iCE Method Development Guide

Introduction

The iCE 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 test mix to make sure your iCE3 is ready to go and a Method Development Guide to help you every step of the way. Taking advantage of iCE3's generic methods for multiple molecules and simple method development has just never been easier — and you can use either the FC or the HT Cartridge.

Kit Contents

Reagents

SOLUTION	REAGENT	DESCRIPTION	QTY/AMT
1	Anolyte	0.08 M H_3PO_4 in 0.1% Methyl Cellulose (MC)	10 mL
2	Catholyte	0.1 M sodium hydroxide in 0.1% MC	10 mL
3	1% MC	1% Methyl Cellulose (MC)	10 mL
4	0.5% MC	0.5% Methyl Cellulose (MC)	3 X 10 mL
5	Cathodic Blocker	500 mM arginine	500 μL
6	System Test Mix	Two pI Markers (4.22 and 9.46) in 8% pH 3–10 Pharmalytes with 0.35% MC	1 mL
7	Urea Powder	Lyophilized urea	5 vials

Ampholytes

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

pI markers

PI	DESCRIPTION	QTY/AMT
3.21	pI Marker 3.21 (ProteinSimple PN 102018)	200 μL
4.22	pI Marker 4.22 (ProteinSimple PN 102350)	200 μL
5.12	pI Marker 5.12 (ProteinSimple PN 102224)	200 μL
6.14	pI Marker 6.14 (ProteinSimple PN 102220)	200 μL
7.05	pI Marker 7.05 (ProteinSimple PN 102226)	200 μL
7.65	pI Marker 7.65 (ProteinSimple PN 102407)	200 μL
8.18	pI Marker 8.18 (ProteinSimple PN 102408)	200 μL
9.46	pI Marker 9.46 (ProteinSimple PN 102349)	200 μL
10.10	pI Marker 10.10 (ProteinSimple PN 102232)	200 μL

Kit Storage

Upon receipt, store all components at 2–8 °C.

Ordering Info

This kit can be reordered by:

- **Phone:** 1-800-206-1027
- **Fax:** 1-416-231-1152
- **Email:** orders@proteinsimple.com

Other Items Needed

GENERAL METHOD DEVELOPMENT

- FC Cartridge (ProteinSimple PN 101701) or HT Cartridge (ProteinSimple PN P-0000035-00)
- Autosampler vials
- DI water: HPLC grade or better
- Pipettes and tips
- Micro centrifuge tubes
- Vortex
- Microcentrifuge

METHOD OPTIMIZATION (SEE FURTHER OPTIMIZATION)

- HT Cartridge

BUFFER EXCHANGE (SEE APPENDIX A)

- Amicon Ultracel 50K membrane centrifugal filter (Millipore PN 4311)
- 1M Tris-HCl buffer pH 7.0 (Life Technologies PN AM9851)

DENATURING SAMPLES (SEE APPENDIX B)

- Urea, electrophoresis grade (Sigma-Aldrich, PN U6504)

NOTE: Urea is only needed if your sample requires 8 M urea to address protein precipitation.

Guidelines to Great Results

- Store all items at 2–8°C
- 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 is good for one day only. Don’t reuse.
- pI markers should be diluted 200-fold into the sample solution.
- Read the entire Method Development Guide before getting started.

Method Development Overview

A successfully defined and optimized iCE method gives you:

- A highly reproducible peak profile
- Satisfactory peak resolution for the targeted application

iCE 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 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 and peak profile reproducibility. Once you have a reproducible profile, resolution can then be addressed if needed by adding narrow pH range ampholytes. The electropherograms in Figure 2 show a successfully completed method development process for a hydrophobic compound with a compact peak profile.

