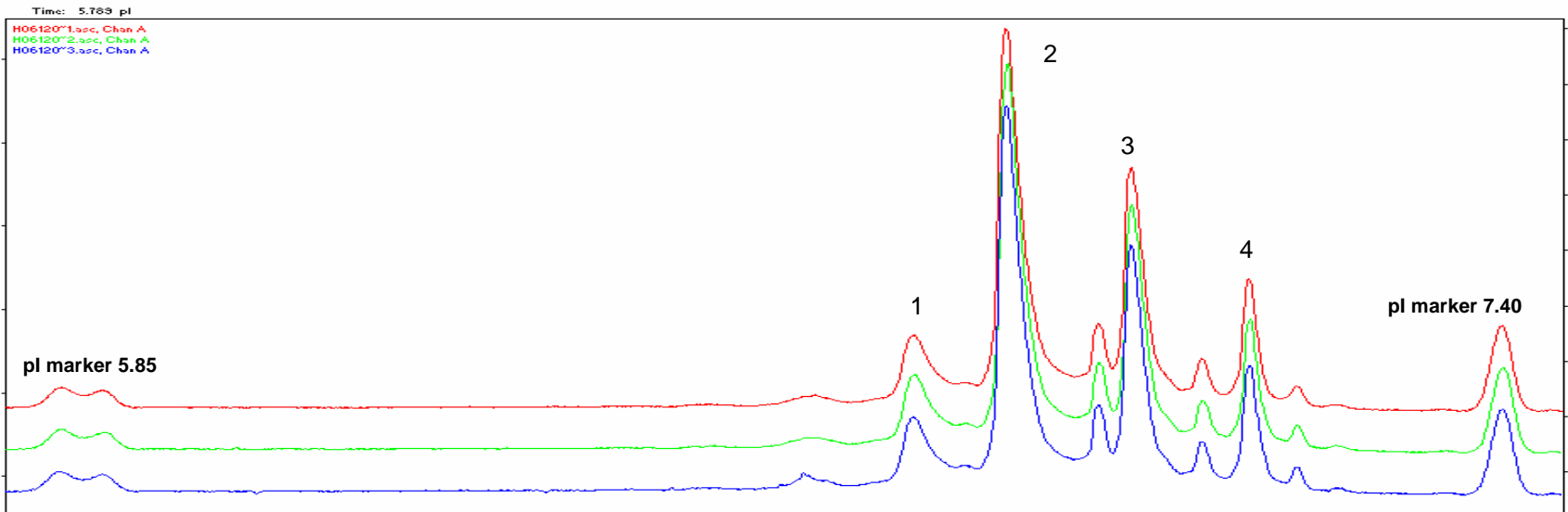
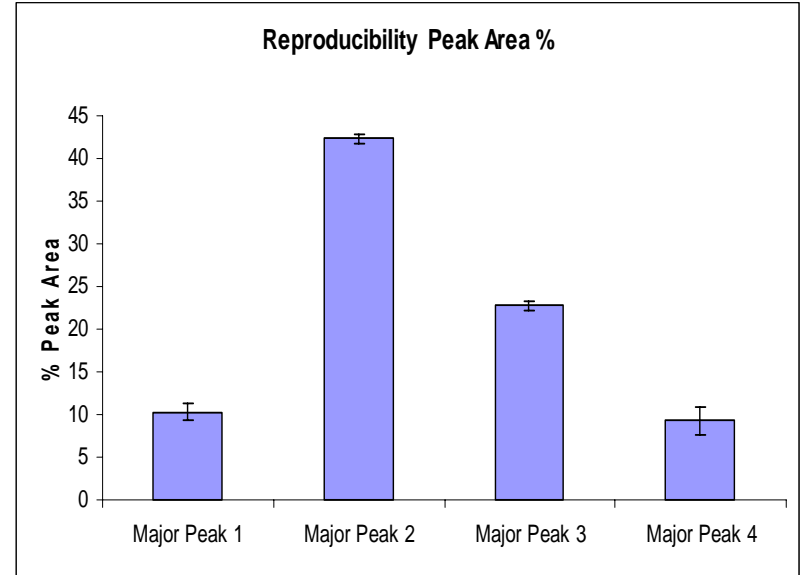
# BMS Mab1 Drug Substance

