Simplifying Charge Heterogeneity Method Development with iCE3

Introduction

As we all know, analyzing charge variants in biopharmaceuticals is a critical part of product development and quality control. Charge heterogeneity can occur as a result of chemical and post-translation modifications like deamidation, oxidation, glycosylation and glycation. And because they can affect the biological activity, patient safety and drug stability, monitoring them is vital.

Imaged capillary isoelectric focusing (icIEF) on iCE3 systems is the go-to method when it comes to measuring charge heterogeneity. It lets you develop and use generic methods for multiple molecules, and gives you high-resolution charge heterogeneity peak profiles in as little as 10 minutes — cutting your development timelines. End result? Your product gets to market faster. In this application note, we'll walk you through how simple and easy it is to develop an icIEF method and also give you a few tips for increasing method robustness.

So What is icIEF?

icIEF is capillary IEF that utilizes whole-column detection, eliminating the lengthy mobilization step needed with traditional cIEF. This not only decreases assay complexity, but the time savings that come with it increase your sample throughput too.

Assays are run in a cartridge that holds the capillary and has integrated electrolyte tanks. After installing the cartridge in your iCE system, prepping it is easy — just fill the two electrolytic tanks at each end of the cartridge with anolyte (acid) and catholyte (base). Then add carrier ampholytes and pI markers to your sample mix and place the prepared sample in your iCE system.

First, your sample is injected into the capillary until the entire capillary is filled. Next, voltage is applied across the capillary, causing a pH gradient to form. The pI markers and your protein of interest will migrate through the capillary across this pH gradient until they reach their isoelectric point — the pH where their net charge is zero. A whole column imaging camera is then used to detect each of the separated proteins across the entire capillary at 280 nm. After a few quick wash steps, the capillary is ready for the next sample. And the full process from sample injection to capillary wash step only takes about 10 minutes.

Developing Your Method

With icIEF, assay optimization is really quick compared to other techniques — all you need to do is follow the simple workflow in **Figure 1** to optimize just a few parameters. To make life even easier, check out our iCE Method Development Kit. It's a one-stop shop for a wide range of Pharmalytes, pI standards, additives and all the sample preparation reagents you'll need to develop a new method.

When you see reproducible peak profiles and satisfactory peak resolution for your molecule, you've got a fully defined and optimized method. And, as seen in **Figure 2**, the data from your optimized method will be hands-down fantastic! Even though the performance of each icIEF cartridge is guaranteed to 100 runs, peak profile reproducibility across 120 consecutive runs of IgG Kappa1 showed minimal drift (<50 pixels) and CVs for

