

# Capillary Isoelectric Focusing (cIEF) Analysis

For the PA 800 Plus Pharmaceutical Analysis System

*Application Guide*



RUO-IDV-05-5862-B  
December 2018

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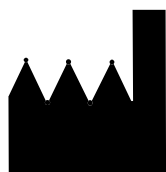
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# Capillary Isoelectric Focusing (cIEF) Analysis



## CAUTION

Prior to using the system, refer to the *System Overview Guide* for detailed information on the safe use and operation of the system.

## Safety

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Refer to the Safety Data Sheets (SDS), available at [sciex.com/tech-regulatory](https://www.sciex.com/tech-regulatory), regarding the proper handling of materials and reagents. Always follow standard laboratory safety guidelines.

## Introduction

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This guide provides instructions on how to separate proteins by their differences in isoelectric point (pI) using the PA 800 Plus Pharmaceutical Analysis System.

**NOTE** This application guide has been validated for use in the PA 800 Plus Pharmaceutical Analysis System.

**NOTE** The PA 800 series systems must be equipped with a UV detector and a 280 nm filter to perform this assay.

## 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 lower 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 higher than that of the ampholytes used with the sample.

**Cathodic Stabilizer** — A high-conductivity molecule that has a pI value higher than the ampholytes but below 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.

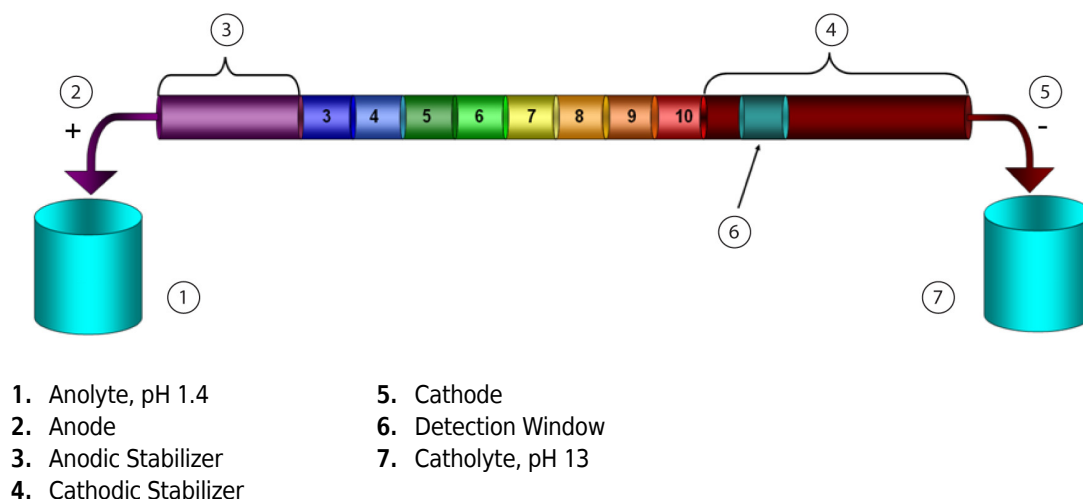
**Anodic Stabilizer** — A high-conductivity molecule that has a pI value lower than the ampholytes but above the pH of the anolyte. The anodic stabilizer is used to minimize distortions on the pH gradient at the anodic side, maximizing resolution while preventing the loss of sample into the anolyte vial.

## Principle of cIEF Separation

At the start of a cIEF separation, the entire capillary is filled with the sample. The cIEF sample is a mixture of ampholytes, stabilizers, pI markers, and the protein of interest.

A cIEF separation consists of two steps, focusing and mobilization. The system performs focusing by first submerging one capillary end in anolyte and the other in catholyte. Next, the system applies voltage across the capillary. A pH gradient forms during focusing through the introduction of hydronium ions from the anolyte and hydroxyl ions from the catholyte at opposite ends of the capillary ([Figure 1](#)). During focusing, 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 is used to fill the outlet side of the capillary, thereby forcing the ampholytes and protein sample to focus before the detection window.<sup>1</sup>

**Figure 1** pH Gradient Inside a Capillary



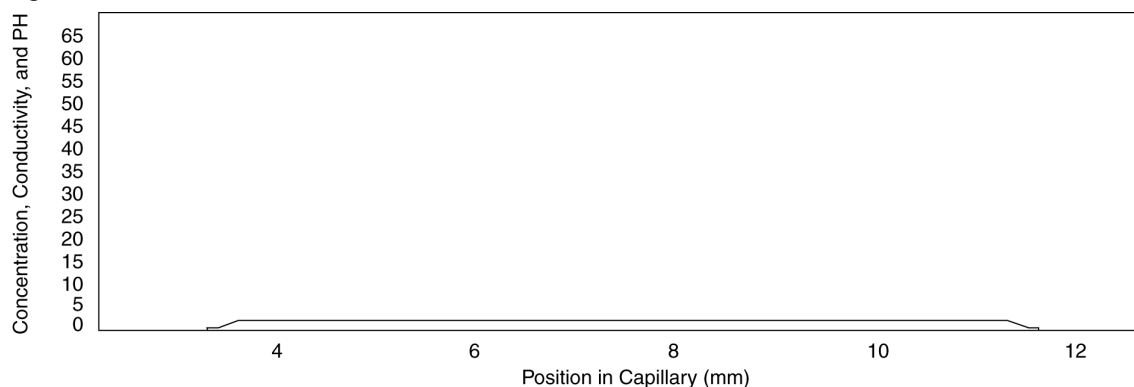
The mechanism of focusing is bi-directional.<sup>2</sup> The pH gradient forms at the capillary ends and then progresses toward the center of the capillary where both anodic and cathodic sides merge. Refer to [Figure 2](#), [Figure 3](#), [Figure 4](#), [Figure 5](#), and [Figure 6](#). With bi-directional focusing, sample peaks are often detected during focusing. Refer to [Figure 3](#) and [Figure 4](#). Detection of unmerged peaks during

mobilization indicate incomplete focusing of the pH gradient. Refer to [Figure 5](#). Focusing time needs to be sufficient to allow for complete formation of the pH gradient. Refer to [Figure 6](#).

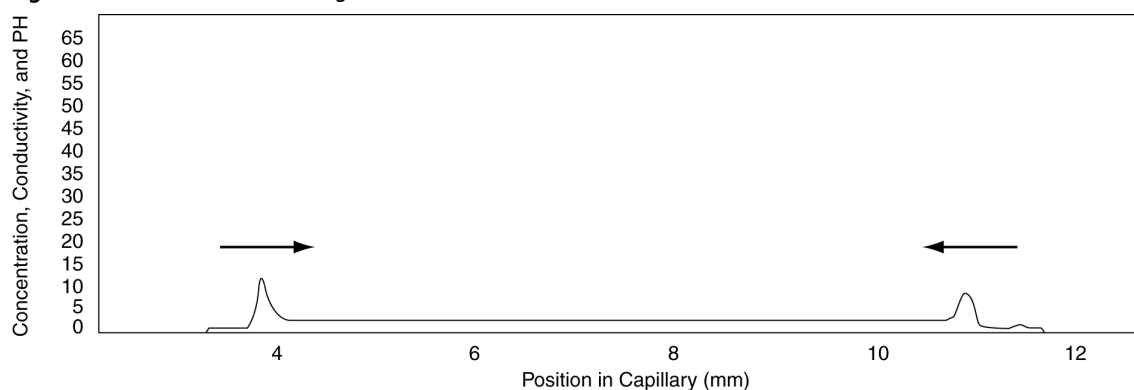
After formation of the pH gradient, mobilization takes place to detect the pI markers and the separated protein sample. Pressure, gravity, or chemical mobilization are methods used to mobilize the pH gradient across the detection window. Both pressure and gravity mobilization techniques create hydrodynamic flow inside the capillary. Hydrodynamic flow in turn causes band broadening. SCIEX recommends the use of acetic acid as a chemical mobilizer.<sup>3, 4</sup> 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 into 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.

Detection in cIEF is performed at 280 nm because ampholytes have low UV-absorbance at this wavelength. Narrow-range ampholytes can be used to maximize resolution.<sup>5</sup> Monoclonal antibodies have been separated with reproducibility by cIEF.<sup>6</sup>

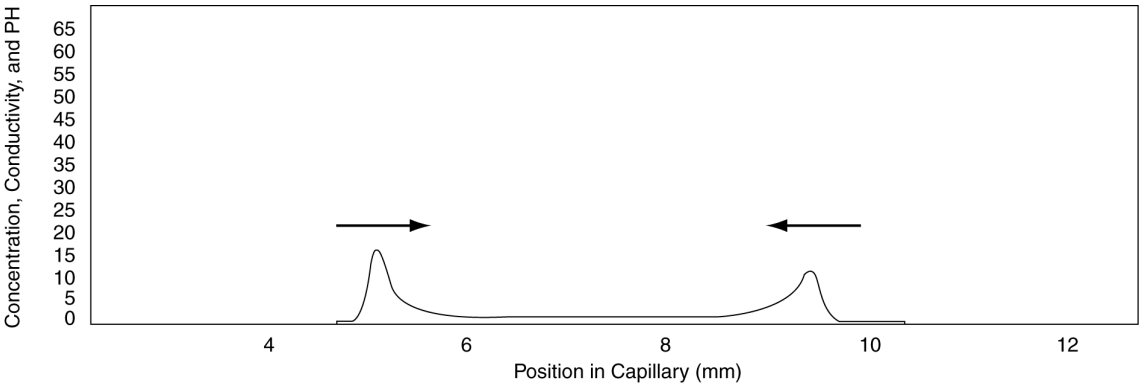
**Figure 2** Simulation of Focusing Mechanism at Time 0