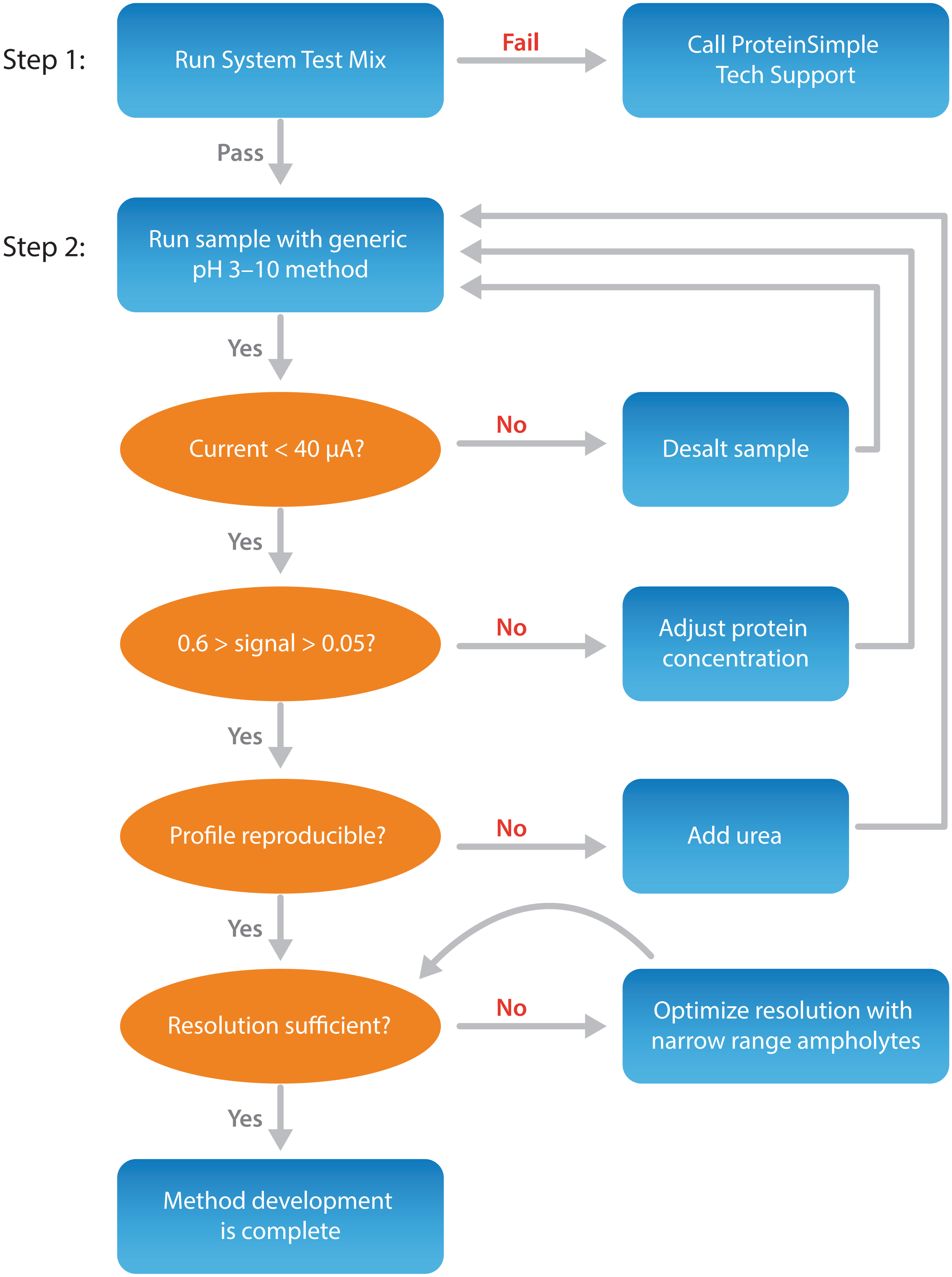


FIGURE 1. Method development strategy.

