# Charge Heterogeneity Analysis of Monoclonal Antibodies: A Comparison of Orthogonal Methods

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## Abstract

Capillary electrophoresis isoelectric focusing has become an increasingly popular choice for the analysis of product quality and charge heterogeneity of biological therapeutics. This technique offers several advantages over the more commonly used gel and column chromatography based methods, the most attractive being ease of development, direct on-line quantification using UV detection, automation of high-speed separation, and enhanced sensitivity and resolution with decreased analysis time.

Three analytical methods were developed to characterize charge heterogeneity of two recombinant monoclonal antibodies: horizontal gel electrophoresis isoelectric focusing (gel-IEF), cation exchange high performance liquid chromatography (CEX-HPLC), and imaging capillary electrophoresis isoelectric focusing (iCE).

A comparison of data obtained from the two standard techniques (gel-IEF and CEX-HPLC) with data from the more recent iCE technology is presented here along with advantages and disadvantages of these methods of analysis. Capillary based isoelectric focusing methods provide many advantages over traditional methods of charge heterogeneity analysis.

## Antibody Preparation & Purity Analysis

Two recombinant monoclonal antibodies were expressed in CHO; each product was purified with a product specific purification process that contains one capture step and two fine purification steps. The drug substances were formulated in product specific formulation buffers; final drug products were evaluated in this study.

Analysis of the two monoclonal antibodies by SEC reports 98.1% monomer for Ab1 and 97.2% monomer for Ab2 (chromatograms not shown). % purity, as evaluated by non-reduced CE-SDS (Figure 1) , is ~94 % for Ab1 and ~88% for Ab2.

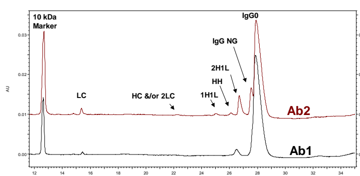


**Figure 1.** ***CE-SDS Electropherograms.*** Non-reduced CE-SDS analysis of **Ab1** and **Ab2**. % purity calculated as sum of % area of intact antibody (glycosylated and non-glycosylated). Intact antibody (IgG0, non-glycosylated intact antibody (IgG NG), two heavy chains + one light chain (2H1L), two heavy chains (HH), one heavy chain + one light chain (1H1L), free heavy chain (HC), two light chain (2LC), free light chain (LC).

## Gel-IEF

### Method

Isoelectric points were analyzed by isoelectric focusing gel electrophoresis. Samples were loaded at 40 µg and run in parallel with IEF Standards (20 µg of lentil lectin (pI 8.8, 8.6, 8.2) and 7 µg trypsinogen (pI 9.3)). An agarose IsoGel pH 7-11 (Cambrex) was used; samples were focused for 2 hours with the following limits: 1000V, 400mA, and 25W.

### Results

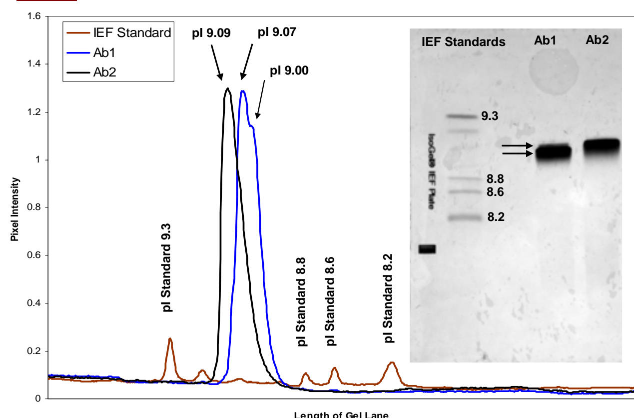


**Figure 2. IEF-gel analysis of Ab1 and Ab2.** Isoelectric points were analyzed by isoelectric focusing gel electrophoresis. Lane densitometry profiles of IEF Standard, Ab1, and Ab2 are overlaid, the IEF-gel is inset. Two species are resolved in the analysis of Ab1 (pIs 9.07 and 9.00), a single species is resolved in the analysis of Ab2 (pI 9.09). Smearing under both samples (seen as peak tailing in the lane profiles) suggests that there are acidic species present.