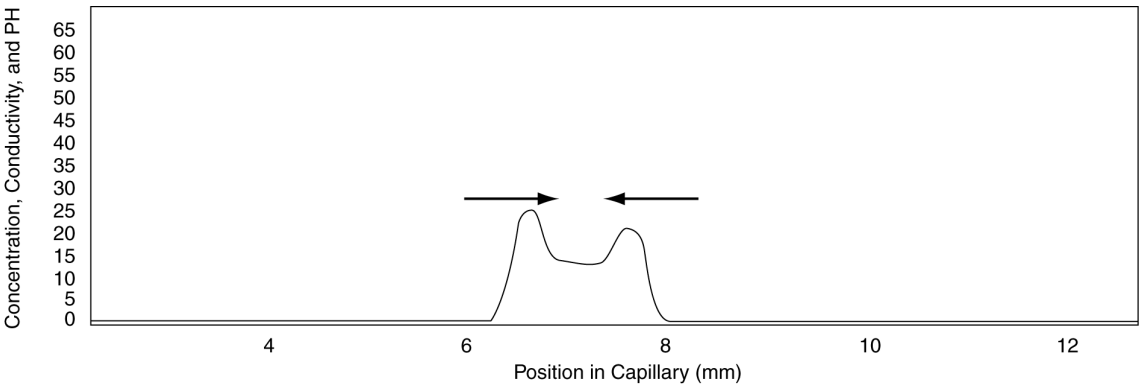
**Figure 3** Simulation of Focusing Mechanism at 30 Seconds



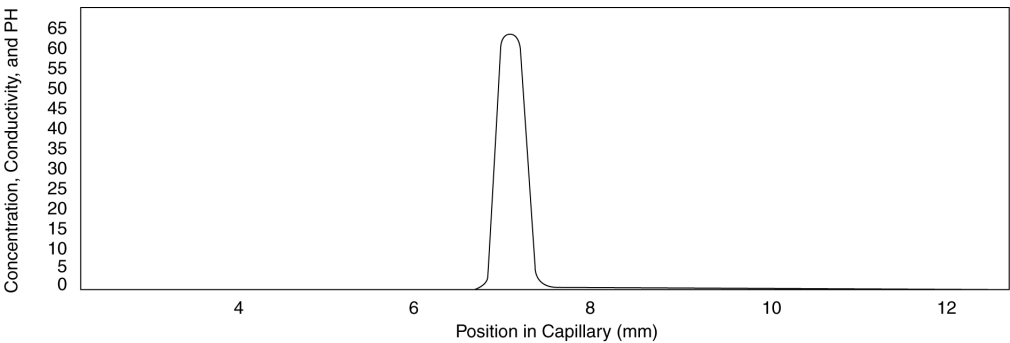
**Figure 4** Simulation of Focusing Mechanism at 2 Minutes



**Figure 5** Simulation of Focusing Mechanism at 4 Minutes



**Figure 6** Simulation of Focusing Mechanism at 6 Minutes



## Intended Use

Capillary Isoelectric Focusing (cIEF) analysis is for laboratory use only.



## Equipment and Materials Required

**Table 1** cIEF Reagents Available from SCIEX

Component	Quantity	Part Number
Neutral Capillary, 50 $\mu$ m i.d. x 45 cm	1	477441
cIEF Gel Polymer Solution	100 mL	477497
pI Marker Kit with peptide markers at pI 4.1, 5.5, 7.0, 9.5, and 10.0	1	A58481
(Optional) eCap 50 mM Tris Buffer at pH 8.0	1	477427
CEQ Sample Loading Solution (SLS)	6 mL	608082

**Table 2** Additional Supplies from SCIEX

Component	Quantity	Part Number
Universal vials	100	A62251
Universal vial caps	100	A62250
Filter, 280 nm (for use with UV detector)	1	144439
Micro vials, 200 $\mu$ L	50	144709

**Table 3** Additional Required Reagents

Reagent	Vendor	Part Number
Arginine	Sigma-Aldrich	A5006
Urea	Sigma-Aldrich	U0631
	GE Healthcare	17-1319-01
Iminodiacetic acid	Sigma-Aldrich	220000
	Spectrum	I-2045
Pharmalyte pH 3-10 carrier ampholytes	GE Healthcare	17-0456-01
Glacial acetic acid	Sigma-Aldrich	A6283
	Spectrum	AC110
Phosphoric acid, 85%	Sigma-Aldrich	345245
Sodium hydroxide, 1 M	Fisher	SS266-1
(Optional) USP IgG Vial	USP	1445550

## Customer-Supplied Equipment and Supplies

- Pipettors and appropriate tips
- Vortex mixer
- Microcentrifuge
- Double-deionized (DDI) water
- Analytical balance

- 5 µm-pore size membrane syringe filters (such as Pall Life Science PN 4199)
- 0.2 µm-pore size membrane syringe filters (such as Pall Life Science PN 4459)
- 10 mL disposable syringes (such as Becton-Dickinson PN 309604)
- Disposable 10 mL and 50 mL plastic Falcon tubes (or equivalent)
- Volumetric flasks (10 mL, 50 mL)
- 0.5 mL centrifuge tubes

## Storage Conditions

Upon receipt, store the neutral capillary and cIEF gel at 2 °C to 8 °C.

For long-term storage, keep the SLS and peptide pI markers frozen at –35 °C to –15 °C.

When in use, store the SLS and peptide pI markers at 2 °C to 8 °C. Store additional cIEF reagents as recommended by each supplier.

## Methods and Sequences



**The cIEF methods and sequence have been updated for robustness. The new methods and sequence are distributed with 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 the SCIEX website.**

Download the latest methods from the SCIEX website at [sciex.com/PA800Plus\\_Methods](https://sciex.com/PA800Plus_Methods).

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

As of publication, the sequence and the following methods are installed with the 32 Karat software and are available on the SCIEX website:

- **cIEF Sequence - PA 800 plus V2.seq** — The sequence.
- **cIEF Conditioning - PA 800 plus V2.met** — To condition the capillary at the start of each day.
- **cIEF Separation - PA 800 plus V2.met** — To perform a cIEF separation.
- **cIEF Shutdown - PA 800 plus V2.met** — To clean the capillary at the end of a sequence, to rinse the capillary for storage, and to turn off the UV lamp.

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## Prepare the Reagents

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- [Anolyte \(200 mM Phosphoric Acid\)](#)
- [Catholyte \(300 mM Sodium Hydroxide\)](#)
- [Chemical Mobilizer \(350 mM Acetic Acid\)](#)
- [Cathodic Stabilizer \(500 mM Arginine\)](#)
- [Anodic Stabilizer \(200 mM Iminodiacetic Acid\)](#)
- [3.75 M Urea-cIEF Gel](#)

### Anolyte (200 mM Phosphoric Acid)

---

- 1 Add 30 mL of DDI water to a clean 50 mL volumetric flask.
  - 2 Add 685  $\mu$ L of 85% phosphoric acid into the volumetric flask.
  - 3 Add DDI water to the volumetric flask so that the volume is 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 as **Anolyte** and record the preparation date.
  - 7 Store the anolyte at 2 °C to 8 °C for up to 14 days.
- 

### Catholyte (300 mM Sodium Hydroxide)

---

- 1 Add 30 mL of DDI water to a clean 50 mL volumetric flask.
  - 2 Add 15 mL of 1 M NaOH to the volumetric flask.
  - 3 Add DDI water to the volumetric flask so that the volume is 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 as **Catholyte** and record the preparation date.

---

**7** Store the catholyte at 2 °C to 8 °C for up to 14 days.

---

#### Chemical Mobilizer (350 mM Acetic Acid)

---

**1** Add 30 mL of DDI water to a clean 50 mL volumetric flask.

---

**2** Add 1.0 mL of glacial acetic acid into the volumetric flask.

---

**3** Add DDI water to the volumetric flask so that the volume is 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 as **Chemical Mobilizer** and record the preparation date.

---

**7** Store the chemical mobilizer at 2 °C to 8 °C for up to 14 days.

---

#### Cathodic Stabilizer (500 mM Arginine)

---

**1** Weigh 0.87 g of arginine using an analytical balance.

---

**2** Transfer the solid into a clean 10 mL volumetric flask.

---

**3** Add 8 mL of DDI water into the volumetric flask.

---

**4** Shake the flask until all solid material is dissolved.

---

**5** Add DDI water to the volumetric flask so that the volume is 10 mL.

---

**6** Transfer this solution to a 10 mL plastic conical tube.

---

---

**7** Label the tube as **Cathodic Stabilizer** and record the preparation date.

---

**8** Store the cathodic stabilizer at 2 °C to 8 °C for up to 14 days.

---

#### **Anodic Stabilizer (200 mM Iminodiacetic Acid)**

---

**1** Weigh 0.27 g of iminodiacetic acid using an analytical balance.

---

**2** Transfer the solid into a clean 10 mL volumetric flask.

---

**3** Add 8 mL of DDI water into the flask.

---

**4** Shake the flask until all solid is dissolved.

---

**5** Add DDI water to the volumetric flask so that the volume is 10 mL.

---

**6** Transfer this solution to a 10 mL plastic conical tube.

---

**7** Label the tube as **Anodic Stabilizer** and record the preparation date.

---

**8** Store the anodic stabilizer at room temperature for up to 30 days.

---

#### **3.75 M Urea-cIEF Gel**

---

**1** Weigh 2.252 g of urea and transfer it to a 10 mL volumetric flask.

---

**2** Add 7 mL of cIEF Gel to the volumetric flask.

---

**3** Vortex the flask for at least 15 minutes until all of the solid material dissolves.

---

**4** Add cIEF Gel to the volumetric flask so that the volume is 10 mL.

---

**5** Vortex the flask by inverting it three times.

---

- 6 Filter the solution through a 5.0  $\mu\text{m}$  membrane syringe filter using a 10 mL disposable plastic syringe and collect the filtered solution in a new 10 mL plastic conical tube.
  - 7 Label the tube as **3.75 M Urea-cIEF Gel** and record the preparation date.
  - 8 Store the 3.75 M Urea-cIEF Gel in the refrigerator between 2 °C and 8 °C for up to 7 days.
- 

## Prepare the UV Lamp

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Turn on the UV lamp and allow the system to warm up for at least 30 minutes.

