Application Guide

PA 800 *plus* Pharmaceutical Analysis System

Capillary Isoelectric Focusing (cIEF) Analysis

A78788AE
April 2010

Beckman Coulter, Inc.
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Brea, CA 92821

BECKMAN COULTER®

Application Guide
PA 800 *plus* Pharmaceutical Analysis System
Capillary Isoelectric Focusing (cIEF) Analysis
PN A78788AE (April 2010)

Find us on the World Wide Web at:
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Replaced Figure 2.14.

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Fourth Issue, A78788AD, February 2010
Updated Table 2.3
Added Appendix B

Fifth Issue, A78788AE, April 2010
Updated rinse steps on page 2-14.

Revision History

Symbols and Labels

Introduction

The following is a description of symbols and labels used on the Beckman Coulter PA 800 *plus* Pharmaceutical Analysis System or shown in this manual.

⚠ WARNING

If the equipment is used in a manner not specified by Beckman Coulter, Inc., the protection provided by the instrument may be impaired.

General Biohazard Symbol

This caution symbol indicates a possible biohazard risk from patient specimen contamination.

A011460L.PNG

Caution, Biohazard Label

This caution symbol indicates a caution to operate only with all covers in position to decrease risk of personal injury or biohazard.

A011459L.EPS

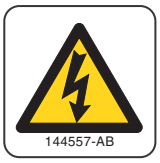
Caution, Moving Parts Label

This caution symbol warns the user of moving parts that can pinch or crush.

A015081L.EPS

High Voltage Electric Shock Risk Symbol

This symbol indicates that there is high voltage and there is a risk of electric shock when the user works in this area.



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Class 1 Laser Caution Label

A label reading “THIS PRODUCT CONFORMS TO APPLICABLE REQUIREMENTS OF 21 CFR 1040 AT THE DATE OF MANUFACTURE” is found near the Name Rating tag. The laser light beam is not visible.

A016350L.EPS

Sharp Object Label

A label reading “CAUTION SHARP OBJECTS” is found on the PA 800 *plus*.

A016351L.EPS

Recycling Label

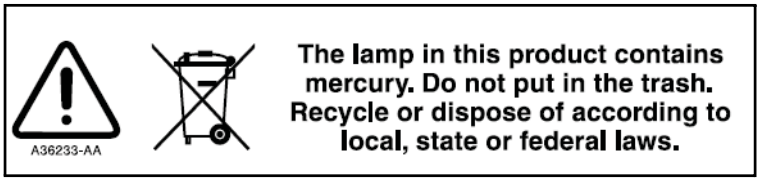
This symbol is required in accordance with the Waste Electrical and Electronic Equipment (WEEE) Directive of the European Union. The presence of this marking on the product indicates:

1. The device was put on the European Market after August 13, 2005.
2. The device is not to be disposed of via the municipal waste collection system of any member state of the European Union.

A016608L.PNG

It is very important that customers understand and follow all laws regarding the proper decontamination and safe disposal of electrical equipment. For Beckman Coulter products bearing this label, please contact your dealer or local Beckman Coulter office for details on the take back program that facilitates the proper collection, treatment, recovery, recycling, and safe disposal of this device.

Disposal of Devices Containing Mercury Components



This product contains a mercury-added part. Recycle or dispose of according to local, state, or federal laws. It is very important that you understand and comply with the safe and proper disposal of devices containing mercury components (switch, lamp, battery, relay, or electrode). The mercury component indicator label can vary depending on the type of device.

Restriction of Hazardous Substances (RoHS) Labels

These labels and materials declaration table (the Table of Hazardous Substance's Name and Concentration) are to meet People's Republic of China Electronic Industry Standard SJ/T11364-2006 "Marking for Control of Pollution Caused by Electronic Information Products" requirements.

RoHS Caution Label

This logo indicates that this electronic information product contains certain toxic or hazardous elements, and can be used safely during its environmental protection use period. The number in the middle of the logo indicates the environmental protection use period for the product. The outer circle indicates that the product can be recycled. The logo also signifies that the product should be recycled immediately after its environmental protection use period has expired. The date on the label indicates the date of manufacture.

RoHS Environmental Label

This logo indicates that the product does not contain any toxic or hazardous substances or elements. The "e" stands for electrical, electronic and environmental electronic information products. This logo indicates that this electronic information product does not contain any toxic or hazardous substances or elements, and is green and is environmental. The outer circle indicates that the product can be recycled. The logo also signifies that the product can be recycled after being discarded, and should not be casually discarded.

Alerts for Warning, Caution, Important, and Note

⚠ WARNING

WARNING indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury. The warning can be used to indicate the possibility of erroneous data that could result in an incorrect diagnosis (does not apply to all products).

⚠ CAUTION

CAUTION indicates a potentially hazardous situation, which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices. The caution can be used to indicate the possibility of erroneous data that could result in an incorrect diagnosis (does not apply to all products).

IMPORTANT IMPORTANT is used for comments that add value to the step or procedure being performed. Following the advice in the IMPORTANT notice adds benefit to the performance of a piece of equipment or to a process.

NOTE NOTE is used to call attention to notable information that should be followed during installation, use, or servicing of this equipment.

Contents

CHAPTER 1

Overview

Introduction

The Beckman Coulter Application Guide for Capillary Isoelectric Focusing (cIEF) Analysis 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 *Enhanced* system and 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.

IMPORTANT This product is for laboratory use only and it is not intended for diagnostic use.

⚠ CAUTION

Refer to the Material Safety Data Sheet (MSDS) information provided with the reagents regarding their proper handling. Always follow standard laboratory safety guidelines.