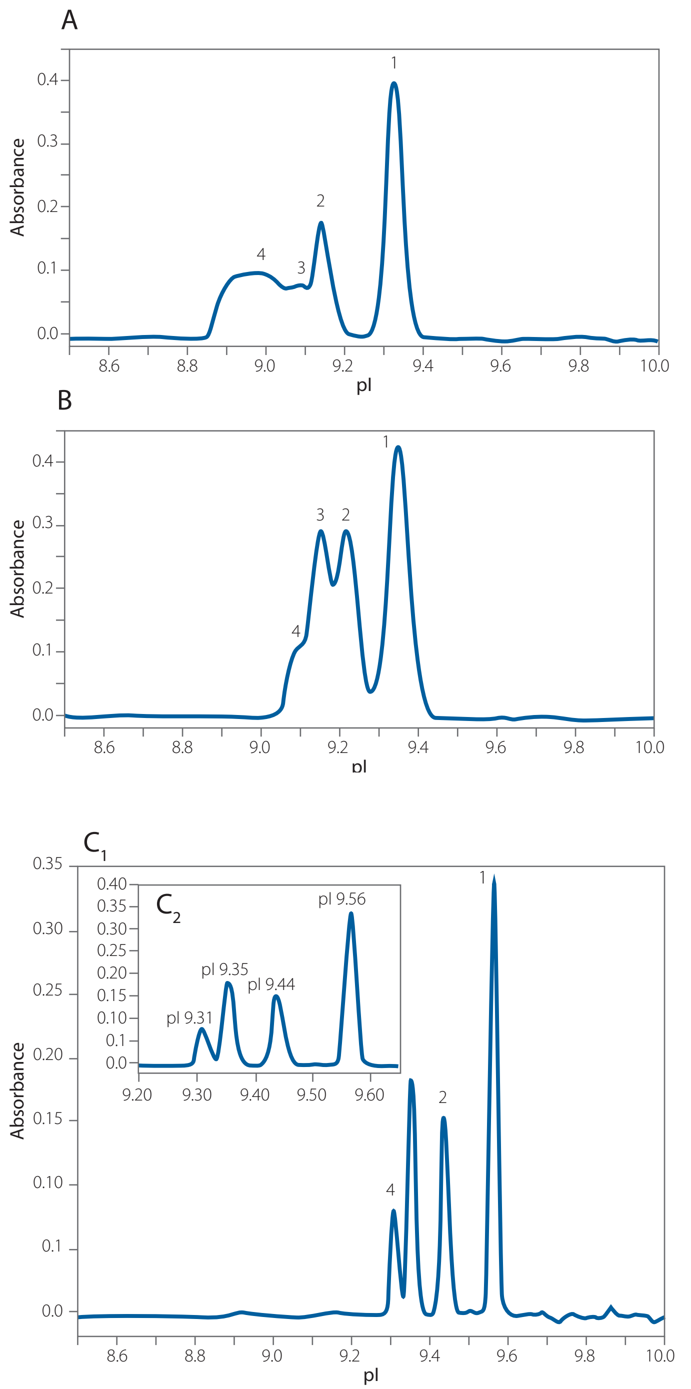


FIGURE 2. Stepwise method development of an iCE method. (A) Shows the results of a general screening method using a 4% 3–10 Pharmalyte gradient. The pI for the compound's charge variants ranged from pH 8.7–9.5 and aggregation is seen between isoforms 2–4. (B) Modifying the sample matrix to include a final concentration of 4 M urea eliminates the aggregation, allowing partial separation of isoforms 2–4. (C) Modifying the conditions from B to include an 8% Pharmalyte 8–10.5 gradient with 10 mM arginine as a cathodic spacer (focused for 1 min at 1500 V followed by 8 min at 3000 V), results in near-baseline separation for all isoforms. The triplicate run overlay shows the separations are highly reproducible with 0.04 pH unit resolution.

iCE3 Performance Check

The iCE Method Development Kit includes a System Test Mix that lets you check iCE3 performance before you begin method development. To run a system performance check:

1. Pipet 200 µL of the System Test Mix into the appropriate autosampler vial.
2. Follow the instructions in iCE CFR software to install an FC Cartridge in your iCE3.
3. When prompted, place the vial with the System Test Mix into the TTM position:
    - iCE3 with PrinCE Next: Buffer position Z15
    - iCE3 with Alcott 720: Vial position 1
4. Once a cartridge is successfully installed, move the System Text Mix vial to sample tray position 1.
5. Program a Batch to do two injections with these parameters:

iCE parameters:

TABLE 1. iCE parameters:

SAMPLE ID	VIAL	FOCUS PERIOD 1		FOCUS PERIOD 2	
		TIME	VOLTAGE	TIME	VOLTAGE
System Test Mix	01	1.00 min	1500 V	4.50 min	3000 V
System Test Mix	01	1.00 min	1500 V	4.50 min	3000 V

Autosampler parameters:

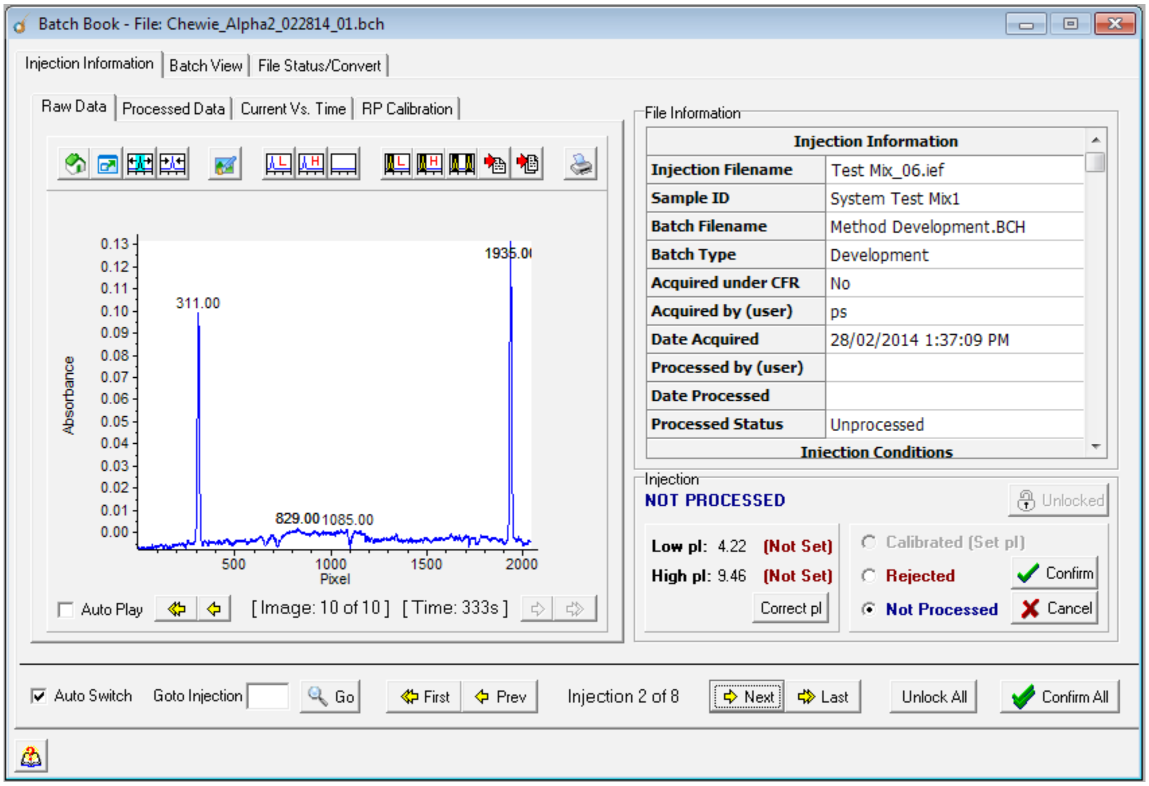
- Turn the temperature control to ON
- Set the temperate at 10 °C and accept all other defaults

Injection conditions:

TABLE 2. Injection conditions:

CARRIER AMPHOLYTES	ADDITIVES	LOW PI MARKER	HIGH PI MARKER
3–10	None	4.22	9.46

6. Save the Batch.
7. Click **Start Acquisition** to run the Batch file.
8. Review your results. If your system is functioning as it should, you will see two peaks corresponding to the



Batch Book - File: Chewie_Alpha2_022814_01.bch

Injection Information	
Injection Filename	Test Mix_06.ief
Sample ID	System Test Mix1
Batch Filename	Method Development.BCH
Batch Type	Development
Acquired under CFR	No
Acquired by (user)	ps
Date Acquired	28/02/2014 1:37:09 PM
Processed by (user)	
Date Processed	
Processed Status	Unprocessed
Injection Conditions	

NOT PROCESSED

Low pI: 4.22 **(Not Set)**
High pI: 9.46 **(Not Set)**

○ Calibrated (Set pI)
○ Rejected
● Not Processed

Injection 2 of 8

FIGURE 3. Acceptable results for System Test Mix.

4.22 and 9.46 pI markers as shown in Figure 3. The height of the 9.46 pI marker should be between 0.05 and 0.15 absorbance units. The position of the pI 9.46 marker (basic marker) should be within the range of 1800 and 2000 pixels. If you don't see any peaks or only see a single peak, please contact Technical Support at support@proteinsimple.com.

Initial Wide pH Range Method

You can screen molecules individually or in groups. If you plan to screen multiple compounds, we recommend you first prepare a large volume of IEF 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 icIEF 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 iCE 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 formulations 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 iCE analysis. For formulations with low protein concentrations (< 10X dilution to final sample concentration), a buffer exchange step may be needed to get your best results.

STEP 1: PREPARE 200 µL OF A 0.2 MG/ML SAMPLE SOLUTION IN 4% PH 3–10 PHARMALYTE

For your initial screen, the protein concentration should be ~ 0.2 mg/mL with a salt concentration < 15 mM. For example, you can dilute a 2 mg/mL sample in 150 mM NaCl 10X into the IEF Master Mix 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. In this case, we recommend desalting your sample. Please see Appendix A for procedures on how to desalt and concentrate samples.

TABLE 3. IEF Master Mix solution.

IEF MASTER MIX SOLUTION	SINGLE SAMPLE	MASTER MIX (SINGLE SAMPLE*X + 10%)				
# of samples	**1**	**2**	**3**	**4**	**5**	**6**
DI water	80 µL	176 µL	264 µL	352 µL	440 µL	528 µL
1% MC	70 µL	154 µL	231 µL	308 µL	385 µL	462 µL
Pharmalyte 3–10	8 µL	17.6 µL	26.4 µL	35.2 µL	44 µL	52.8 µL
pI marker 9.46	1 µL	2.2 µL	3.3 µL	4.4 µL	5.5 µL	6.6 µL
pI marker 4.22	1 µL	2.2 µL	3.3 µL	4.4 µL	5.5 µL	6.6 µL
Total volume	**160 µL**	**352 µL**	**528 µL**	**704 µL**	**880 µL**	**1056 µL**

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 A for the procedure.
2. Prepare a Master Mix based the quantities in Table 3, where the # of samples to screen = X.
    a. Add 160 µL of IEF Master Mix solution to an individual microcentrifuge tube for each sample.
    b. Add 40 µL of 1.0 mg/mL protein sample to the 160 µL IEF Master Mix in the tube.
    c. Vortex each sample to mix completely.
    d. Centrifuge at 13,000 x g for 5 minutes to remove air bubbles and minimize air spikes.
    e. Transfer 150 µL to the appropriate autosampler vial.

STEP 2: PROGRAM A BATCH

Program a Batch that does duplicate injections of each sample using these parameters:

iCE parameters:

TABLE 4. iCE parameters:

FOCUS PERIOD 1		FOCUS PERIOD 2	
TIME	VOLTAGE	TIME	VOLTAGE
1.00 min	1500 V	8.00 min	3000 V

Autosampler parameters:

- Turn the temperature control to ON
- Set the temperate at 10 °C and accept all other defaults

Injection conditions:

TABLE 5. Injection conditions:

CARRIER AMPHOLYTES	ADDITIVES	LOW PI MARKER	HIGH PI MARKER	CONC.
3–10	None	4.22	9.46	0.2 mg/mL

STEP 3: RUN THE BATCH

1. Save the Batch.
2. Click **Start Acquisition** to run the Batch file.

STEP 4: REVIEW RESULTS

1. Optimize your sample concentration. Review the initial results for each of your samples and adjust the sample concentration so that the largest/major peak is not above 0.6 AU. All major peaks should be above 0.05 AU and below 0.60 AU.

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

2. Optimize your sample matrix. Check the current profile of the duplicate samples you ran in the Batch. If the current is above 40 µA, the ionic strength of your sample needs to be reduced. If the ionic strength of your sample can't be reduced by dilution, you'll need to desalt. Please see Appendix A for the procedure.

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 4. 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 (see Figures 5 and 6). Sample aggregation is induced by isoelectric focusing so doesn't necessarily originate in the sample, and more than 50% of non-antibody proteins may precipitate during isoelectric focusing.