## Clean the Interface Block

---

Carefully clean the system electrodes and the interface block as described in Chapter 2 of the *System Maintenance Guide*. Repeat this procedure at the start of each working day.

## Build and Install the Capillary Cartridge

---

- 1 Install a neutral capillary (50  $\mu\text{m}$  i.d., 30.2 cm long, 20 cm from injection site to detector) into a capillary cartridge using the *PA 800 Plus and P/ACE MDQ Plus Systems Capillary Cartridge Rebuild Instructions*.
  - 2 Install a 200  $\mu\text{m}$  aperture in the cartridge. This aperture is labeled with a **2**. Inspect the aperture for the signs of deterioration with a microscope or magnifying glass each time you replace a capillary; change if necessary.
  - 3 Install the cartridge into the PA 800 Plus System.
  - 4 Place 2 capped vials with 1.5 ml of DDI water at positions BI:A1 and BO:A1.
  - 5 Close the outer door.
-

**CAUTION**

**Potential System Damage.** Do not expose the neutral capillary ends to air for more than five minutes. After five minutes of exposure causes irreversible damage to the capillary coating. When the capillary is not in use, submerge the capillary ends in vials filled with DDI water.

## Prepare the Samples

**NOTE** The protein solution should not contain more than 50 mM of salt. [APPENDIX C](#) describes a procedure for buffer exchanging an IgG sample with a low salt buffer. SCIEX recommends the use of three pI markers close to the pI of the protein sample to verify the linearity of the pH gradient and to determine the pI value of the protein sample.

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

- 200 µL of 3.75 M Urea-cIEF Gel
- 12.0 µL of Pharmalyte 3-10 carrier ampholytes
- 20.0 µL of cathodic stabilizer
- 2.0 µL of anodic stabilizer
- 2.0 µL of each pI marker

## Best Practices When Preparing Multiple Samples

When analyzing multiple samples, SCIEX recommends preparing a master mix, to simplify sample preparation and minimize pipetting errors. [Table 4](#) provides the amounts required to prepare a master mix when running multiple samples using Pharmalyte 3-10 carrier ampholytes.

Start by entering the number of samples to be prepared in the table. Add one to the number of samples, then multiply each reagent volume by that number of samples, and record the result. As needed, add or remove pI markers (from those provided in the pI Marker Kit).

- 1 Prepare the master mix. Pipet each calculated reagent volume from [Table 4](#) into a centrifuge tube.

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

Reagent	Volume per Sample (µL)	Number of Samples	Total Volume to be Measured (µL)
3.75 M Urea-cIEF Gel	200	x ____ + 1 =	
Pharmalyte 3-10 Carrier Ampholytes	12	x ____ + 1 =	
Cathodic Stabilizer	20	x ____ + 1 =	
Anodic Stabilizer	2	x ____ + 1 =	
pI marker A	2	x ____ + 1 =	

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

Reagent	Volume per Sample (μL)	Number of Samples	Total Volume to be Measured (μL)
pI marker B	2	x ____ + 1 =	
pI marker C	2	x ____ + 1 =	

- 2 Vortex the master mix for 15 seconds to mix thoroughly, and then spin down. Store the master mix at 2 °C to 8 °C and discard at the end of the day.
- 3 Mix 240 μL of master mix with a volume of sample containing 50 μg to 100 μg of protein in a volume no greater than 10 μL.
- 4 Vortex the cIEF sample (master mix with protein) for 30 seconds.
- 5 Transfer 200 μL of the cIEF sample into a micro vial and then centrifuge the vial in a microcentrifuge for 20 seconds at low speed to remove any air bubbles.
- 6 Put the micro vial into a universal vial and cap it with a blue cap.
- 7 Put the sample vial in the inlet sample tray.

## Set Up the System



**DANGER! Toxic Chemical Hazard. Read the *Safety Data Sheet* for CEQ Sample Loading Solution (SLS) prior to use.**

Refer to [APPENDIX A](#) for additional information.

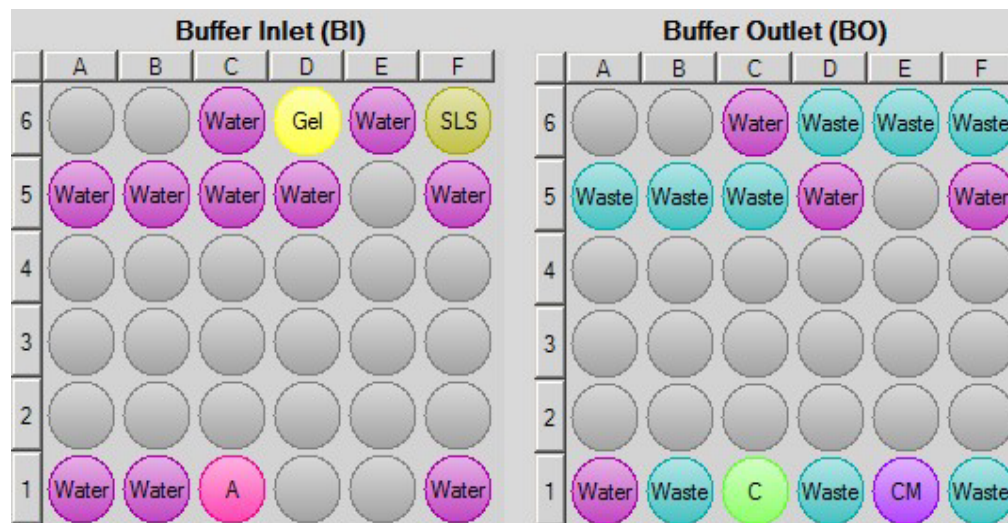


## Prepare the Buffer Trays

- 1 Add 1.5 mL of reagent per vial and cap each vial with a blue cap. Refer to [Figure 7](#).

**NOTE** In the following figure, rows 5 and 6 contain the reagent vials for the capillary conditioning and shutdown methods. Row 1 contains the reagent vials for the separation. The vials contain sufficient reagents for ten runs. For 11 to 20 runs (20 is the maximum number of runs allowed), duplicate the reagent vials in row 1 in row 2.

**Figure 7** Buffer Tray Map for cIEF Analysis for 10 Samples




- |                                      |                            |
|--------------------------------------|----------------------------|
| 1. Water - DDI Water                 | 5. Waste                   |
| 2. A - Analyte                       | 6. C - Catholyte           |
| 3. SLS - CEQ Sample Loading Solution | 7. CM - Chemical Mobilizer |
| 4. Gel - cIEF Gel                    |                            |

- 2 Fill the vials with 1.0 mL DDI water. Cap and place vials in the buffer tray in all the Waste positions as shown in [Figure 7](#).
- 3 Put the inlet and outlet buffer trays inside the instrument.
- 4 When sample preparation is complete, put the cIEF samples on the inlet sample tray.

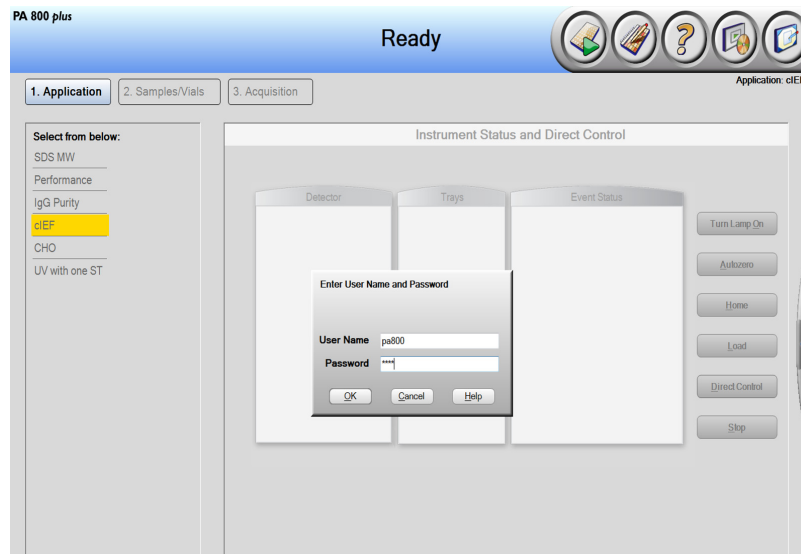
## Create the Sequence and Start the Run

- 1 Double-click the PA 800 Plus software icon on the desktop.

- 2 In the PA 800 Plus window, click  (Run) in the upper right corner of the window.
- 3 In the **Application** list, click **cIEF**. In the **Sequence** list, click **Browse** and then browse to **cIEF Sequence - PA 800 plus V2**.

If system administration is enabled, type the user name and the password when prompted, and then click **OK** (Figure 8). The default user name is **pa800**, and the default password is **plus**.





**Figure 8** Instrument Status and Direct Control Window: Ready



The Instrument Status and Direct Control window appears (Figure 9).

