cIEF Method Development Guide
Maurice and Maurice C.

proteinsimple
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Introduction

The Maurice cIEF Method Development Kit is a one-stop shop for all your method development needs! It includes all sample preparation reagents and a wide range of ampholytes, pI markers and additives that'll let you tackle any protein. We've even included a System Suitability Kit to make sure your Maurice is ready to go. This Method Development Guide will help you every step of the way, and with Maurice's generic methods for multiple molecules, method development has just never been easier.

cIEF Method Development Kit contents (P/N PS-MDK01-C)

pI Markers

Store at -20 °C.

PN	DESCRIPTION	QTY/AMT*
046-028	Maurice cIEF pI Marker - 3.38	210 µL
046-029	Maurice cIEF pI Marker - 4.05	210 µL
046-030	Maurice cIEF pI Marker - 5.85	210 µL
046-031	Maurice cIEF pI Marker - 6.14	210 µL
046-032	Maurice cIEF pI Marker - 7.05	210 µL
046-033	Maurice cIEF pI Marker - 8.40	210 µL
046-034	Maurice cIEF pI Marker - 9.99	210 µL
046-035	Maurice cIEF pI Marker - 10.17	210 µL

**After reconstitution.*

REAGENTS

Store at 2–8 °C.

PN	REAGENT	DESCRIPTION	QTY/AMT
102506	iCE Electrolyte Kit	Anolyte solution: 0.08 M H_3PO_4 in 0.1% Methyl Cellulose Catholyte solution: 0.1 M NaOH in 0.1% Methyl Cellulose	2 x 10 mL each
101876	1% MC	1% Methyl Cellulose	10 mL
102505	0.5% MC	0.5% Methyl Cellulose	2 x 10 mL
046-025	Maurice cIEF Fluorescence Calibration Standard	Fluorescence standard for cIEF calibration	1 x 5.5 mL
042-691	500 mM arginine	500 mM arginine. For use as a cathodic spacer.	500 µL
046-574	SimpleSol Protein Solubilizer	Stock solution of SimpleSol protein solubilizer for cIEF	24 mL
Not sold separately	Urea	Lyophilized urea	5 vials
046-044	Maurice cIEF System Suitability Kit	The kit contains 8 tubes of lyophilized System Suitability Peptide Panel and 1 vial of System Suitability Test Mix	1 mL each

AMPHOLYTES

Store at 2–8 °C.

pH RANGE	DESCRIPTION	QTY/AMT
3–10	Pharmalyte pH 3–10 (Pharmalyte PN 17-0456-01)	200 µL
5–8	Pharmalyte pH 5–8 (Pharmalyte PN 17-0453-01)	100 µL
8–10.5	Pharmalyte pH 8–10.5 (Pharmalyte PN 17-0455-01)	200 µL
2.5–5	Pharmalyte pH 2.5–5 (Pharmalyte PN 17-0451-01)	100 µL
2–9	Servalyte 2–9 (seed grade) (Servalyte PN 42935)	50 µL

Ordering info

This kit can be reordered by:

- **Phone:** 1-888-607-9692, option 1
- **Fax:** 1-408-520-4831
- **Email:** orders@proteinsimple.com

Other things you'll need

- cIEF Cartridges, PN PS-MC02-C
- Maurice sample vials with integrated inserts, 0.2 mL, PN 046-083 or 96-well plates, PN 046-021
- Maurice glass reagent vials, 2 mL, PN 046-017
- Maurice clear screw caps for sample and reagent vials, PN 046-138
- Maurice cIEF blue pressure caps, PN 046-573
- Deionized (DI) water
- Pipettes and tips
- Microcentrifuge and tubes
- Ice and ice bucket
- Vortex
- Centrifuge with plate or vials adapter (12 mm, 2 mL vials)

***NOTE:** If you need to seal the 96-well plate during your run, we recommend the 4titude Pierceable Film (PN 4ti-0566, 4titude). It can be used in both absorbance and native fluorescence modes. If you're currently using X-Pierce adhesive film (PN XP-100, Excel Scientific), we recommend using it for absorbance mode only.*

BUFFER EXCHANGE

- Amicon Ultracel 50K membrane centrifugal filter (Millipore, PN 4311)

***Note:** Select a filter with a molecular weight cutoff appropriate for your protein.*

- 1 M Tris-HCl buffer pH 7.0 (Life Technologies, PN AM9851)

DENATURING SAMPLES

- Urea, electrophoresis grade (Sigma-Aldrich, PN U6504)
- SimpleSol Protein Solubilizer (PN 046-574, 046-575)

***NOTE:** A secondary source of urea is only needed if your sample requires 8 M urea to address protein precipitation.*

Guidelines to Great Results

- Lyophilized urea must be stored dry, and is provided in a foil pouch with desiccant. Please be sure to reseal the pouch after opening.
- Urea should be prepared fresh. Once the urea is reconstituted, it's good for one day only. Don't reuse.
- Lyophilized peptides should be aliquoted and stored at -20 °C after reconstitution. Reconstituted peptides have a 1-month shelf-life at 4 °C and a 6-month shelf-life at -20 °C
- pI markers should be diluted 100-fold into the sample solution.
- Make sure you read the entire Method Development Guide before getting started.

Method development overview

A successfully defined and optimized cIEF method gives you:

- A robust signal in both native fluorescence and/or absorbance detection modes
- A highly reproducible peak profile
- Satisfactory peak resolution for the targeted application

Maurice cIEF method development starts with a broad pH range method, and you'll typically only need to optimize a few parameters from there. For many molecules, methods with this broad pH range provide sufficient performance and don't need further development. For more challenging molecules with complex peak profiles, signal deviations between absorbance and fluorescence detection modes, and/or limited solubility, method optimization is accomplished using the strategy in Figure 1. The first step is to screen new compounds with a generic pH 3–10 method and evaluate the separation current, peak height in fluorescence and/or absorbance, and peak profile reproducibility. Once you have a reproducible profile, resolution can then be addressed if needed by increasing the concentration of broad range ampholytes or by adding narrow pH range ampholytes.