Peak pl	Major Peak 1	Major Peak 2	Major Peak 3	Major Peak 4
Replicate 1	6.767	6.868	7.003	7.13
Replicate 2	6.765	6.868	7.002	7.129
Replicate 3	6.767	6.867	7.002	7.128
Mean	6.77	6.87	7.00	7.13
STD	0.00	0.00	0.00	0.00
%RSD	0.02	0.01	0.01	0.01

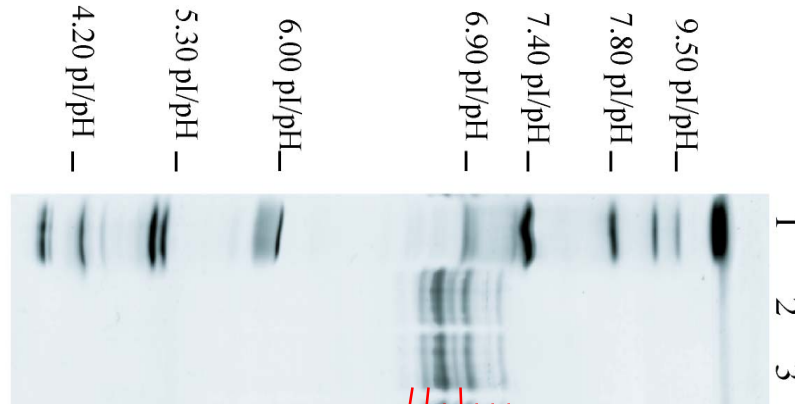


# BMS Mab1 Drug Product

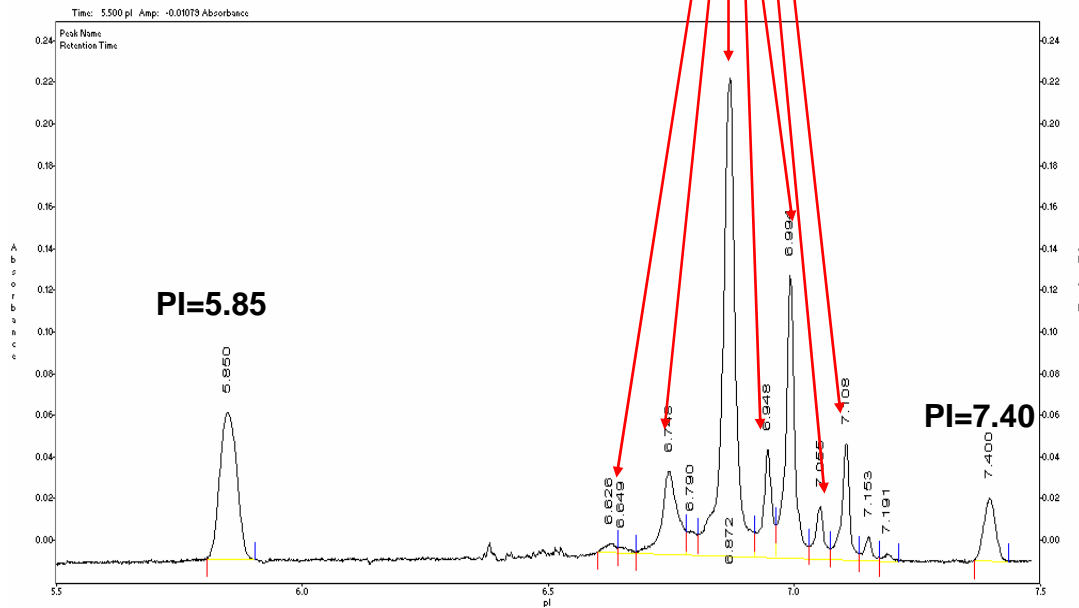
Peak pl	Major Peak 1	Major Peak 2	Major Peak 3	Major Peak 4
Replicate 1	6.766	6.866	7.001	7.127
Replicate 2	6.767	6.868	7	7.129
Replicate 3	6.766	6.867	7.001	7.129
Mean	6.77	6.87	7.00	7.13
STD	0.00	0.00	0.00	0.00
%RSD	0.01	0.01	0.01	0.02