**Figure 9** Instrument Status and Direct Control Window: Idle



- 4 In the **Instrument Status and Direct Control** window, 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 In the **Application** list, click **cIEF**, click **Browse** and then browse to **cIEF Sequence - PA 800 plus V2**.  
If prompted, type a user name and password.  
The page updates to show the sequence, with all rows in the sequence designated as samples.
- 7 (Optional) Edit the **Sample ID** and the **Data File Name** as desired.
- 8 Set the type for the first and last rows in the sequence. The first row is for the capillary conditioning method and the last row is for the shutdown method.
  - a. Click the first row (with the conditioning method) to select it and then click  (Always) in the **Rows** area.
  - b. Click the last row (with the shutdown method) to select it and then click  (Always) in the **Rows** area.
 The icon in the Type column in the first and last rows in the sequence is now a triangle.

**Figure 10** Describe sequence rows and columns Window – Conditioning Method Set to “Always”

**Describe sequence rows and columns**

Application:


Sequence:

Rows: ☒ Sample ☐ Control ☒ Always

Columns: ☐ Optional ☒ Required ☐ Fixed

Verification:

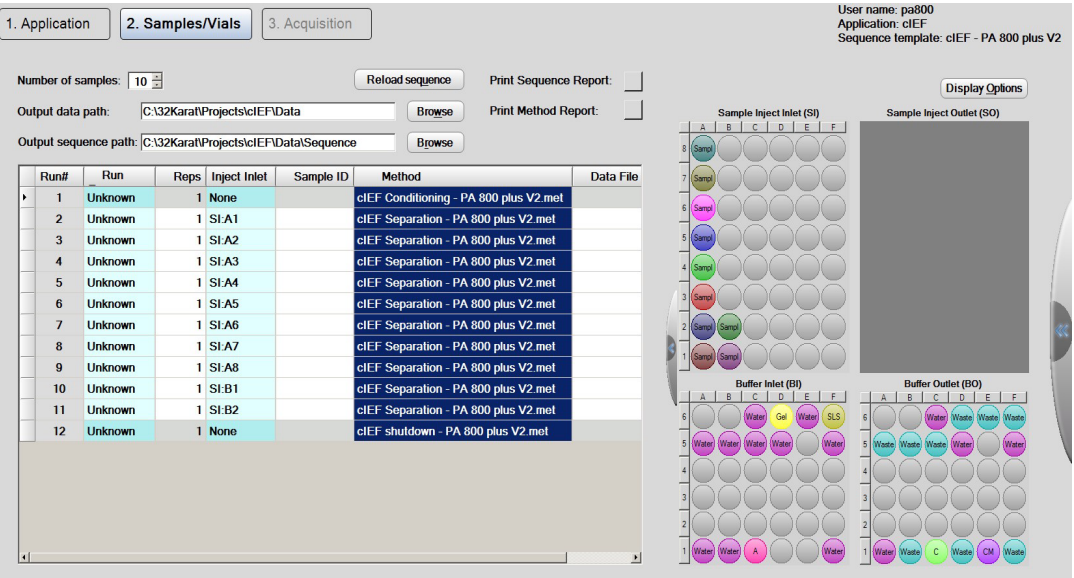
Run#	Type	Run Type	Reps	Inject Inlet	Sample ID	Method
1	▲	Unknown	1	None		cIEF Conditioning ...
2	●	Unknown	1	SI:A1		cIEF Separation - ...
3	●	Unknown	1	SI:A2		cIEF Separation - ...
4	●	Unknown	1	SI:A3		cIEF Separation - ...
5	●	Unknown	1	SI:A4		cIEF Separation - ...
6	●	Unknown	1	SI:A5		cIEF Separation - ...
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...

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

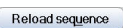
**NOTE** Some columns can be set as Optional, Required, or Fixed. In [Figure 10](#), the Sample ID column is Optional indicating that an ID is not required.

10 In the lower right corner of the window, click  (Save) and then click  (Finish).


**Figure 11** Describe sequence rows and columns Window – Reload Sequence



**NOTE** In the upper left corner next to Run #1 of the table shown in [Figure 11](#), a blinking exclamation mark (not shown) will indicate that the sequence has changed indicating that the software expects an action from the user. The required action can be accessed by hovering the mouse over the exclamation mark.

11 Click  (Reload sequence). This sequence table updates to display the appropriate number of runs. Newly described sequences will be saved on a data path with a time stamp.

12 In the **Output data path** field, click **Browse** and select the location where the data will be saved.

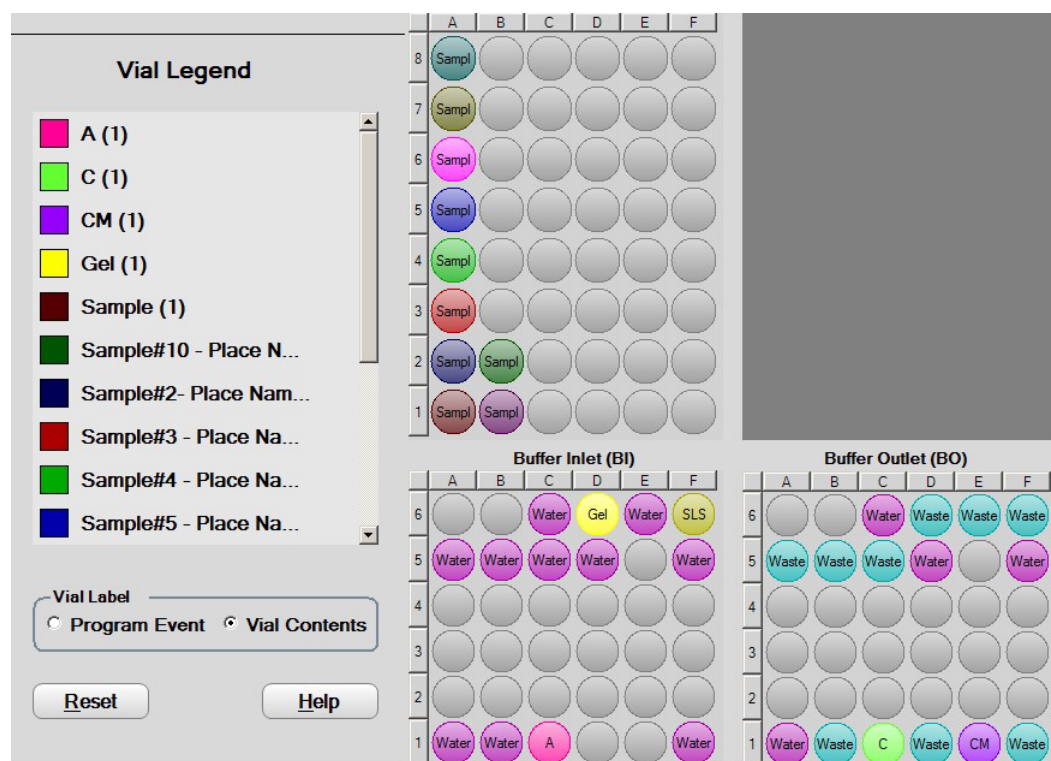
13 In the **Number of samples** field , click the arrow buttons to set the number of samples for the run.

When the number of samples changes, the images of the buffer and sample trays on the right ([Figure 11](#)) update to show the number of vials and their locations.


(Optional) Increase the number of replicates as desired.

**NOTE** The reagent tray map updates with the quantity of reagents needed in order to accommodate the number of replicates. SCIEX does not recommend running more than 20 runs per sequence, including replicates.

**Figure 12** Samples/Vials Window



**14** (Optional) To view the contents of each vial, open the tray and click on the **Vial Content** radio button.

**15** If the buffer and sample trays have not been loaded, click  (Load), put the trays in the PA 800 Plus system, and then close the door.

**16** Click  (Next) and then click **Yes - run now.**

## Store the Capillary

---

At the end of the sequence run:

- For short-term storage (less than 24 hours): leave the cartridge in the system. If you wish to take the cartridge out of the instrument, then put the capillary cartridge in the storage box and put two vials containing 1.5 mL of DDI water at either of the exposed ends of the capillary.
- For long-term storage (more than 24 hours): put the capillary cartridge in the original storage box with both ends immersed in the cIEF gel, and then place the box vertically inside a refrigerator (2 °C to 8 °C) for at least 18 hours before being used in cIEF again. The capillary run-life will be decreased significantly if this procedure is not followed. Do not let the capillary ends dry out; otherwise, the capillary may become plugged or the coating might be permanently damaged.

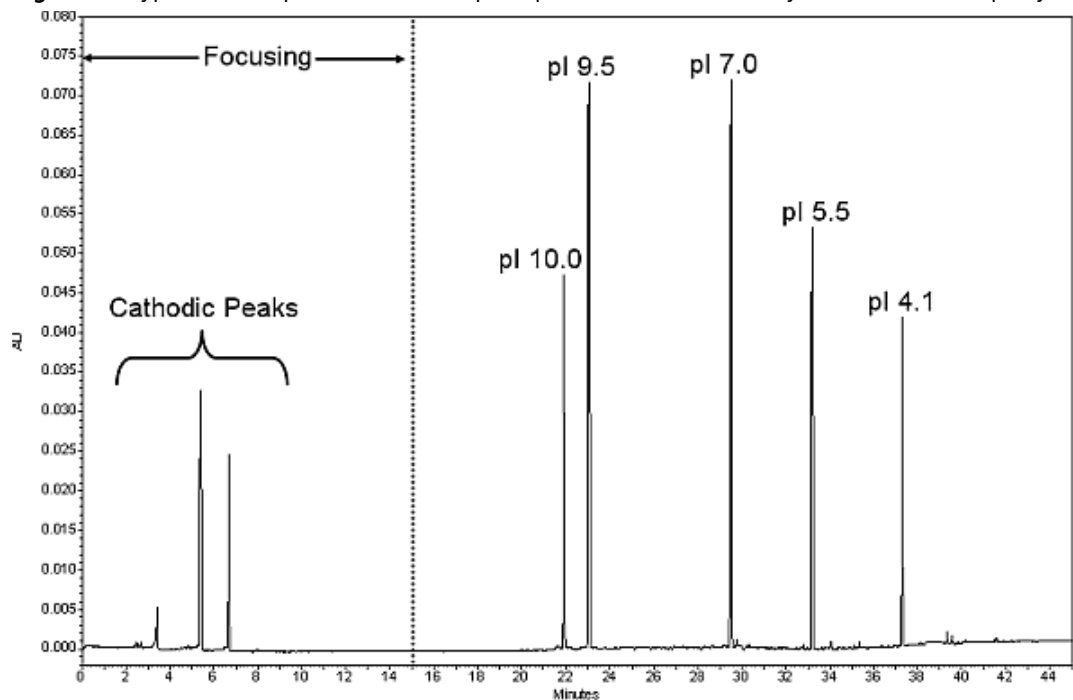
At the start of the next cIEF testing day, condition the capillary using the capillary conditioning method.

