

Capillary Isoelectric Focusing (cIEF) Kit

For the PA 800 Plus Pharmaceutical Analysis System

Application Guide

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cIEF Kit

Capillary isoelectric focusing (cIEF) is a technique that allows quantitative, experimental analysis of the isoelectric focusing point (pI) and charge variants of a protein. Many of the reagents and supplies required to prepare samples for cIEF analysis are available from SCIEX.

This document provides instructions for separation of proteins by their differences in pI using the PA 800 Plus Pharmaceutical Analysis system and a UV detector. It also provides instructions for data acquisition and data analysis using the PA 800 Plus software and the Waters Empower™ 3 (FR4) software.

Use the information in this application guide as a starting point. As needed, modify injection time, voltage, injection type, or other parameters to find the best conditions for your needs.

Note: For instructions for safe use of the system, refer to the document: *System Overview Guide*.

Note: It is strongly recommended that cIEF analysis be done with a PA 800 Plus Pharmaceutical Analysis system that has been qualified with an *Operational Qualification*.

Safety

Refer to the Safety Data Sheets (SDS), available at [sciex.com/tech-regulatory](https://www.sciex.com/tech-regulatory), for information about the proper handling of materials and reagents. Always follow standard laboratory safety guidelines. For hazardous substance information, refer to the section: [Hazardous Substance Information](#).

Intended Use

Capillary isoelectric focusing (cIEF) analysis is for laboratory use only.

Introduction

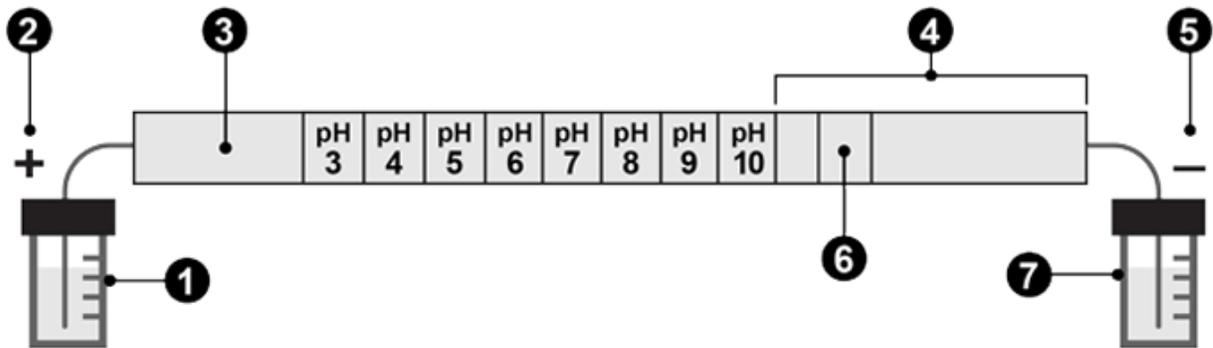
A cIEF separation consists of two steps, focusing and mobilization. Focusing creates a pH gradient across the capillary. Mobilization is the movement of the sample and the pH gradient past the detection window. At the start of a cIEF separation, the entire capillary is filled with the sample, a mixture of ampholytes, stabilizers, pI markers, and the protein of interest. During focusing, one capillary end is submerged in anolyte and the other in catholyte. Next, the system applies voltage across the capillary. A pH gradient forms through the introduction of hydronium ions from the anodic side of the capillary and hydroxyl ions from the cathodic side. The cathodic stabilizer migrates toward the cathodic side of the capillary. The cathodic stabilizer fills the outlet side of the capillary, thereby forcing the ampholytes and protein sample to focus between the

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capillary inlet and the detection window. The cathodic stabilizer migrates toward the cathodic side of the capillary, and the anodic stabilizer migrates toward the anodic side of the capillary. The cathodic stabilizer fills the outlet side of the capillary, thereby forcing the ampholytes and protein sample to focus before and the detection window.¹

The mechanism of focusing is bi-directional.² The pH gradient forms at the capillary ends and then progresses toward the center of the capillary where both anodic and cathodic sides merge. With bi-directional focusing, sample peaks are often detected during focusing. Unmerged peaks detected during mobilization indicate incomplete focusing of the pH gradient. Focusing time must be sufficient to allow for complete formation of the pH gradient.

Figure 1 pH Gradient Inside a Capillary



Item	Description
1	Anolyte, pH 1.4
2	Anode
3	Anodic Stabilizer
4	Cathodic Stabilizer
5	Cathode
6	Detection window
7	Catholyte, pH 13

The following figures show a simulation of the focusing the process over time. Refer to the figures: [Figure 2](#) to [Figure 6](#).

¹ Cruzado-Park, I. D., Mack, S., and Ratnayake, C. K., *Application Information Bulletin A-11634A: Identification of System Parameters Critical for High Performance cIEF*, Beckman Coulter, Inc., Fullerton, CA, 2008.

² Hjerten, S., Liao, J. L., and Yao, K. Q., *J Chromatogr*, Volume 387, pp 127, 1987.

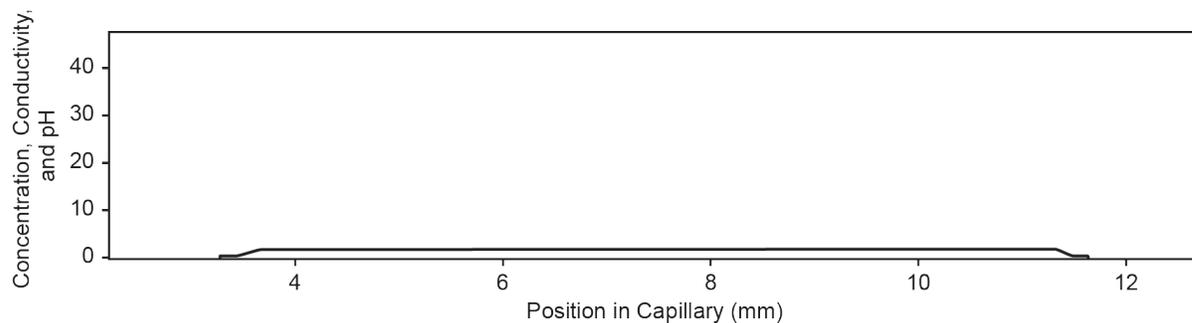
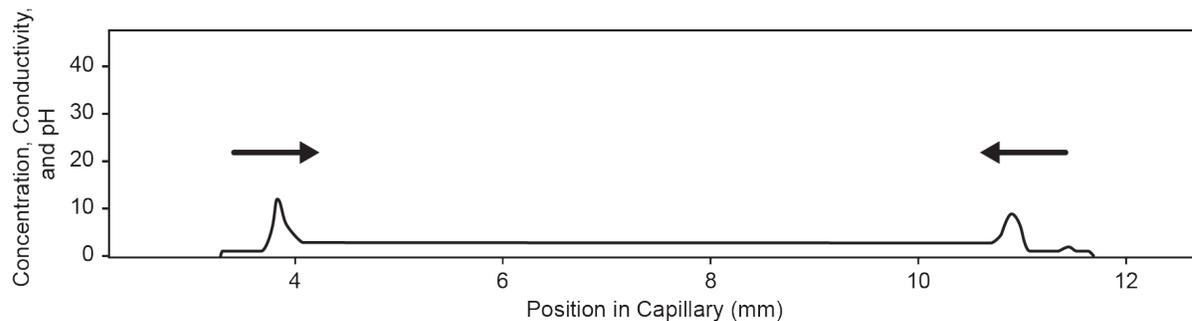
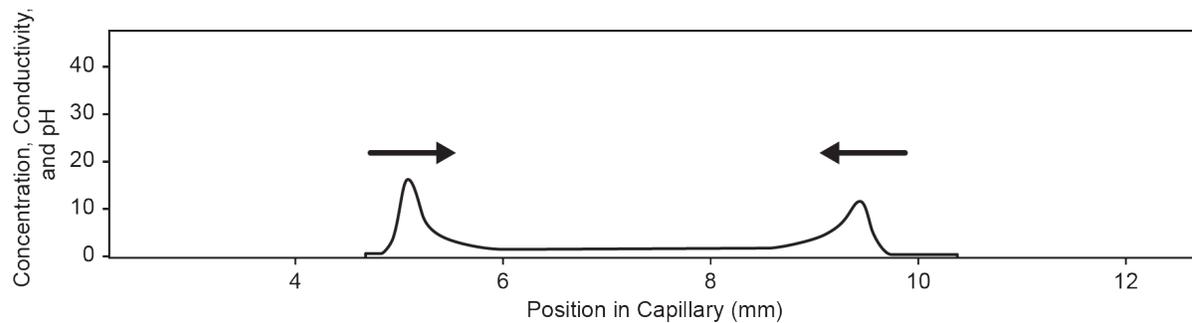
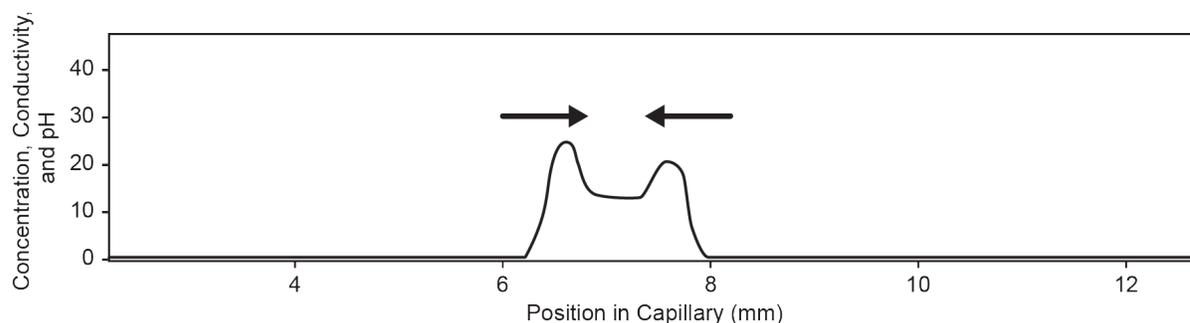
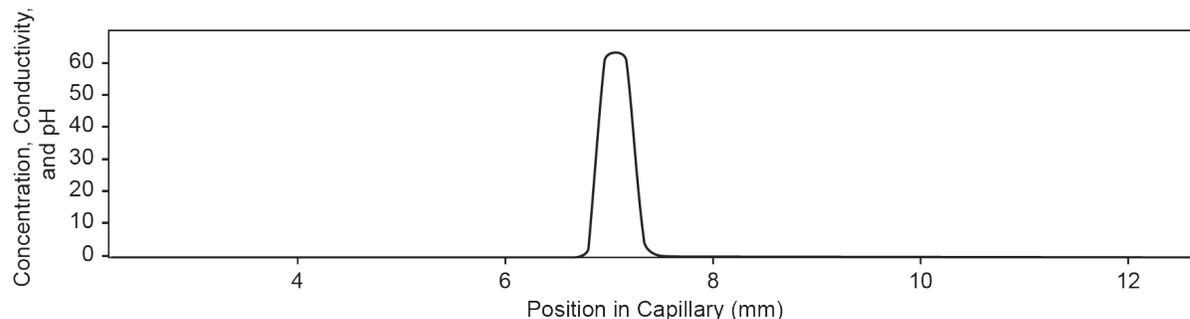
Figure 2 Simulation of Focusing Process at Time 0**Figure 3 Simulation of Focusing Process at 30 Seconds****Figure 4 Simulation of Focusing Process at 2 Minutes**

Figure 5 Simulation of Focusing Process at 4 Minutes**Figure 6 Simulation of Focusing Process at 6 Minutes**

After the pH gradient is formed, the capillary contents are mobilized toward the outlet to detect the pI markers and the separated protein sample. Pressure, gravity, or chemical mobilization can be used to mobilize the pH gradient past the detection window. The procedure described in this guide uses chemical mobilization. Both pressure and gravity mobilization techniques create hydrodynamic flow inside the capillary. Hydrodynamic flow in turn causes band broadening. We recommend the use of acetic acid as a chemical mobilizer.^{3, 4} To start the mobilization step, first replace the catholyte vial with a vial filled with chemical mobilizing solution. Next, apply voltage across the capillary. During mobilization, hydronium ions are introduced from the anolyte in the capillary, while acetate ions are introduced at the cathodic side. As a result, the pH gradient is titrated from basic to acidic and the protein sample bands are detected as they obtain a positive charge and migrate toward the cathode.

At the beginning of the mobilization step, the capillary outlet moves from the well with a catholyte to a well with chemical mobilizer and then the system applies a voltage across the capillary. During mobilization using acetic acid, hydronium ions are introduced from the anodic side of the capillary, while acetate ions are introduced at the cathodic side. As a result, the pH gradient is

³ Manabe, T., Miyamoto, H., and Iwasaki, A., *Electrophoresis*, Volume 18, pp 92, 1997.

⁴ *Application Information Bulletin A-12015A: A Robust cIEF Method: Intermediate Precision for the pH 5-7 Range*, Beckman Coulter, Inc., Fullerton, CA, 2008.

titrated from basic to acidic and the proteins become positively charged. The sample is detected as it passes the capillary window and migrates toward the cathode.

Detection in cIEF is performed at 280 nm because ampholytes have low UV absorbances at this wavelength. Narrow-range ampholytes can be used to maximize resolution.⁵ Monoclonal antibodies have been reproducibly separated by cIEF.⁶

Note: This application guide has been validated with the PA 800 Plus Pharmaceutical Analysis system.

Terms and Definitions

pI	The pH at which a molecule is neutral or has zero net charge. The total number of negative charges is equal to the total number of positive charges at this pH.
Ampholytes	Molecules that contain both acidic and basic groups and become zwitterionic at and near their pI values. Ampholytes are used to establish a pH gradient in cIEF.
Anolyte	An acidic solution placed at the anode (positively-charged electrode). The pH of the anolyte is less than that of the ampholytes used with the sample.
Catholyte	A basic solution placed at the cathode (negatively-charged electrode). The pH of the catholyte is greater than that of the ampholytes used with the sample.
Cathodic Stabilizer	A high-conductivity molecule that has a pI value greater than that of the ampholytes, but less than the pH of the catholyte. The cathodic stabilizer is used to fill the portion of the capillary from detector to outlet, forcing the sample and ampholytes to focus before the capillary window. The cathodic stabilizer is also used to minimize distortions of the pH gradient at the cathodic side, maximizing resolution and reproducibility.
Anodic Stabilizer	A high-conductivity molecule that has a pI value less than that of the ampholytes, but greater than the pH of the anolyte. The anodic stabilizer is used to minimize distortions on the pH gradient at the anodic side, maximizing resolution while preventing the loss of sample in the anolyte vial.

Equipment and Materials Required

Note: For items with a reorder part number, sometimes the reorder quantity is different than the quantity in the kit.

⁵ Mack, S., Cruzado-Park, I. D., and Ratnayake, C. K., *Application Information Bulletin A-12026A: High Resolution cIEF of Therapeutic Monoclonal Antibodies: A Platform Method Covering pH 4-10*, Beckman Coulter, Inc., Fullerton, CA, 2008.