## iCE280 Analysis

### Method

Imaging isoelectric focusing was performed with Convergent Biosciences iCE280. Samples were prefocused for 1 minute at 1500V and focused for 8 minutes at 3000V. Samples contained 0.35% methyl cellulose, ~0.3 mg/ml sample, and 4% Pharmalyte 8-10.5; markers used to flank the sample were at a pI of 8.79 and 9.5 (Convergent Biosciences).

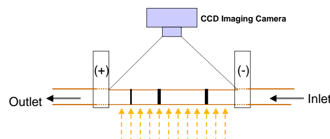


**Fig. 3.** ***iCE280 Schematic.*** Representation of iCE280. Samples are injected into capillary, voltage is applied to focus sample, and the progression of sample focusing is monitored by illuminating the capillary from underneath and taking an image of the capillary with a CCD camera. The focused proteins absorb the 280 nm light and the intensity is measured by the CCD camera.

### Results

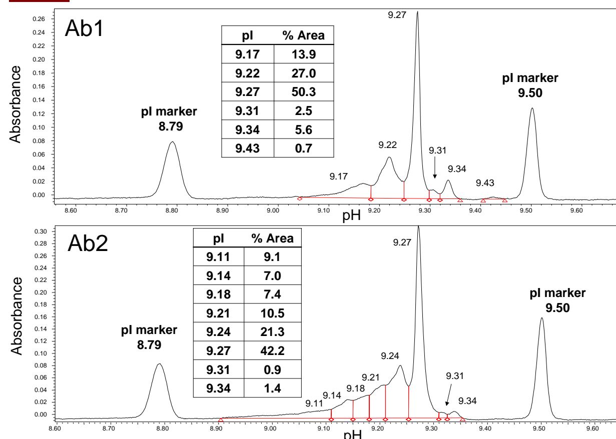


**Figure 4.** ***iCE280 Analysis of Ab1 and Ab2.*** Samples were analyzed in a fused silica capillary to examine charge heterogeneity. Species focus in order of decreasing acidity, the most acidic focusing to the left and the least acidic focusing to the right. The two flanking peaks (at pH 8.79 and 9.50) are pI standards used to calculate pI values for the sample. The inset tables detail the % composition of each species in the total sample. The Ab1 sample contains 50.3% main species, 40.9% acidic species, and 8.8% basic species. Ab2 contains 42.2% main species, 55.4% acidic species, and 2.4% basic species.

## Data Summary

| Ab1 | IEF-gel | CEX-HPLC | iCE280 |
|---|---|---|---|
| % main | - | 48.9 | 50.3 |
| % acidic | - | 21.8 | 40.9 |
| % basic | - | 29.3 | 8.8 |
| pI main peak | 9.07 | - | 9.27 |

| Ab2 | IEF-gel | CEX-HPLC | iCE280 |
|---|---|---|---|
| % main | - | 49.4 | 42.2 |
| % acidic | - | 28.9 | 55.3 |
| % basic | - | 21.7 | 2.3 |
| pI main peak | 9.09 | - | 9.27 |

**Table 2. Summary of Charge Heterogeneity Analysis of two monoclonal antibodies.** Samples were analyzed by IEF-gel (Figure 2), iCE280 (Figure 4), and CEX-HPLC (Figure 5). Charged species are grouped into three categories for simplicity; main species, acidic species (relative to main species), and basic species (relative to main species).

### Summary

A summary of the data for the three separation methods is presented in Table 2. The difference in the pIs calculated from the IEF-gel and iCE280 can be attributed to the use of different pI markers, different pH ranges, and different ampholytes. Despite these differences, pI values obtained from the two methods are quite similar; differing by only ± 0.2 pH units.