**NOTE** Do not share capillaries between applications. If the capillary has been used for cIEF analysis, do not use it with another application.

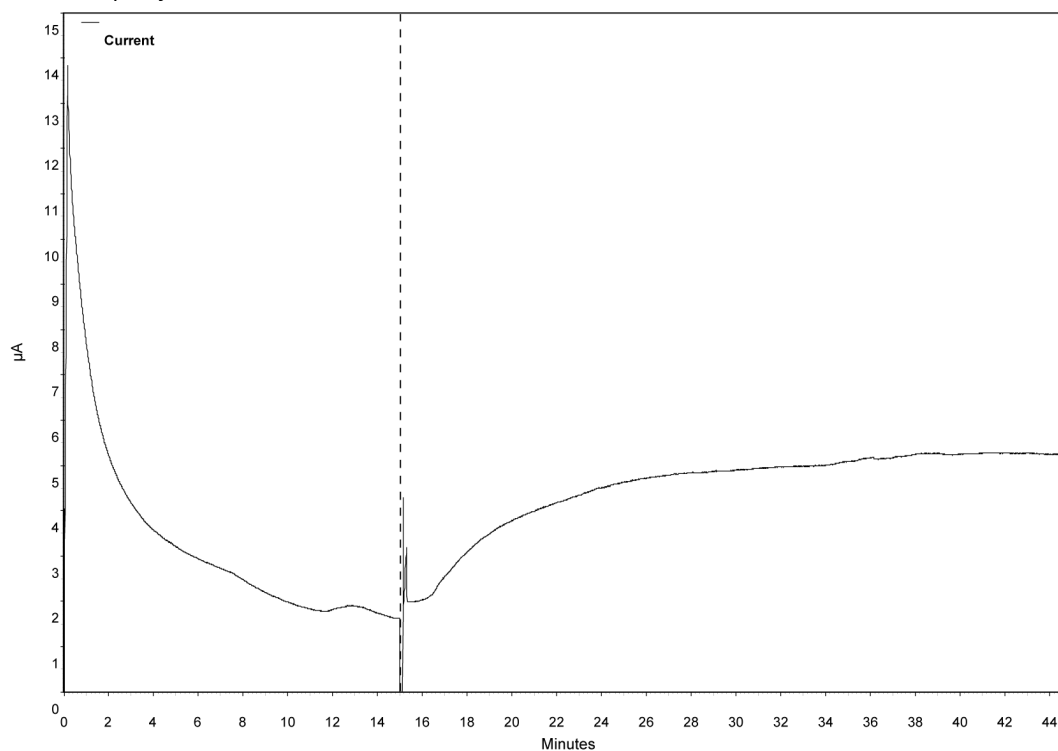
## Check System Performance with Peptide pI Markers and Pharmalyte 3-10 Carrier Ampholytes

---

To check the performance of the PA 800 Plus System, perform a cIEF separation of the five peptide markers. Compare the electropherogram obtained with the one in [Figure 13](#). The electrical current should be similar to [Figure 14](#).

**Figure 13** Typical cIEF Separation of Five Peptide pI Markers with Pharmalyte 3-10 Carrier Ampholytes

**NOTE** The cathodic peaks observed in the electropherogram are due to the bi-directional migration of the sample and ampholytes that occurs during focusing. Refer to [Figure 2](#). The absence of cathodic peaks can indicate incomplete focusing.

**Figure 14** Typical Electrical Current Profile of cIEF Separation of Peptide pI Markers with Pharmalyte 3-10 Carrier Ampholytes



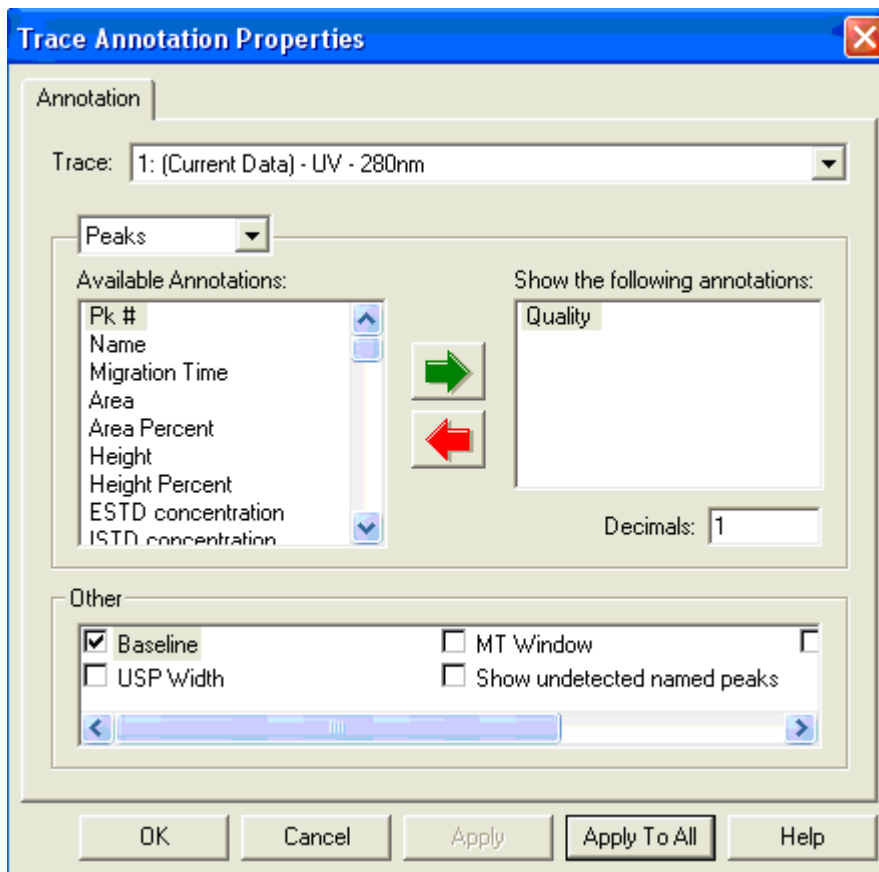
The vertical dashed line shown in [Figure 13](#) and [Figure 14](#) separates the focusing data from the mobilization data. Focusing data is very helpful in troubleshooting cIEF separations. For example, variations in the initial electrical current value at the start of focusing can indicate problems in pipetting or in the preparation of the cIEF reagents.

The separation method using 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. In order to pass system suitability, the five markers must be detected during the mobilization step (15 minutes to 45 minutes). If it fails, refer to the [Troubleshooting](#) section in this guide. Refer to [APPENDIX B](#) for more information regarding the System Suitability method in the cIEF instrument.

## Optimize Integration Parameters

- 1 Right-click an electropherogram and select **Annotation**. From **Available Annotations**, click **Migration Time** and then click the green arrow shown in [Figure 15](#) to move Migration Time to the **Show the following annotations** pane on the right.

**Figure 15** Trace Annotation Properties Dialog



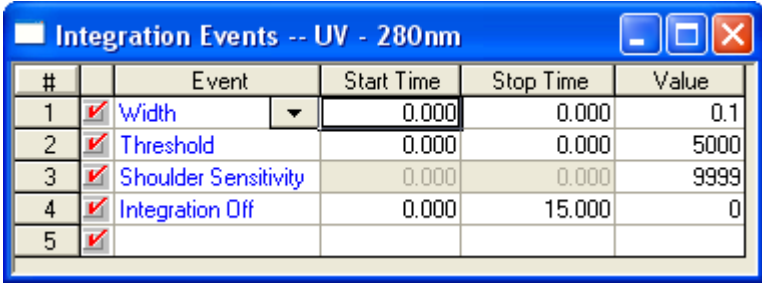


- Click **OK** to save the changes in the Trace Annotation Properties dialog.

SCIEX recommends optimizing the integration parameters in the analysis method for each sample. As a starting point, use the integration values in [Figure 16](#). These integration parameters are for the cIEF separation of the peptide pI markers:

- **Width** = 0.1
- **Shoulder Sensitivity** = 9999
- **Threshold** = 5000
- **Integration Off** from 0 minutes to 15 minutes (during focusing)

**Figure 16** Recommended Integration Parameters and Initial Values



#		Event	Start Time	Stop Time	Value
1	<input checked="" type="checkbox"/>	Width	0.000	0.000	0.1
2	<input checked="" type="checkbox"/>	Threshold	0.000	0.000	5000
3	<input checked="" type="checkbox"/>	Shoulder Sensitivity	0.000	0.000	9999
4	<input checked="" type="checkbox"/>	Integration Off	0.000	15.000	0
5	<input checked="" type="checkbox"/>				

**Width** sets the sensitivity of the peak detection in regards to changes in the baseline.

**Threshold** determines how high a peak must rise above the baseline noise before it is recognized as a peak.

**Shoulder Sensitivity** enables the detection of shoulders in large peaks. The value specifies the slope value for splitting a peak.