⁶ Mack, S., Cruzado-Park, I. D., Chapman, J., Ratnayake, C., and Vigh, G., *Electrophoresis*, Volume 30, pp 4049, 2009.

cIEF Kit

Table 1 cIEF Kit for PA 800 Plus (PN A80976)

Component	Quantity	Reorder Part Number
Neutral capillary, 50 µm i.d. × 45 cm	1	47441
CE Grade Water (140 mL)	1	C48034
cIEF gel	100 mL	477497
cIEF Peptide Marker Kit (pI 4.1, pI 5.5, pI 7.0, pI 9.5, pI 10.0), (140 µL each)	1	A58481
Sample loading solution (SLS)	6 mL	608082

Table 2 Additional Supplies from SCIEX

Component	Quantity	Part Number
(Optional) eCap Tris Buffer (50 mM, pH 8) (100 mL)	1	477427
Capillary cartridge, blank	1	144738
Filter, 280 nm, for use with UV detector	1	144439
Micro vials, 200 µL	100	144709
Universal vial caps, blue	100	A62250
Universal vials	100	A62251

Table 3 Additional Required Reagents or Supplies

Description	Vendor	Part Number
(Optional) MicroCon-10 kDa Centrifugal Filter Unit with Ultracel-10 membrane	MilliporeSigma	MRCPR010
(Optional) Monoclonal IgG System Suitability	United States Pharmacopoeia	1445550
Arginine	Sigma-Aldrich	A5006
Glacial acetic acid	Sigma-Aldrich	A6283
	Spectrum	AC110
Iminodiacetic acid	Sigma-Aldrich	220000
	Spectrum	I-2045
Pharmalyte pH 3-10 carrier ampholytes	Cytiva	17-0456-01

Table 3 Additional Required Reagents or Supplies (continued)

Description	Vendor	Part Number
Phosphoric acid, 85%	Sigma-Aldrich	345245
Sodium hydroxide (NaOH), 1 M	Fisher	SS266-1
Urea	Sigma-Aldrich	U0631
	GE Healthcare	17-1319-01

Storage Conditions

Note: For storage conditions for prepared reagents, refer to the preparation instructions.

- Upon receipt, store the cIEF Peptide Marker kit at $-35\text{ }^{\circ}\text{C}$ to $-15\text{ }^{\circ}\text{C}$.
- Upon receipt, store the following at $2\text{ }^{\circ}\text{C}$ to $8\text{ }^{\circ}\text{C}$:
 - cIEF gel
 - Neutral capillary
- Upon receipt, store the sample loading solution (SLS) at $-20\text{ }^{\circ}\text{C}$
- Upon receipt, store the CE Grade Water at room temperature.

Customer-Supplied Equipment and Supplies

- Powder-free gloves, neoprene or nitrile recommended
- Safety glasses
- Laboratory coat
- Table-top mini centrifuge
- Microcentrifuge, or equivalent, and microcentrifuge tubes
- Vortex mixer
- Pipettes and appropriate tips.
- Parafilm
- Disposable syringes, 10 mL
- Membrane syringe filters, $5\text{ }\mu\text{m}$ pores
- Membrane syringe filters, $0.2\text{ }\mu\text{m}$ pores
- Volumetric flasks, 10 mL and 50 mL
- Disposable plastic Falcon tubes, 10 mL and 50 mL (or equivalent)

cIEF Kit

- Analytical balance
- Spatula

Required Detector

A UV detector with a 280 nm filter is required.

Required Cartridge or Capillary

CAUTION: Potential Wrong Result. If a capillary is being used with the cIEF kit, then do not use the same capillary for another application. Different buffers and types of samples can lead to sample carryover, non-specific binding, or poor separation.

A capillary cartridge (PN 144738) and a neutral capillary, 50 μm i.d. \times 67 cm (PN 477441) are required. The capillary must be trimmed to a total length of 30.2 cm and an effective length of 20 cm.

Note: The capillary is included with the kit.

Methods and Sequences

Note: This section applies to users using the PA 800 Plus system with the PA 800 Plus and 32 Karat software. If the system will be used with the Empower™ software, then the methods are different. Refer to [Run the Samples with the Waters Empower™ Software](#).

Note: The methods and sequence have been updated for robustness. The new methods and sequence are distributed with the 32 Karat software version 10.3 or higher. For systems using an earlier version of the software, the new methods and sequence can be downloaded from sciex.com.

Download the methods and sequences from sciex.com/products/methods. The methods and sequences can also be created manually using the 32 Karat software. Refer to the section: [Methods](#).

Save the methods to the PA 800 Plus controller: C:\32Karat\projects\cIEF\Method.

Save the sequence to: C:\32Karat\projects\cIEF\Sequence.

At the time of publication, the following methods and sequence are available on the SCIEX website:

- Methods:
 - cIEF Conditioning - PA 800 plus V2.met: Conditions the capillary before first use.
-

-
- `cIEF Separation - PA 800 plus V2.met`: Performs a cIEF separation.
 - `cIEF Shutdown - PA 800 plus V2.met`: Cleans the capillary at the end of a sequence and turns off the light source.
 - `cIEFSequence - PA 800 plus V2.seq`: Contains the sequence table.

Prepare the Reagents

Prepare the Anolyte (200 mM Phosphoric Acid)

1. Add 30 mL of CE Grade Water to a clean 50 mL volumetric flask.
2. Add 685 μ L of 85% phosphoric acid to the volumetric flask.
3. Add CE Grade Water to the volumetric flask, for a total volume of 50 mL.
4. Shake the flask to mix the contents.
5. Transfer the anolyte solution to a 50 mL plastic tube.
6. Label the tube `Anolyte`, and then record the preparation date.
7. Store the solution at 2 °C to 8 °C for up to 1 month.

Prepare the Catholyte (300 mM Sodium Hydroxide)

1. Add 30 mL of CE Grade Water to a clean 50 mL volumetric flask.
2. Add 15 mL of 1 M sodium hydroxide to the volumetric flask.
3. Add CE Grade Water to the volumetric flask, for a total volume of 50 mL.
4. Shake the flask to mix the contents.
5. Transfer the catholyte solution to a 50 mL plastic tube.
6. Label the tube `Catholyte`, and then record the preparation date.
7. Store the solution at 2 °C to 8 °C for up to 1 month.

Prepare the Chemical Mobilizer (350 mM Acetic Acid)

1. Add 30 mL of CE Grade Water to a clean 50 mL volumetric flask.
2. Add 1.0 mL of glacial acetic acid to the volumetric flask.
3. Add CE Grade Water to the volumetric flask, for a total volume of 50 mL.
4. Shake the flask to mix the contents.
5. Transfer the acetic acid solution to a 50 mL plastic tube.
6. Label the tube `Chemical Mobilizer`, and then record the preparation date.
7. Store the solution at 2 °C to 8 °C for up to 1 month.

Prepare the Cathodic Stabilizer (500 mM Arginine)

1. Weigh 0.87 g of arginine using an analytical balance, and then transfer it to a clean 10 mL volumetric flask.
2. Add 8 mL of CE Grade Water to the volumetric flask.
3. Shake the flask until all solid material is dissolved.
4. Add CE Grade Water to the volumetric flask, for a total volume of 10 mL.
5. Transfer this solution to a 10 mL plastic conical tube.
6. Label the tube as `Cathodic Stabilizer` and record the preparation date.
7. Store the solution at 2 °C to 8 °C for up to 1 month.

Prepare the Anodic Stabilizer (200 mM Iminodiacetic Acid)

1. Weigh 0.27 g of iminodiacetic acid using an analytical balance, and then transfer it to a clean 10 mL volumetric flask.
2. Add 8 mL of CE Grade Water to the flask.
3. Shake the flask until all solid is dissolved.
4. Add CE Grade Water to the volumetric flask, for a total volume of 10 mL.
5. Transfer the solution to a 10 mL plastic conical tube.
6. Label the tube `Anodic Stabilizer`, and then record the preparation date.
7. Store the solution at room temperature for up to 7 days.

Prepare the Urea-cIEF Gel

1. Weigh 2.252 g of urea, and then transfer it to a 10 mL volumetric flask.
2. Add 7 mL of the cIEF gel to the volumetric flask.
3. Mix the flask, using a vortex mixer, for at least 15 minutes, until all of the solid material dissolves.
4. Add the cIEF gel to the volumetric flask, for a total volume of 10 mL.
5. Mix the solution by inverting the flask three times.
6. Use a 10 mL disposable plastic syringe to filter the solution through a 5.0 µm membrane syringe filter, and then collect the filtered solution in a new 10 mL plastic conical tube.
7. Label the tube `3.75 M Urea-cIEF Gel`, and then record the preparation date.
8. Store the solution at 2 °C to 8 °C for up to 7 days.

Prepare the Samples

Note: The protein solution should not contain more than 50 mM of salt. For an IgG sample, to exchange the buffer with a low salt buffer, refer to the section: [Perform a Buffer Exchange](#).

We recommend the use of three pI markers close to the pI of the protein sample to make sure that the pH gradient is linear, and to determine the pI value of the protein sample.

To prepare one cIEF sample, mix the following reagents in a 0.5 mL microcentrifuge tube:

- 200 mL of Urea-cIEF gel
- 12.0 mL of Pharmalyte pH 3-10 carrier ampholytes
- 20.0 mL of cathodic stabilizer
- 2.0 mL of anodic stabilizer
- 2.0 mL of each pI marker

Best Practices for Preparing Multiple Samples

To simplify sample preparation and minimize pipetting errors, we recommend that the user prepare a master mix when analyzing multiple samples. The amounts required to prepare a master mix when running multiple samples using Pharmalyte pH 3-10 carrier ampholytes are shown in the table: [Table 4](#).

Start by entering the number of samples to be prepared in the table. Increment the number of samples by one, multiply each reagent volume by that number of samples, and then record the result. As required, add or remove pI markers from those provided in the pI marker kit. Refer to the table: [Table 4](#).

Prepare the cIEF Sample

1. Prepare the master mix:
 - a. Use a pipette to add each calculated reagent volume shown in the following table to a centrifuge tube.

Table 4 Preparation of a cIEF Master Mix for Analyzing Multiple Samples

Reagent	Volume per Sample (mL)	Number of Samples	Total Volume to be Measured (mL)
3.75 M Urea-cIEF Gel	200	× _____ + 1 =	
Pharmalyte pH 3-10 carrier ampholytes	12	× _____ + 1 =	
Cathodic stabilizer	20	× _____ + 1 =	

Table 4 Preparation of a cIEF Master Mix for Analyzing Multiple Samples (continued)

Reagent	Volume per Sample (mL)	Number of Samples	Total Volume to be Measured (mL)
Anodic stabilizer	2	× _____ + 1 =	
pI marker 10.0	2	× _____ + 1 =	
pI marker 9.5	2	× _____ + 1 =	
pI marker 5.5	2	× _____ + 1 =	
pI marker 4.1	2	× _____ + 1 =	

- b. Use a vortex mixer to mix the master mix for 15 seconds, and then use the centrifuge to spin it down. Store the master mix at 2 °C to 8 °C, and then discard it at the end of the day.
2. Mix 240 µL of the cIEF master mix with a volume of sample containing 50 µg to 100 µg of the protein in up to 10 µL total volume.
3. Use a vortex mixer to mix the cIEF sample (master mix with protein) for 30 seconds, and then spin it using a table-top microcentrifuge for 3 minutes at 3,500 g to remove any precipitates.

For preparation of the cIEF sample vial, refer to the section: [Load the Sample Tray](#).

Prepare the Monoclonal IgG Reference Standard Sample

Note: Because of its low salt content, this IgG standard sample does not require buffer exchange.

1. Upon receipt, open the IgG vial, and then add 400 µL of CE Grade Water to the lyophilized material.
2. Mix the vial contents until the solution is clear.

Tip! To avoid multiple freeze-thaw cycles, make 10 µL aliquots (equivalent to 50 µg) and store them at –35 °C to –15 °C. Record the preparation date for reference.

3. Mix 240 µL of the cIEF master mix with 10 µL of the reconstituted IgG in a 0.5 mL microcentrifuge tube, for 30 seconds using a vortex mixer, and then spin using a table-top microcentrifuge for 3 minutes at 3,500 g to remove any precipitates.

For preparation of the IgG reference sample vial, refer to the section: [Load the Sample Tray](#).

Prepare the PA 800 Plus System

Use the procedures in this section to prepare the PA 800 Plus system to acquire data.

The procedures in this section assume that the system has already been properly installed and initialized.

Install the UV Detector

1. Turn off the PA 800 Plus System and then install the UV detector. Refer to the document: *System Maintenance Guide*.
2. Turn on the system and let the lamp warm up for at least 30 minutes.

Clean the Interface Block

CAUTION: Potential System Damage. Do not allow the gel to accumulate on the electrodes, opening levers, capillary ends, and interface block. Gel accumulation might cause broken capillaries, bent electrodes, jammed vials, or missed injections.

Clean the electrodes, opening levers, capillary ends, and interface block after every use or when changing chemistries. For detailed instructions, refer to the document: *System Maintenance Guide*.

The Urea-cIEF gel can be very viscous and can accumulate in the system unless regular and thorough cleaning is performed.

Install the Cartridge

1. Remove the cartridge from the box.

Note: Make sure to use the 100 mm × 200 mm aperture for the cIEF assay.

2. Use the components in the capillary cartridge kit to install the neutral capillary.
3. Trim the ends of the capillary carefully, and then put both ends in universal vials that are filled with CE Grade Water and capped with blue universal vial caps.
4. Install the cartridge in the PA 800 Plus system. Refer to the document: *System Maintenance Guide*.
5. Close the front panel.

Load the Buffer Trays



DANGER! Toxic Chemical Hazard. Read the safety data sheets for the Sample Loading Solution (SLS) before use. Refer to the section: [Hazardous Substance Information](#).

CAUTION: Potential System Damage. Do not fill any vial with more than 1.8 mL of liquid. Also, do not allow more than 1.8 mL to accumulate in the waste vials. If a vial contains more than 1.8 mL, then the pressure system might be damaged.

cIEF Kit

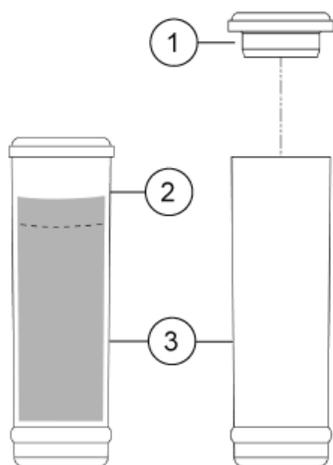
1. Depending on the number of samples to be run, add 1.5 mL of reagent per vial, and then cap each vial with a blue universal vial cap. For all sequence runs containing 8 to 20 samples, including replicates, prepare the following:

- 1 universal vial, containing 1.5 mL of Sample Loading Solution, for the SLS position

Note: If 8 or fewer samples are being used, then insert a PCR microvial in the universal vial for the SLS, and fill it with 100 μ L of SLS. This prevents wasting the SLS on small sample runs.