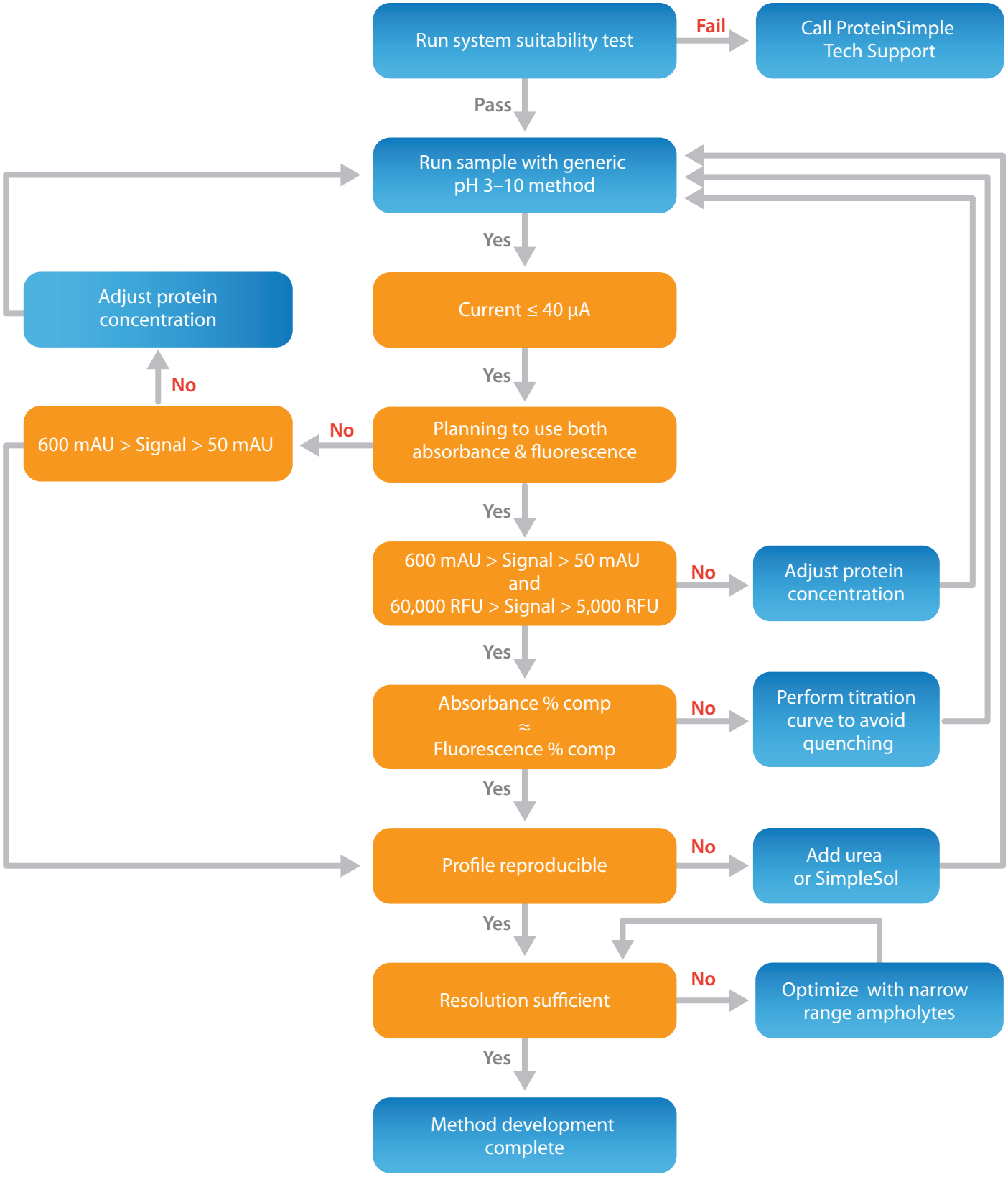

FIGURE 1. Method development workflow.

Step 1: Maurice system performance check

The Maurice cIEF Method Development Kit includes a System Suitability Test Sample that lets you check Maurice performance before you begin method development.

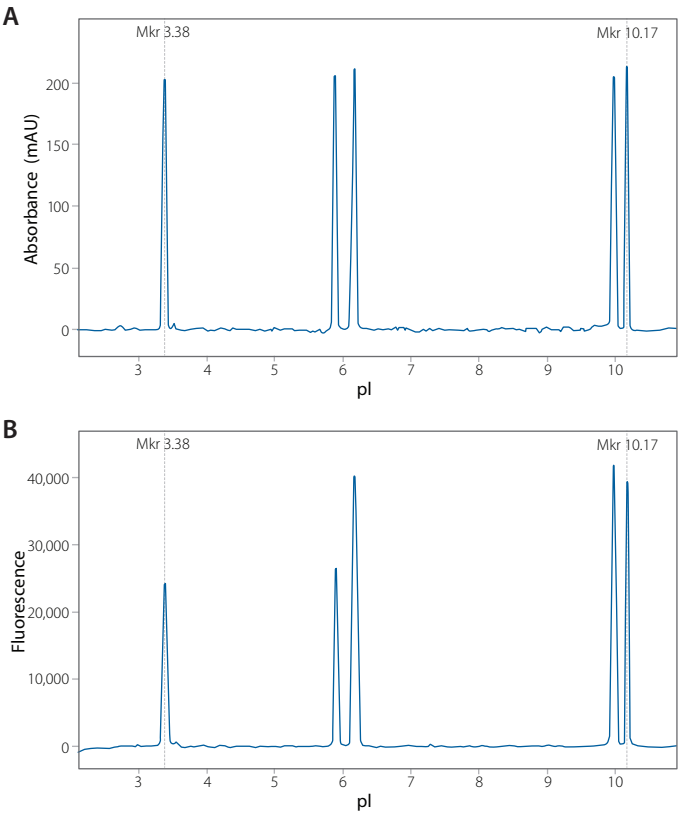

FIGURE 2. System Suitability test UV absorbance result (A) and fluorescence result (B).