CHAPTER 2

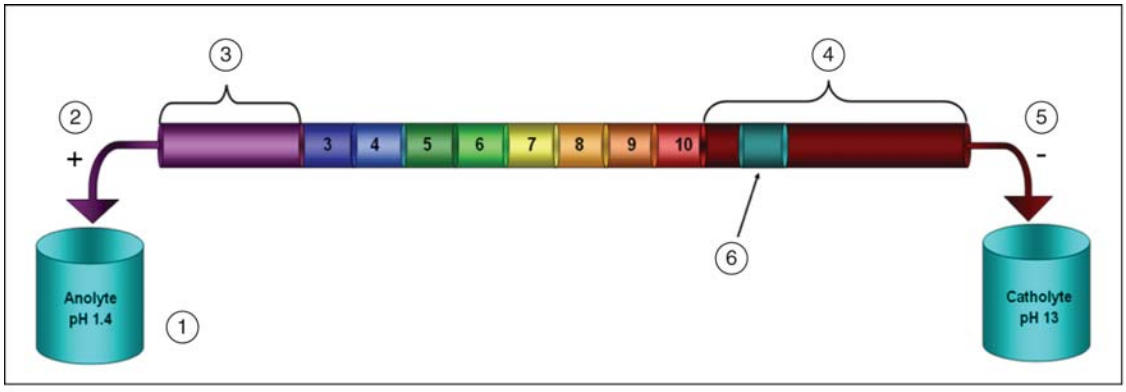
Capillary Isoelectric Focusing (cIEF) Analysis

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. The protein solution should not contain more than 50 mM of salt. Beckman Coulter 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.

The 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. The 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, see Figure 2.1. 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. A large amount of cathodic stabilizer in cIEF is used to fill the outlet side of the capillary. This forces the ampholytes and protein sample to focus before the detection window and ensures their detection during mobilization.[1]

Figure 2.1 pH Gradient Inside a Capillary



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1. Anolyte, pH 1.4
2. Anode
3. Anodic Stabilizer
4. Cathodic Stabilizer
5. Cathode
6. Detection Window
7. Catholyte, pH 13

The mechanism of focusing is bi-directional. The pH gradient forms at the capillary ends and then progresses toward the center of the capillary where both anodic and cathodic sides merge, see Figure 2.2, Figure 2.3, Figure 2.4, Figure 2.5, and Figure 2.6. With bi-directional focusing, sample peaks are often detected during focusing, see Figure 2.3 and Figure 2.4. Detection of unmerged peaks during mobilization indicate incomplete focusing of the pH gradient, see Figure 2.5. Focusing time needs to be sufficient to allow for complete formation of the pH gradient, see Figure 2.6.

After formation of the pH gradient, mobilization takes place to detect the pI markers and the separated protein sample. Use a pressure, gravity, or chemical method 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. Beckman Coulter recommends the use of acetic acid with chemical mobilization.[2, 3] 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.[4] Monoclonal antibodies have been separated with reproducibility by cIEF.[5]

Figure 2.2 Simulation of Focusing Mechanism at Time 0

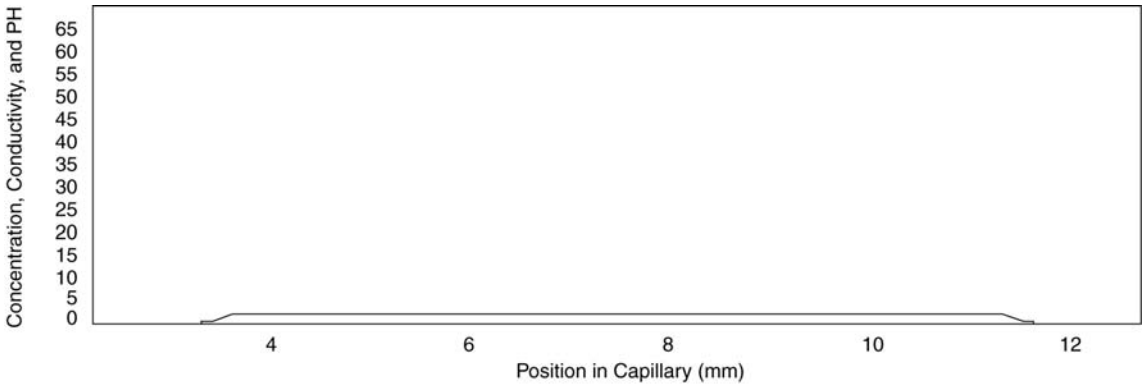

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Figure 2.3 Simulation of Focusing Mechanism at 30 Seconds

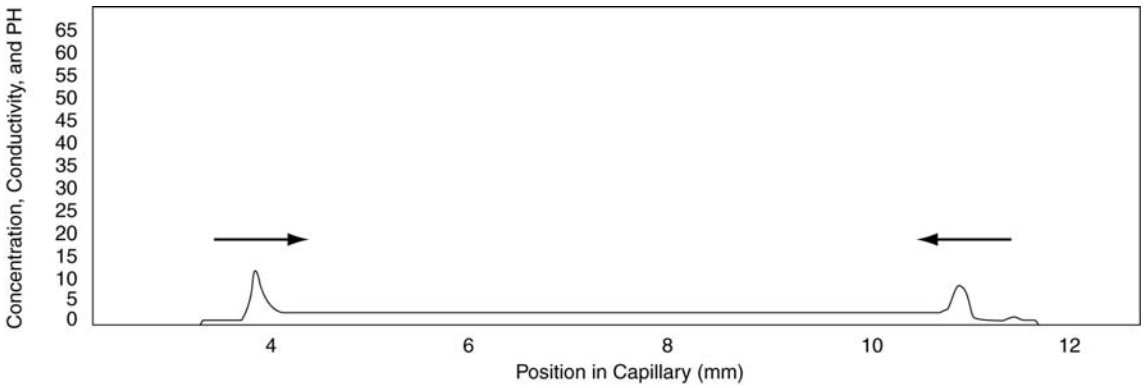

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Figure 2.4 Simulation of Focusing Mechanism at 2 Minutes

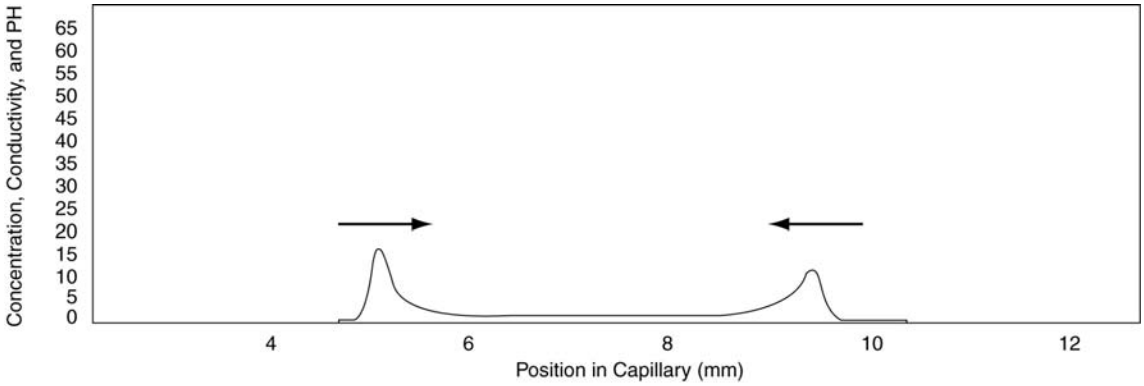

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Figure 2.5 Simulation of Focusing Mechanism at 4 Minutes