Non-reproducible peak profiles are easily tackled with urea. Adding 4 M urea to your sample solution will

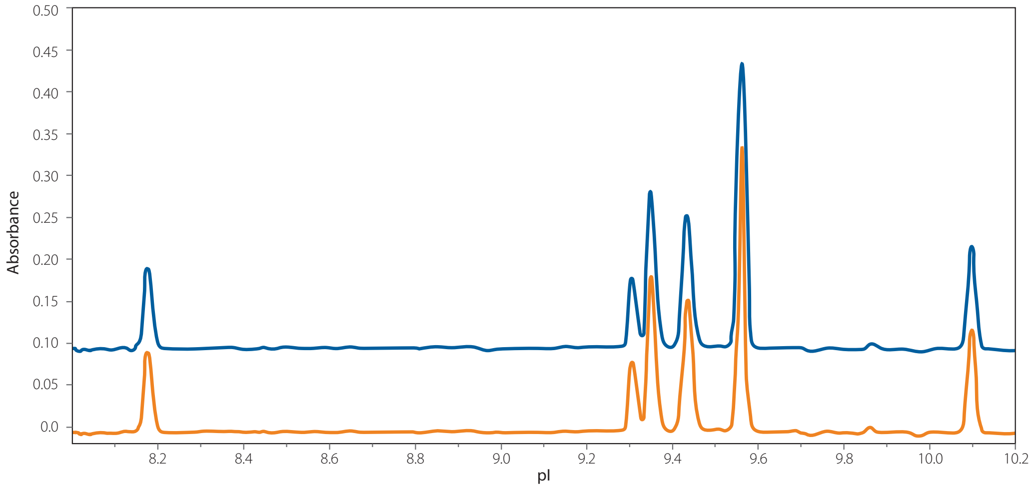


FIGURE 4. Reproducible peak profile.

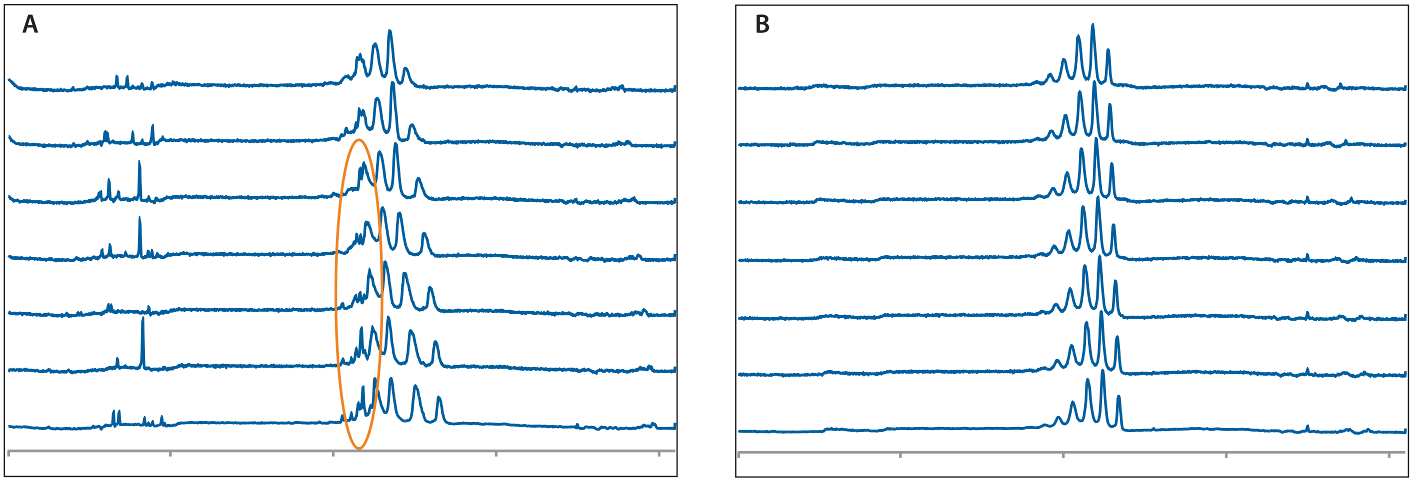


FIGURE 5. mAb separation before and after adding urea. (A) Separation conditions are 0.2 mg/mL mAb in 1% pH 3–10, 3% pH 5–8 Pharmalytes, 0.35% Methyl Cellulose, and (B) shows a standard protein aggregation profile. When focusing begins, peak shapes are normal but later in the focusing process, sharp spikes start to appear. These spikes will never stabilize during the focusing process. But, part of the peak profile other than the spikes may stabilize and become somewhat reproducible. (B) 4 M urea was added to the separation mix to eliminate aggregation, and results in a stable peak profile.