- 1 universal vial, containing 1.5 mL of analyte, for the A position
- 14 universal vials, containing 1.5 mL of CE Grade Water, for the Water positions
- 1 universal vial, containing 1.5 mL of cIEF gel, for the Gel position
- 1 universal vial, containing 1.5 mL of catholyte, for the C position
- 1 universal vial, containing 1.5 mL of chemical mobilizer, for the CM position
- 9 universal vials, containing 1.0 mL of CE Grade Water, for the Waste positions in the outlet buffer tray

Figure 7 Universal Vial and Cap Setup



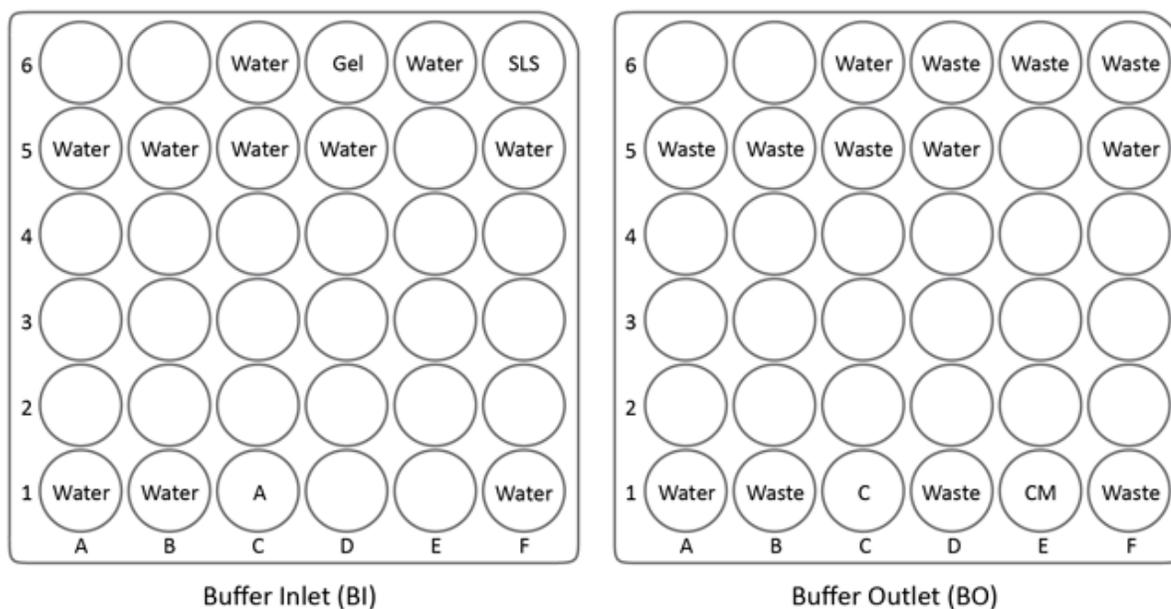
Item	Description
1	Universal vial cap
2	Maximum fill line
3	Universal vial

2. Put the vials in the buffer trays.

Note: In the following figure, rows 5 and 6 have the reagent vials for the capillary conditioning and shutdown methods. Row 1 has the reagent vials for the separation. The vials have sufficient reagents for ten runs. For 11 to 20 runs, duplicate the row 1 reagent vials in row 2. The maximum number of runs allowed is 20.

Note: For this application, all vials and caps are designed for a maximum of 20 runs, including replicates. Do not reuse the caps, because they might be contaminated with dried gel and other chemicals.

Figure 8 Buffer Tray Layout for 16 Runs



Item	Description
Water	CE Grade Water
A	Anolyte
SLS	Sample Loading Solution
Gel	cIEF gel
Waste	CE Grade Water
C	Catholyte
CM	Chemical mobilizer

Note: During electrophoresis the ionic strength of the buffer changes. The separation method is programed to increment the buffer vials after ten runs to avoid ionic depletion.

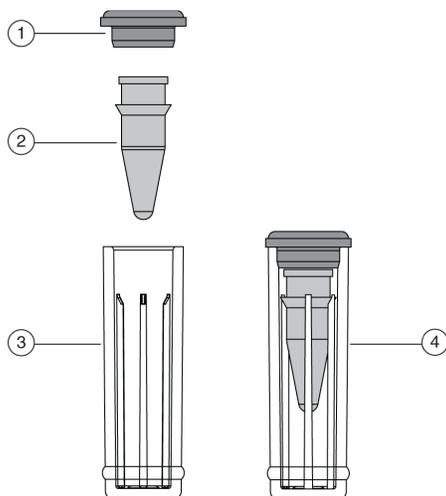
Load the Sample Tray

1. Prepare the cIEF test samples and the IgG reference test sample:

Note: The minimum sample volume for the PA 800 Plus System is 50 μL . If less than 200 μL is available, then make sure that the sample volume is at least 50 μL .

- a. Carefully transfer 200 μL of each previously prepared cIEF test sample to a micro vial without disturbing the pellet, and then remove air bubbles by spinning it for 30 seconds at 3,500 g with a table-top microcentrifuge
 - b. Carefully transfer 200 μL of the IgG reference sample to a micro vial without disturbing the pellet, and then remove air bubbles by spinning it for 30 seconds at 3,500 g with a table-top microcentrifuge.
 - c. Make sure that no bubbles are present at the bottom of the vials. If bubbles are present, then spin the vial again, with the same parameters.
2. Put the micro vial in a universal vial, and then cap the vial with a blue universal vial cap.

Figure 9 Sample Vial Setup

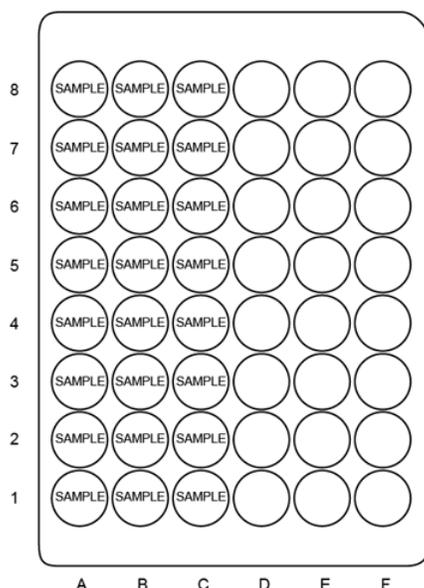


Item	Description
1	Universal vial cap
2	Micro vial
3	Universal vial

Item	Description
4	Micro vial inside universal vial

- Put the universal vials in positions A1:C8 in the inlet sample tray. Refer to the figure: [Figure 10](#). For fewer than 24 samples, start at position A1 and fill all of the A wells before filling any other wells.

Figure 10 Sample Tray Layout



Run the Samples

Create the Sequence and Start the Run

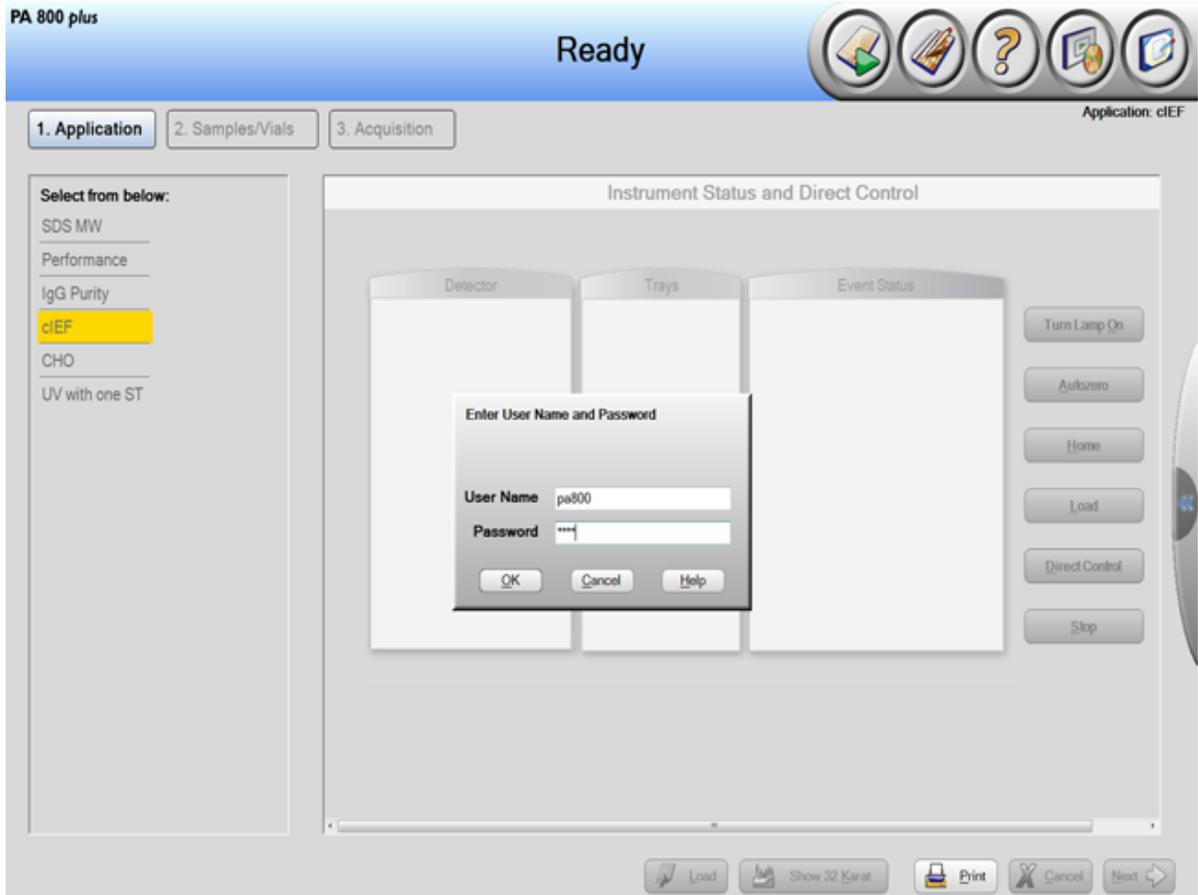
To use the Empower™ software, refer to the section: [Run the Samples with the Waters Empower™ Software](#).

- Double-click the PA 800 Plus Software icon on the desktop to open the PA 800 Plus Software.
- In the **PA 800 plus** window, click  (**Run**) in the upper right corner of the window.
- In the **Application** list, click **cIEF**. In the **Sequence** list, click **Browse**, and then browse to and select **cIEF Sequence - PA 800 plus V2**.

If prompted, type a user name and password.

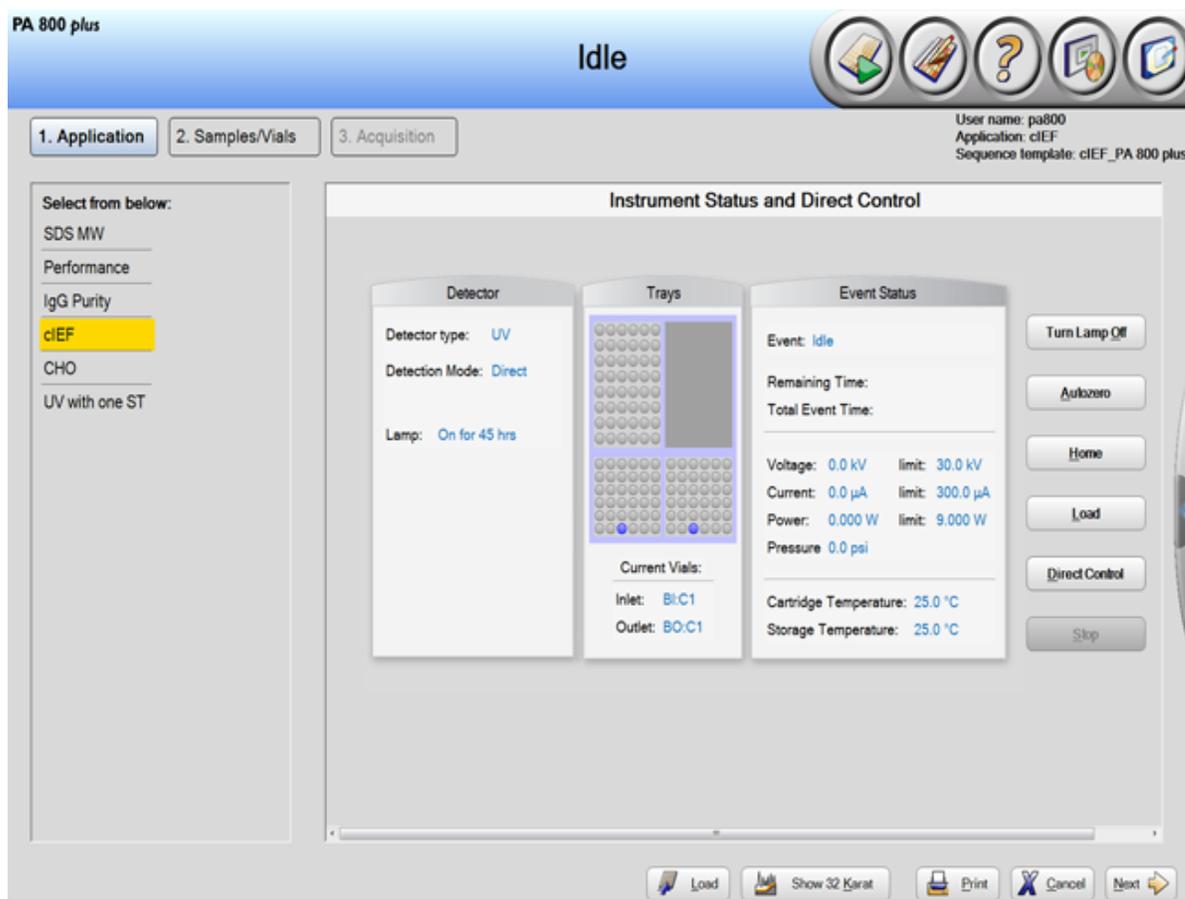
If system administration is enabled, type a user name and password when prompted, and then click **OK**. The default user name is `pa800`, and the default password is `plus`.

Figure 11 Instrument Status and Direct Control Window: Ready



The Instrument Status and Direct Control page opens.

Figure 12 Instrument Status and Direct Control Page



4. Click  (**Next**) in the bottom right corner of the window. The sequence opens.
5. Click  (**Describe**) in the upper right corner of the window.
6. (Optional) Edit the **Sample ID** and the **Data File Name** as required. Editable fields such as **Sample ID** and **Data File Name** can be set to **Mandatory**, **Optional**, or **Fixed**.
7. Set the type for the first and last rows in the sequence. The first row is for capillary conditioning and the last row is for system shutdown.
 - a. Click the first row, the row with the cIEF Conditioning method, to select it, and then click  (**Always**) in the **Rows** area.
 - b. Click the last row, the row with the cIEF Shutdown method, to select it, and then click  (**Always**) in the **Rows** area.

The icon in the **Type** column first and last rows in the sequence is now a triangle.

Figure 13 Describe sequence rows and columns Page with Conditioning Method Set to "Always"

Describe sequence rows and columns

Application:

Sequence:

Rows: Sample Control Always

Columns: Optional Required Fixed

Verification: Samples

Run#	Type	Run Type	Reps	Inject Inlet	Sample ID	Method	Data F
1	▲	Unknown	1	None		cIEF Conditioning ...	
2	●	Unknown	1	SI:A1		cIEF Separation - ...	
3	●	Unknown	1	SI:A2		cIEF Separation - ...	
4	●	Unknown	1	SI:A3		cIEF Separation - ...	
5	●	Unknown	1	SI:A4		cIEF Separation - ...	
6	●	Unknown	1	SI:A5		cIEF Separation - ...	
7	●	Unknown	1	SI:A6		cIEF Separation - ...	
8	●	Unknown	1	SI:A7		cIEF Separation - ...	
9	●	Unknown	1	SI:A8		cIEF Separation - ...	
10	●	Unknown	1	SI:B1		cIEF Separation - ...	
11	●	Unknown	1	SI:B2		cIEF Separation - ...	
12	▲	Unknown	1	None		cIEF shutdown - P...	