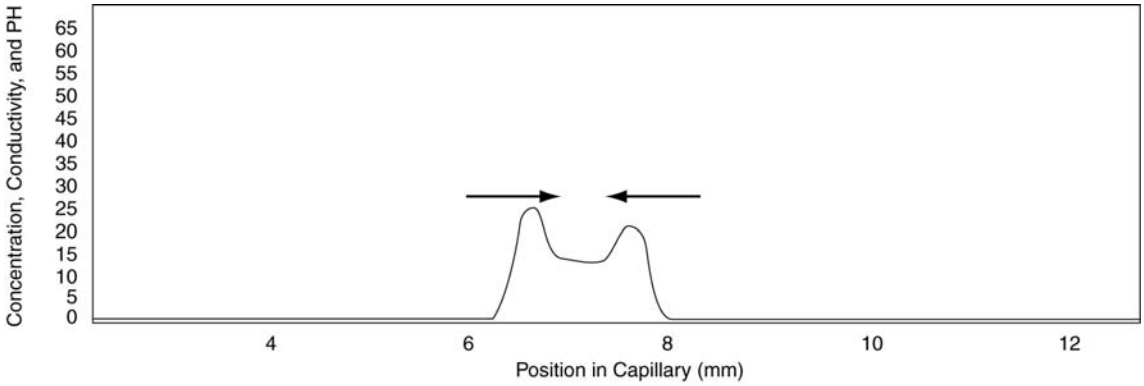

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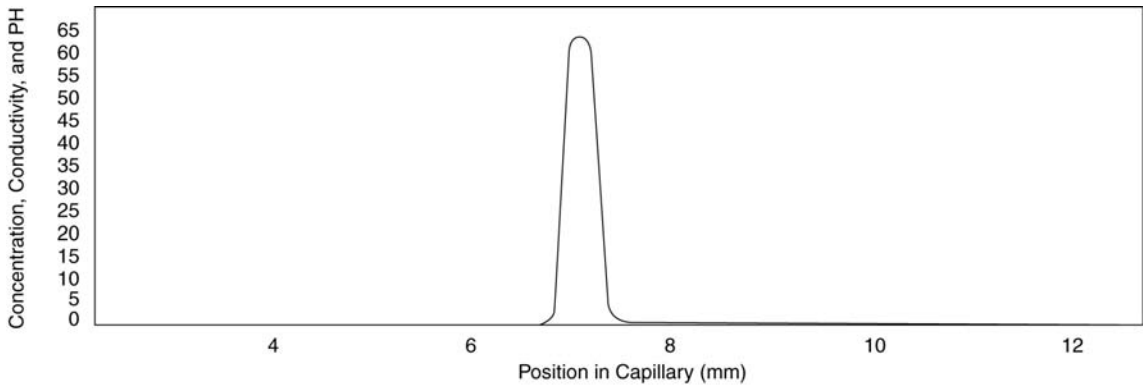
Figure 2.6 Simulation of Focusing Mechanism at 6 Minutes



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Materials and Reagents

Table 2.1 cIEF reagents available from Beckman Coulter, Inc.

Component	Quantity	PN
Neutral Capillary, 50 µm i.d. x 45 cm	1	477441
cIEF Gel (Polymer solution)	100 mL	477497
cIEF Peptide Marker, pI 10.0	1	A58481[a]
cIEF Peptide Marker, pI 9.5	1	A58481[a]
cIEF Peptide Marker, pI 7.0	1	A58481[a]
cIEF Peptide Marker, pI 5.5	1	A58481[a]
cIEF Peptide Marker, pI 4.1	1	A58481[a]
eCap 50 mM Tris buffer at pH 8.0	1	477427

a. Provided as a set of five peptide markers.

Table 2.2 Additional Chemical Reagents Required for cIEF

Reagent	Vendor	PN
Arginine	Sigma-Aldrich	A5006
Urea	Sigma-Aldrich	U0631
	GE Healthcare	17-1319-01
Iminodiacetic acid	Sigma-Aldrich	220,000
	Spectrum	I-2045
Pharmalyte 3-10 carrier ampholytes	GE Healthcare	17-0456-01
Glacial acetic acid	Sigma-Aldrich	537,020
	Spectrum	AC110

Table 2.2 Additional Chemical Reagents Required for cIEF *(Continued)*

Reagent	Vendor	PN
Phosphoric acid, 85%	Sigma-Aldrich	345,245
Sodium hydroxide, 1 M	Sigma-Aldrich	72082

Table 2.3 Additional Supplies from Beckman Coulter, Inc.

Component	Quantity	PN
Universal Plastic Vials	100	A62251
Universal Rubber Vial caps-blue	100	A62250
Filter 280 nm (for use with UV detector)	1	144439
200 µL micro vials	50	144709

Additional materials:

- Pipettes and pipette 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, 50 mL)
- 0.5 mL centrifuge tubes

Storage of Reagents

Upon receipt, store the Neutral Capillary and cIEF gel at 2°C to 8°C. For long-term storage, keep the peptide pI markers frozen at -35°C to -15°C. When in use, store the peptide pI markers at 2°C to 8°C. Store additional cIEF reagents as recommended by each supplier.

Preparation of cIEF Solutions

Preparation of cIEF solutions include:

- 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)
- Urea Solution (4.3 M Urea)
- 3 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 µL 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 room temperature for up to 30 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 room temperature for up to 30 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 room temperature for up to 30 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 room temperature for up to 30 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.

Urea Solution (4.3 M Urea)

1 Weigh 10.8 g of urea and transfer it to a 50 mL plastic conical tube.

2 Add 30.0 mL of DDI water to the tube containing urea.

3 Shake the flask for at least 15 minutes, until all the solid material is dissolved.

4 Filter the solution through a 5 µm pore-size membrane filter and collect the filtered solution in a 50 mL plastic conical tube.