PREPARE THE SYSTEM SUITABILITY PEPTIDE PANEL

1. Using scissors, carefully cut the top of the foil package leaving the sealing strip intact.
2. Take out the strip of tubes and cut one clear tube of lyophilized System Suitability Peptide Panel from the strip. Return the remaining tubes to the original package, reseal tightly and store at 2–8 °C.
3. Pierce the foil on the tube with a pipette tip.
4. Add 40 µL of DI water to the tube. Gently resuspend by pipetting the solution up and down to mix.
5. Add 160 µL of the System Suitability Test Mix to the freshly reconstituted peptide panel. Gently mix by pipetting up and down. Transfer this solution to a 1.5 mL microcentrifuge tube.
6. Vortex the tube 3 times, 5 seconds each.
7. Centrifuge the tube at 10,000 x g for 3 minutes to sediment any particulates.
8. Carefully aspirate the top 160 µL of the solution and pipette it into a sample vial with integrated insert or well of a 96-well plate. You'll want to insert the pipette tip all the way to the bottom of the insert or well when you dispense the solution to avoid introducing bubbles.

***Note:** Make sure to check for and remove any bubbles at the bottom of the sample vial or well.*

9. If you're using vials, close the sample vial with a clear screw cap.
10. Spin your sample plate or sample vials for 5 minutes at 1000 xg using the appropriate centrifuge adapter.

MAURICE SET UP AND START

1. Prepare your batch reagents and place them in Maurice. See "Appendix A: Reagent preparation" for prep details.
2. Place the metal 96-well plate insert or the metal vials insert in Maurice and then place your sample plate or vials in the insert.
3. Prepare your cartridge using the procedure in "Appendix B: cIEF Cartridge preparation", then install the cartridge in Maurice.
4. Launch Compass for iCE.
5. Click the **Batch** screen.
6. In the File menu, click **New Batch**. If your Maurice runs both size and charge, select **Maurice cIEF**.
7. Add samples by highlighting the sample location(s) in the Layout pane and clicking **Add**.

8. Use the default System Suitability method that uses these parameters:

METHOD PARAMETER	SETTING
Focus Period 1	Time: 1.0 min Voltage: 1500 V
Focus Period 2	Time: 4.5 min Voltage: 3000 V
Detection	Absorbance: 0.005 s Fluorescence: 3, 5, 10, 20 s
Sample Load Duration	55 s
Standards	pI 3.38: 300 pixels pI 10.17: 1800 pixels

9. Do two sequential replicate injections. Highlight the injection in the Injections pane and click **Replicate** to add a second injection.

10. Save your batch.

11. Click **Start**.

EXPECTED RESULTS

1. Review your results. If your system is functioning correctly you will see:

   - Five peaks for the pI Markers 3.38, 5.85, 6.14, 9.99, and 10.17 as shown in Figure 2A and 2B.
   - The estimated pI for the 5.85 and 6.14 markers should be between the ranges specified by the COA.
   - Peak heights of >100 mAU (absorbance mode with 0.005 s exposure time) and <60,000 RFU (fluorescence mode with 10 s exposure time).
   - Resolution of ≥2.1 for the 6.14 marker and ≥1.7 for the 10.17 marker (fluorescence mode with 10 s exposure time).

***NOTE:** Relative peak heights for the system suitability peptides will change between absorbance and fluorescence measurements as they have different primary amino acid sequences which affects fluorescence emissions.*

2. If the System Suitability test results are outside the specified range, we recommend replacing the cIEF Cartridge and repeating the test. In cases where the new cartridge provides the same result, carefully prep a new System Suitability Test Sample and rerun. If the System Suitability test continues to not meet specifications, please contact ProteinSimple Technical Support at support@proteinsimple.com.

Step 2: Method Development

You can screen molecules individually or in groups. If you plan to screen multiple compounds, we recommend you first prepare a large volume of cIEF Master Mix. When you do this, you can easily add aliquots of the Master Mix to the 1.0 mg/mL sample protein solutions in a 4 to 1 volume ratio to generate sample solutions. The instructions you'll need to prepare either one or multiple samples follow in the next sections.

Sample components, especially salts, can compromise both resolution and robustness in cIEF methods. Salt concentrations above 15 mM in the sample solution compress the pH gradient and generate a higher separation current that can damage your cartridge. When you have samples with high protein concentration, diluting the protein down to the final working concentration in the sample solution (0.2 mg/mL) will eliminate enough ionic strength for successful Maurice cIEF analysis. For the high salt-containing samples with low protein concentrations, you can use one of the following approaches to optimize your methods:

- A buffer exchange step to lower or remove salt, or
- Dilute the sample and use the improved sensitivity of the fluorescence detection mode.

PREPARE SAMPLE SOLUTION

For your initial screen, the sample's final protein concentration should be ~ 0.2 mg/mL with a salt concentration <15 mM. For example, a 2 mg/mL sample in 150 mM NaCl will need to be diluted 2-fold so you'll have 0.2 mg/ mL protein and 15 mM NaCl in the final mix.

If your sample is low in protein concentration or is high in salt, you may not be able to reach the recommended salt concentration. If you have a high salt concentration and/or low protein concentration, we highly recommend using the fluorescence detection mode which lets you dilute protein samples further without sacrificing method

cIEF MASTER MIX	SINGLE SAMPLE	MASTER MIX ((SINGLE SAMPLE*X) + 20%)				
# of samples	**1**	**2**	**3**	**4**	**5**	**6**
DI water	74 µL	178 µL	266 µL	355 µL	444 µL	459 µL
1% Methyl Cellulose	70 µL	168 µL	252 µL	336 µL	420 µL	504 µL
Pharmalyte 3–10	8 µL	19.2 µL	29 µL	38 µL	48 µL	58 µL
500 mM arginine	4 µL	9.6 µL	14.4 µL	19.2 µL	24 µL	29 µL
pI Marker 9.99	2 µL	4.8 µL	7.2 µL	9.6µL	12 µL	14.4 µL
pI Marker 4.05	2 µL	4.8 µL	7.2 µL	9.6 µL	12 µL	14.4 µL
Total volume	**160 µL**	**384 µL**	**576 µL**	**768 µL**	**960 µL**	**1152 µL**

TABLE 1. cIEF Master Mix solution.

sensitivity. In cases where further dilution does not generate the desired result, we recommend desalting your sample. Please see "Appendix C: Desalting and concentrating samples" for procedures on how to desalt and concentrate samples.