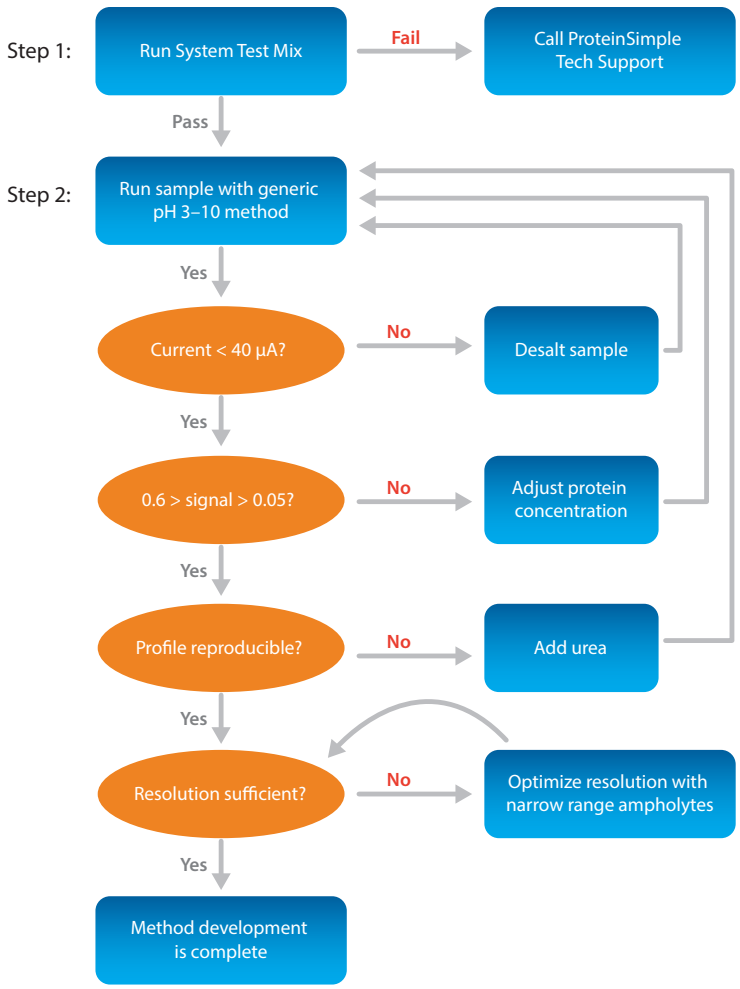

FIGURE 1. Method optimization workflow.

peak clusters greater than 5% of the total area were all below 10%.

To optimize your method, you'll first want to screen with a generic method using a Pharmalyte 3–10 pH gradient to get a sense of where your molecule focuses **(Figure 3A)**. For most molecules, this method with the broad pH gradient will give you the separation profiles you need. For trickier molecules, like those with complex peak profiles and/or limited solubility, method optimization is easily tackled with a few simple strategies.

In IEF, proteins simultaneously lose surface charge as they are being focused into very concentrated sample zones. When this happens, hydrophobic regions may interact or aggregate, which affects the resolution and reproducibility of the charge heterogeneity profile. Adding a solubilizer like urea to your sample will eliminate aggregates and improve your separation **(Figure 3B)**.

After your protein's peak profile has stabilized, you can improve the resolution further, if needed. Adding narrow pH-range ampholytes to the sample matrix will help get you near-baseline resolution of all isoforms **(Figure 3C$_1$)**, with high resolution of 0.04 pH units (**Figure 3C$_2$**).

Method development with iCE systems is so straightforward and fast that the entire process from screening conditions **(Figure 3A)** to a final, optimized and reproducible analytical method **(Figure 3C)** only takes 2.5 hours! And if you plan to transfer and run your method in a QC or GMP environment after it's developed, iCE CFR software has all the required functionality needed to ensure compliance with 21 CFR Part 11.[1]

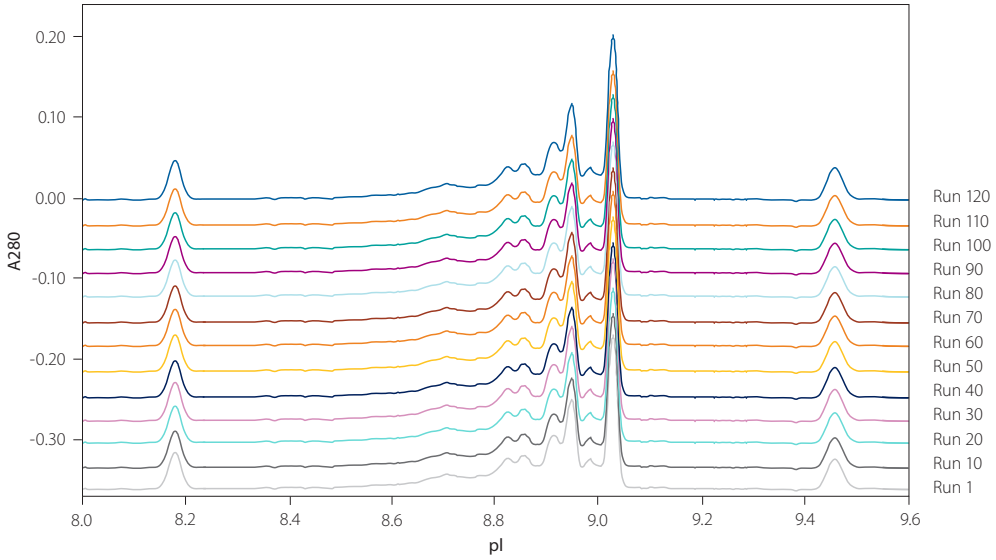

FIGURE 2. Example of the data robustness on the iCE3 system after method optimization. Overlaid electropherograms clearly demonstrate the reproducible peak profiles over 120 injections.