# Comparison of IEF Gel Banding Pattern and Imaged iCIEF Profile of BMS MAb1

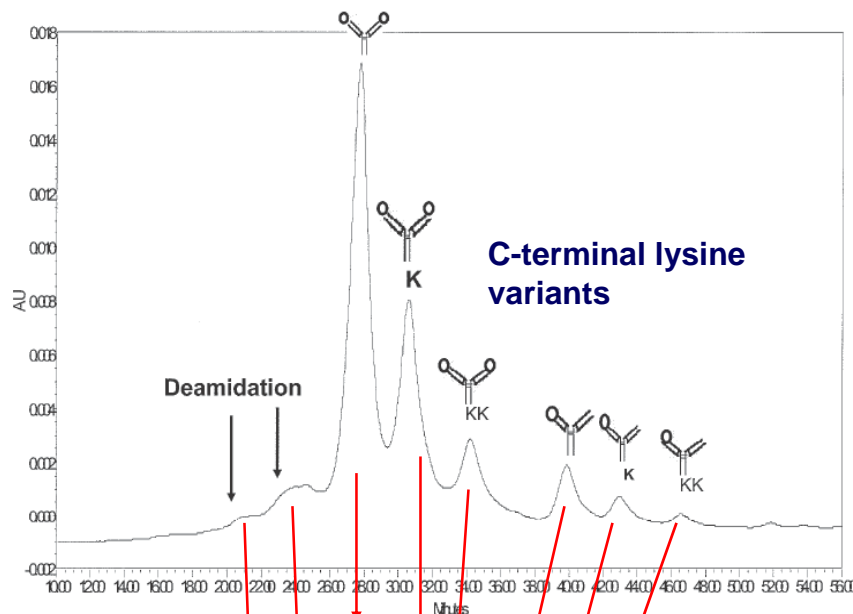


**Gel IEF**



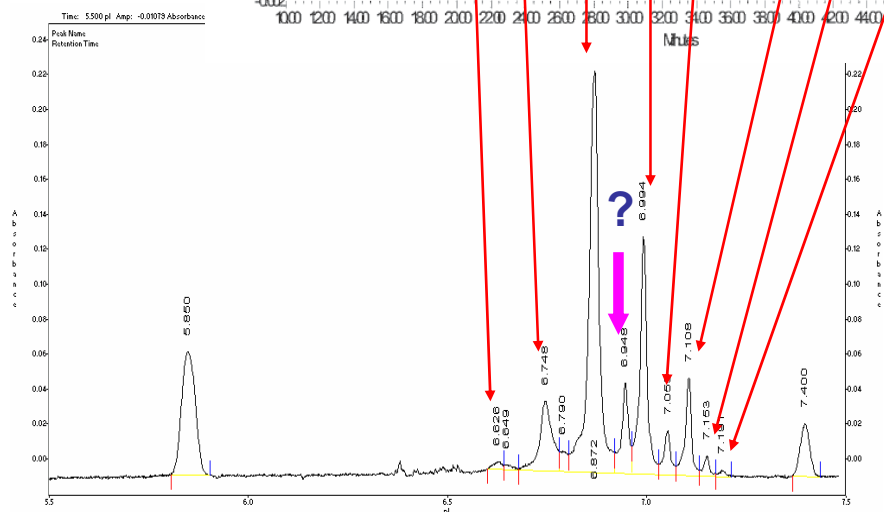
**Imaged CIEF**

# Comparison of Ion Exchange HPLC Profile and Imaged iCIEF Profile of BMS MAb1



Ion exchange  
~ 1 hour

CoElution of peaks  
due to lack of  
resolving ability



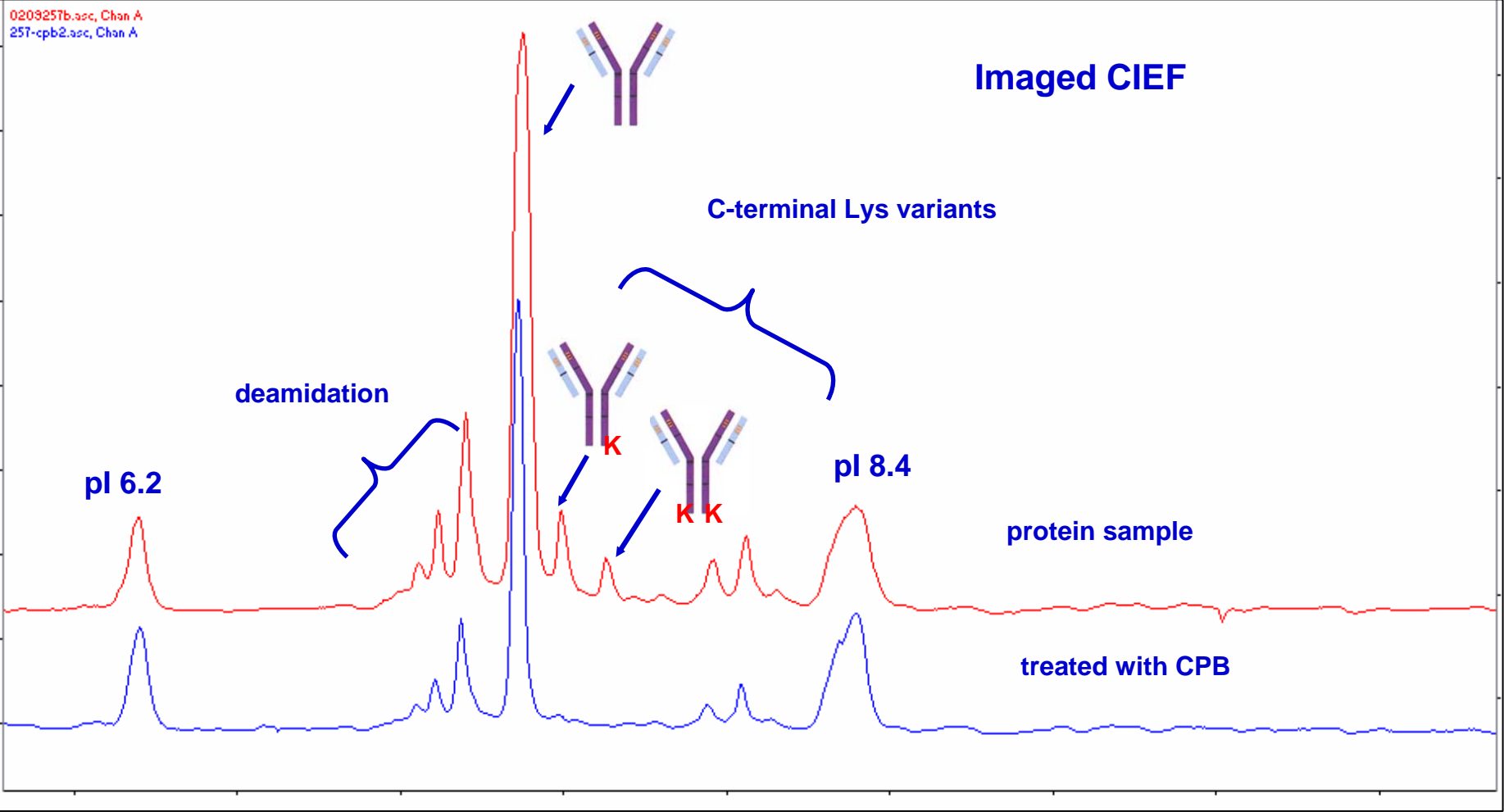
Imaged iCIEF  
~10 min

Increased resolution,  
identifies additional  
peaks

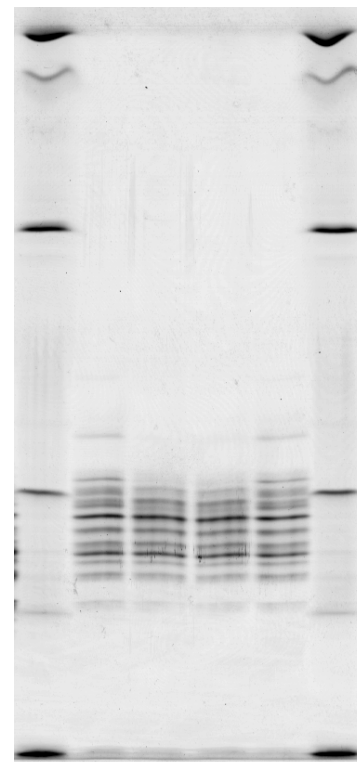
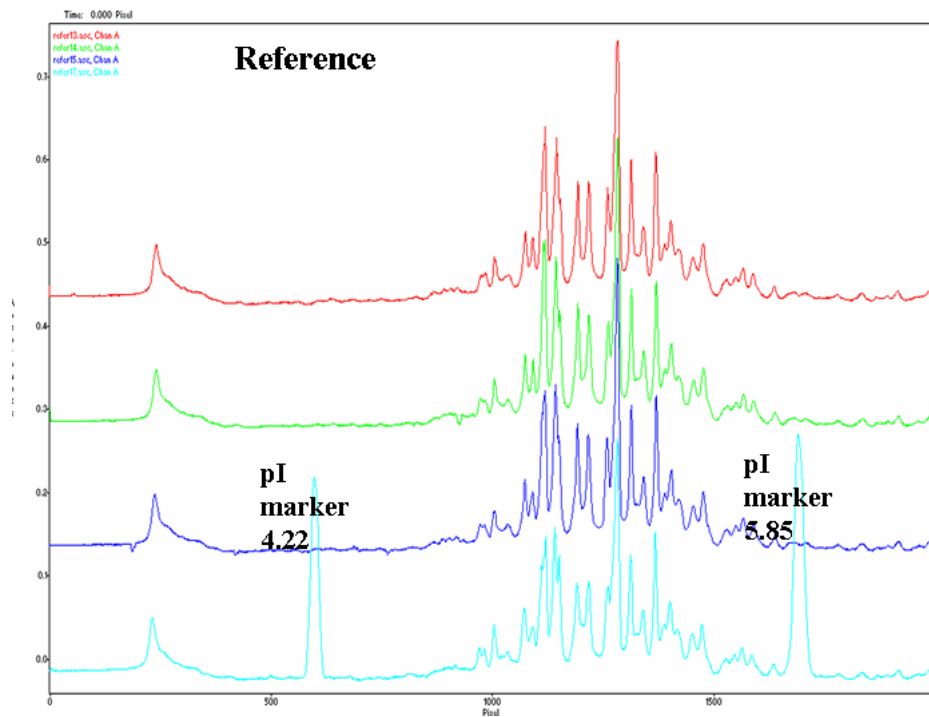
# **Complexity of Protein Glycosylation, Variants, and Phosphorylation**

- **Microheterogeneity**
  - The variation seen in glycosylation at a given glycosylation site. A site may be unoccupied or may be occupied by different sugars.