1. Dilute each sample to 1 mg/mL in water. If your protein is <1 mg/mL we suggest concentrating the sample. Please see "Appendix C: Desalting and concentrating samples" for the procedure.
2. Prepare a Master Mix based on the quantities in Table 1, where the # of samples to screen = X.
3. Add 160 µL of cIEF Master Mix solution to an individual microcentrifuge tube for each sample.
4. Add 40 µL of 1.0 mg/mL protein sample to the 160 µL cIEF Master Mix in the tube.
5. Vortex each sample to mix completely.
6. Centrifuge at 13,000 x g for 5 minutes to remove air bubbles and minimize air spikes.
7. Carefully aspirate the top 160 µL of the solution and pipette it into a sample vial with integrated insert or well of a 96-well plate. You'll want to insert the pipette tip all the way to the bottom of the insert or well when you dispense the solution to avoid introducing bubbles.
8. Spin your sample plate or sample vials for 5 minutes at 1000 x g using the appropriate centrifuge adapter.

MAURICE SET UP AND START

1. Prepare your batch reagents and place them in Maurice. See "Appendix A: Reagent preparation" for prep details.
2. Place the metal 96-well plate insert or the metal vials insert in Maurice and then place your sample plate or vials in the insert.
3. Prepare your cartridge using the procedure in "Appendix B: cIEF Cartridge preparation", then install the cartridge in Maurice.
4. Launch Compass for iCE.
5. Click the **Batch** screen.
6. In the File menu, click **New Batch**. If your Maurice runs both size and charge, select **Maurice cIEF**.
7. Add samples by highlighting the sample location(s) in the Layout pane and clicking **Add**.
8. Create a new method and set up parameters as shown in the table below.

METHOD PARAMETER	SETTING
Focus Period 1	Time: 1.0 min Voltage: 1500 V
Focus Period 2	Time: 8.0 min Voltage: 3000 V
Detection	Absorbance: 0.005 s Fluorescence: 3, 5, 10, 20 s
Sample Load Duration	55 s
Standards	pI 4.05: 250 pixels pI 9.99: 1800 pixels

9. Do two sequential replicate injections per sample. Highlight the injection in the Injections pane and click **Replicate** to add a second injection.

10. Save your batch.

11. Click **Start**.

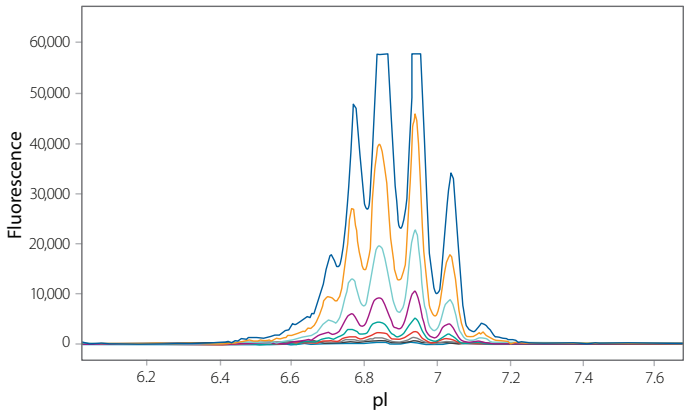

FIGURE 3. mAb signal saturation in fluorescence detection mode. At 250 µg/mL and a 30-second fluorescence exposure time (blue trace), the two most prominent isoforms show saturation. The traces are from a two-fold serial dilution of a mAb from 0.977 to 250 µg/mL. Each sample contained 0.35% methyl cellulose, 4% Pharmalyte 3 to 10, 10 mM arginine as a cathodic spacer, and 10 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 6 minutes.

REVIEW AND OPTIMIZE YOUR RESULTS

1. **Optimize your sample concentration for absorbance.** Maurice has been designed to give you comparable cIEF results to iCE280 and iCE3 systems in terms of absorbance signal sensitivity and linear dynamic range. The only difference between the three systems is that Maurice reports absorbance in mAU where iCE280 and iCE3 report absorbance in AU (1000 mAU = 1 AU). Review the initial results for each of your samples and adjust the sample concentration so that the largest/major peak is not above 600 mAU. All major peaks should be above 50 mAU and below 600 mAU.

***NOTE:** Major peaks are any peak that equals at least 20% of the total peak area.*

2. **Optimize your sample concentration for fluorescence**. When optimizing fluorescence signal, it's important to avoid artifacts caused by signal saturation and quenching. Both of these events will affect the linear response of your signal to protein concentration. Signal saturation can be identified by peak shapes that flatten near their maxima, resulting in inaccurate quantitative results (Figure 3, blue trace). Signal saturation can easily be avoided by reducing the exposure time so that the most intense peak is less than 60,000 fluorescence units. Maurice can take fluorescence measurements at multiple exposures times, making it easier to identify and eliminate saturation effects. Alternatively, lowering the protein concentration in the sample will reduce the signal, eliminating saturation effects and improving peak shape and quantitation.

   Signal quenching is a compound-dependent phenomenon that can occur in the most abundant charge isoforms, suppressing their fluorescence emissions resulting in lower signal (Figure 4). Quenching can be easily detected by comparing the peak height and total peak area results between the absorbance and fluorescence detection modes. As shown in Figure 4, the relative intensity of the main peak to the less abundant isoforms is reduced when compared to the absorbance profile. Reducing the sample concentration is required to restore linear signal production of the main peak. To identify the best concentration range for fluorescence measurements, run a dilution curve on a compound and then plot the

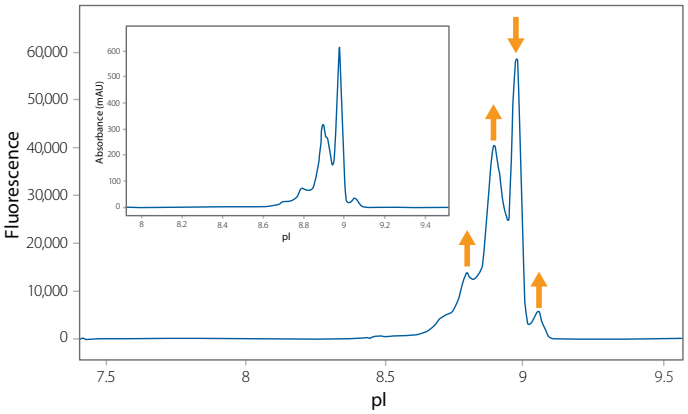

FIGURE 4. mAb signal quenching in fluorescence detection mode. At 250 µg/mL and a 20-second fluorescence exposure time, the suppression of the main peak's fluorescence signal is due to quenching. The sample contained 0.35% methyl cellulose, 2M urea, 6% Pharmalyte 3 to 10, 10 mM arginine as a cathodic spacer, and 10 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 7 minutes.