- In the **Verification** field, click the arrow buttons to set the number of samples for the run.

Note: Some columns can be set to **Optional**, **Required**, or **Fixed**. In the preceding figure, the **Sample ID** column is **Optional**, indicating that an ID is not required.

Figure 14 Describe sequence rows and columns Window: Reload Sequence

1. Application 2. Samples/Vials 3. Acquisition

User name: pa800
Application: cIEF
Sequence template: cIEF - PA 800 plus V2

Number of samples: 10 Reload sequence Print Sequence Report:

Output data path: C:\32Karat\Projects\cIEF\Data Browse Print Method Report:

Output sequence path: C:\32Karat\Projects\cIEF\Data\Sequence Browse Display Options

Run#	Run	Reps	Inject Inlet	Sample ID	Method	Data File
1	Unknown	1	None		cIEF Conditioning - PA 800 plus V2.met	
2	Unknown	1	SI.A1		cIEF Separation - PA 800 plus V2.met	
3	Unknown	1	SI.A2		cIEF Separation - PA 800 plus V2.met	
4	Unknown	1	SI.A3		cIEF Separation - PA 800 plus V2.met	
5	Unknown	1	SI.A4		cIEF Separation - PA 800 plus V2.met	
6	Unknown	1	SI.A5		cIEF Separation - PA 800 plus V2.met	
7	Unknown	1	SI.A6		cIEF Separation - PA 800 plus V2.met	
8	Unknown	1	SI.A7		cIEF Separation - PA 800 plus V2.met	
9	Unknown	1	SI.A8		cIEF Separation - PA 800 plus V2.met	
10	Unknown	1	SI.B1		cIEF Separation - PA 800 plus V2.met	
11	Unknown	1	SI.B2		cIEF Separation - PA 800 plus V2.met	
12	Unknown	1	None		cIEF shutdown - PA 800 plus V2.met	

Back Load Show 32 Karat Print Cancel Next

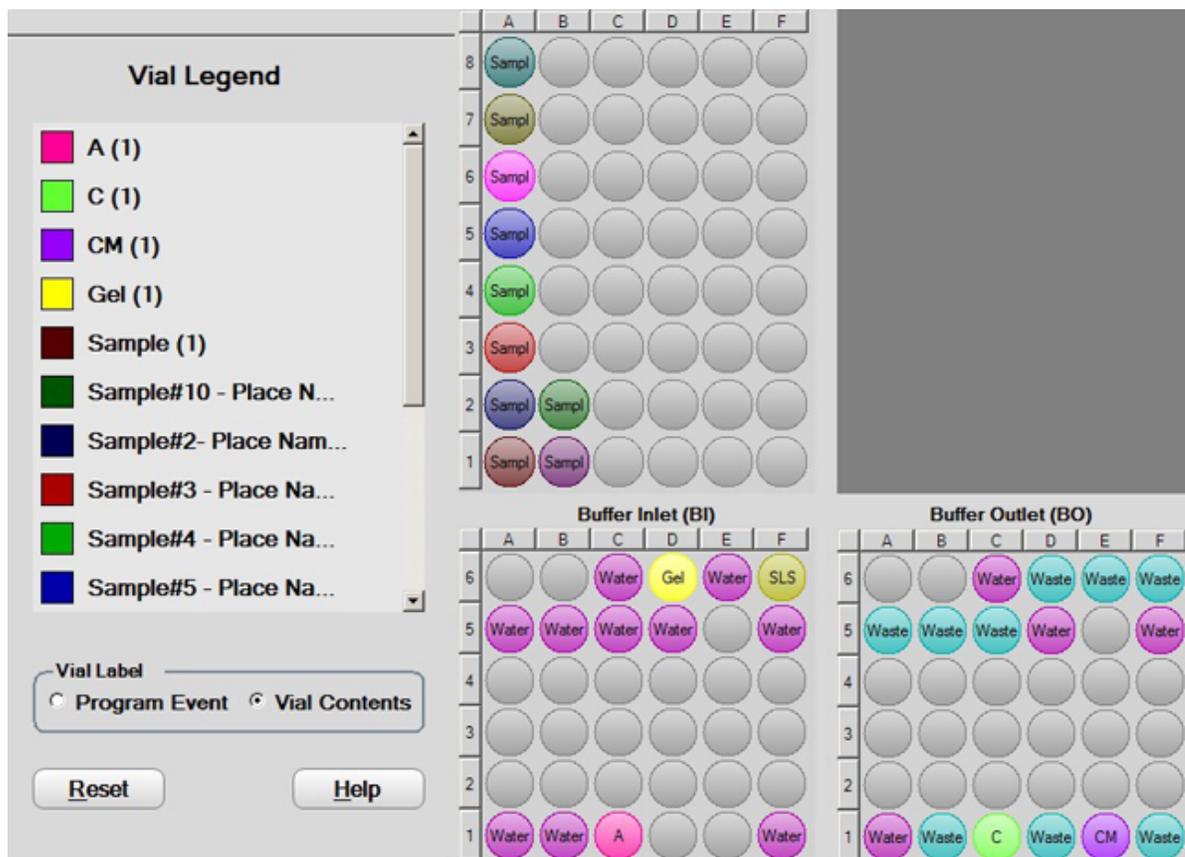
Note: In the upper left corner, next to Run #1, of the table shown in the preceding figure, a blinking exclamation mark (not shown) indicates that the sequence has changed and that the software expects an action from the user. Hover over the exclamation point to view a tooltip with the required action.

9. Click **Reload sequence**.
The sequence table updates to show the appropriate number of runs. Newly described sequences are saved on a data path with a time stamp.
10. In the **Output data path** field, click **Browse** and then select the location where the data will be saved.
11. In the **Number of samples** field, click the arrow buttons to set the number of samples for the run.
As the number of samples changes, the images of the buffer and sample trays on the right are updated to show the correct number of vials and their locations for the run.
12. (Optional) Increase the number of replicates as required.

The buffer tray map updates with the quantity of reagents required to accommodate the number of replicates.

Note: We do not recommend running more than 20 runs per sequence, including replicates.

Figure 15 Samples/Vials Window

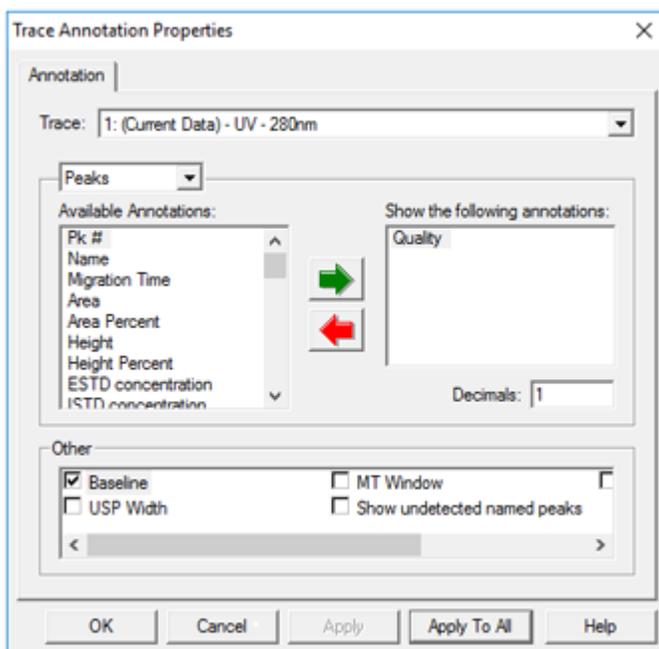


Tip! To view the contents of each vial, open the tray and then click **Vial Contents**.

13. If the buffer and sample trays have not been loaded, then click  **(Load)**, load the buffer and sample trays in the PA 800 Plus system, and then close the door.
14. Click  **(Next)** and then click **Yes - run now**.

Optimize Integration Parameters

1. Right-click an electropherogram and then select **Annotation**. From **Available Annotations**, click **Migration Time** and then click the green arrow shown in the following figure to move **Migration Time** to the Show the following annotations pane on the right.

Figure 16 Trace Annotation Properties Dialog

2. Click **OK** to save the changes in the Trace Annotation Properties dialog.

We recommend optimizing the integration parameters in the analysis method for each sample. As a starting point, use the recommended integration values, shown in the following table. These integration parameters are for the cIEF separation of the peptide pI markers.

Table 5 Recommended Values for Integration of Peptide pI Markers

Setting	Value	Description
Width	0.1	Sets the sensitivity of the peak detection to changes in the baseline.
Threshold	5000	Determines how high a peak must rise above the baseline noise before it is recognized as a peak.
Shoulder Sensitivity	9999	Enables the detection of shoulders in large peaks. The value specifies the slope value for splitting a peak.
Integration Off	0 minutes to 15 minutes (during focusing)	Sets the time interval in the electropherogram that is not integrated.

Figure 17 Integration Events Table

#	Event	Start Time	Stop Time	Value
1	Width	0.000	0.000	0.1
2	Threshold	0.000	0.000	5000
3	Shoulder Sensitivity	0.000	0.000	9999
4	Integration Off	0.000	15.000	0
5				

Use the integration parameter **Minimum Cluster Distance** to split peaks when shoulder sensitivity does not provide proper integration. **Minimum Cluster Distance** specifies the distance between non-baseline separated peaks so they are not identified as one peak.

Waste Disposal



WARNING! Biohazard or Toxic Chemical Hazard. Follow local directives when disposing of chemicals, vials and caps, and the remains of the prepared samples, if applicable. They might contain regulated compounds and biohazardous agents.

Store the Cartridge

Store the Cartridge for Less Than 24 Hours

1. Use the shutdown method to clean the capillary.
The shutdown method rinses the capillaries with SLS, CE Grade Water, and cIEF gel at 50 psi for 3, 10, and 3 minutes, respectively. The capillary is filled with cIEF gel.
2. Store the cartridge for up to 24 hours in the system, with the capillary ends immersed in vials of CE Grade Water.

Store the Cartridge for More Than 24 Hours

1. Perform the shutdown method to clean the capillary.
The capillary is filled with cIEF gel.
2. Remove the cartridge from the system.
3. Put the cartridge in the cartridge storage box with the capillary ends immersed in vials of CE Grade Water.
4. Store the cartridge storage box upright in the refrigerator between 2 °C and 8 °C.

Prepare the Cartridge After Storage

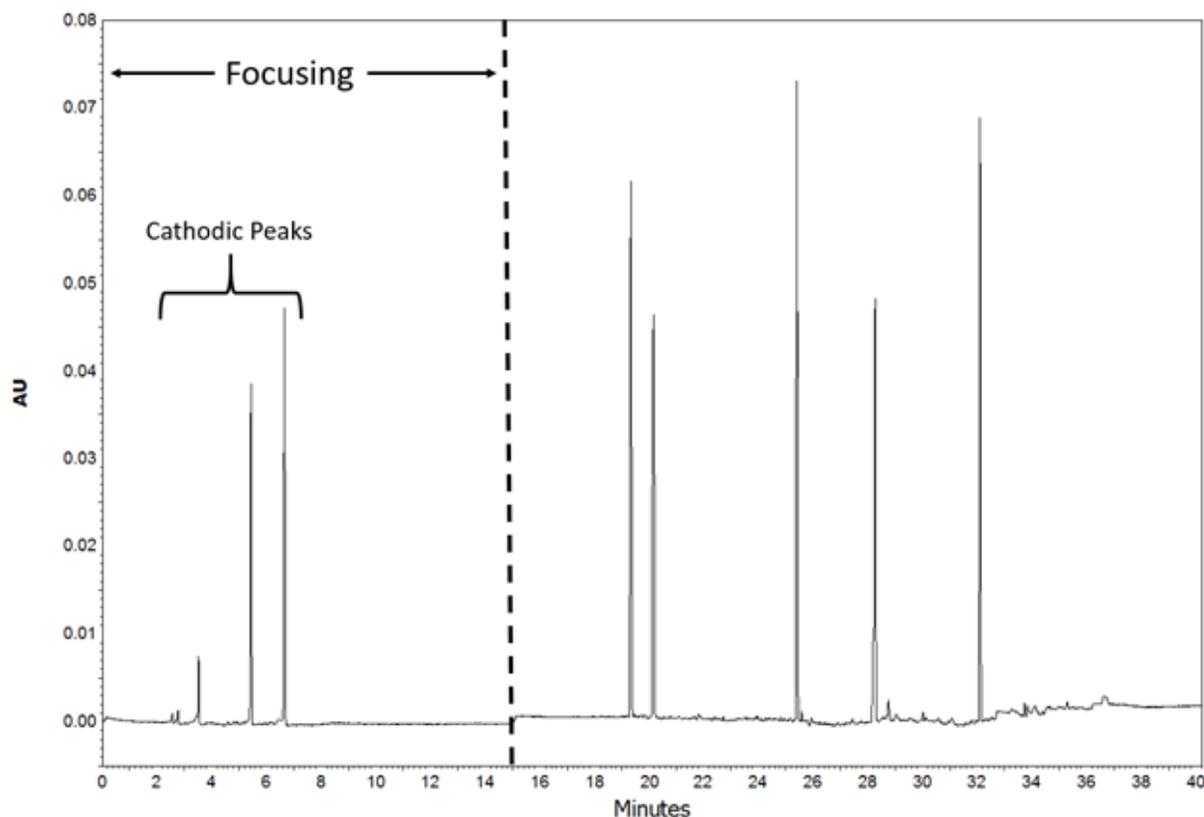
If the cartridge has not been used for more than a day or it has been stored for an extended time, then condition the capillary using the cIEF Conditioning method.