Things to Consider for a Robust Method

If you want the most robust method possible, you can optimize your method further by adding computational tools such as Central Composite Design of Experiment (DOE) into the mix. We've outlined how you can execute DOE and fine-tune your method already in our Computer-aided Assay Development for Charge Heterogeneity Analysis by iCE application note.[2]

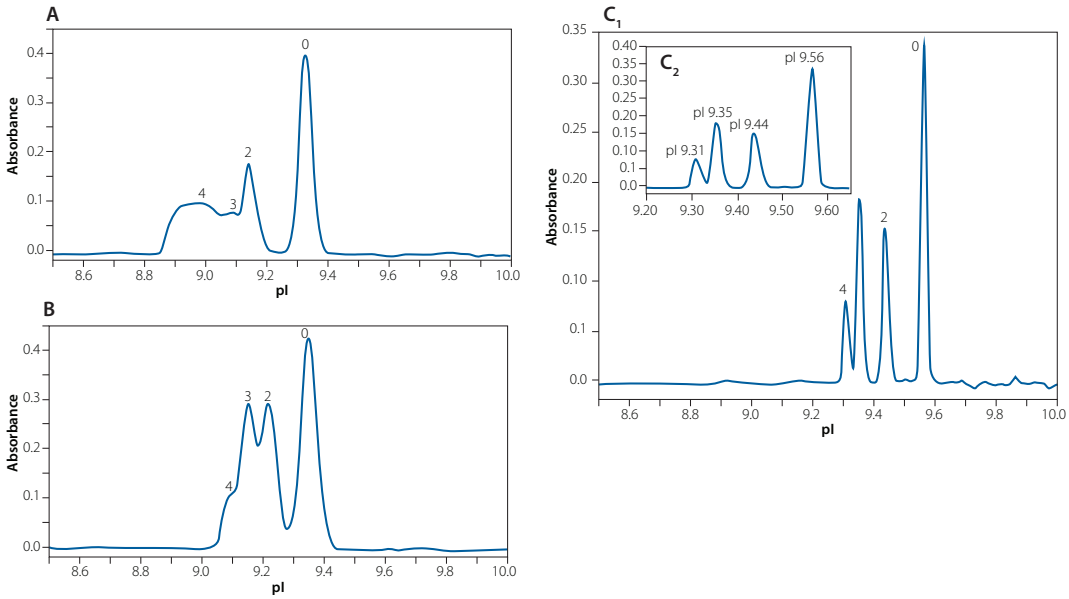

FIGURE 3. The complete icIEF method development process only takes 2.5 hours from (A) screening of the molecule in a pH gradient, to (B) the addition of solubilizers, to (C) the addition of narrow-range ampholytes.

Sample components like salt can affect the resolution and robustness of your method, as ions that don't have a zwitterionic or neutral charge state can be driven out of the capillary by electrokinesis during the focusing process. And as these charged compounds leave the capillary, they're replaced by the anolyte's hydronium and catholyte's hydroxyl ions to maintain electroneutrality, which can result in a high separation current and compression of the pH gradient. But don't worry, this is easily remedied.

To give you an example of how high salt can adversely affect analysis, we separated IgG1 Kappa in various salt concentrations **(Figure 4A)**. The pH gradient compression results in a loss of resolution for the IgG1 Kappa charge isoforms and a shift of the 9.46 pI marker. Replicate runs show how salt can also affect method reproducibility **(Figure 4B)**. At the highest salt concentration, IgG1 Kappa charge isoforms migrate towards the lower pH and form an unresolved mound as they either degrade and/or aggregate in this extreme separation.