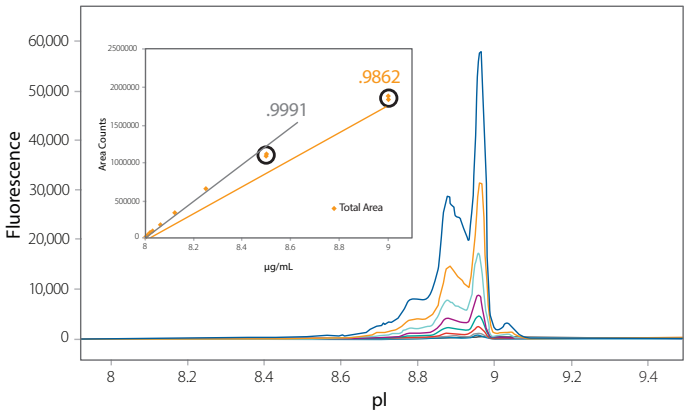

FIGURE 5. Characterizing a mAb native fluorescence signal's linear dynamic range. Graph of protein concentration vs. fluorescence total peak area showing 2 linear fits: orange line includes all samples and gray line excludes the 250 and 500 µg/mL samples. The peak traces are from a two-fold serial dilution of a mAb from 0.488 to 125 µg/mL. Each sample contained 0.35% methyl cellulose, 2M urea, 6% Pharmalyte 3 to 10, 10 mM arginine as a cathodic spacer, and 10 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 7 minutes.

total peak area versus the protein concentration. The optimal sample concentrations should have a strong linear correlation between protein concentration and total peak area. The graph in Figure 5 shows that signal for samples at 250 and 500 µg/mL (circled points) significantly deviate from a linear relationship due to quenching as demonstrated by the linear fit in orange. Running this mAb at a concentration ≤125 µg/mL avoids the quenching phenomenon and allows for linear quantitation of all peaks in the charge profile as demonstrated by the linear fit in gray (Figure 5).

3. **Evaluate your injection profiles**. For each sample, review both injections and compare the peak profile. Your peak profiles should be reproducible as shown in Figure 8. If the duplicate sample injections don't have the same peak profile, move on to the next section. If the duplicate peak profiles are reproducible, skip to the "Resolution optimization" section.

Non-reproducible peak profiles

There are two major causes of a non-reproducible peak profile: sample aggregation and sample precipitation (Figures 6 and 7). Sample aggregation is induced by isoelectric focusing and doesn't necessarily originate in the sample, while more than 50% of non-antibody proteins may precipitate during isoelectric focusing. The sample shown in Figure 6 shows non-reproducible spikes due to protein precipitation occurring near the isoelectric point.

Non-reproducible peak profiles are easily tackled with protein solubilizers. Adding 40% (final concentration in the prepared sample) of SimpleSol protein solubilizer or 4 M urea to your sample solution will eliminate sample aggregation almost 100% of the time. We recommend you start with 40% of SimpleSol or 4 M urea. If this gives you a reproducible peak profile but you prefer to limit the amount of additives used in your samples, you can then screen samples using lower concentrations. If you would like to screen for both 20% and 40% SimpleSol or 2 M and 4 M urea in one batch, see the instructions in the next section.

After preparing your samples, set up a batch that performs duplicate injections of each sample, with the same method parameters used previously. When the batch is done, review both injections and compare the peak profiles (which should be reproducible) as shown in Figure 8. If your

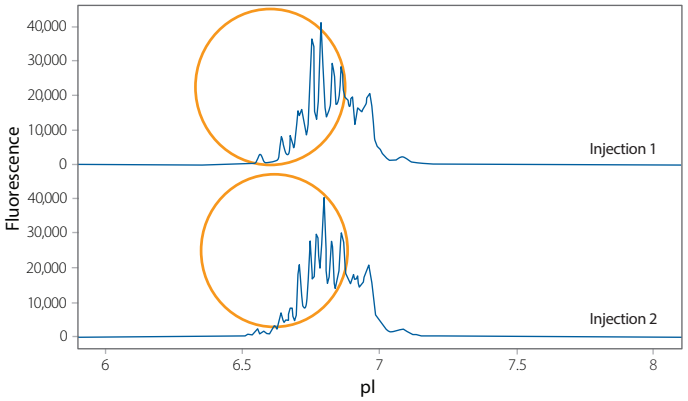

FIGURE 6. Precipitation/aggregation affecting charge profile reproducibility. At 250 µg/mL and a 20-second native fluorescence exposure time, two replicate injections show irreproducible spikes in the charge profile of this mAb. The sample contained 0.35% methyl cellulose, 4% Pharmalyte 3 to 10, 10 mM arginine as a cathodic spacer, and 10 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 6 minutes.