5 Label the tube as **Urea Solution** and record the preparation date.

6 Store the urea solution in the refrigerator between 2°C and 8°C for up to 30 days.

3 M Urea- cIEF Gel

1 Weigh 1.80 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 µm membrane syringe filter using a 10 mL disposable plastic syringe and collect the filtered solution in a new 15 mL plastic conical tube.

7 Label the tube as **3 M urea-cIEF Gel** and record the preparation date.

8 Store the 3 M urea-cIEF Gel in the refrigerator between 2°C and 8°C for up to 30 days.

UV Lamp

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

Clean the Capillary Interface

Carefully clean the system electrodes and the interface block as described in the Maintenance Procedure section of the instrument manual. Repeat this general maintenance procedure at the start of each working day.

Install the Capillary in the Cartridge

1. Install a neutral-coated, 50 µm I.D., 30.2 cm long (20 cm from injection site to detector) fused-silica capillary into a capillary cartridge using the Capillary Cartridge Rebuild Procedure.

2. Use a 200 µm aperture in the cartridge. This aperture is labeled with a **2**. To ensure integrity, inspect all apertures yearly with a microscope or magnifying glass.

3. After the capillary has been installed in the cartridge, insert the cartridge into the PA 800 System, then close the front panel.

NOTE Do not expose the neutral-coated capillary ends to air for more than five minutes. Over 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.

Store the Capillary

At the end of each working day, run the shutdown method, see Time Program for cIEF Shutdown Method. For short term storage (1 to 3 days), leave the capillary on the instrument with both ends submerged in DDI water. For long term storage (over 3 days), place the capillary cartridge in the original capillary cartridge storage box with both ends submerged in DDI water, and then place the box vertically inside a refrigerator (2°C to 8°C). Do not let the capillary ends dry out, the capillary may become plugged or the coating may incur permanent damage.

At the start of each day or after a long storage period, condition the capillary using the capillary conditioning method, see Time Program for cIEF Conditioning Method.

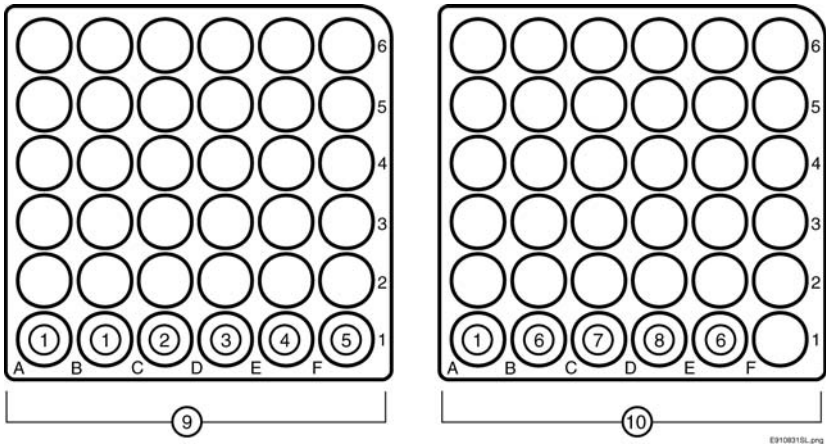
NOTE Do not share capillaries between applications. If the capillary has been used for cIEF analysis, do not use it with another application.

Prepare the Buffer Trays

1. Fill the indicated vials with each reagent and close each vial with a cap, see Figure 2.7. Place 1.5 mL of reagent per vial and cap each vial with a blue cap.

   NOTE Each set of buffer vials is good for 6 consecutive runs or for 24 hours inside the instrument.

Figure 2.7 Buffer Tray Configurations for cIEF Analysis



1. DDI water	**6.** Waste (positions B1 and E1)
2. Anolyte	**7.** Catholyte
3. Urea Solution	**8.** Chemical Mobilizer
4. cIEF Gel	**9.** BI (Inlet Buffer Tray)
5. Chemical Mobilizer Rinse	**10.** BO (Outlet Buffer Tray)

2 For waste, place capped vials filled with 0.8 mL of DDI water in the tray.

3 Load the inlet and outlet buffer trays inside the instrument.

4 Replace all vials after 24 hours inside the instrument. The increment option in the cIEF separation method can be used to automatically increment the vials every 6 consecutive runs on both buffer trays.

5 Once sample preparation is complete, place the cIEF samples on the inlet sample tray.

Sample Preparation

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

- 200 µL of 3 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

When analyzing multiple samples, Beckman Coulter recommends preparing a master mix, see Table 2.4. A master mix simplifies sample preparation and minimizes pipetting errors. Table 2.4

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 (no less than three) 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.

Table 2.4 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 M urea-cIEF Gel	200	x _____ + 1 =	
Pharmalyte 3-10	12	x _____ + 1 =	
Cathodic Stabilizer	20	x _____ + 1 =	
Anodic Stabilizer	2	x _____ + 1 =	
pI marker A	2	x _____ + 1 =	
pI marker B	2	x _____ + 1 =	
pI marker C	2	x _____ + 1 =	

Pipet each calculated reagent volume into a centrifuge tube. Vortex the master mix for 15 seconds to ensure complete mixing. Store the master mix at 2°C to 8°C and discard it at the end of the day.

When preparing a protein sample for cIEF analysis, mix 240 µL of master mix with 10 µL of protein with a concentration of 5 mg/mL to 10 mg/mL. The protein sample must have a salt concentration below 50 mM since high salt content is detrimental to cIEF separation. The protein sample can be buffer exchanged before use. Please go to APPENDIX B for buffer exchange and desalting procedure.

Vortex the cIEF sample (master mix with protein) for 15 seconds. Transfer 200 µL of the sample into a micro vial and then centrifuge the vial in a microcentrifuge for 20 seconds at low speed to remove any air bubbles. Place the micro vial into a universal plastic vial and cap it with a blue cap. Then, place the sample vial in the inlet sample tray.

