

# Characterization of Charge Based Heterogeneity in a Recombinant Monoclonal Antibody by Imaging Capillary Isoelectric Focusing and Comparison to Weak Cation-Exchange Chromatography

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## ABSTRACT

Characterizing and quantifying charge-based heterogeneity in biopharmaceuticals is a critical component of product development and product quality. Variations in protein charge (i.e. increase or decrease in acidic and basic species) can indicate issues with product stability and/or pre-formulation and cell-culture development. The cause of this heterogeneity can result from multiple sources such as deamidation, oxidation, amino acid substitution/deletion, differential glycosylation, glycation, and proteolytic degradation, all of which can negatively influence a products behavior (1). Therefore, it is essential to have robust and reliable methods for detecting changes in charged isoforms.

Capillary isoelectric focusing (cIEF) has been utilized as a tool for detecting acidic and basic isoforms in proteins for many years as it offers many benefits with its largely unchallenged resolving power. In this work, we report on development of an imaged capillary isoelectric focusing (icIEF) method for the characterization and quantification of charged-based heterogeneity in a recombinant monoclonal antibody (mAb). We examined the effects of pH and temperatures as a function of time using a Convergent Biosciences iCE280 instrument and compared to a conventional weak-cation exchange chromatographic method (CIEX). Qualitative and quantitative aspects of each method are presented.

## INTRODUCTION

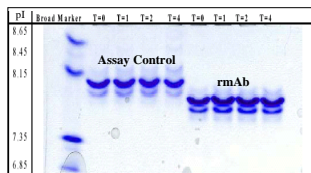
Charge heterogeneity exhibited by recombinant monoclonal antibodies has long been demonstrated by ion-exchange chromatography and/or by isoelectric focusing (IEF). Conventionally, IEF is accomplished using slab gels to determine isoelectric points (pI) and in recent years IEF has been achieved in a capillary format (cIEF), introducing improvements in quantitation, resolution and sensitivity. However, cIEF is not without its drawbacks, requiring mobilization of the focused proteins to a fixed detection point, which can have an effect on reproducibility. Imaging cIEF (icIEF), developed by Wu and Pawliszyn (2,3,4), removes the mobility by enabling the whole-column detection as the proteins are being focused. In this presentation we demonstrate the approach our lab has taken in measuring charge heterogeneity of a mAb by icIEF and compare results to a traditional CIEX approach.

## MATERIALS AND METHODS

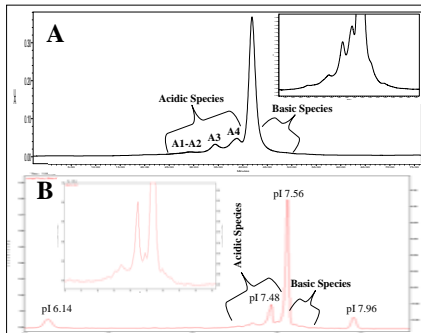
All chemicals were obtained from Sigma (Sigma Chemicals, St. Louis, MO) unless otherwise noted. Proteins were produced by Biogen Idec, Inc. Imaging cIEF analysis was performed using an ICE280 Analyzer with PrinCE (Convergent Biosciences). Ampholyte solutions were prepared as follows: 0.5 and/or 0.25 mg/ml intact mAb, 0.5% methyl cellulose (Convergent Biosciences), 2% 5-8 pharmlalyte and 2% 8-10.5 pharmlalyte (GE Healthcare) and 6.14 and 7.96 pI markers (Convergent Biosciences). Focusing was conducted with 1 minute at 1500 V and 8 minutes at 3000 V using 0.26 % NaOH and 80 mM H<sub>2</sub>PO<sub>4</sub>, UV detection at 280 nm. Weak cation-exchange chromatography (CIEX) was performed on an HP 1090 HPLC system. Antibody samples were buffer exchanged prior to chromatographic analysis into Buffer A (10mM Na Phosphate pH 6.5) by centrifugation using a 10K MWCO Amicon Biomax membrane filter from Millipore. Separation was achieved using a Dionex ProPacTM WCX-10, 4x250mm column with 1 mL/min flow and a linear gradient of buffer A and buffer B (10mM Na Phosphate, pH6.5, 200mM NaCl). A wash buffer, buffer C (10mM Na Phosphate, pH6.5, 1 M NaCl), was used to clean the column after each injection. The column effluent was monitored at 280nm

## RESULTS

- A reference mAb sample was analyzed by IEF, CIEX and icIEF (Figures 1, 2A and 2B illustrate a gel image and representative traces).
- Precision data from CIEX and icIEF analysis on different columns/capillaries over several days are summarized in Table 1.
- Temperature and pH stressed samples were analyzed by CIEX and icIEF (Figures 3 and 4) and results shown Table 2.
- Acidic and main isoforms of mAb were collected by CIEX and a peak identity correlation with icIEF is illustrated in Figures 5 and 6.