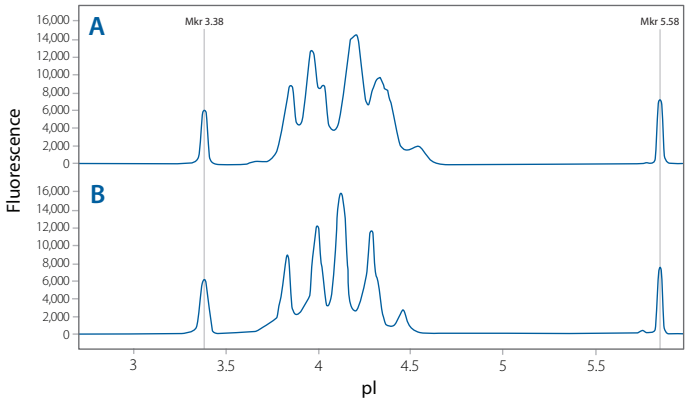

FIGURE 7. Eliminating precipitation/aggregation for recombinant human erythropoietin (EPO; R&D Systems PN 286-EP) by adding SimpleSol to the sample solution. (A) Injection without SimpleSol. (B) Injection with 40% SimpleSol. At 200 µg/mL and a 20 second native fluorescence exposure time, an injection without SimpleSol in the sample shows poor resolution (A). Including 40% SimpleSol in the sample solution results in well resolved peaks for a reproducible charge profile (B). The sample contained 0.35% methyl cellulose, 3.5% Pharmalyte 2.5 to 5, 0.5% Servalyte 3 to 5, 10 mM iminodiacetic acid as an anodic spacer, with and without 40% SimpleSol. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 12 minutes.

duplicate sample injections aren't reproducible, you'll need to increase the amount of urea (see Appendix D for the sample denaturing procedure). If your duplicate injections are reproducible and the resolution is acceptable, your method development is complete (Figures 7 and 8). If you need greater peak separation, move on to the "Resolution optimization" section.

ADDRESSING NON-REPRODUCIBLE PEAK PROFILES

Add appropriate volume of SimpleSol (for final concentration of 20-40% in the prepared sample) OR reconstitute the urea:

1. Add 320 µL of DI water to one vial of lyophilized urea.
2. Vortex to dissolve. This will result in a total volume of 560 µL of 10 M urea.

***NOTE:** Prepare urea fresh each time. Once urea is reconstituted, it's only good for one day. Don't reuse.*

3. Prepare 200 µL of sample solution in 4% pH 3–10 Pharmalyte with SimpleSol or urea:
   a. Dilute each sample to the optimal protein concentration determined in your initial sample screen.
   b. Prepare a cIEF Master Mix solution using the amounts in Table 2 or Table 3, where the # of samples to screen = X.

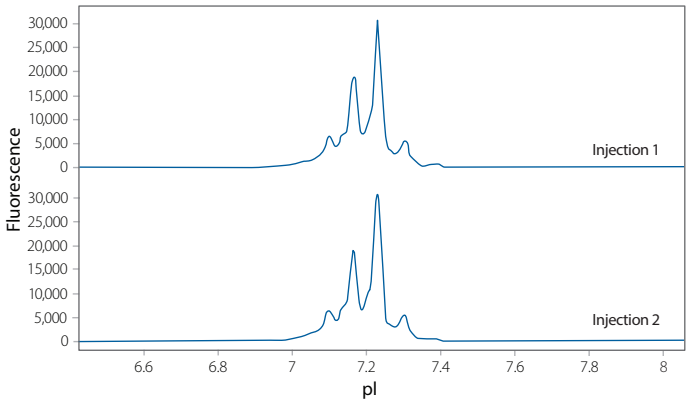

FIGURE 8. Eliminating mAb precipitation/aggregation by adding urea to the sample solution. At 250 µg/mL and a 20-second native fluorescence exposure time, two replicate injections show a highly reproducible charge profile after addition of 2M urea to the mAb sample solution. The sample contained 0.35% methyl cellulose, 2M urea, 4% Pharmalyte 3 to 10, 10 mM arginine as a cathodic spacer, and 10 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 7 minutes.

   c. Add 166 µL of cIEF Master Mix to individual microcentrifuge tubes for each sample.
   d. Add 34 µL of the 1.0 mg/mL protein sample to the 166 µL cIEF Master Mix in the tube.
   e. Vortex each sample to mix completely.

cIEF MASTER MIX	FOR A SINGLE SAMPLE	MASTER MIX ((SINGLE SAMPLE*X) + 20%)			
# of samples	**1**	**2**	**3**	**4**	**5**
DI water	0 µL	0 µL	0 µL	0 µL	0 µL
1% MC	70 µL	168 µL	252 µL	336 µL	420 µL
*10 M urea/SimpleSol	80 µL	192 µL	288 µL	384 µL	480 µL
Pharmalyte 3–10	8 µL	19.2 µL	29 µL	38 µL	48 µL
500 mM arginine	4 µL	9.6 µL	14.4 µL	19.2 µL	24 µL
pI marker 9.99	2 µL	4.8 µL	7.2 µL	9.6 µL	12 µL
pI marker 4.05	2 µL	4.8 µL	7.2 µL	9.6 µL	12 µL
Total volume	**166 µL**	**398 µL**	**598 µL**	**797 µL**	**996 µL**

TABLE 2. 4% 3–10 pH Pharmalyte with 40% SimpleSol or 4 M urea in cIEF Master Mix (maximum protein sample volume = 34 µL). *Note: Choose either SimpleSol or urea as your protein solubilizer. Do not mix both.

cIEF MASTER MIX	FOR A SINGLE SAMPLE	MASTER MIX ((SINGLE SAMPLE*X) + 20%)			
# of samples	**1**	**2**	**3**	**4**	**5**
DI water	40 µL	88 µL	132 µL	176 µL	208 µL
1% MC	70 µL	168 µL	252 µL	336 µL	420 µL
*10 M urea/SimpleSol	40 µL	96 µL	144 µL	192 µL	240 µL
Pharmalyte 3–10	8 µL	19.2 µL	29 µL	38 µL	48 µL
500 mM arginine	4 µL	9.6 µL	14.4 µL	19.2 µL	24 µL
pI marker 9.99	2 µL	4.8 µL	7.2 µL	9.6 µL	12 µL
pI marker 4.05	2 µL	4.8 µL	7.2 µL	9.6 µL	12 µL
Total volume	**166 µL**	**390 µL**	**586 µL**	**781 µL**	**964 µL**

TABLE 3. 4% 3–10 pH Pharmalyte with 20% SimpleSol or 2 M urea in cIEF Master Mix (maximum protein sample volume = 34 µL). *Note: Choose either SimpleSol or urea as your protein solubilizer. Do not mix both.

f. Centrifuge at 13,000 x g for 5 minutes to remove air bubbles and minimize air spikes.

g. Carefully aspirate the top 160 µL of the solution and pipette it into a sample vial with integrated insert or well of a 96-well plate. You'll want to insert the pipette tip all the way to the bottom of the insert or well when you dispense the solution to avoid introducing bubbles.