Analyze the Data

Verify System Performance with Peptide pI Markers and Pharmalyte Carrier Ampholytes

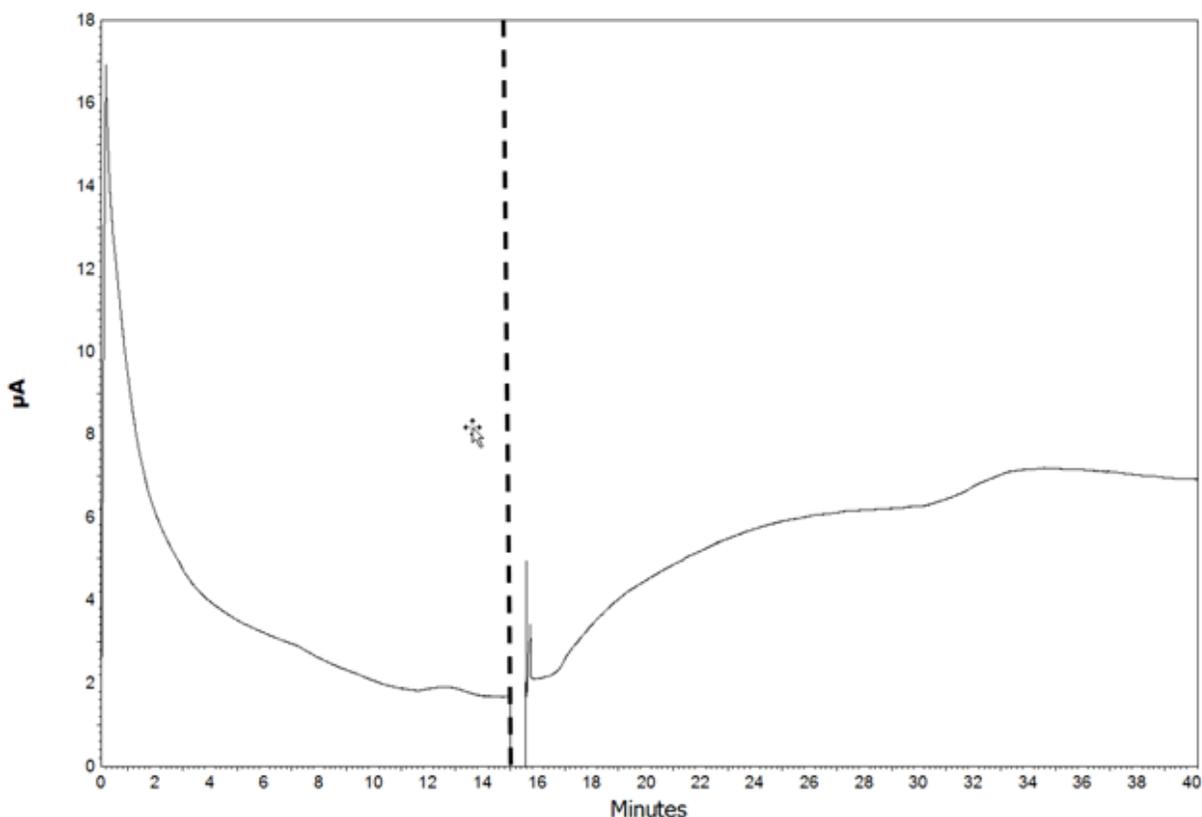
To verify the performance of the PA 800 Plus system, do a cIEF separation of the five peptide markers. Compare the electropherogram obtained with the electropherogram for the peptide pI markers. Refer to [Figure 18](#). The electrical current should be similar to that shown for the peptide pI markers. Refer to [Figure 19](#).

Figure 18 Typical cIEF Separation of Five Peptide pI Markers with Pharmalyte pH 3-10 Carrier Ampholytes



Note: The cathodic peaks shown in the electropherogram are caused by the bi-directional migration of the sample and the ampholytes that occurs during focusing. Refer to the figure: [Figure 2](#). The absence of cathodic peaks can indicate incomplete focusing.

Figure 19 Typical Electrical Current Profile of cIEF Separation of Peptide pI Markers with Pharmalyte pH 3-10 Carrier Ampholytes



The vertical dashed line separates the focusing data from the mobilization data. Refer to the figures: [Figure 18](#) and [Figure 19](#).

Focusing data is very helpful in troubleshooting cIEF separations. For example, variations in the initial electrical current value at the start of focusing can indicate problems in pipetting or in the preparation of the cIEF reagents.

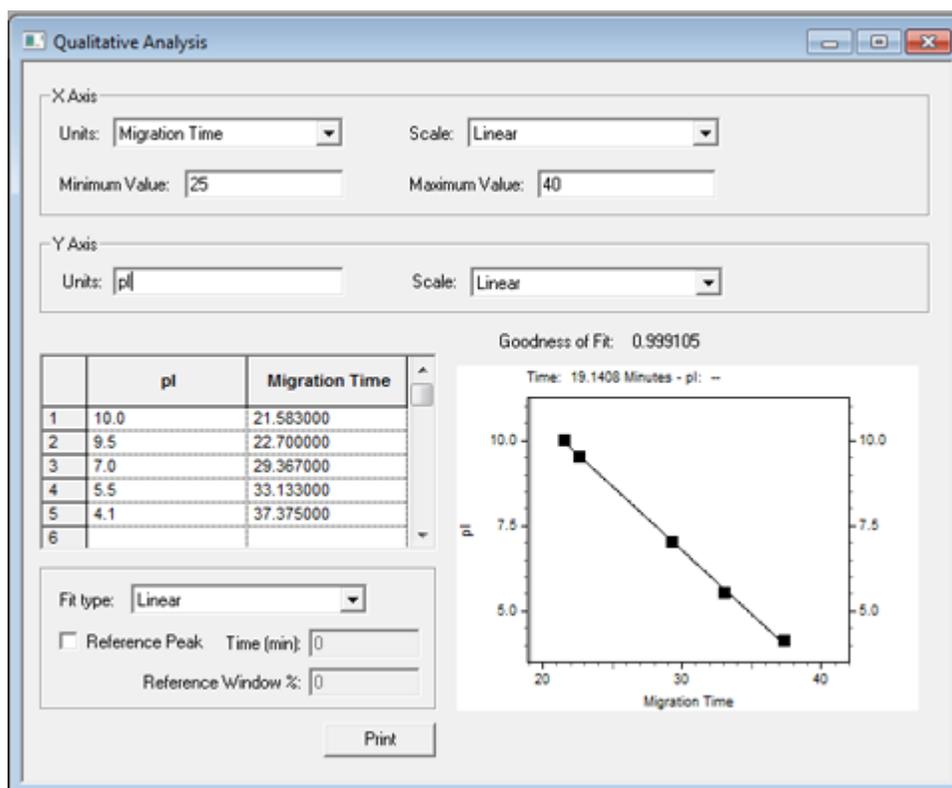
The separation of the cIEF peptide markers can be used as a System Suitability method to make sure that the whole system, including the reagents, is working properly. To pass system suitability, the five markers must be detected during the mobilization step. In the example separation method, mobilization begins at about 15 minutes. If the markers are not detected during mobilization, then refer to the section: [Troubleshooting](#). Refer to the section [System Suitability Method](#).

Determine the pI Value

Use the 32 Karat software to calculate the experimental pI value of a sample.

1. Click **Method > Qualitative Analysis**.
The Qualitative Analysis dialog opens.
2. In the Qualitative Analysis table, type the theoretical **pI** values of the markers detected during the mobilization step with their corresponding **Migration Time**, in minutes.

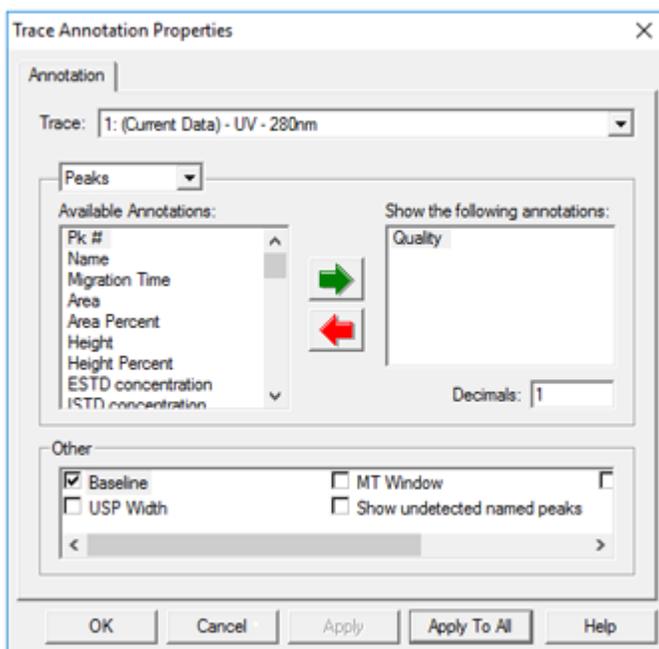
Figure 20 Qualitative Analysis Dialog for pI Determination



3. To save the method, click **File > Method > Save**.
4. Click **Analysis > Analyze**.
5. To open the calculated pI values in the cIEF separation (UV trace), right-click in the UV trace and then click **Annotations**. In **Available Annotations**, click **Quality**, and then click **Add**.

Note: In this analysis, quality corresponds to the calculated pI value.

Figure 21 Trace Annotation Properties Dialog



6. To save the changes in the Trace Annotation Properties dialog, click **OK**.

Analyze a cIEF Separation of the Monoclonal IgG Reference Standard

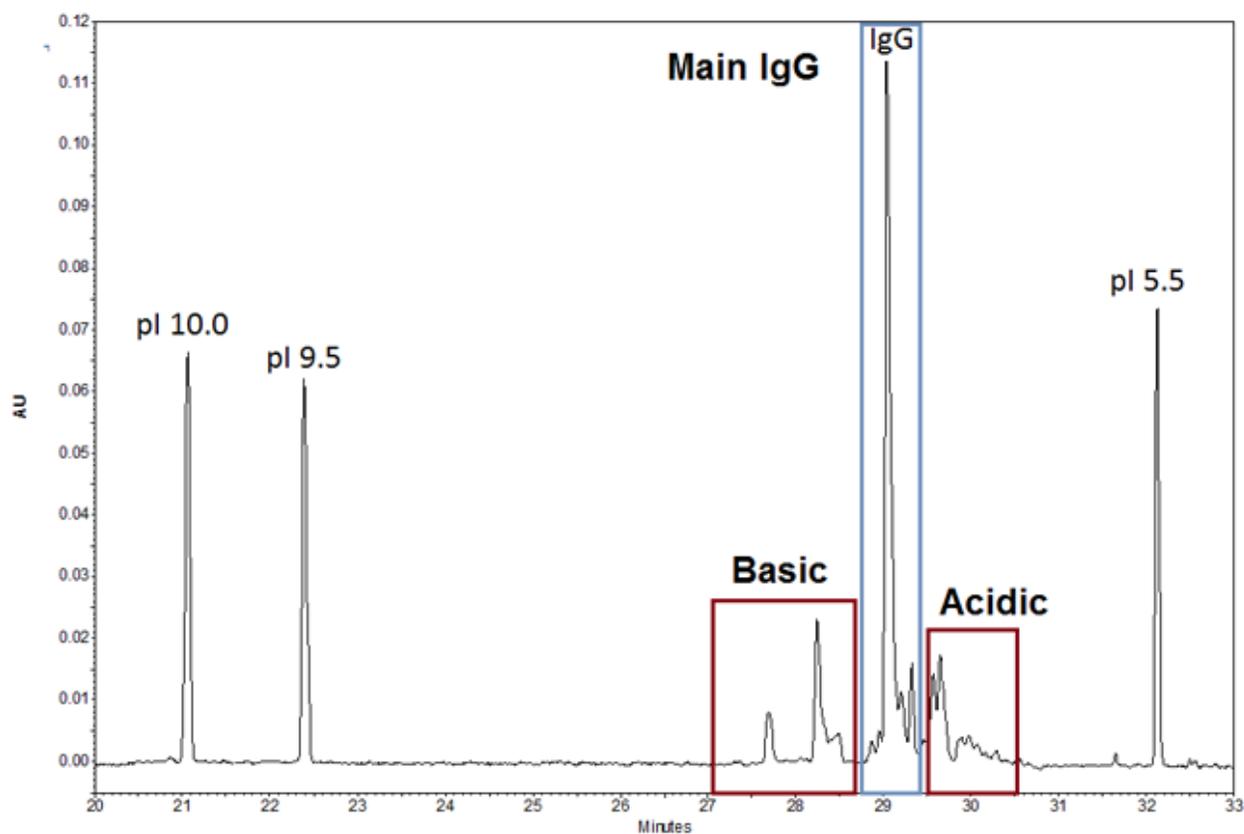
The following figure shows a close-up of a cIEF separation of the IgG Reference with Pharmalyte pH 3-10 carrier ampholytes and peptide pI markers 10.0, 9.5, and 5.5. Focusing occurred from 0 minutes to 15 minutes, and mobilization occurred from 15 minutes to 40 minutes. Refer to the figure: [Figure 22](#).

Note: The cIEF separation profile of an IgG sample might vary because of changes in glycosylation and other post-translational modifications as well as ampholyte lot⁷ and manufacturer. To simplify peak integration, we recommend dividing the IgG cIEF profile into three areas:

- Main: The major IgG peak
- Basic: All IgG peaks at the left side of the main peak, which are more basic than the main peak
- Acidic: All IgG peaks at the right side of the main peak, which are more acidic than the main peak

⁷ Righetti P. G., Simó C., Sebastiano R., Citterio A., Electrophoresis Volume 28, 3799-3810, 2007.

Figure 22 cIEF Separation of Monoclonal IgG Reference Standard



Troubleshooting

Symptom	Possible Cause	Corrective Action
Electrical current at the start of the focusing step changes between replicate runs.	<ol style="list-style-type: none"> The sample was not completely mixed. The capillary coating has degraded and the electroosmotic flow (EOF) is significant. 	<ol style="list-style-type: none"> Prepare a new sample and repeat the analysis. Replace the capillary.
Low resolution	<ol style="list-style-type: none"> The protein profile is shown as a single broad peak. 	<ol style="list-style-type: none"> The protein is precipitating or aggregating. Increase the urea content in the cIEF sample.

cIEF Kit

Symptom	Possible Cause	Corrective Action
Loss of resolution	1. The Urea-cIEF gel solution has high conductivity because of thermal degradation.	1. Prepare a new Urea-cIEF gel solution. Store this solution at 2 °C to 8 °C to prevent thermal degradation.
Missing peaks	1. A pipetting error occurred during sample or master mix preparation.	1. Prepare a new sample or master mix.
No peaks	<ol style="list-style-type: none"> 1. The method polarity is incorrect. 2. The sample is missing or not in the correct position in the sample tray. 3. The buffer vials are at the incorrect location. 4. The sample has a high salt concentration. 5. The capillary window is not centered on the aperture. 6. The fiber optic cable in the UV detector is loose. 	<ol style="list-style-type: none"> 1. Use the normal polarity in the method. 2. Make sure that the samples are in the correct locations in the sample tray. 3. Make sure that the buffer vials are in the location indicated in the method. 4. Exchange the sample buffer so that the salt concentration is less than 50 mM. 5. Adjust the capillary window inside the cartridge. Make sure that light passes through both the aperture and the capillary window by shining a flashlight on the back of the aperture. 6. Tighten both ends of the fiber optic cable.
Changes to the peak profile between consecutive runs	<ol style="list-style-type: none"> 1. Focusing is incomplete. 2. The protein is precipitating or aggregating. 3. The protein is denatured. 	<ol style="list-style-type: none"> 1. Increase the focusing time. 2. Increase the urea concentration in the sample, and increase the focusing time. 3. Try a cIEF separation without urea in the sample.

Hazardous Substance Information

A

The following information must be noted and the relevant safety measures taken. Refer to the respective safety data sheets for more information. The safety data sheets are available upon request or can be downloaded from our website, at sciex.com/tech-regulatory.

Hazard classification according to HCS 2012.

Sample Loading Solution



DANGER! May damage fertility or the unborn child.

Other Reagents

These components are not classified as hazardous:

- CE Grade Water
- cIEF gel
- cIEF Peptide Markers
- eCAP 50 mM Tris Buffer at pH 8.0

For reagents from other vendors, read the *Safety Data Sheet* from the vendor before use.

Note: The following information applies to users using the PA 800 Plus system with the PA 800 Plus and 32 Karat software. If the system is used with the Waters Empower™ software, then the methods are different. Refer to the section: [Run the Samples with the Waters Empower™ Software](#).

The cIEF application requires three methods.

Note: The values on the Initial Conditions and UV Detector Initial Conditions tabs are the same for all of the methods.