Running Methods

To perform cIEF, use the following methods:

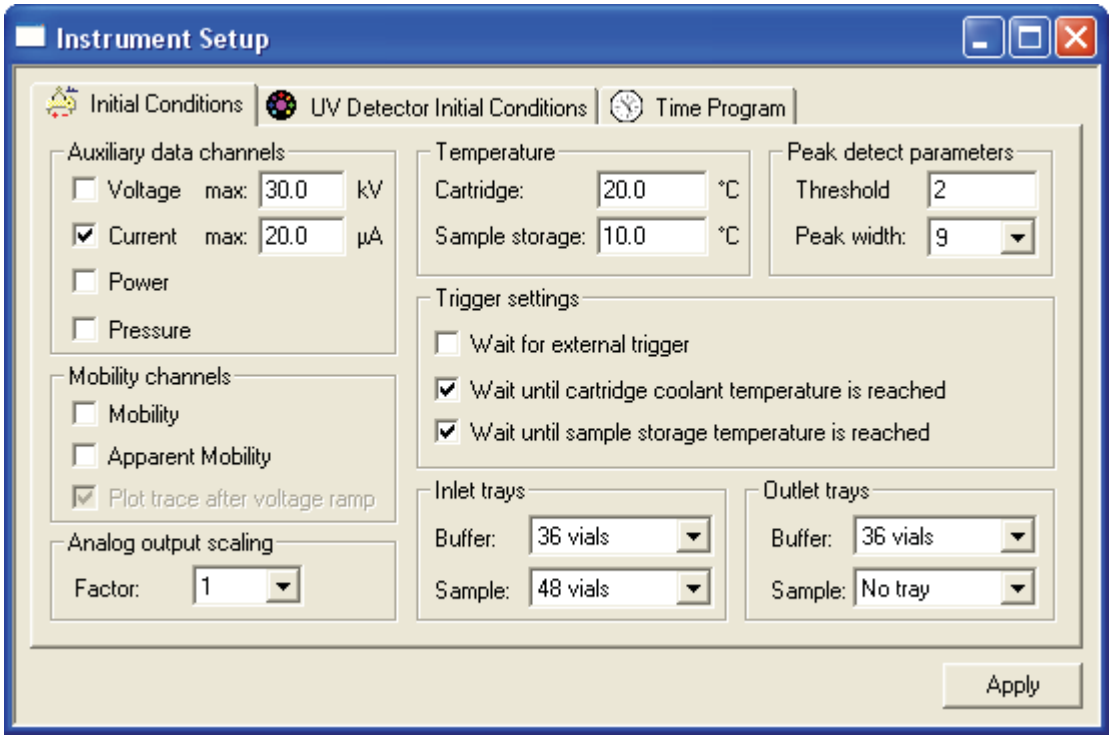
- **cIEF Conditioning - PA 800 plus.met** - To condition the capillary at the start of each day.
- **cIEF Separation - PA 800 plus.met** - To perform a cIEF separation using Pharmalyte 3-10.
- **cIEF Shutdown - PA 800 plus.met** - To rinse the capillary for storage and turn off the UV lamp.

All three methods use the same initial conditions, see Initial Conditions, and UV detector settings, see UV Detector Initial Conditions. However, each method has a different time program, see Time Program for cIEF Conditioning Method, Time Program for cIEF Separation Method, and Time Program for cIEF Shutdown Method. All three methods can be run using the cIEF system configuration provided in the 32 Karat software.

Initial Conditions

- Temperature
  - **Cartridge** (Coolant) = 20°C
  - **Sample Storage** = 10°C
- Auxiliary Data Channels
  - **Maximum Current** = 20 µA
- Analog Output Scaling
  - **Factor** = 1
- Peak Detect Parameters
  - **Threshold** = 2
  - **Peak Width** = 9
- Inlet Trays
  - **Buffer** = 36 vials
  - **Sample** = 48 vials
- Outlet Trays
  - **Buffer** = 36 vials
  - **Sample** = No Tray

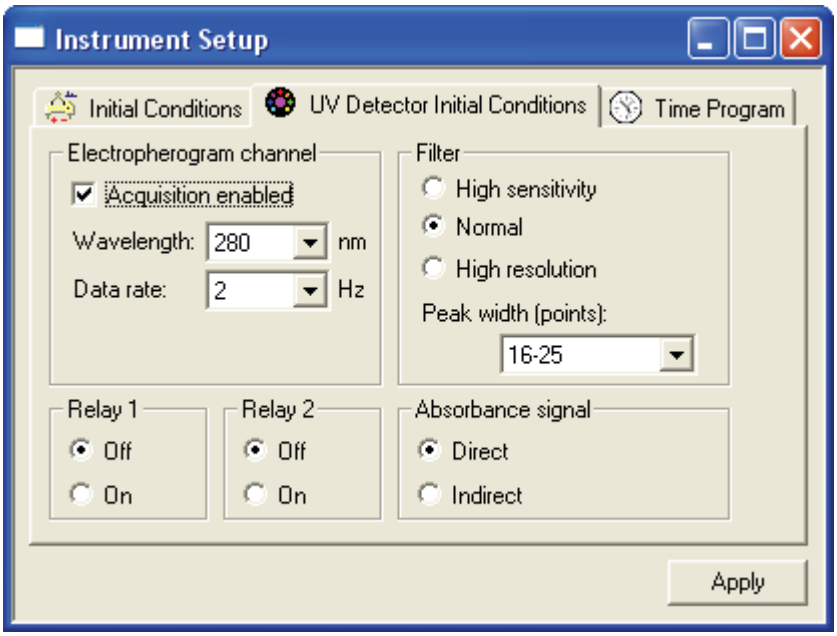
Figure 2.8 Initial Conditions for cIEF Methods



UV Detector Initial Conditions

- Electropherogram Channel
  - **Acquisition Enabled** = selected
  - **Wavelength** = 280 nm
  - **Data Rate** = 2 Hz
- Relay 1 = **Off**
- Relay 2 = **Off**
- Filter
  - **Normal**
  - **Peak Width** = 16 - 25 points
- Absorbance Signal = **Direct**

Figure 2.9 UV Detector Initial Conditions for cIEF MEthods



Time Program for cIEF Conditioning Method

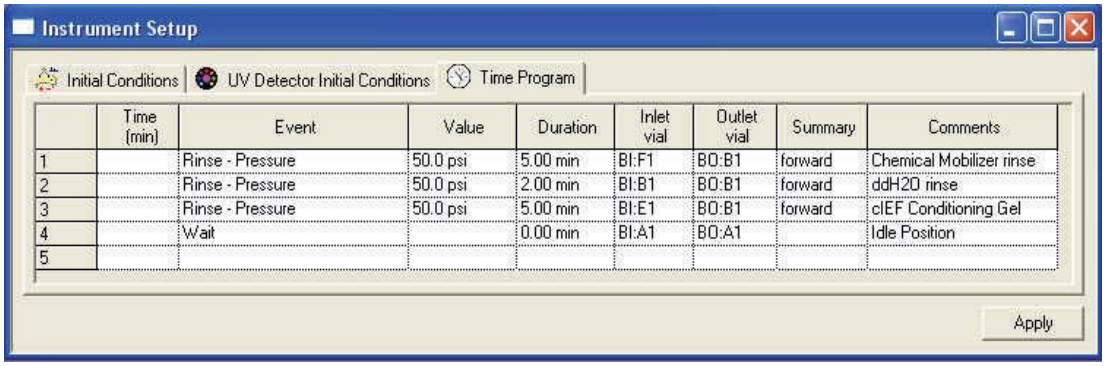
1 Rinse for 5 minutes at 50 psi with Chemical Mobilizer, see Figure 2.10.