- **Glycoforms**
  - A glycoconjugate may have different glycoforms. The non-carbohydrate portion remains the same, but variances in the carbohydrate portion creates different glycoforms of a glycoconjugate.
- **Deamidation, oxidation, C-terminal lysine variants, pyroglutamation**

# Imaged CIEF Utilized for Characterization of C-Terminal Lysine Variants in BAS Mab1



# Profiling of Glycoforms of Protein Sample by iCIEF and Gel IEF



Lane 1 - IEF Marker  
Lane 2 - Reference Material  
Lane 3 - Drug Substance  
Lane 4 - Drug Substance  
Lane 5 - Reference Material  
Lane 6 - IEF Marker

# Conclusions

- An iCIEF method for the analysis of BMS-Mab1 was developed based on the Convergent Biosciences iCE280. The results of the optimization experiments suggest that sample dilution at 1mg/mL in Milli-Q water, 8% Pharmalyte, 2M Urea, and 0.35% Methyl Cellulose (MC) prefocused for 1 minute at 300V/cm and focused for 9 minutes at 600V/cm generate highly reproducible results.
- The iCIEF peak profile aligned with both the band migration pattern and band intensity distribution of the conventional slab gel analysis, demonstrating the method to be accurate and may be used to replace the gel IEF methodology.
- iCIEF analysis demonstrated superior resolution to Ion Exchange Chromatography, discriminating multiple isoform species which coeluted within the IEX method.
- iCIEF demonstrated characterization abilities, defining Mab structure post enzymatic digestion.