**Integration Off** sets the time interval in the electropherogram that is not integrated.

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.

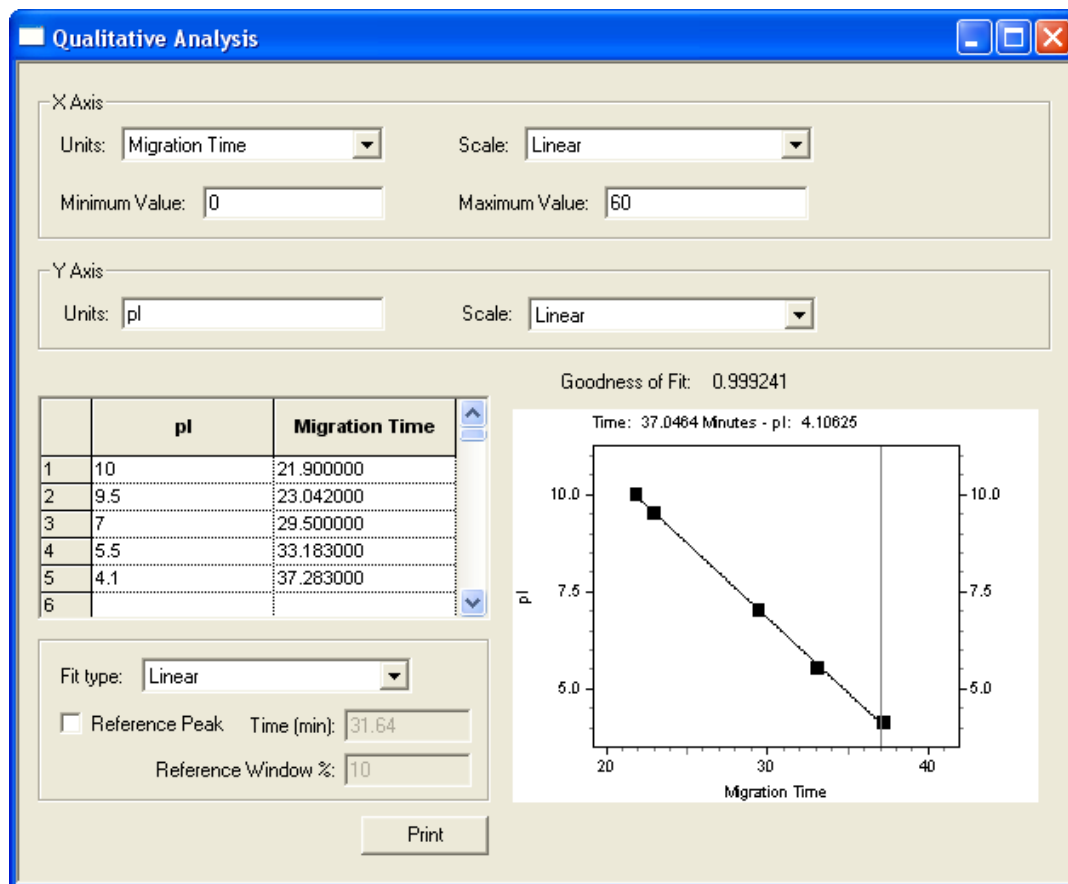
## Determine the pI Value

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

- Click **Method > Qualitative Analysis** to open the Qualitative Analysis window.

- 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. Refer to [Figure 17](#).

**Figure 17** Qualitative Analysis Window for pI Determination

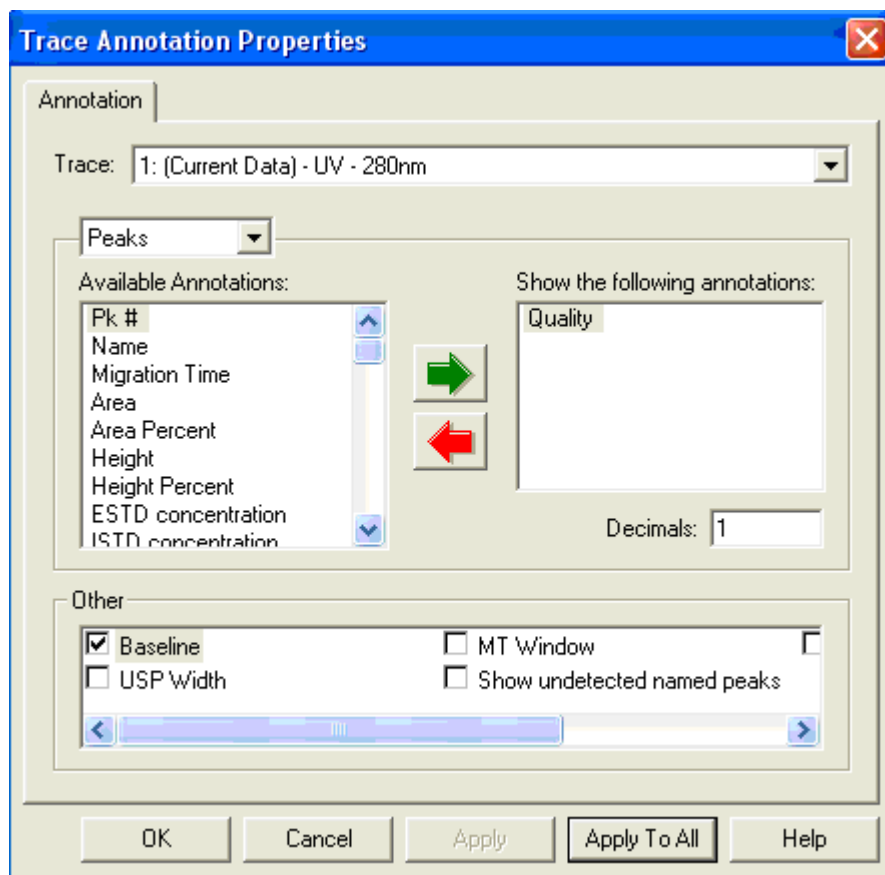


- 3 Click **File > Method > Save** to save the method.

- 4 Click **Analysis > Analyze**.

- 5 To open the calculated pI values in the cIEF separation (UV trace), right-click inside the UV trace and select **Annotations**. In **Available Annotations**, click **Quality** and then click **Add**. Refer to [Figure 18](#). In this analysis, quality corresponds to calculated pI value.

**Figure 18** Trace Annotation Properties Dialog



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

## Perform a cIEF Separation of the USP Monoclonal IgG Reference Standard

Upon receipt, open the USP IgG vial (PN 1445550) and add 400  $\mu$ L of DDI water to the lyophilized material. Mix vial contents until the solution is clear. To avoid multiple freeze-thaw cycles, make 10  $\mu$ L aliquots (equivalent to 5  $\mu$ g) and store at  $-35^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ . Record the preparation date for reference.

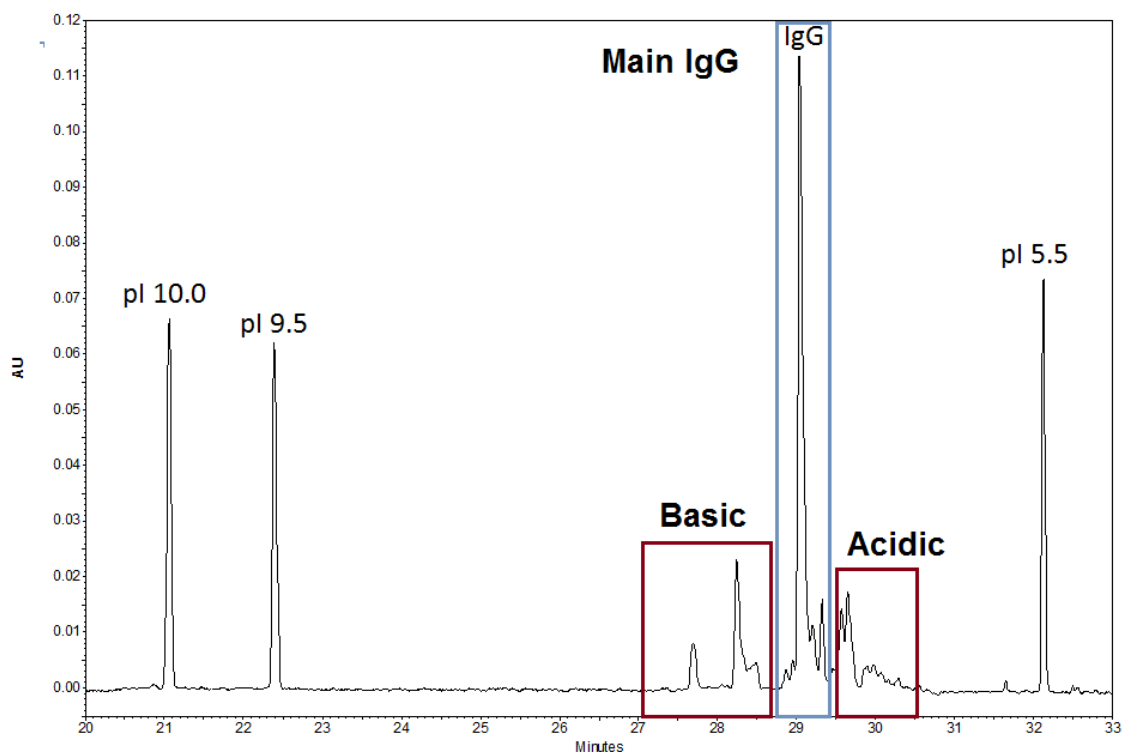
**NOTE** This IgG sample does not require buffer exchange due to its low salt content.