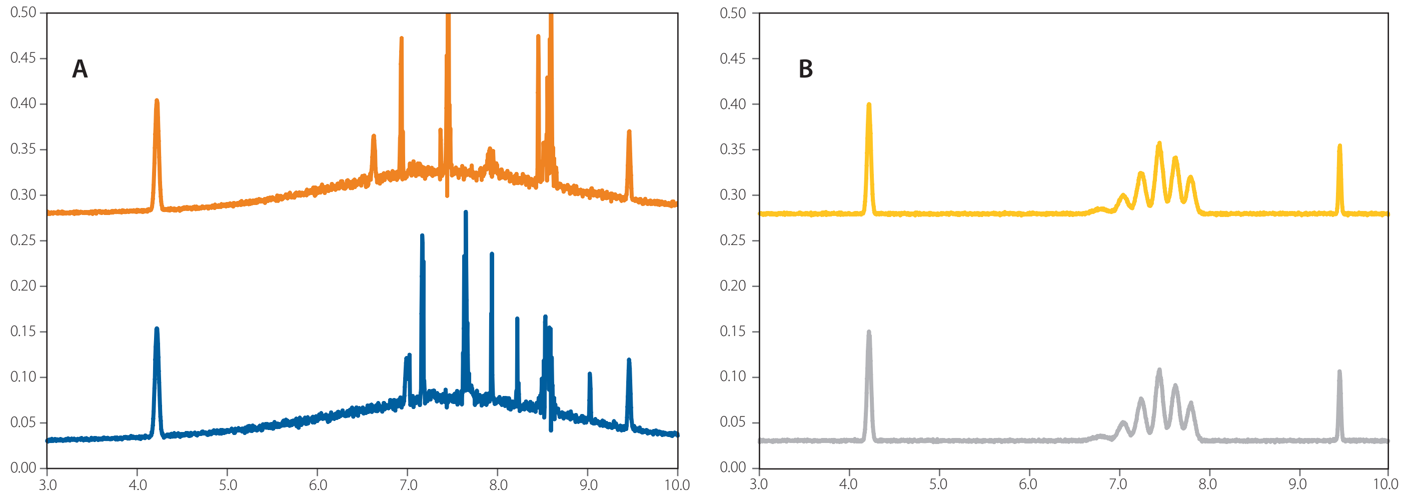


FIGURE 6. mAb separation before and after adding urea. (A) Separation conditions are 0.2 mg/mL mAb in 1% pH 3–10, 3% pH 5–8 Pharmalytes, 0.35% Methyl Cellulose, (B) shows a standard protein precipitation profile. When focusing begins, sharp spikes start to appear almost immediately and no part of the profile is reproducible. These spikes will never stabilize during the focusing process. In extreme cases, precipitation may require adding 8 M urea. (B) Includes 4 M urea in the sample matrix.

eliminate sample aggregation almost 100% of the time. We recommend you start with 4 M urea. If this gives you a reproducible peak profile but you prefer to limit the amount of urea used in your samples, you can then screen samples using lower urea concentrations. If you would like to screen for both 2 M and 4 M urea in one Batch, see the instructions in the next section.

After preparing your samples, program a Batch file that does duplicate injections of each sample, with the same parameters used previously. When the Batch is complete, review both injections and compare the peak profiles (which should be reproducible) as shown in Figure 4. If your duplicate sample injections aren't reproducible, you'll need to increase the amount of urea (see Appendix B for the sample denaturing procedure). If your duplicate injections are reproducible and the resolution is acceptable, your method development is complete. If you need greater peak separation, move on to the Resolution Optimization section.

Addressing Non-reproducible Peak Profiles

1. Reconstitute the urea:
    a. Add 320 µL of DI water to one vial of lyophilized urea.
    b. 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.

2. Prepare 200 µL of sample solution in 4% pH 3–10 Pharmalyte with 2 M or 4 M urea:
    a. Dilute each sample to the optimal protein concentration determined in your initial sample screen.
    b. Prepare an IEF Master Mix solution using the amounts in Table 6 or Table 7, where the # of samples to screen = X.
    c. Add 160 µL of IEF Master Mix to individual microcentrifuge tubes for each sample.
    d. Add 40 µL of the 1.0 mg/mL protein sample to the 160 µL IEF Master Mix in the tube.
    e. Vortex each sample to mix completely.
    f. Centrifuge at 13,000 x g for 5 minutes to remove air bubbles and minimize air spikes.
    g. Transfer 150 µL to the appropriate autosampler vial.

TABLE 6. 4% 3–10 pH Pharmalyte with 4 M urea in IEF Master Mix

IEF MASTER MIX SOLUTION	FOR A SINGLE SAMPLE	MASTER MIX (SINGLE SAMPLE*X + 10%)			
# of samples	**1**	**2**	**3**	**4**	**5**
DI water	0 µL	0 µL	0 µL	0 µL	0 µL
1% MC	70 µL	154 µL	231 µL	308 µL	385 µL
10 M urea	80 µL	176 µL	264 µL	352 µL	440 µL
Pharmalyte 3–10	8 µL	17.6 µL	26.4 µL	35.2 µL	44 µL
pI marker 9.46	1 µL	2.2 µL	3.3 µL	4.4 µL	5.5 µL
pI marker 4.22	1 µL	2.2 µL	3.3 µL	4.4 µL	5.5 µL
Total volume	**160 µL**	**352 µL**	**528 µL**	**704 µL**	**880 µL**