Differences in the CEX chromatograms and the iCE280 electropherograms can be attributed to the mechanisms of separation. Although both techniques look at charge heterogeneity, CEX-HPLC analysis reflects the distribution of bound species at a specific pH and their inherent differences in ionic strength as exemplified by their elution at different salt concentrations. On the other hand, iCE280 analysis is representative of the species that are free in solution and separates molecules based upon their effective isoelectric point.

## CEX-HPLC

### Method

| | |
|---|---|
| System | Waters 2695 HPLC System |
| Column | Sepax Technologies WCX-NP3 |
| Column Temperature | 25 °C |
| Flow Rate | 1.2 ml/min |
| Equilibration Buffer | Phosphate Buffer System, pH 6 |
| Sample Load | 50 µg |
| Elution Conditions | Phosphate Buffer System + 200 mM salt, pH 6 |
| Strip Buffer | 1M salt |
| Cleaning Buffers | Sodium Hydroxide / Acetic Acid |

### Results

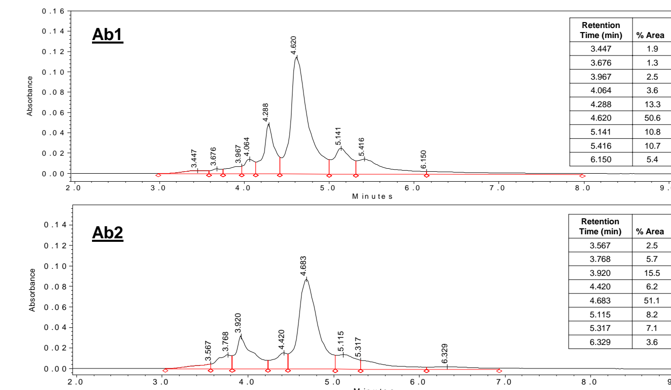


**Fig. 5.** ***CEX-HPLC Analysis of Ab1 and Ab2.*** Samples were analyzed on a weak cation exchange analytical column to examine charge heterogeneity. Species elute in order of decreasing acidity, the most acidic eluting first the least acidic eluting last. The last two peaks (at ~14 min and 17 min) are cleaning peaks. The inset tables detail the % composition of each species in the total sample. The Ab1 sample contains 48.9% main species, 21.8 % acidic species (elute prior to the main peak), and 29.3% basic species (elute after the main peak). Ab2 contains 49.4% main species, 28.9 % acidic species, and 21.7% basic species.

## Comparison of Methods

| | gel-IEF | CEX | iCE280 |
|---|---|---|---|
| Resolution | - | ++ | ++ |
| Quantitative - pI | yes | no | yes |
| Quantitative - Composition | no | yes | yes |
| Amount of Material Required* | 40 µg | 50µg | 60µg |
| Analysis Time | 1 day | 60 min | 8 min |

**Table 3. Advantages & Disadvantages of Analysis Technique.** The advantages and disadvantages of each of the methods of analysis are presented. The (traditional) gel-IEF method resolves fewer species than either of the other methods and is much more time consuming. Both the CEX and iCE280 methods resolve multiple species, and the iCE280 method allows quantitation of both pI and composition.

* Amount required for analysis of n=1 by IEF-gel and CEX, multiple replicates when analyzing by iCE280.

## Conclusions

1. Comparable Isoelectric point values are obtained with the iCE280 and IEF-gel methods.
2. Both iCE280 and CEX-HPLC permit the resolution of multiple molecular species. The numbers and relative distributions of species depend on the details of the separation methods and the molecular species involved. Purification of specific isoforms and spike studies will aid in resolving these issues.
3. Imaged capillary isoelectric focusing (iCE280) offers several advantages over other methods of analyzing charge heterogeneity. The method:
   - allows quantitation of both pI and composition
   - offers a much shorter analysis time (8 minutes compared to 60 minutes or 1 day for CEX-HPLC and IEF-gel, respectively)
   - requires a lower sample amount while compromising only a little on resolution
   - provides ease of development and shortened development times (parameters to optimize are clear and tangible, development time-line shortened from months to weeks