Prepare the cIEF sample by mixing 240  $\mu\text{L}$  of Master Mix with 10  $\mu\text{L}$  of reconstituted USP IgG in a 0.5 mL centrifuge tube, vortexing it for 15 seconds, and then centrifuging it for 10 seconds.

Figure 19 shows a close up of the cIEF separation of USP IgG Reference with Pharmalyte 3-10 carrier ampholytes and peptide pI markers 10.0, 9.5, and 5.5. In Figure 19, focusing occurred from 0 minutes to 15 minutes, and mobilization was from 15 minutes to 40 minutes.

**NOTE** The cIEF separation profile of an IgG sample may vary due to changes in glycosylation and other post-translational modifications as well as ampholyte lot<sup>7</sup> and manufacturer. To simplify peak integration, SCIEX recommends 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), and Acidic (all IgG peaks at the right side of the main peak, which are more acidic than the main peak).

**Figure 19** cIEF Separation of USP Monoclonal IgG Reference Standard



## Troubleshooting

**Table 5** Troubleshooting

Problem	Possible Cause	Corrective Action
Electrical current at the start of the focusing step is changing between replicate runs.	Sample was not completely mixed.	Prepare new sample and repeat the analysis.
	Capillary coating has degraded and the electroosmotic flow (EOF) is significant.	Replace the capillary.
No peaks	Incorrect polarity in the method.	Use normal polarity in the method.
	No sample vial or sample at the incorrect location.	Check the sample vial position.
	The lamp is off.	Turn on the lamp.
	Buffer vials are at the incorrect location.	Check the vial positions as indicated in the method.
	Sample has high salt concentration.	Buffer exchange the sample so that it is below a salt concentration of 50 mM.
	Capillary window is not centered on the aperture.	Readjust the capillary window inside the cartridge. Make sure that light passes through both the aperture and capillary window by shining a flashlight on the back of the aperture.
	UV fiber optic of the UV detector is loose.	Tighten both ends of the fiber optic.
No current	The capillary is broken.	Replace the capillary.
	Electrode is broken or bent.	Replace the electrode.
	The capillary is plugged.	Replace the capillary.
	Buffer vials are at the incorrect position.	Check the vial positions as indicated in the method.
	The capillary is filled with air.	Fill the sample vial with 200 $\mu$ L of cIEF sample. Make sure that all of the buffer vials have 1.5 mL of reagent.
Low Resolution	The protein profile appears as a single broad peak.	Protein is precipitating or aggregating. Increase the urea content in the cIEF sample.
Loss of resolution	Urea-cIEF gel solution has high conductivity due to thermal degradation.	Prepare a new Urea-cIEF gel solution. Store this solution at 2 °C to 8 °C to prevent thermal degradation.
Missing peaks	Pipetting error during sample or master mix preparation.	Prepare a new cIEF sample or master mix.
Peak profile changes between consecutive runs	Incomplete focusing.	Increase focusing time.
	Protein is precipitating or aggregating.	Increase urea concentration in the sample and focusing time.
	Protein is denatured.	Try cIEF separation without urea in the sample.



# Hazardous Substance Information

The following information must be noted and the relevant safety measures taken. Refer to the respective safety data sheets for more information. These are available upon request or can be downloaded from our website, [sciex.com](https://www.sciex.com).

Hazard classification according to HCS 2012.

## Hazardous Substances

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### CEQ Sample Loading Solution (SLS)



---

**DANGER!** May damage fertility or the unborn child.

---

### Citrate Buffer

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**WARNING!** May be harmful if swallowed.

---

### Citrate/MES Buffer

---

**WARNING!** May be harmful if swallowed. Causes mild skin irritation.

---

## Histamine Reference Marker



**DANGER!** Causes mild skin irritation. May cause an allergic skin reaction. May cause allergy or asthma symptoms or breathing difficulties if inhaled.

---

## Protein Test Mix



**DANGER!** May cause allergy or asthma symptoms or breathing difficulties if inhaled.

---

## Other Reagents

---

These components are not classified as hazardous:

- cIEF Gel Polymer Solution
- cIEF Peptide Markers
- cIEF Gel Polymer Solution
- eCAP 50 mM Tris Buffer at pH 8.0

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



# System Suitability Method

## System Suitability Method Overview

---

A System Suitability method can be used to determine if 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, instrumentation settings, chemistries, and environment to perform the analysis.

## Activation of System Suitability

---

System Suitability must be activated in the cIEF instrument to use this feature.

- 1 Start by closing all the PA 800 software windows except for the 32 Karat software main window.
- 2 In the 32 Karat software main window, click **Tools > Enterprise Login**.
- 3 Enter 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 click **Configure > Instrument**.
- 5 Click **Configure**. The PA 800 System Configuration dialog opens.
- 6 Click **Options**.
- 7 On the General tab, click **System Suitability**, **Qualitative Analysis**, and **Caesar Integration**.

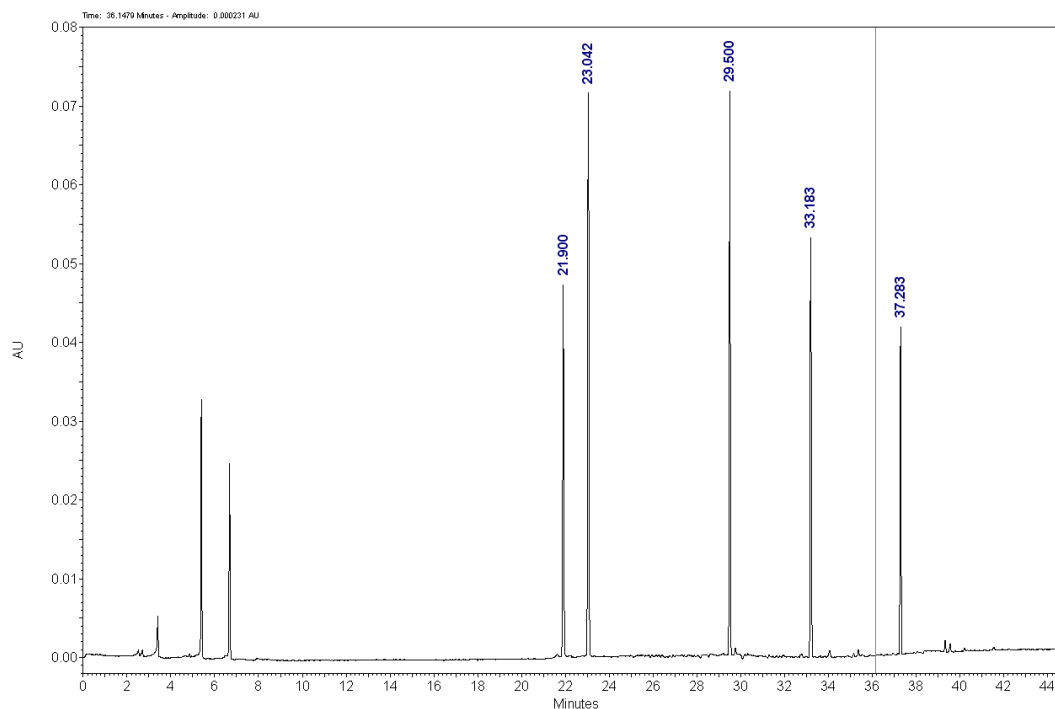
- 8 Click **OK** in the next three dialogs.

## Making a cIEF System Suitability Method

**NOTE** This example uses the separation of the five cIEF Peptide Markers. Refer to [Figure B.1](#).

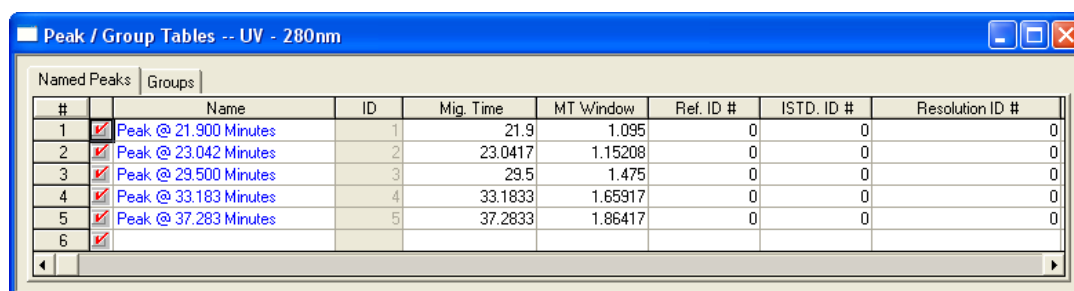
- 1 Double-click the **cIEF** icon (found on the Enterprise window in the 32 Karat software) to open the cIEF instrument.
- 2 Open the cIEF method that will be converted into a System Suitability method, and save it as **System Suitability**.
- 3 Click **File > Data > Open**. Browse to the cIEF Examples folder and select **Marker-1**, then go to **Options** (bottom left corner of the Open Data File dialog) and from the Method menu, select **Current**.
- 4 Open the system suitability data.

**Figure B.1** Separation with Five cIEF Peptide Markers



- 5 For instructions on data integration including how to display the migration times on the open data, refer to [Optimize Integration Parameters](#).
- 6 To enter the integrated peaks on the method peak table, right-click inside the UV trace and click **Graphical Programming > Define Peaks**.
- 7 Select the beginning of the marker peaks and then select the end of the marker peaks.  
In this example, select at 20 minutes and then at 39 minutes to include the five peptide markers.
- 8 Click **Method > Peaks/Groups** to open the peak table.  
In this example, the table containing the Named Peaks is shown. Refer to [Figure B.2](#). Peaks are labeled according to their migration time.