2 Rinse for 2 minutes at 50 psi with DDI water.

3 Rinse for 5 minutes at 50 psi with cIEF gel.

4 Submerge both of the capillary ends in vials filled with DDI water.

Figure 2.10 Time Program for the cIEF Conditioning Method

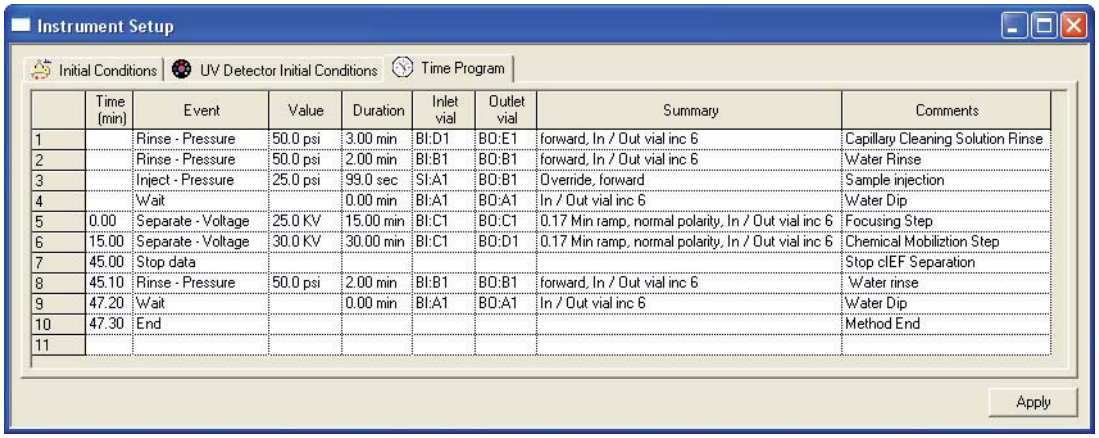


	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	5.00 min	BI:F1	BO:B1	forward	Chemical Mobilizer rinse
2		Rinse - Pressure	50.0 psi	2.00 min	BI:B1	BO:B1	forward	ddH20 rinse
3		Rinse - Pressure	50.0 psi	5.00 min	BI:E1	BO:B1	forward	cIEF Conditioning Gel
4		Wait		0.00 min	BI:A1	BO:A1		Idle Position
5								

Time Program for cIEF Separation Method

1. Rinse for 3 minutes at 50 psi with Urea Solution, see Figure 2.11.
2. Rinse for 2 minutes at 50 psi with DDI water.
3. Inject sample for 99.9 seconds at 25 psi.
4. Water dip by submerging both capillary ends in DDI water.
5. Focusing step, 15 minutes at 25 kV under normal polarity (Time = 0).
6. Chemical mobilization, 30 minutes at 30 kV under normal polarity (Time = 15 minutes).
7. Stop data collection (Time = 45 minutes).
8. Rinse for 2 minutes at 50 psi with DDI water (Time = 45 minutes).
9. Submerged both of the capillary ends in DDI water (Time = 47 minutes).
10. End the method (Time = 47.10 minutes).

Figure 2.11 Time Program for the cIEF Separation Method

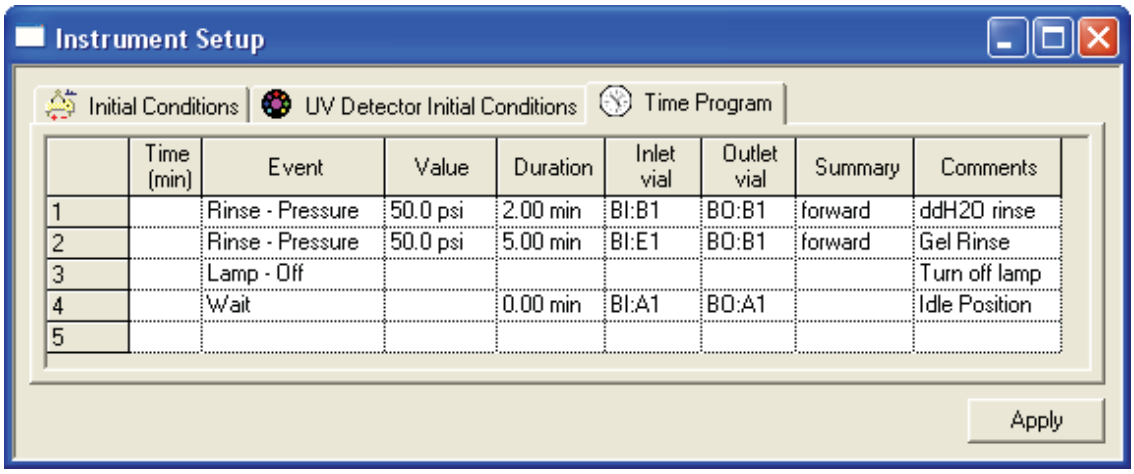


	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	3.00 min	BI:D1	BO:E1	forward, In / Out vial inc 6	Capillary Cleaning Solution Rinse
2		Rinse - Pressure	50.0 psi	2.00 min	BI:B1	BO:B1	forward, In / Out vial inc 6	Water Rinse
3		Inject - Pressure	25.0 psi	99.0 sec	SI:A1	BO:B1	Override, forward	Sample injection
4		Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 6	Water Dip
5	0.00	Separate - Voltage	25.0 KV	15.00 min	BI:C1	BO:C1	0.17 Min ramp, normal polarity, In / Out vial inc 6	Focusing Step
6	15.00	Separate - Voltage	30.0 KV	30.00 min	BI:C1	BO:D1	0.17 Min ramp, normal polarity, In / Out vial inc 6	Chemical Mobiliztion Step
7	45.00	Stop data						Stop cIEF Separation
8	45.10	Rinse - Pressure	50.0 psi	2.00 min	BI:B1	BO:B1	forward, In / Out vial inc 6	Water rinse
9	47.20	Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 6	Water Dip
10	47.30	End						Method End
11								

Time Program for cIEF Shutdown Method

1. Rinse for 2 minutes at 50 psi with DDI water, see Figure 2.12.
2. Rinse for 5 minutes at 50 psi with cIEF gel.
3. Turn off the UV lamp.
4. Submerge both of the capillary ends in vials filled with DDI water.