Capillary Conditioning Method

Figure B-1 Initial Conditions Tab

The screenshot shows the 'Initial Conditions' tab of a software interface. It contains several sections with various settings:

- Auxiliary data channels:** Includes checkboxes for Voltage (unchecked), Current (checked), Power (unchecked), and Pressure (unchecked). Voltage max is 30.0 kV, and Current max is 20.0 μA.
- Mobility channels:** Includes checkboxes for Mobility (unchecked), Apparent Mobility (unchecked), and Plot trace after voltage ramp (checked).
- Analog output scaling:** Factor is set to 1.
- Temperature:** Cartridge is 20.0 °C and Sample storage is 10.0 °C.
- Peak detect parameters:** Threshold is 2 and Peak width is 9.
- Trigger settings:** Includes checkboxes for Wait for external trigger (unchecked), Wait until cartridge coolant temperature is reached (checked), and Wait until sample storage temperature is reached (checked).
- Inlet trays:** Buffer is 36 vials and Sample is 48 vials.
- Outlet trays:** Buffer is 36 vials and Sample is No tray.

Figure B-2 UV Detector Initial Conditions Tab

Figure B-3 Capillary Conditioning Method Time Program Tab

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	5.00 min	BI:A5	BO:A5	forward	Water Rinse 1
2		Rinse - Pressure	50.0 psi	5.00 min	BI:F6	BO:F6	forward	SLS Rinse
3		Rinse - Pressure	50.0 psi	3.00 min	BI:B5	BO:B5	forward	Water Rinse 2
4	0.00	Separate - Pressure	50.0 psi	3.00 min	BI:C5	BO:C5	forward	Water Rinse 3
5	3.00	Wait		0.00 min	BI:D5	BO:D5		Water dip
6								

Separation Method

Figure B-4 Initial Conditions Tab

The screenshot shows the 'Initial Conditions' tab with three sub-tabs: 'Initial Conditions', 'UV Detector Initial Conditions', and 'Time Program'. The 'Initial Conditions' sub-tab is active and contains the following settings:

- Auxiliary data channels:**
 - Voltage max: 30.0 kV
 - Current max: 20.0 μ A
 - Power
 - Pressure
- Mobility channels:**
 - Mobility
 - Apparent Mobility
 - Plot trace after voltage ramp
- Analog output scaling:**
 - Factor: 1
- Temperature:**
 - Cartridge: 20.0 $^{\circ}$ C
 - Sample storage: 10.0 $^{\circ}$ C
- Peak detect parameters:**
 - Threshold: 2
 - Peak width: 9
- Trigger settings:**
 - Wait for external trigger
 - Wait until cartridge coolant temperature is reached
 - Wait until sample storage temperature is reached
- Inlet trays:**
 - Buffer: 36 vials
 - Sample: 48 vials
- Outlet trays:**
 - Buffer: 36 vials
 - Sample: No tray

Figure B-5 UV Detector Initial Conditions Tab

The screenshot shows the 'UV Detector Initial Conditions' sub-tab. The settings are as follows:

- Electropherogram channel:**
 - Acquisition enabled
 - Wavelength: 280 nm
 - Data rate: 4 Hz
- Filter:**
 - High sensitivity
 - Normal
 - High resolution
 - Peak width (points): 16-25
- Relay 1:**
 - Off
 - On
- Relay 2:**
 - Off
 - On
- Absorbance signal:**
 - Direct
 - Indirect

Figure B-6 Separation Method Time Program Tab

Initial Conditions UV Detector Initial Conditions Time Program								
	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	1.00 min	BI:F6	BO:F6	forward	SLS rinse
2		Rinse - Pressure	20.0 psi	3.00 min	BI:F1	BO:F1	forward, In / Out vial inc 10	Water Rinse 1
3		Rinse - Pressure	50.0 psi	2.00 min	BI:B1	BO:B1	forward, In / Out vial inc 10	Water Rinse 2
4		Inject - Pressure	15.0 psi	150.0 sec	SI:A1	BO:B1	Override, forward	Sample Injection
5		Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	Water dip 1
6	0.00	Separate - Voltage	25.0 kV	15.00 min	BI:C1	BO:C1	0.17 Min ramp, normal polarity, In / Out vial inc 10	Focusing step
7	1.00	Autozero						
8	15.10	Wait		0.00 min	BI:C1	BO:A1	In / Out vial inc 10	Water dip 2
9	15.20	Separate - Voltage	30.0 kV	25.00 min	BI:C1	BO:E1	0.17 Min ramp, normal polarity, In / Out vial inc 10	Mobilization step
10	40.20	Stop data						Stop cIEF separation
11	40.30	Rinse - Pressure	50.0 psi	3.00 min	BI:B1	BO:D1	forward, In / Out vial inc 10	Water Rinse 3
12	43.40	Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	Water dip 3
13	43.50	End						Method end
14								

Shutdown Method

Figure B-7 Initial Conditions Tab

Initial Conditions UV Detector Initial Conditions Time Program

Auxiliary data channels

Voltage max: 30.0 kV

Current max: 20.0 μ A

Power

Pressure

Mobility channels

Mobility

Apparent Mobility

Plot trace after voltage ramp

Analog output scaling

Factor: 1

Temperature

Cartridge: 20.0 $^{\circ}$ C

Sample storage: 10.0 $^{\circ}$ C

Peak detect parameters

Threshold: 2

Peak width: 9

Trigger settings

Wait for external trigger

Wait until cartridge coolant temperature is reached

Wait until sample storage temperature is reached

Inlet trays

Buffer: 36 vials

Sample: 48 vials

Outlet trays

Buffer: 36 vials

Sample: No tray

Methods

Figure B-8 UV Detector Initial Conditions Tab

Figure B-9 Shutdown Method Time Program Tab

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	3.00 min	BI:F6	BO:F6	forward	SLS Rinse
2		Wait		0.00 min	BI:C6	BO:C6		Water dip
3		Rinse - Pressure	50.0 psi	10.00 min	BI:E6	BO:E6	forward	Water Rinse
4	0.00	Separate - Pressure	50.0 psi	3.00 min	BI:D6	BO:D6	forward	cIEF gel Rinse
5	3.10	Lamp - Off						Turn off the lamp
6	3.20	Wait		0.00 min	BI:F5	BO:F5		Water dip
7								

A System Suitability method can be used to determine whether an electrophoretic system is suitable for a particular analysis. This type of method involves running a mixture of analytes and examining the parameters that describe the suitability of the sample preparation procedure, instrument settings, chemistries, and environment to perform the analysis.

Activate System Suitability

To use this feature, make sure that System Suitability is activated in the cIEF instrument.

1. Close all of the PA 800 Plus software windows except for the 32 Karat Software Enterprise window.
2. In the 32 Karat software main window, click **Tools > Enterprise Login**.
3. Type the user name and the password and then click **OK**.
The default user name is `PA800`, and the default password is `Plus`.
4. Right-click the cIEF icon and then click **Configure > Instrument**.
5. Click **Configure**.
The PA 800 Plus System Configuration dialog opens.
6. Click **Options**.
7. On the General tab, click **System Suitability**, **Qualitative Analysis**, and **Caesar Integration**.
8. Click **OK** in the next three dialogs.

Make a cIEF System Suitability Method

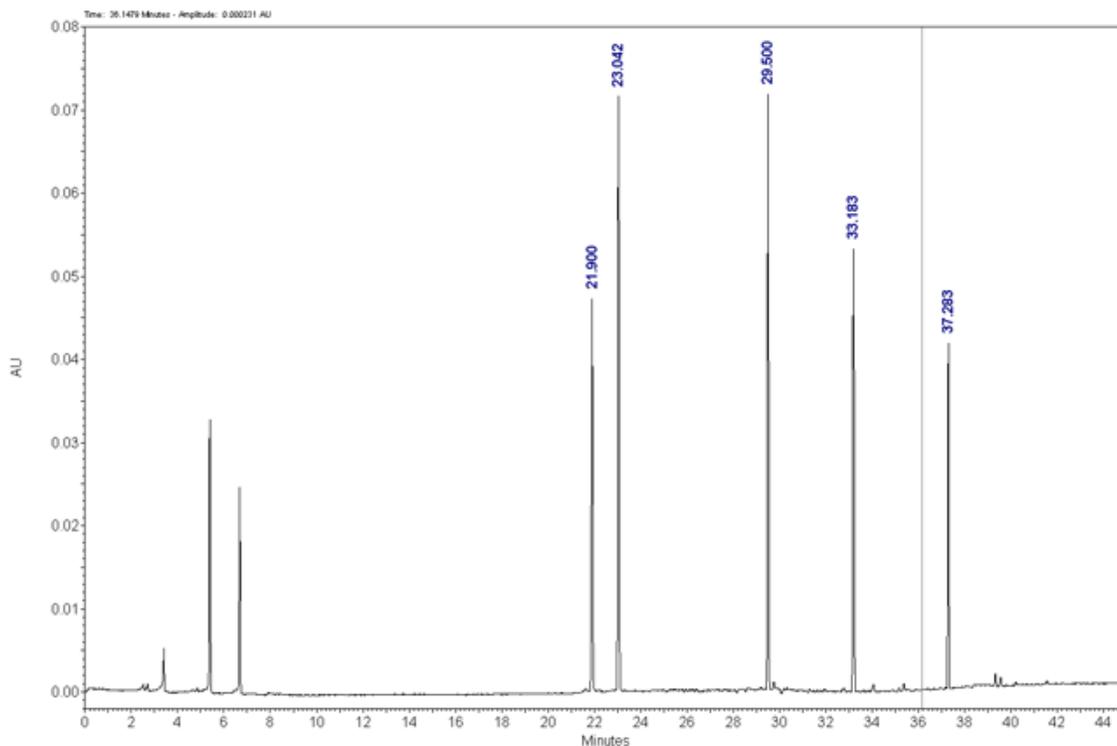
Note: This example uses the separation of the five cIEF Peptide Markers. Refer to the figure: [Figure C-1](#).

1. To open the cIEF instrument, double-click the cIEF icon on the 32 Karat Software Enterprise window in the 32 Karat software.
2. Open the cIEF method that will be converted to a System Suitability method, and then save it as `System Suitability`.
3. Open the system suitability data:
 - a. Click **File > Data > Open**.
The Open Data File dialog opens.

System Suitability Method

- b. Browse to the `cIEF Examples` folder, and then select `Marker-1`.
- c. In the Options section, select **Current** from the **Method** list.
- d. Click **Open**.

Figure C-1 Separation with Five cIEF Peptide Markers



Note: For instructions on data integration, including how to show the migration times on the open data, refer to the section: [Optimize Integration Parameters](#).

4. To add the integrated peaks to the method peak table, right-click inside the UV trace and then click **Graphical Programming > Define Peaks**.
5. Click the beginning of the marker peaks, and then click the end of the marker peaks. In this example, click at 20 minutes and then at 39 minutes to include the five peptide markers.
6. To open the the peak table, click **Method > Peaks/Groups**. In this example, the table containing the Named Peaks tab is shown. Peaks are labeled according to their migration time.

Figure C-2 Named Peaks Tab

#	Name	ID	MT Window	Ref. ID #	ISTD. ID #	Resolution ID #	Units
1	Peak @ 21.900	1	1.095	0	0	0	
2	Peak @ 23.042	2	1.05208	0	0	0	
3	Peak @ 29.500	3	1.475	0	0	0	
4	Peak @ 33.183	4	1.65917	0	0	0	
5	Peak @ 37.283	5	1.86417	0	0	0	
6							

- (Optional) Rename the peaks in the table. For example, name the marker peaks after their corresponding pI value.
- Click **Method > System Suitability**.

Figure C-3 System Suitability Setup Window

Compound:

- pl 10.0
- pl 9.5
- pl 7.0
- pl 5.5
- pl 4.1

#	Parameter	Min	Max	%RSD
1				

#	Test	Start	End	Value
1				

- Select the criteria in the **Parameter** list that the data must meet to be considered a pass for each analyte. For example, click **Quality**, which corresponds to the **pI** in the cIEF software module.
- Click **Method > Qualitative Analysis**.
The Qualitative Analysis window opens. Refer to the figure: [Figure 20](#).
- In the Qualitative Analysis table, type the theoretical pI values for the markers detected during the mobilization step with their corresponding **Migration Time**, in minutes.
- To save the method, click **File > Method > Save Method**.

Generate a System Suitability Report

1. Open a new sequence.
2. In row 1 of the sequence, in the **Method** column, click the **System Suitability Method**.
3. In row 1 of the sequence, in the **Filename** column, click the data to be checked by the System Suitability.

Note: Make sure that the migration time of the data file selected here is typed manually in the Qualitative Analysis window.

4. In row 1 of the sequence, right-click the **Row Number** column, and then click **Run Types > System Suitability**.

Both **Begin System Suitability** and **End System Suitability** (SSB SSE) are selected.

Note: The default System Suitability report template is `SysSuit.brp`.

5. To close the dialog, click **OK**.
6. To save the sequence, click **File > Sequence > Save Sequence**.
7. Click **Sequence > Process**.
The Process Sequence dialog opens. The open sequence is shown as sequence name.
8. To select the rows for analysis, set the **Run Range** to **All**.
9. To print the results, select the options in the Printing section.
10. For the **Processing Mode**, click **Reintegrate**.
11. Click **Start**.

After the analysis completes successfully, the row is labeled **Complete** in the **Status** column.

Tip! To see the result after reintegration, click **Review**.

12. To view the System Suitability report, click **Reports > View > Sequence Custom Reports**.
13. To open the report, click **System Suitability > View**.

Note: For additional information on setting and performing System Suitability, refer to the Help file in the 32 Karat software.

Perform a Buffer Exchange

D

In cIEF, the presence of salts greater than 50 mM in the sample can lead to compression of the pH gradient, altered focusing conditions, and damage to the capillary coating. To reduce the negative effects that sample buffer components can have on cIEF separations, we recommend that the buffer exchange be performed.

Note: This procedure was validated using a centrifugal filter unit with a 10 kDa molecular weight cutoff. If a different molecular weight cutoff is required, choose a different filter and adjust the centrifugation parameters as required.

Note: If a buffer other than eCap Tris Buffer is used, make sure that the buffer does not contain any components that are ampholytic. Those components will focus during the run and might interfere with the separation of the sample and affect the linearity of the pH gradient.

1. Add 4 mL of the eCap Tris Buffer to 6 mL of CE Grade Water.
2. Add 500 μ L of the protein (between 5 mg/mL and 10 mg/mL) to a centrifugal filter unit and then spin the vial for 5 minutes at 12,000 g .
3. Add 250 μ L of the diluted eCap Tris Buffer prepared in step 1 to the retentate. Discard the permeate.
4. Using a centrifuge, spin the vial for 10 minutes at 12,000 g .
5. Repeat from step 2 to step 3, twice.
6. Put the centrifugal filter unit in an inverted position in a clean microcentrifuge vial and then mix the vial using a centrifuge for 3 minutes at 2,000 g .
7. Prepare 50 μ L aliquots at a protein concentration of 5 mg/mL to 10 mg/mL.
Store the aliquots at or below -15 $^{\circ}$ C.
8. Prepare aliquots of 5 μ g.
Store the aliquots between -35 $^{\circ}$ C and -15 $^{\circ}$ C for up to 3 months.