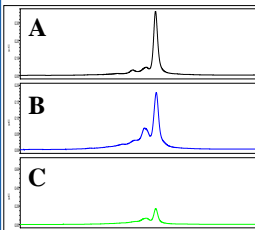
**Figure 1.** Slab gel IEF of mAb using Invitrogen minigel system. mAb was diluted with sample buffer prior to loading 15 µg/lane. Electrophoresis was performed at constant voltage, 100 V for 1 hour, 200 V for 1 hour and 500 V for 30 minutes. Gels were fixed for 30 minutes and protein bands were stained with 0.302% PhastGel Blue R. The background was reduced with an ethanol/acetic acid/water solution. Pharmacia calibration markers were used for pI comparison. mAb discussed in this presentation is labeled on gel as mAb.



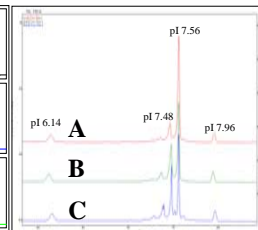
**Figure 2.** UV 280 nm IEC chromatogram (A). The acidic species are labeled A1 through A4 and elute prior to the main peak, which elutes at approximately 43 minutes. The icIEF electropherogram (B). The acidic species migrate to the left of the main peak (pI 7.56, as determined by software using two pI marker, pI 7.96 and 6.14 respectively). Further experimental conditions are described in Materials and Methods Section.

Measurement	CIEX (n=37)		icIEF (n=39)	
	Average	% RSD	Average	% RSD
% Acidic Species	26.5	1.1	23.0	3.0
% Main Peak	71.6	0.7	73.4	1.0
% Basic Species	2.0	20.0	3.6	8.2
Retention of main peak by CIEX (min)	42.8	1.1	----	----
Retention of main acidic peak by CIEX (min)	41.0	1.6	----	----
pI of main peak by icIEF	----	----	7.57	0.2
pI of main acidic peak	----	----	7.47	0.2

**Table 1.** Peak area intermediate precision of CIEX and icIEF analysis of a mAb. Total number of analyzed samples were n=37 for CIEX and n=39 for icIEF over several days



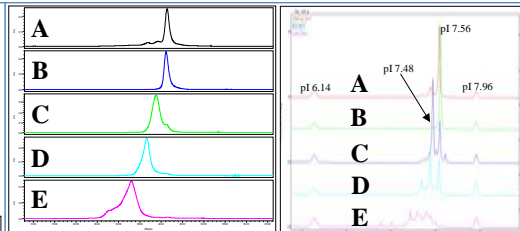
**Figure 3.** UV 280 IEC chromatogram of temperature/pH stressed mAb. (A) Assay control. (B) Assay control buffer exchanged and incubated in 50mM CAPS pH 9.5 at 37°C for 48 hours. (C) Assay control incubated at 45°C for 48 hours.



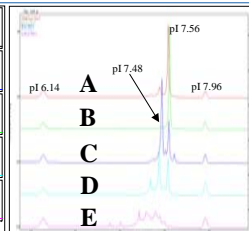
**Figure 4.** UV 280 nm icIEF electropherograms of temperature/pH stressed mAb. (A) Assay Control. (B) Assay control buffer exchanged and incubated in 50mM CAPS pH 9.5 at 37°C for 48 hours. (C) Assay control incubated at 45°C for 48 hours.

Measurement	CIEX			icIEF		
	Control	pH Stress	Temp. Stress	Control	pH Stressed	Temp. Stress
% Acidic Species	26.7	46.9	51.8	22.8	48.2	62.7
% Main Species	71.7	50.4	45.7	73.6	48.9	32.6
% Basic Species	1.6	2.8	2.5	3.6	2.9	4.7

**Table 2.** Peak area analysis of three main CIEX and icIEF isoforms (acidic, main and basic) from pH and temperature stressed mAb samples. pH stressed samples were buffer exchanged into 50 mM CAPS pH 9.5 and incubated at 37°C for 48 hours then buffer exchanged back into formulation buffer before analysis. Temperature stressed samples were incubated at 45°C for 48 hours before analysis. The ach sample was analyzed in triplicate



**Figure 5.** UV 280 CIEX chromatograms of re-injected mAb fractions collected by CIEX. (A) Assay Control. (B) CIEX main peak. (C) CIEX Acidic peak 4. (D) CIEX Acidic peak 3. (E) CIEX Acidic peak 1-2.



**Figure 6.** UV 280 icIEF electropherograms of CIEX collected mAb fractions. The icIEF electropherograms were generated as described in Figure 2. (A) Assay Control. (B) CIEX main peak. (C) CIEX Acidic peak 4. (D) CIEX Acidic peak 3. (E) CIEX Acidic peak 1-2.

## CONCLUSIONS

- Charge heterogeneity of a mAb has been assessed by icIEF and compared to CIEX analysis.
- Precision of icIEF method performance has been assessed, and the highest variability was observed for basic peaks (% RSD for peak area was 8.2 % by icIEF compared to 20 % by CIEX).
- icIEF has been shown to be stability indicating and icIEF peak assignments correlated to CIEX.
- icIEF methodology offers a faster alternative to CIEX (15 minutes compared to >90 minutes for CIEX) and increased separation resolution for assessing mAb charge heterogeneity.
- Additional work to determine the source of the mAb charge heterogeneity will be performed by collection and analysis of each peak by mass spectrometry.

## REFERENCES

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