Figure 2.12 Time Program for the cIEF Shutdown Method

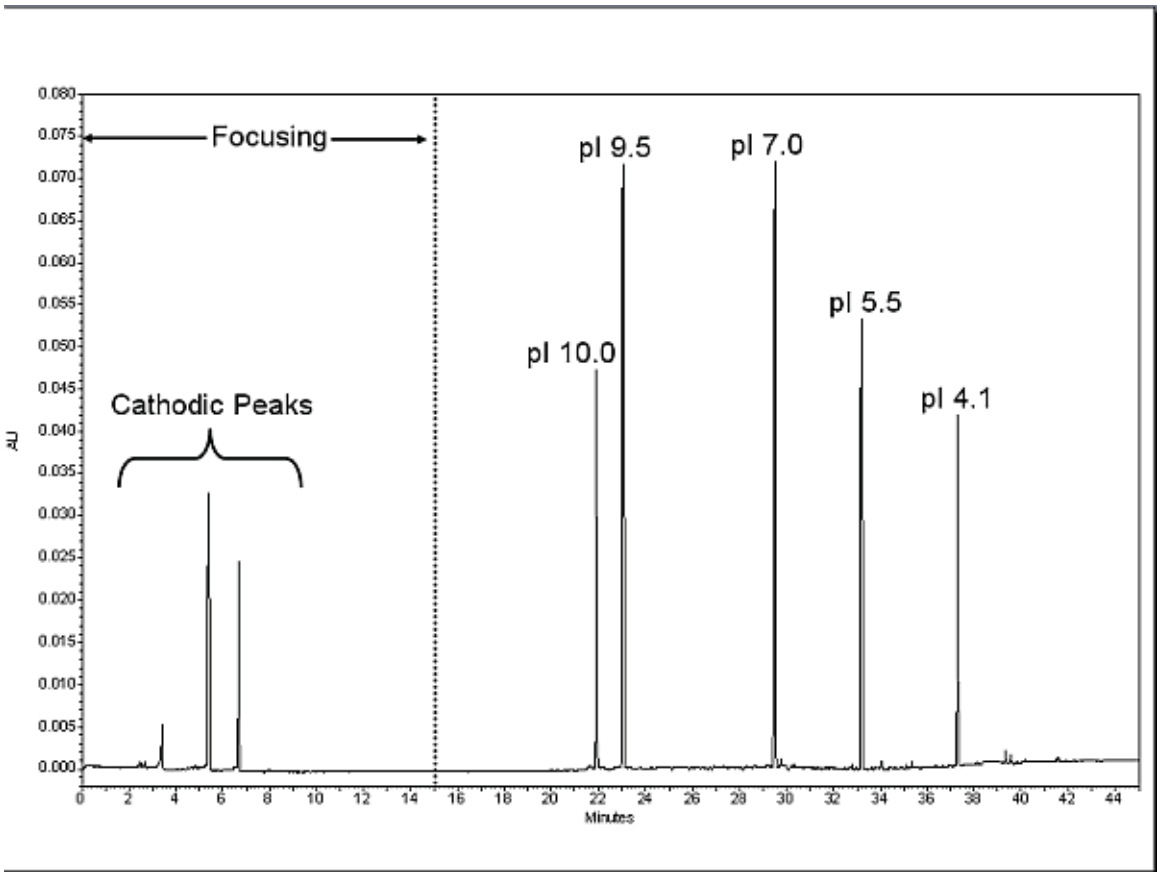


	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	2.00 min	BI:B1	BO:B1	forward	ddH20 rinse
2		Rinse - Pressure	50.0 psi	5.00 min	BI:E1	BO:B1	forward	Gel Rinse
3		Lamp - Off						Turn off lamp
4		Wait		0.00 min	BI:A1	BO:A1		Idle Position
5								

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

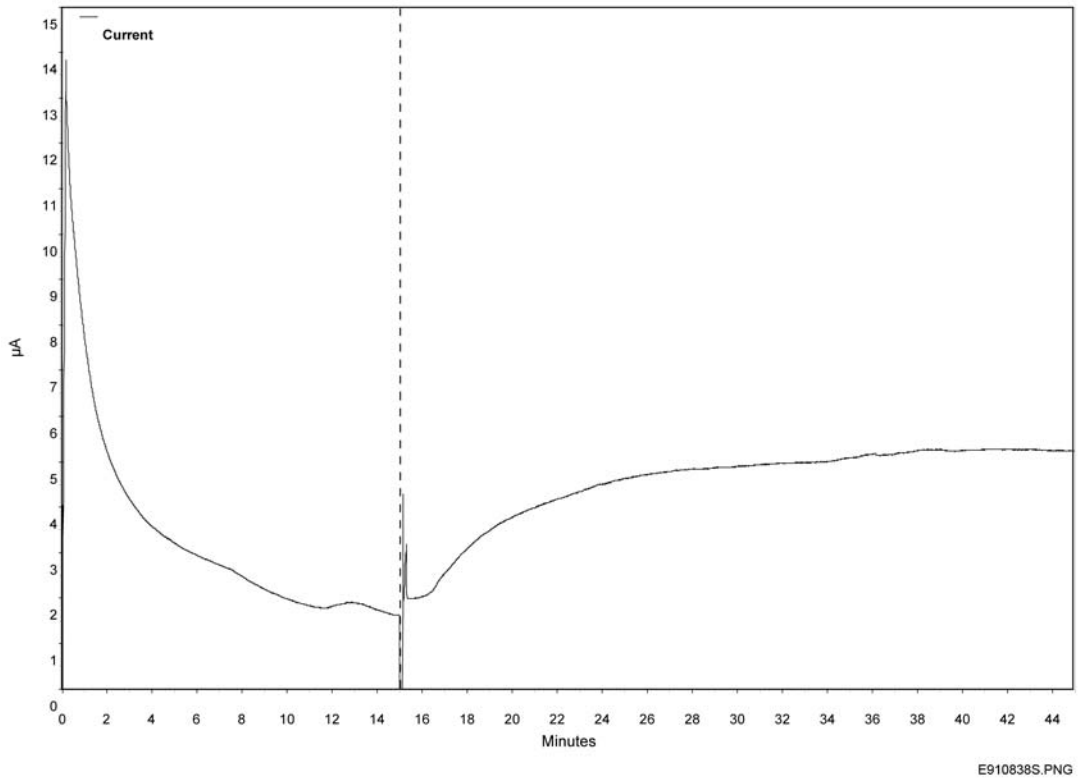
To check the performance of the PA 800 System, perform a cIEF separation of the five peptide markers. Compare the electropherogram obtained with the one shown in Figure 2.13. The electrical current should have a profile similar to that in Figure 2.14.