You can get around the salt effects by reducing the concentration of salt components in the sample before analysis. If your formulation has a high protein concentration, all you need to do is dilute the protein down to the final working concentration in sample solution, typically in the 200–250 µg/mL range for mAbs. This will reduce the ionic strength enough to let you successfully analyze your molecule. For formulations with

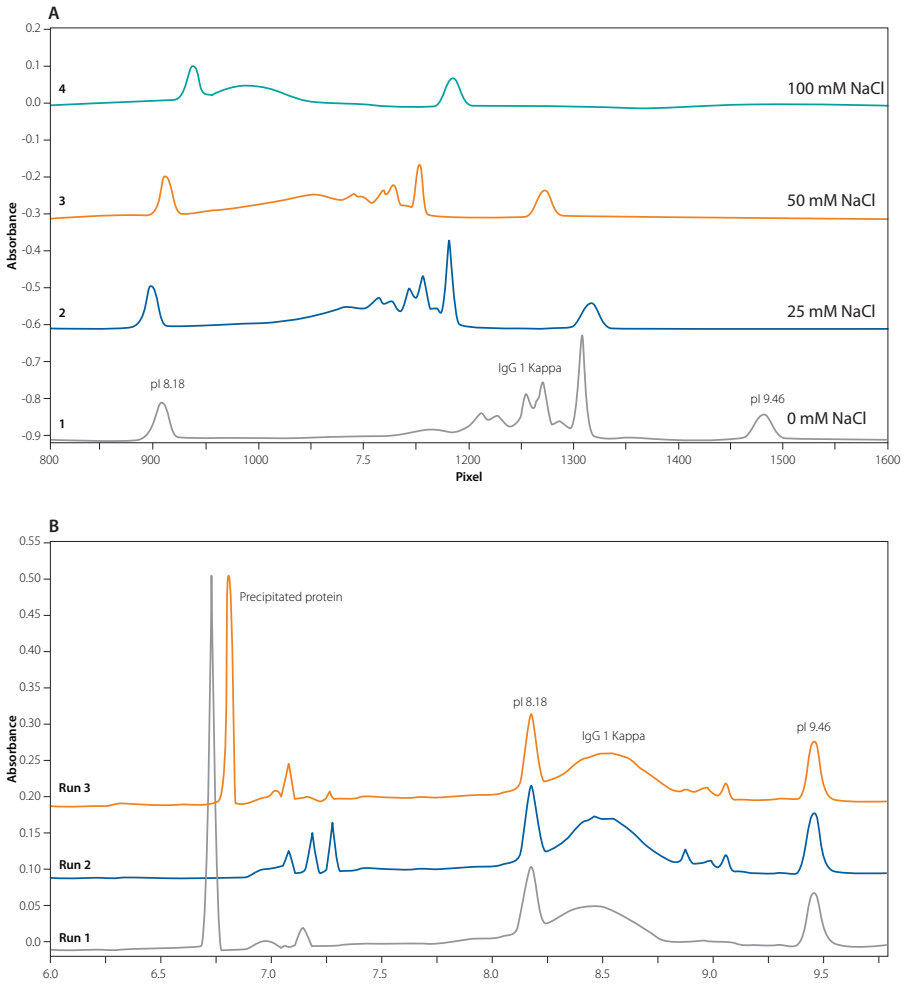

FIGURE 4. Separation of IgG1 Kappa in various salt concentrations. (A) Increasing salt concentration shows the adverse effects on resolution. (B) Triplicate runs at 100 mM NaCl show that salt also affects reproducibility.

low protein concentrations (<10x dilution to the final sample concentration), a quick buffer exchange will get you the best results.

And like all other separation techniques, you always want to use the highest quality reagents to ensure consistent results. Improperly stored or expired consumables and reagents will affect assay performance. This is especially true for methods that use urea to eliminate aggregation **(Figure 5A)**. Always make your urea solutions are fresh, and keep them away from heat to avoid thermal degradation. Otherwise, one of the thermal degradation products of urea, isocyanic acid, can rapidly react with amino groups to artificially increase the percent composition of a protein's acidic species **(Figure 5B)**.