TABLE 7. 4% 3–10 pH Pharmalyte with 2 M urea in IEF Master Mix

IEF MASTER MIX SOLUTION	FOR A SINGLE SAMPLE	MASTER MIX (SINGLE SAMPLE*X + 10%)			
# of samples	**1**	**2**	**3**	**4**	**5**
DI water	40 µL	88 µL	132 µL	176 µL	220 µL
1% MC	70 µL	154 µL	231 µL	308 µL	385 µL
10 M urea	40 µL	88 µL	132 µL	176 µL	220 µL
Pharmalyte 3–10	8 µL	17.6 µL	26.4 µL	35.2 µL	44 µL
pI marker 9.46	1 µL	2.2 µL	3.3 µL	4.4 µL	5.5 µL
pI marker 4.22	1 µL	2.2 µL	3.3 µL	4.4 µL	5.0 µL
Total volume	**160 µL**	**352 µL**	**528 µL**	**704 µL**	**880 µL**

Resolution Optimization

If you need higher peak resolution, you can optimize it by adding narrow range ampholytes — but your peak pattern needs to 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 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 straight narrow range 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 1.5 kV followed by a 10-minute focus step at 3 kV, and then optimizing the focusing time from there. The tables in the next section include a few common narrow range methods.

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

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 below 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 Table 8, Table 9 and Table 10 for method recipes.
5. Prefocus at 1.5 kV for 1 minute and increase the focus time at 3 kV to 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%.

TABLE 8. Acidic range methods.

	AMPHOLYTE RANGE (PH)									
ACIDIC METHOD	3–10	2.5–5	LOW PI MARKER	HIGH PI MARKER	1% MC	DI WATER	10 M UREA	SAMPLE	PREFOCUS @ 1.5 KV	FOCUS @ 3 KV
4% Ampholyte 1:1 Ratio No Urea	4 µL	4 µL	1 µL	1 µL	70 µL	80 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 2 M Urea	4 µL	4 µL	1 µL	1 µL	70 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 4 M Urea	4 µL	4 µL	1 µL	1 µL	70 µL	0 µL	80 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio No Urea	2 µL	6 µL	1 µL	1 µL	70 µL	80 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 2 M Urea	2 µL	6 µL	1 µL	1 µL	70 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 4 M Urea	2 µL	6 µL	1 µL	1 µL	70 µL	80 µL	0 µL	40 µL	1 min	10 min

TABLE 9. Neutral range methods.

NEUTRAL METHOD	AMPHOLYTE RANGE (PH)		LOW PI MARKER	HIGH PI MARKER	1% MC	DI WATER	10 M UREA	SAMPLE	PREFOCUS @ 1.5 KV	FOCUS @ 3 KV
	3–10	5–8								
4% Ampholyte 1:1 Ratio No Urea	4 µL	4 µL	1 µL	1 µL	70 µL	80 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 2 M Urea	4 µL	4 µL	1 µL	1 µL	70 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 4 M Urea	4 µL	4 µL	1 µL	1 µL	70 µL	0 µL	80 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio No Urea	2 µL	6 µL	1 µL	1 µL	70 µL	80 µL	0 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 2 M Urea	2 µL	6 µL	1 µL	1 µL	70 µL	40 µL	40 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 4 M Urea	2 µL	6 µL	1 µL	1 µL	70 µL	80 µL	0 µL	40 µL	1 min	10 min

TABLE 10. Basic range methods.

BASIC METHOD	AMPHOLYTE RANGE (PH)		LOW PI MARKER	HIGH PI MARKER	1% MC	DI WATER	10 M UREA	500 MM ARGININE	SAMPLE	PREFOCUS @ 1.5 KV	FOCUS @ 3 KV
	3–10	8–10.5									
4% Ampholyte 1:1 Ratio No Urea	4 µL	4 µL	1 µL	1 µL	70 µL	76 µL	0 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 2 M Urea	4 µL	4 µL	1 µL	1 µL	70 µL	36 µL	40 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:1 Ratio 4 M Urea	4 µL	4 µL	1 µL	1 µL	70 µL	0 µL	80 µL	4 µL	36 µL	1 min	10 min
4% Ampholyte 1:3 Ratio No Urea	2 µL	6 µL	1 µL	1 µL	70 µL	76 µL	0 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 2 M Urea	2 µL	6 µL	1 µL	1 µL	70 µL	36 µL	40 µL	4 µL	40 µL	1 min	10 min
4% Ampholyte 1:3 Ratio 4 M Urea	2 µL	6 µL	1 µL	1 µL	70 µL	0 µL	80 µL	4 µL	36 µL	1 min	10 min
8% Narrow range No Urea	0 µL	12 µL	1 µL	1 µL	70 µL	76 µL	0 µL	4 µL	36 µL	1 min	10 min
8% Narrow range 2 M Urea	0 µL	12 µL	1 µL	1 µL	70 µL	36 µL	40 µL	4 µL	30 µL	1 min	10 min

NOTE: Increasing the concentration of ampholytes will also increase your baseline noise. Pharmalytes have the lowest baseline noise so we prefer them for this reason. Servalyte produces higher background noise, but we've found that a small amount of Servalyte added to Pharmalytes is very useful for separating fusion proteins.

7. Once you have the resolution you need, you can optimize the focusing time to reduce assay time. You can do this in one of two ways:
    - Set up a Batch to test your sample at different focusing times. Start by decreasing focusing by 1 minute intervals, 10 min, 9 min, 8 min… etc.
    - Observe the sample while it's focusing. When a stable profile is achieved with no split peaks, focusing is complete.

If these steps don't provide sufficient resolution, please see the Further Optimization section.