***Note:** Make sure to check for and remove any bubbles at the bottom of the sample vial or well.*

h. If you're using vials, close the sample vial with a clear screw cap.

i. Spin your sample plate or sample vials for 5 minutes at 1000 x g using the appropriate centrifuge adapter.

Resolution optimization

If you need higher peak resolution, you can optimize it by increasing the concentration of broad range ampholytes (Figure 9) or by adding narrow range ampholytes (Figure 10) — however, your peak pattern needs to be reproducible before doing so. A fast way to improve resolution is to add narrow pH range carrier ampholytes, which are usually mixed with the wide pH range ampholytes in ratios ranging from 1:1 to 5:1. We recommend starting with a 1:1 ratio of narrow range ampholytes to 3–10 ampholytes. It's also possible to use

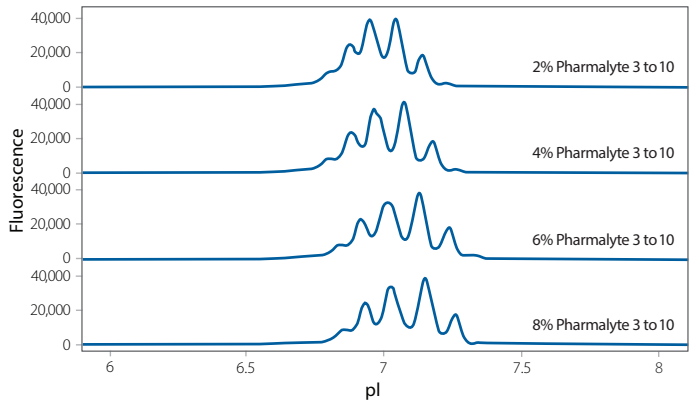

FIGURE 9. Improving peak resolution of an mAb by increasing broad range ampholyte concentration. At 250 µg/mL and a 20-second native fluorescence exposure time profile, resolution improves with increasing concentration of Pharmalyte 3 to 10. The traces are from a Pharmalyte 3 to 10 response curve on a mAb. Each sample contained 0.35% methyl cellulose, between 2 to 8% Pharmalyte 3 to 10, 5 to 20 mM arginine as a cathodic spacer, and 5 to 20 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 7 minutes.

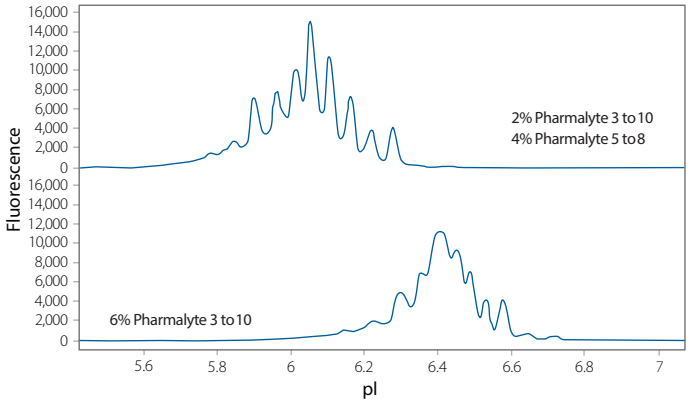

FIGURE 10. Improving peak resolution of a fusion protein by adding narrow range ampholytes. At 400 µg/mL and a 20-second native fluorescence exposure time, a fusion protein's profile resolution improves with the addition of Pharmalyte 5 to 8 narrow range ampholytes. The traces are from a Pharmalyte 3 to 10 response curve on a mAb. Each sample contained 0.35% methyl cellulose, 2 M urea, between 2 to 6% Pharmalyte 3 to 10 and between 0 to 4% Pharmalyte 5 to 8, 10 mM arginine as a cathodic spacer, and 10 mM iminodiacetic acid as an anodic spacer. Sample solutions were separated by first applying 1500 V for 1 minute, followed by 3000 V for 7 or 10 minutes.

only narrow range ampholytes but this requires using an anodic or cathodic spacer. 500 mM arginine is included in the Method Development Kit as a cathodic spacer.

Using narrow range ampholytes will increase the required focusing time. We recommend starting with a 1-minute prefocus at 1500 V followed by a 10-minute focus step at 3000 V, and then optimizing the focusing time from there. Tables 4, 5 and 6 include a few common narrow range methods.

Many cIEF methods have been published, so you can check these journal publications for additional information on cIEF method development and peak resolution optimization.[1,2,3,4,5]

Method of resolution optimization

1. Determine the pI of your sample.
2. Choose the narrow range ampholytes in your pH range of interest.
3. Begin with a total 4% ampholytes concentration (8 µL total ampholytes per 200 µL sample solution) and a 1:1 ratio of narrow range to 3–10 wide range ampholytes. See Tables 4, 5 and 6 for method recipes.
4. Select two pI markers from those included in the kit that will closely bracket the peaks in your sample. These markers need to be within the pH range of the narrow range ampholytes for accurate pI determination. Add 1 µL of both the low and high pI marker to the sample solution. See Tables 4, 5 and 6 for sample preparation and method parameters.
5. Prefocus at 1500 V for 1 minute and focus at 3000 V for 10 minutes.
6. If you need more resolution, increase the amount of the narrow range ampholytes in the ampholyte mix. Increasing the concentration of ampholytes can also improve resolution. Ampholyte concentration should be no higher than 8%.

Robustness

You can also optimize method robustness using a Design of Experiment (DOE) approach with statistical analysis software packages. Find more information on applying

ACIDIC METHOD	AMPHOLYTE RANGE (pH) 3–10	2.5–5	LOW pI MARKER	HIGH pI MARKER	1% MC	DI WATER	*10 M UREA	*SIMPLESOL	SAMPLE (1 mg/mL)	PREFOCUS @ 1500 V	FOCUS @ 3000 V
4% Ampholyte 1:1 Ratio No Urea / SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	78 µL	0 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 2 M Urea /20% SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	38 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 4 M Urea /40% SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	0 µL	80 µL	80 µL	38 µL	1 min	10 min
4% Ampholyte 1:3 Ratio No Urea / SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	78 µL	0 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 2 M Urea /20% SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	38 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 4 M Urea /40% SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	0 µL	80 µL	80 µL	38 µL	1 min	10 min

TABLE 4. Acidic range methods. *Note: Choose either SimpleSol or urea as your protein solubilizer. Do not mix both.