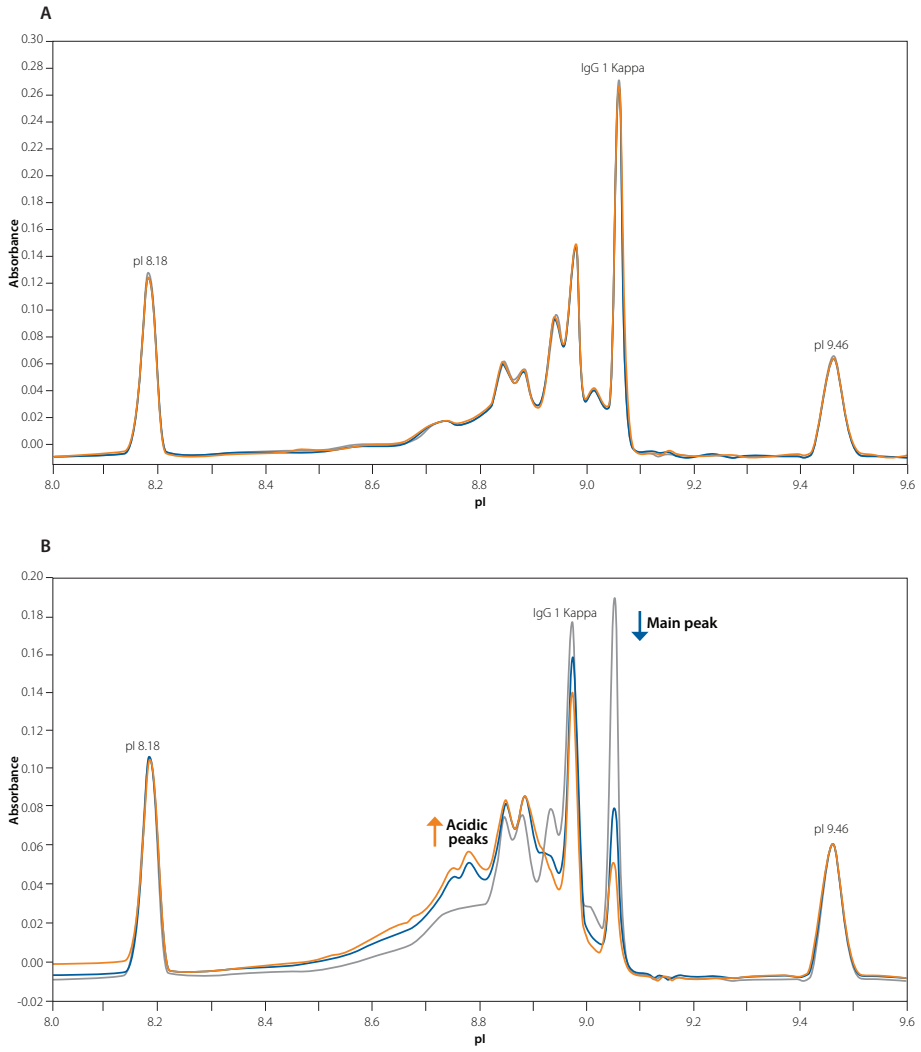

FIGURE 5. Comparison of injections over time using degraded urea. (A) Use of freshly made urea ensures consistent results across all injections. (B) Artificial increases in a protein's acidic species percent composition are seen between injections at 0 min (grey), 1.5 hours (blue), and 3.0 hours (orange).

Conclusion

It's really quick and easy to develop icIEF methods with iCE systems — just follow these few simple procedures and guidelines. Add our iCE Method Development Kit into the mix, and even new users can develop really robust charge heterogeneity methods in an afternoon! Of course, there are a few things to look out for along the way, but we've spent years developing these methods ourselves and have a great game plan with best bet options for you to try in case you need them.

Once you have an optimized method, the high resolution, 10-minute cIEF separations are ideal for characterizing and monitoring charge variants in biopharmaceutical development, manufacturing and formulations. And if you need to analyze samples in a QC or GMP environment, iCE systems and software have fully-compliant ready 21 CFR Part 11 features too!

References

1. Achieving 21 CFR Part 11 Compliance with the iCE3, ProteinSimple Application Note.
2. Computer-aided Assay Development for Charge Heterogeneity Analysis by iCE, ProteinSimple Application Note.

proteinsimple
a bio-techne brand

Toll-free: (888) 607-9692
Tel: (408) 510-5500
Fax: (408) 510-5599
info@proteinsimple.com
proteinsimple.com

98-4002-00 / Rev A