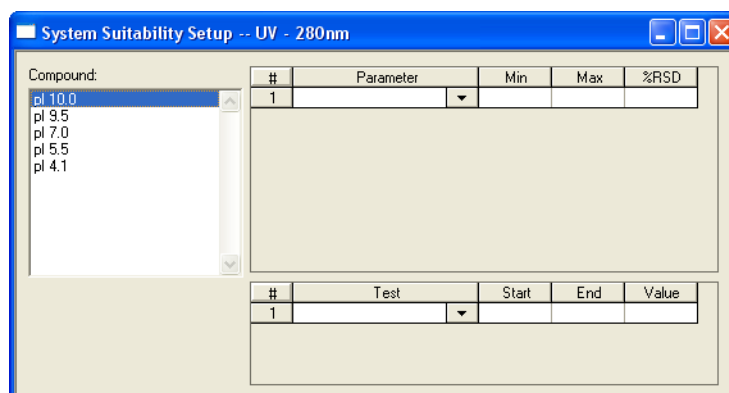
Figure B.2 Named Peaks Table



Peak / Group Tables -- UV - 280nm								
Named Peaks		Groups						
#		Name	ID	Mig. Time	MT Window	Ref. ID #	ISTD. ID #	Resolution ID #
1	<input checked="" type="checkbox"/>	Peak @ 21.900 Minutes	1	21.9	1.095	0	0	0
2	<input checked="" type="checkbox"/>	Peak @ 23.042 Minutes	2	23.0417	1.15208	0	0	0
3	<input checked="" type="checkbox"/>	Peak @ 29.500 Minutes	3	29.5	1.475	0	0	0
4	<input checked="" type="checkbox"/>	Peak @ 33.183 Minutes	4	33.1833	1.65917	0	0	0
5	<input checked="" type="checkbox"/>	Peak @ 37.283 Minutes	5	37.2833	1.86417	0	0	0
6	<input checked="" type="checkbox"/>							

- 9 (Optional) Rename the peaks in the table. For example, name the marker peaks after their corresponding pI value.
- 10 Click **Method > System Suitability** to open the System Suitability window. Refer to [Figure B.3](#).

Figure B.3 System Suitability Window



System Suitability Setup -- UV - 280nm				
Compound:				
#	Parameter	Min	Max	%RSD
1				

#	Test	Start	End	Value
1				

- 11 Click the parameter 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.
- 12 Click **Method > Qualitative Analysis** to open the Qualitative Analysis window. Refer to [Figure 17](#).  
In the Qualitative Analysis table, enter the theoretical pI values of the markers detected during the mobilization step with their corresponding Migration Time in minutes. Refer to [Figure 17](#).
- 13 Click **File > Method > Save Method** to save the method.

## Generating 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 the Migration time of the data file selected here is entered manually in the [Qualitative Analysis Window for pI Determination](#).
- 4 In row 1 of the sequence, right-click the **Row Number** column and click **Run Types > System Suitability**.
- 5 Both **Begin System Suitability** and **End System Suitability** will be selected (SSB SSE).  
**NOTE** The default System Suitability report template is *SysSuit.brp*.
- 6 Click **OK** to close the dialog.
- 7 Click **File > Sequence > Save Sequence** to save the sequence.
- 8 Click **Sequence > Process**. The Process Sequence dialog opens.
- 9 The open sequence will display in **sequence name**.

**10** Use **Run Range All** to specify the row for analysis.

**11** To print the results, select the options under **Printing**.

**12** For the Processing Mode, click **Reintegrate**. To see the result after reintegration, click **Review**.

**13** Click **Start** to reintegrate the data.

The row will be labeled as **Complete** in the Status column after successful analysis.

**14** Click **Reports > View > Sequence Custom Reports** to view the System Suitability report.

**15** Click **System Suitability > View** to open the report.

**NOTE** Refer to the Help file in the 32 Karat software for additional information on setting and performing System Suitability.



# Buffer Exchange

## Buffer Exchange Overview

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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 capillary coating damage. To reduce the negative effects that sample buffer components can have on cIEF separations, performing a buffer exchange prior to analysis is highly recommended.

## Buffer Exchange Procedure

---

- 1 Prepare the Exchange Buffer; the 20 mM Tris buffer replacement solution is prepared by diluting 4 mL of eCap 50 mM Tris buffer at pH 8.0 in 6 mL of DDI water.
- 2 Load 500  $\mu$ L of the protein to be desalted (5 mg/mL to 10 mg/mL) into a concentrator device (Microcon Ultracell YM 10; Millipore PN A11530).
- 3 Centrifuge for 5 minutes at 12 000 *g*.
- 4 Add 250  $\mu$ L of 20 mM Tris buffer to the retentate. Discard the permeate.
- 5 Centrifuge for 10 minutes at 12 000 *g*.
- 6 Repeat the centrifugation and buffer replacement cycles twice.
- 7 Retrieve the retentate (which contains the desalted antibody) by inverting the concentrator into a clean microcentrifuge tube. Centrifuge for 3 minutes at 2000 *g*.


---

**8** Determine the protein concentration by direct absorbance at 280 nm.

---

**9** Prepare aliquots of 5 µg and store at -35 °C to -15 °C or below for up to 3 months.

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# References

## Publications

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**NOTE** Please search online for additional recent publications.

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



The cIEF analysis requires three methods. The methods are installed with the software and can be downloaded from the SCIEX web site. The following figures are provided for reference.

## Initial Conditions

The Initial Conditions and the UV Detector Initial Conditions are the same for all of the methods.

**Figure E.1** Initial Conditions for All Methods

The screenshot shows the 'Initial Conditions' dialog box with the following settings:

- Auxiliary data channels:**
  - ☐ Voltage max: 30.0 kV
  - ☒ Current max: 20.0  $\mu$ 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 E.2** UV Detector Initial Conditions for All Methods

Initial Conditions | UV Detector Initial Conditions | Time Program

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

## Time Programs

Each of the three methods has a different time program.

**Figure E.3** Time Program for cIEF Conditioning-PA 800 plus V2 Method

	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								

**Figure E.4** Time Program for cIEF Separation-PA 800 plus V2 Method

	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								

**Figure E.5** Time Program for cIEF Shutdown-PA 800 plus V2 Method

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	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								



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# Revision History

**Initial Issue, A78788AA, April 2009**

32 Karat Software version 9.1

PA 800 *plus* Software version 1.1

PA 800 *plus* Firmware version 9.0

**First Revision, A78788AB, May 2009**

Replaced Figure 2.14

**Second Revision, A78788AC, December 2009**

Replaced Table 2.2

Replaced Figure 2.7

Replaced Figure 2.10

**Third Revision, A78788AD, February 2010**

Updated Table 2.3

Added Appendix B

**Fourth Revision, A78788AE, February 2011**

32 Karat Software version 9.1 patch

PA 800 *plus* Software version 1.1 patch

PA 800 *plus* Firmware version 9.2

Updated rinse steps 1 and 2 on page 2-14

Numerous syntax and grammatical edits

**Fifth Revision, A78788AF, January 2014**

Formatting update.

**Sixth Revision, A78788AG, February 2018**

PA 800 Plus Software version 10.1

32 Karat Software version 10.1

Rebranded. Applied new template. Legal content updated. Safety chapter removed and a reference to the safety content found in the System Overview Guide added. Best Practices added to Prepare the Samples. Replaced instructions for creating methods with instructions for using the PA 800 Plus software. Added Perform a cIEF Separation of a USP Monoclonal IgG Reference Standard. Added Hazardous Substance Information appendix. Updated System Suitability Method appendix.

**Seventh Revision, A78788AH, December 2018**

PA 800 Plus Software version 10.3

32 Karat Software version 10.3

In the Methods and Sequences section, updated the names of the methods and the sequence. As needed throughout, updated method and sequence names. In the Prepare the Samples section, corrected the mass of protein to add. Added additional waste vial to Figure 7 and edited the associated note for clarity. Updated Figures 10, 11, and 12 to show new method names. Added Methods appendix and Contact Us chapter.

*This guide applies to the latest software and firmware listed above, and any higher subsequent versions. When a subsequent software or firmware version affects the information in this guide, a new issue will be released to the SCIEX website. For updates, go to [www.sciex.com](http://www.sciex.com) and download the latest version of the guide.*



## Customer Training

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- In North America: [NA.CustomerTraining@sciex.com](mailto:NA.CustomerTraining@sciex.com)
- In Europe: [Europe.CustomerTraining@sciex.com](mailto:Europe.CustomerTraining@sciex.com)
- Outside the EU and North America, visit [sciex.com/education](https://sciex.com/education) for contact information.

## Online Learning Center

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- [SCIEXUniversity](#)

## SCIEX Support

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SCIEX and its representatives maintain a staff of fully-trained service and technical specialists located throughout the world. They can answer questions about the system or any technical issues that might arise. For more information, visit the SCIEX website at [sciex.com](https://sciex.com) or contact us in one of the following ways.

- [sciex.com/contact-us](https://sciex.com/contact-us)
- [sciex.com/request-support](https://sciex.com/request-support)

## CyberSecurity

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For the latest guidance on cybersecurity for SCIEX products, visit [sciex.com/productsecurity](https://sciex.com/productsecurity).

## Documentation

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

To view this document electronically, Adobe Acrobat Reader is required. To download the latest version, go to <https://get.adobe.com/reader>.

To find software product documentation, refer to the release notes or software installation guide that comes with the software. Documentation for the hardware products can be found on the Customer Reference DVD that comes with the system or component.

For the latest versions of the documentation, visit the SCIEX website at [sciex.com](http://sciex.com).

**NOTE** To request a free, printed version of this document, contact [sciex.com/contact-us](http://sciex.com/contact-us).