NEUTRAL METHOD	AMPHOLYTE RANGE (pH) 3–10	5–8	LOW pI MARKER	HIGH pI MARKER	1% MC	DI WATER	*10 M UREA	*SIMPLESOL	SAMPLE (1 mg/mL)	PREFOCUS @ 1500 V	FOCUS @ 3000 V
4% Ampholyte 1:1 Ratio No Urea/ SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	78 µL	0 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 2 M Urea/20% SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	38 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 4 M Urea/40% SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	0 µL	80 µL	80 µL	38 µL	1 min	10 min
4% Ampholyte 1:3 Ratio No Urea/ SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	78 µL	0 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 2 M Urea/20% SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	38 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 4 M Urea/40% SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	0 µL	80 µL	80 µL	38 µL	1 min	10 min

TABLE 5. Neutral range methods. *Note: Choose either SimpleSol or urea as your protein solubilizer. Do not mix both.

BASIC METHOD	AMPHOLYTE RANGE (pH) 3–10	8–10.5	LOW pI MARKER	HIGH pI MARKER	1% MC	DI WATER	*10 M UREA	*SIMPLESOL	500 mM ARGININE (1 mg/mL)	SAMPLE	PREFOCUS @ 1500 V	FOCUS @ 3000 V
4% Ampholyte 1:1 Ratio No Urea/ SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	74 µL	0 µL	0 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 2 M Urea/20% SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	34 µL	40 µL	40 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 4 M Urea/40% SimpleSol	4 µL	4 µL	2 µL	2 µL	70 µL	0 µL	80 µL	80 µL	4 µL	34 µL	1 min	10 min
4% Ampholyte 1:3 Ratio No Urea/ SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	74 µL	0 µL	0 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 2 M Urea/20% SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	34 µL	40 µL	40 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 4 M Urea/40% SimpleSol	2 µL	6 µL	2 µL	2 µL	70 µL	0 µL	80 µL	80 µL	4 µL	34 µL	1 min	10 min
8% Narrow range No Urea/SimpleSol	0 µL	12 µL	2 µL	2 µL	70 µL	74 µL	0 µL	0 µL	4 µL	36 µL	1 min	10 min
8% Narrow range 2 M Urea/20% SimpleSol	0 µL	12 µL	2 µL	2 µL	70 µL	34 µL	40 µL	40 µL	4 µL	30 µL	1 min	10 min

TABLE 6. Basic range methods. *Note: Choose either SimpleSol or urea as your protein solubilizer. Do not mix both.

DOE principles in the Application Note *Computer-aided Assay Development for Charge Heterogeneity Analysis by iCE* (download at http:// www.proteinsimple.com/ documents/Application_Note_ Computer-Aided_Assay_ Development_for_iCE.pdf).

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Appendix A: Reagent preparation

Prepare your batch reagents as shown in Table 7 and place the reagent vials in Maurice as shown in Figure 11.

REAGENT	VOLUME	CAP	POSITION
0.5% Methyl Cellulose	2.0 mL	**Blue** pressure cap	P1
Fluorescence Calibration Standard	500 µL	**Blue** pressure cap	P2
DI water	2.0 mL	**Blue** pressure cap	P3
Empty vial (air)	N/A	**Blue** pressure cap	P6
DI water	2.0 mL	**Clear** screw cap	N1

TABLE 7. Batch reagent preparation.

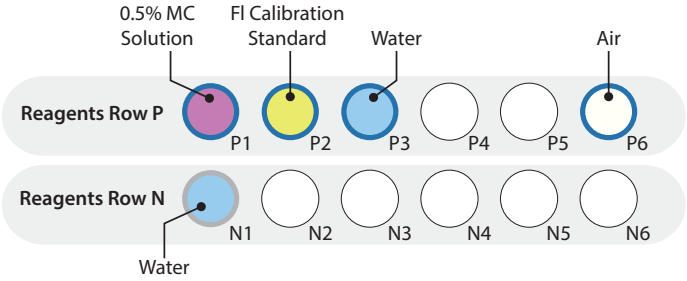

FIGURE 11. Reagent vial placement.

Appendix B: cIEF Cartridge preparation

1. Take the cartridge out of its packaging. Save the packaging, you'll need it later.
2. Place the cartridge on a flat surface with its electrolyte tanks facing up.
3. Remove the stoppers from both electrolyte tanks.
4. Add 2 mL of Catholyte solution to the OH^- electrolyte tank (white port).
5. Add 2 mL of Anolyte solution to the H^+ electrolyte tank (red port).

***Note:** Make sure you don't overfill the electrolyte tanks.*

6. Seal each tank with the rubber stoppers. Use the grey stopper for the OH^- tank and the red one for the H^+ tank. If excess liquid comes out of the tank, make sure to wipe it with a lint-free laboratory wipe.

Appendix C: Desalting and concentrating samples

1. Add 500 µL of your sample into an Amicon Ultracel 50K Membrane Centrifugal Filter (Millipore PN 4311).
2. Centrifuge for 5 minutes at 12K rcf.
3. Replace the filtered volume with 20 mM Tris buffer pH 7.0 (Life Technologies PN AM9851).
4. Do two additional cycles of centrifugation and buffer replacement.
5. For simple desalting, replace the filtered volume to 500 µL. If you need to concentrate the sample, store the remaining 100 µL of buffer-exchanged sample at -20 °C or below if you won't use it immediately.

Appendix D: Denaturing sample preparation

In extreme cases, some proteins need a higher concentration than 40% SimpleSol or 4 M urea to stay in solution during cIEF analysis. When this happens, you'll need to denature the samples using 8 M urea in the cIEF Master Mix solution. To make sure the final urea concentration in the sample is 8 M, follow the example preparation.

The total sample volume should be 200 µL. You'll need the following amount of components per 160 µL of master mix prepared with 96 mg urea powder

- 70 µL of 1% Methyl Cellulose
- 8 µL of carrier ampholytes (total)
- 2 µL of each pI marker
- q.s. DI water

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046-296, Rev D