Run the Samples with the Waters Empower™ Software

E

This section gives instructions on data acquisition using the Waters Empower™ software. For data analysis instructions, refer to the Waters Empower™ software guides and help file.

Note: Calibrate the UV detector before acquiring data. For instructions, refer to the document: *PA 800 Plus Empower™ Driver User Guide*.

Create the Instrument Methods

Note: The validated instrument methods are included on the PA 800 Plus Empower™ Driver DVD. The methods can be imported instead of being created manually. Refer to the section: [Import the Instrument Methods](#). If the methods are missing, then use the following instructions to create them.

Three instrument methods are required:

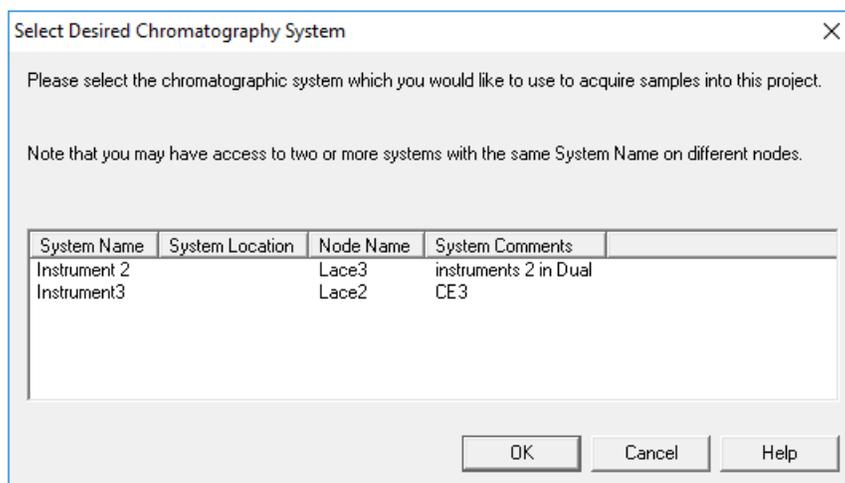
- cIEF_CONDITIONING
- cIEF_SEPARATION
- cIEF_SHUTDOWN

Note: The values on the General and Detector tabs are the same for all of the methods.

Note: Pressure values can be shown in millibar (mbar) or pounds per square inch (psi), depending on a registry setting for the Waters Empower™ software. The default unit is millibar. To change the units, refer to the document: *PA 800 Plus Empower™ Driver Release Notes*.

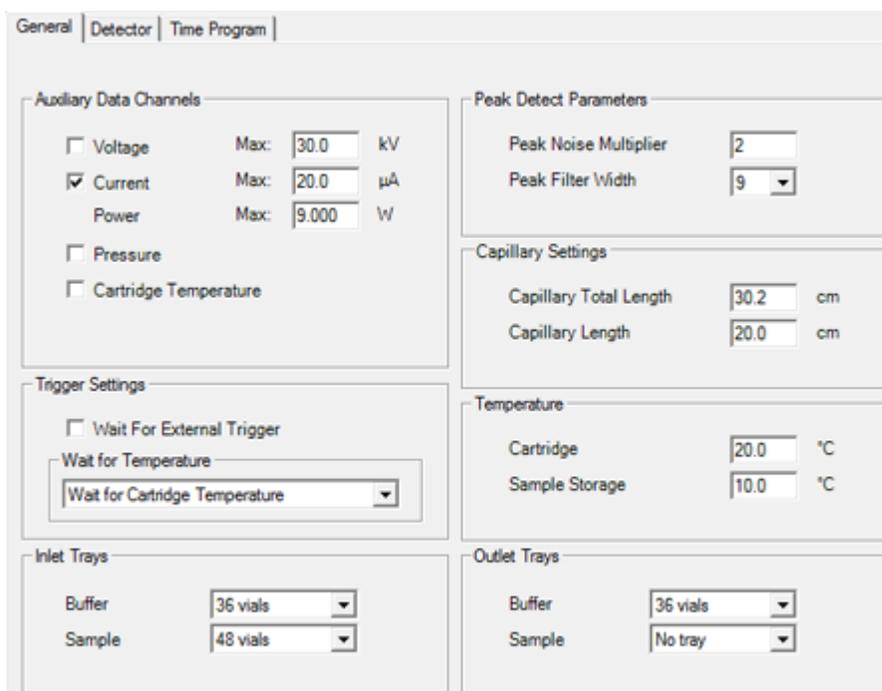
1. In the Waters Empower™ Software Project window, click **File > New Method > Instrument Method**.

Figure E-1 Select Desired Chromatography System Dialog



2. Click the system to be used and then click **OK**.
Make sure that the instrument is configured with a UV detector.
The Instrument Method Editor opens.
3. Set the parameters on the General tab.

Figure E-2 General Parameters for cIEF_CONDITIONING Instrument Method



Run the Samples with the Waters Empower™ Software

- Open the Detector tab, click **UV** in the **Detector Type** list, and then set the parameters.

Note: For 3D data, in the Electropherogram Channel Data section, set the **Data Rate** to **On**.

Figure E-3 Detector Parameters for cIEF_CONDITIONING Instrument Method

The screenshot shows the 'Detector' tab in the software. The 'Detector Type' dropdown is set to 'UV'. Below it, the 'Filter' dropdown is set to 'General Purpose 16-25'. The 'Electropherogram Channel Data' section contains two dropdowns: 'Data Rate' is set to '4 Hz' and 'Wavelength' is set to '280 nm'. The 'Relays' section has two dropdowns, 'Relay 1' and 'Relay 2', both set to 'Closed'. The 'Absorbance Signal' section has a 'Signal' dropdown set to 'Direct'.

- Add the events in the following figure to the time program.

Figure E-4 Time Program for cIEF_CONDITIONING Instrument Method

	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
▶ 1		Rinse Pressure	50.0 psi	5.00 min	A5	Buffer	A5	Buffer	Forward;0;0	WATER rinse
2		Rinse Pressure	50.0 psi	5.00 min	F6	Buffer	F6	Buffer	Forward;0;0	SLS
3		Rinse Pressure	50.0 psi	3.00 min	B5	Buffer	B5	Buffer	Forward;0;0	water
4	0.00	Separate Pressure	50.0 psi	3.00 min	C5	Buffer	C5	Buffer	Forward;0;0	WATER
5	3.00	Stop Data								
6	3.00	Wait		0.00	D5	Buffer	D5	Buffer	0;0	water rinse
7	3.00	End	25.0 kV							
* 8										

Note: If the system is using mbar as the units for pressure, then type the following:

- For the pressure in the **Rinse Pressure** events (steps 1, 2, and 3), type 3447.4.
- For the pressure in the **Separate Pressure** event (step 4), type 3447.4.

6. Save the instrument method.
 - a. Click **File > Save**.
The Save current Instrument Method dialog opens.
 - b. Type `cIEF_CONDITIONING` in the **Name** field.
 - c. (Optional) Type information in the **Method Comments** field.
 - d. If prompted, type the Waters Empower™ software password for the current user in the **Password** field and then click **Save**.

The instrument method is saved to the current project.

7. Create the separation instrument method.
 - a. Set the parameters on the General tab. Refer to the figure: [Figure E-2](#).
 - b. Set the parameters on the Detector tab. Refer to the figure: [Figure E-3](#).
 - c. Add the events in the following figure to the time program.

Figure E-5 Time Program for cIEF_SEPARATION Instrument Method

Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments	
1	Rinse Pressure	50.0 psi	1.00 min	F6	Buffer	F6	Buffer	Forward;0:0	SLS rinse	
2	Rinse Pressure	20.0 psi	3.00 min	F1	Buffer	F1	Buffer	Forward;10:10	water rinse 1	
3	Rinse Pressure	50.0 psi	2.00 min	B1	Buffer	B1	Buffer	Forward;10:10	water rinse 2	
4	Inject Pressure Capillary Fill	15.0 psi	150.0 s	A1	Sample	B1	Buffer	Forward;1:10	sample injection	
5	Wait		0.00	A1	Buffer	A1	Buffer	10:10	water dip	
6	0.00	Separate Voltage	25.0 kV	15.00 min	C1	Buffer	C1	Buffer	Normal (+);0.17;10:10	focusing step C
7	1.00	Autozero								
8	15.00	Wait	0.10	C1	Buffer	A1	Buffer	10:10	water dip	
9	15.10	Separate Voltage	30.0 kV	25.00 min	C1	Buffer	E1	Buffer	Normal (+);0.17;10:10	Mobilization step
10	40.00	Stop Data								
11	40.10	Rinse Pressure	50.0 psi	3.00 min	B1	Buffer	D1	Buffer	Forward;10:10	water rinse
12	43.10	Wait	0.00	A1	Buffer	A1	Buffer	10:10	water dip	
13	43.10	End								

Note: If the system is using mbar as the units for pressure, then type the following:

- For the pressure in the **Rinse Pressure** events (steps 1 and 3), type 3447.4.
- For the pressure in the **Rinse Pressure** event (step 2), type 1379.0.
- For the pressure in the **Inject Pressure Capillary Fill** event (steps 4), type 1034.2.

Run the Samples with the Waters Empower™ Software

- d. Save the method as cIEF_SEPARATION.
8. Create the shutdown instrument method.
 - a. Set the parameters on the General tab. Refer to the figure: [Figure E-2](#).
 - b. Set the parameters on the Detector tab. Refer to the figure: [Figure E-3](#).
 - c. Add the events in the following figure to the time program.

Figure E-6 Time Program for cIEF_SHUTDOWN Instrument Method

	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
▶ 1		Rinse Pressure	50.0 psi	3.00 min	F6	Buffer	F6	Buffer	Forward:0.0	SLS rinse
2		Wait		0.00	C6	Buffer	C6	Buffer	0.0	
3		Rinse Pressure	50.0 psi	10.00 min	E6	Buffer	E6	Buffer	Forward:0.0	WATER
4	0.00	Separate Pressure	50.0 psi	3.00 min	D6	Buffer	D6	Buffer	Forward:0.0	
5	3.00	Stop Data								
6	3.00	Lamp Off								
7	3.00	Wait		0.00	F5	Buffer	F5	Buffer	0.0	water
8	3.00	End	25.0 kV							
* 9										

Note: If the system is using mbar as the units for pressure, then type the following:

- For the pressure in the **Rinse Pressure** events (steps 1 and 3), type 3447.4.
 - For the pressure in the **Separate Pressue** event (steps 4), type 3447.4.
-

- d. Save the method as cIEF_SHUTDOWN.

Create the Method Sets

Three method sets are required:

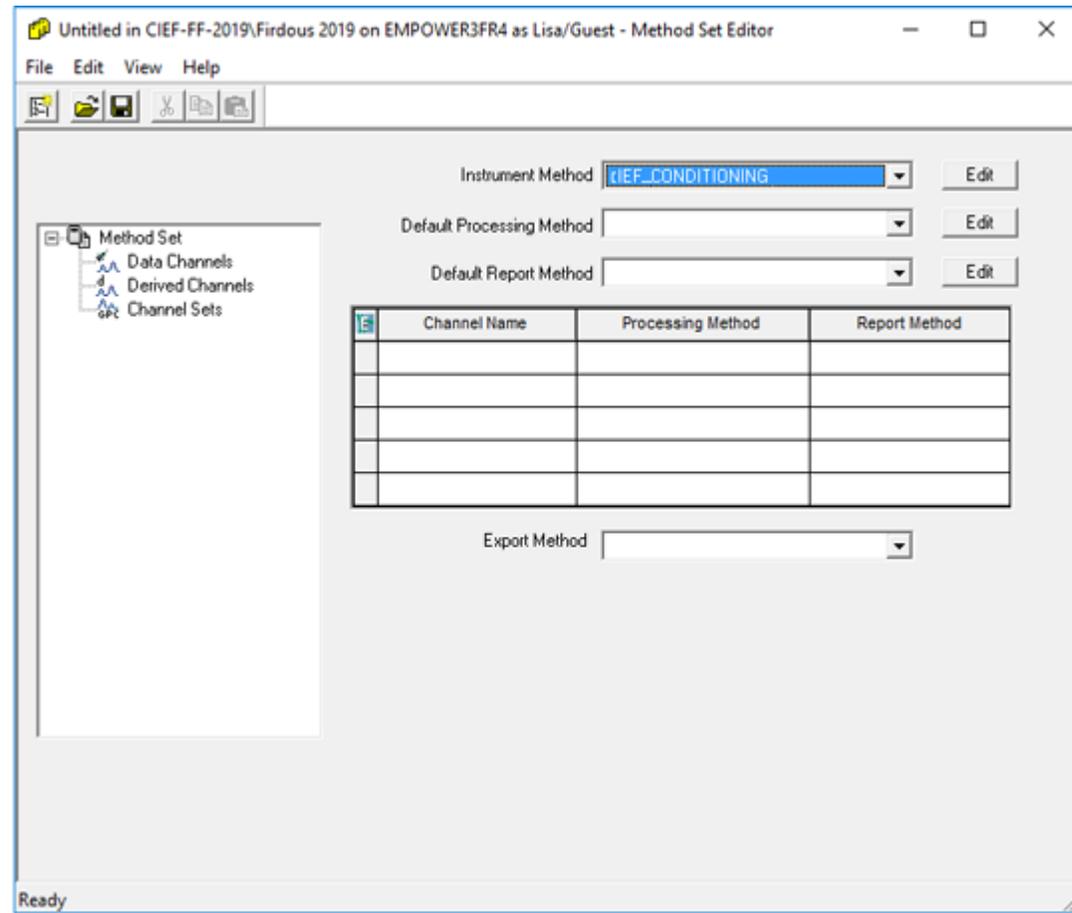
- cIEF Conditioning Method Set
 - cIEF Separation Method Set
 - cIEF Shutdown Method Set
-

Note: A method set can also include processing and report. To create these methods, refer to the documentation supplied with the Waters Empower™ software.

1. In the Waters Empower™ Software Project window, click **File > New Method > Method Set**.
 2. Click **No** in the message.
The Method Set Editor window opens.
-

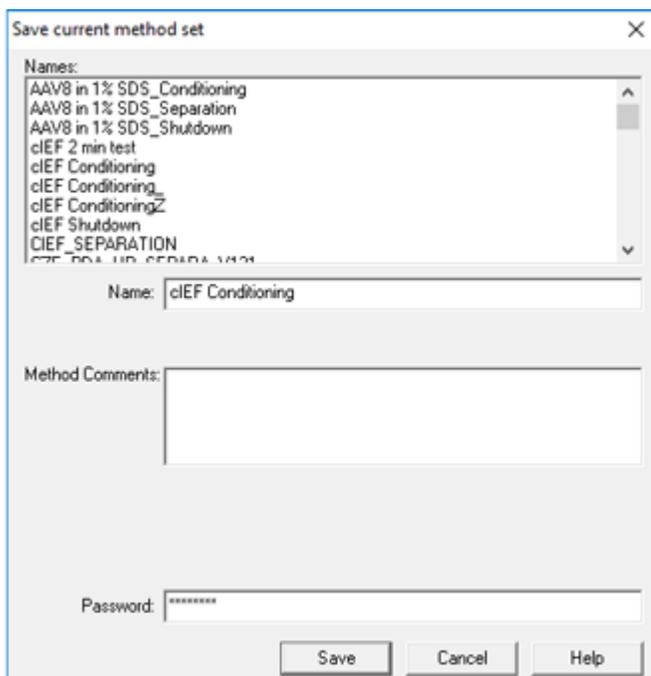
3. In the **Instrument Method** list, click **cIEF_CONDITIONING**. Do not make any other changes.