Basic pI Marker Considerations

It's important to observe both pI markers in the imaged portion of the capillary throughout the entire focusing period when estimating the pI of an unknown protein. Some shift of the marker's pixel positions due to variable sample compositions, reagent lots, and different cartridges or instruments is normal. pI marker pixel shift tends to be greater at the basic (cathodic) region of the capillary so be sure to keep markers on the imaged portion of the capillary so pI calculations aren't affected.

The probability a basic pI marker will stay on the imaged portion of the capillary based on the pixel position during a normal 6–12 minute focusing period:

- 1890 Pixel Units = 95%
- 1860 Pixel Units = 99%
- 1810 Pixel Units = 99.99%
- 1740 Pixel Units = 99.99997%

If your method's basic pI marker is at a pixel position that might not stay in the imaged portion of the capillary, add either 5 to 10 mM L-arginine or 1 to 2% Pharmalyte 8 to 10.5 to the sample solution to reduce the marker's pixel position.

NOTE: If you need any assistance optimizing your peak resolution, please contact your ProteinSimple Field Application Specialist or our Technical Support team at support@proteinsimple.com.

Guidance for Specific Protein Types

The methods detailed in the previous sections will let you successfully analyze most monoclonal antibodies and glycoproteins. But, certain protein types such as fusion proteins and antibody drug conjugates may have specific requirements.

FUSION PROTEINS

You can get better resolution for some fusion proteins by using Servalyte instead of Pharmalyte ampholytes. Servalyte produces a high background so we recommend using a low percentage of Servalyte in the ampholytes mix when needed. A sample method for fusion proteins using Servalyte is shown in Table 11. To optimize your resolution, just add narrow range ampholyte in the appropriate range keeping the overall % ampholytes between 4–8%. Many methods for fusion proteins have been published that you can refer to when developing your own methods.[2,4]

ANTIBODY DRUG CONJUGATES

Antibody drug conjugates (ADC) are a monoclonal antibody linked to a chemotherapeutic small molecule. The linker and the drug are generally very hydrophobic and may require using at least 4 M urea. There are also many recent journal publications describing iCE methods

TABLE 11. Example fusion protein method using Servalyte.

FUSION PROTEIN METHOD	PH 2–9	LOW PI MARKER	HIGH PI MARKER	1% MC	DI WATER	10 M UREA	SAMPLE	PREFOCUS @ 1.5 KV	FOCUS @ 3 KV
Wide Range 2% Ampholyte 4 M Urea	2 µL	1 µL	1 µL	70 µL	6 µL	80 µL	40 µL	1 min	10 min

for ADC that you can refer to when developing your own methods.[5,6]

Further Optimization

RESOLUTION

You can optimize resolution even more by mixing different narrow range ampholytes together, or by blending ampholytes from different vendors. The overall % of ampholytes should not be higher than 8%.

HT CARTRIDGE

If you need to optimize your resolution and speed even farther, try using the HT Cartridge. It doesn't need methyl cellulose in the sample solution, so it's ideal for separating proteins with hydrophobic features or moieties. And the reduced sample viscosity allows faster sample loading and focusing. To transfer a developed method to the HT Cartridge, just remove the methyl cellulose in the sample solution and decrease the focusing time. You can find more information on the HT Cartridge in the Application Note *Faster and Easier Charge Heterogeneity Analysis with the iCE3* (download at www.proteinsimple.com/documents/App_Note_iCE_web3.pdf).

ROBUSTNESS

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

References

1. Analysis of charge heterogeneities in mAbs using imaged CE, X He, A Que and J Mo, *Electrophoresis*, Mar 2009; 23(11):1605–11.
2. Applications of imaged capillary isoelectric focusing technique in development of biopharmaceutical glycoprotein-based products, C Anderson, Y Wang, R Rustandi, *Electrophoresis*, Jun 2012; 33(11):1538–44.
3. Application of capillary electrophoresis in glycoprotein analysis, R Rustandi, C Anderson, M Hamm, *Methods Mol Biol,* 2013; 988:181–97.
4. Application of imaged capillary IEF for characterization and quantitative analysis of recombinant protein charge heterogeneity, Z Sosic, D Houde, A Blum, T Carlage, Y Lyubarskaya, *Electrophoresis,* Nov 2008; 29(21):4368–4376.
5. Determination of charge heterogeneity and level of unconjugated antibody by imaged cIEF, J Lin and A Lazar, *Methods Mol Bio,* 2013; 1045:295–302.
6. Charge-based analysis of antibodies with engineered cysteines: from multiple peaks to a single main peak, X Chen, M Nguyen, F Jacobson and J Ouyang, *mAbs,* Nov/Dec 2009; 1:6, 563–571.

Appendix A: Desalting and Concentrating Samples

1. Add 500 µL of your sample into a 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 B: Denaturing Samples

In extreme cases, some proteins need a higher concentration than 4 M urea to stay in solution during iCE analysis. When this happens, you'll need to denature the samples using 8 M urea in the IEF 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. This requires adding 96 mg of urea powder to 120 µL of solution.

Example preparation: Add 96 mg urea powder to a solution containing:

- 22 µL of DI water
- 70 µL of 1% Methyl Cellulose
- 8 µL of carrier ampholytes (total)
- 20 µL of your sample

proteinsimple
a bio-techne brand

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