Figure E-7 Method Set Editor Window



4. Save the method set.
 - a. Click **File > Save**.
 - b. Type **cIEF Conditioning Method Set** in the **Name** field.
 - c. (Optional) Type information in the **Method Comments** field.
 - d. If prompted, type the Waters Empower™ software password for the current user in the **Password** field and then click **Save**.

Figure E-8 Save current method set Dialog



The method set is saved to the current project.

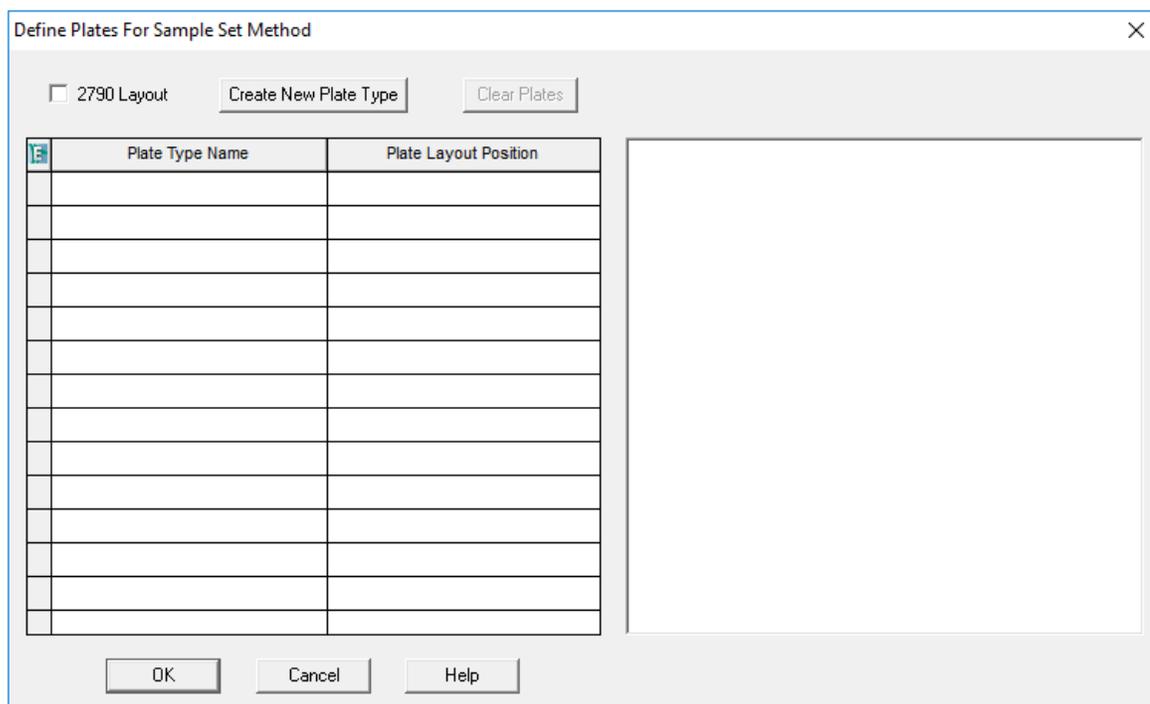
5. Repeat the previous steps to create the other method sets.
 - a. Create the separation method set by selecting **cIEF_SEPARATION** in the **Instrument Method** list. Save the method set as **cIEF Separation Method Set**.
 - b. Create the shutdown method set by selecting **cIEF_SHUTDOWN** in the **Instrument Method** list. Save the method set as **cIEF Shutdown Method Set**.

Configure the Software to Use Multiple Plates

The Waters Empower™ software is designed for chromatography systems that do not have buffer trays. To use the buffer trays, configure the Waters Empower™ software as follows.

1. In the Waters Empower™ software Run Samples window, click **Edit > Plates**.

Figure E-9 Define Plates for Sample Set Method Dialog



Note: If the dialog does not look like the preceding figure, then clear the **2790 Layout** check box.

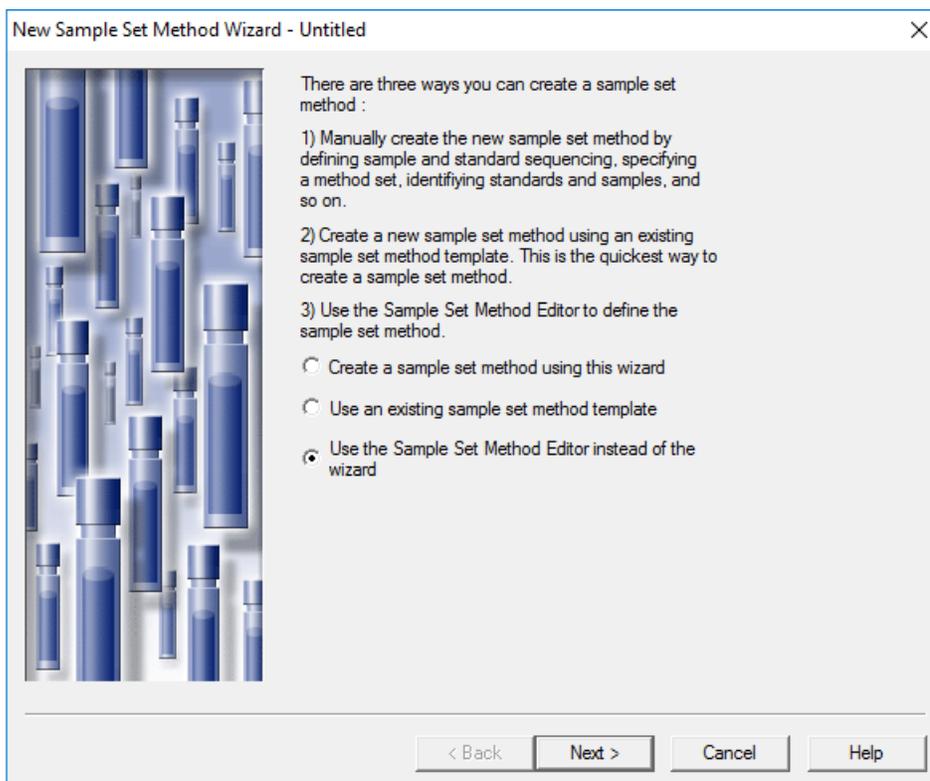
2. In the first row, configure the buffer inlet tray.
 - a. Click the **Plate Type Name** cell and then select **PA 800 Plus Buffer Tray**.

Note: If **PA 800 Plus Buffer Tray** is missing, then the buffer and sample trays might not have been defined. Refer to the document: *PA 800 Plus Empower™ Driver User Guide*.

The dialog updates with an image of the plate and buttons for the plate sequencing mode.

- b. Click the **Plate Layout Position** cell and then type BI.
 - c. Click  (**Vertical Discontinuous Plate Sequencing Mode**) to indicate the order in which the vials are accessed during the run.

Figure E-12 New Sample Set Method Wizard



The Sample Set Method Editor opens.

3. Set up the sample set method.
 - a. In the first row, select **cIEF_CONDITIONING** in the **Method Set/Report or Export Method** cell.
 - b. For rows 2 through 17, select **cIEF_SEPARATION** in the **Method Set/Report or Export Method** cell.
 - c. For row 18, select **cIEF_SHUTDOWN** in the **Method Set/Report or Export Method** cell.
 - d. Add the required information for the samples. Refer to the table: [Table E-1](#).

Table E-1 Required Fields for a Sample Set Method

Name	Description
Plate/Well	The position of the sample in the sample tray.
# of Injs	The number of times the sample is to be injected.
SampleName	The name of the sample.

Table E-1 Required Fields for a Sample Set Method (continued)

Name	Description
Run Time (Minutes)	The duration of the run. CAUTION: Potential Wrong Result. Make sure that the Run Time is greater than or equal to the duration of the time program in the instrument method. If the Run Time is shorter, the system stops the run before the time program is complete.

The completed sample set is shown in the following figure.

Note: The **Level** and **Label Reference** columns are hidden in the following figure.

Figure E-13 Sample Set Method

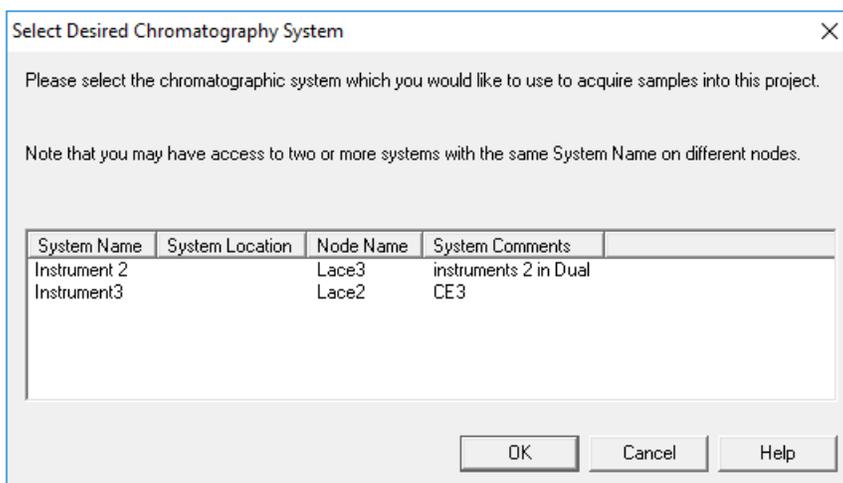
	Plate/Well	Inj Vol (uL)	# of Injs	Label	SampleName	Function	Method Set / Report or Export Method	Processing	Run Time (Minutes)	Data Start (Minutes)	Next Inj. Delay (Minutes)
1	B/A,1	10.0	1		Conditioning	Inject Samples	CIEF_CONDITIONING	Normal	10.00	0.00	0.00
2	S/A,1	10.0	1		pl Marker _ 5	Inject Samples	CIEF_SEPARATION	Normal	40.00	0.00	0.00
3	S/A,1	10.0	1		pl Marker _ 5	Inject Samples	CIEF_SEPARATION	Normal	40.00	0.00	0.00
4	S/A,2	10.0	1		pl Marker _ 5	Inject Samples	CIEF_SEPARATION	Normal	40.00	0.00	0.00
5	S/A,2	10.0	1		pl Marker _ 5	Inject Samples	CIEF_SEPARATION	Normal	40.00	0.00	0.00
6	S/A,3	10.0	1		pl Marker _ 5	Inject Samples	CIEF_SEPARATION	Normal	40.00	0.00	0.00
7	S/A,3	10.0	1		pl Marker _ 5	Inject Samples	CIEF_SEPARATION	Normal	40.00	0.00	0.00
8	B/A,1	10.0	1		Shutdown	Inject Samples	CIEF_SHUTDOWN	Normal	10.00	0.00	0.00

4. Save the sample set method.
 - a. Click **File > Save**.
The Save current sample set method dialog opens.
 - b. Type `cIEF Sample Set Method` in the **Name** field.
 - c. (Optional) Type information in the **Method Comments** field.
 - d. If prompted, type the Waters Empower™ software password for the current user in the **Password** field and then click **Save**.

The method set is saved to the current project.

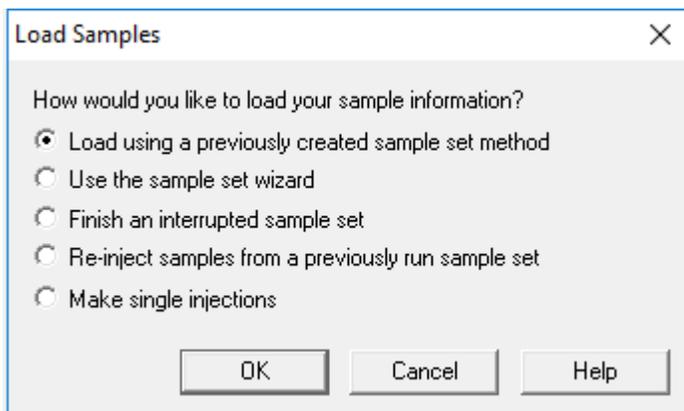
5. Click **Tools > Run Samples**.

Figure E-14 Select Desired Chromatography System Dialog



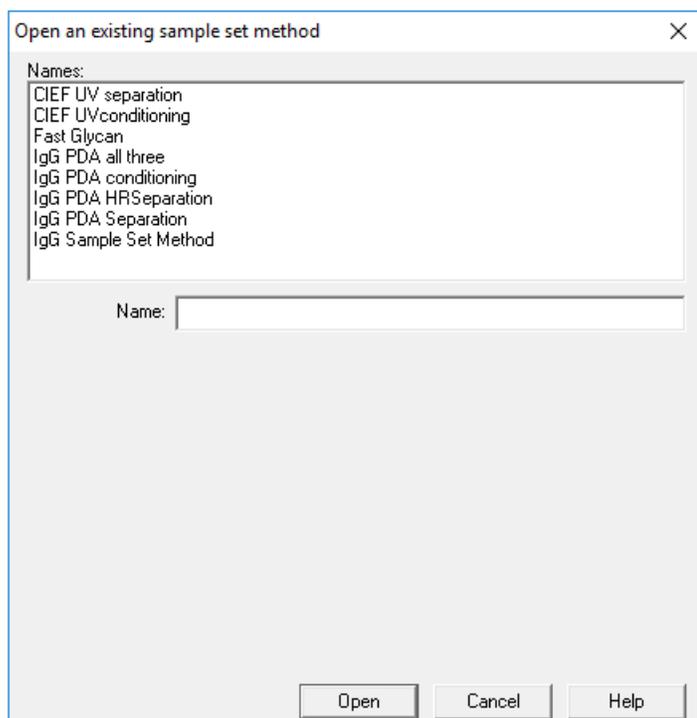
6. Click the system to be used and then click **OK**.
Make sure that the instrument is configured with a UV detector.
The Run Samples window opens.
7. Click  (**Load Sample Set**).

Figure E-15 Load Samples Dialog



8. Click **Load using a previously created sample set method** and then click **OK**.

Figure E-16 Open an existing sample set method Dialog



9. Click **cIEF Sample Set Method** in the list and then click **Open**.
The sample set method opens in the Samples tab.
10. In the Waters Empower™ Software Project window, click  (**Start**).
Data acquisition starts. During the run, the text in the row in the Sample Set Method window for the sample being acquired is red.
11. Do the following:
 - (Optional) Click  (**Stop**) to halt data acquisition.
 - View the voltage and current data.

When the run ends, the text in all rows in the Sample Set Method window is red.

Import the Instrument Methods

1. Open the **Methods** folder on the PA 800 Plus Empower™ Driver DVD.
2. In the Waters Empower™ Software Pro Interface window, click **Browse Projects**, click the project of interest, and then click **OK**.

Run the Samples with the Waters Empower™ Software

Figure E-17 Waters Empower™ Software Pro Interface Window



The Project window opens.

3. Open the Methods tab.
4. On the Windows desktop, click each min file in the `Methods` folder and then drag it to the Project window.
The instrument method is added to the project and can be edited and added to a method set like any other method.

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Documentation

This version of the document supersedes all previous versions of this document.

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To find software product documentation, refer to the release notes or software installation guide that comes with the software.

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