Figure 2.13 Typical cIEF Separation of Five Peptide pI Markers with Pharmalyte 3-10



NOTE The cathodic peaks observed in the electropherogram are due to the bi-directional migration of the sample and ampholytes that occurs during focusing, see Figure 2.2. The absence of cathodic peaks can indicate incomplete focusing.

Figure 2.14 Typical Electrical Current Profile of cIEF Separation of Peptide pI Markers with Pharmalyte 3-10



The vertical dashed line shown in Figure 2.13 and Figure 2.14 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. However, you do not have to analyze the focusing data because the cIEF separation is generated during mobilization, not during focusing.

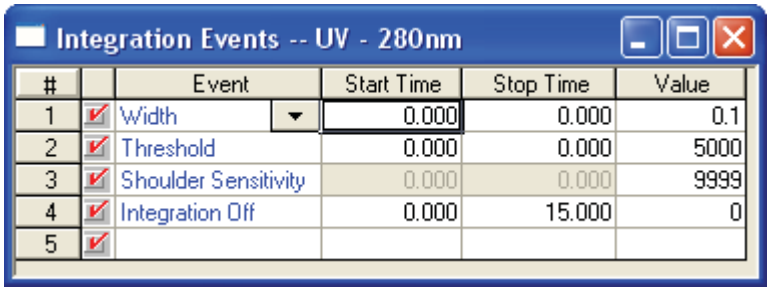
The separation method using the cIEF Peptide Markers can be used as a System Suitability Method to ensure that the whole system, including the reagents, is working properly. In order to pass, the five markers must be detected during the mobilization step (15 to 45 minutes). If it fails, refer to the Troubleshooting section in this guide. Consult APPENDIX A, System Suitability Method for more information regarding the set-up of a System Suitability Method in the cIEF module.

Integration Parameters

You should optimize the integration parameters in the analysis method for each sample. As a starting point, use the integration values shown in Figure 2.15. 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 to 15 minutes (during focusing)

Figure 2.15 Recommended Integration Parameters and Initial Values



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.

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

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

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.

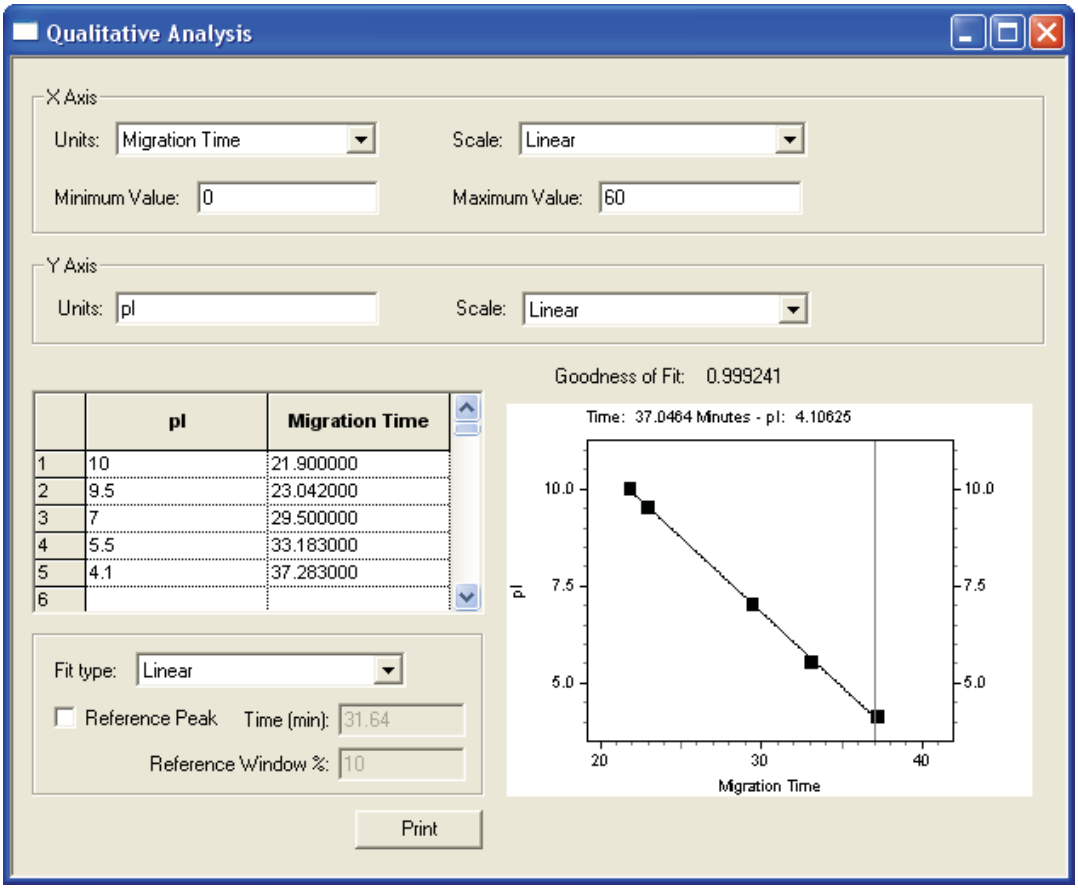
Determination of the pI Value

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

1 Select **Method > Qualitative Analysis** to open the Qualitative Analysis window, see Figure 2.16.

2 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, see Figure 2.16

Figure 2.16 Qualitative Analysis Window for pI Determination

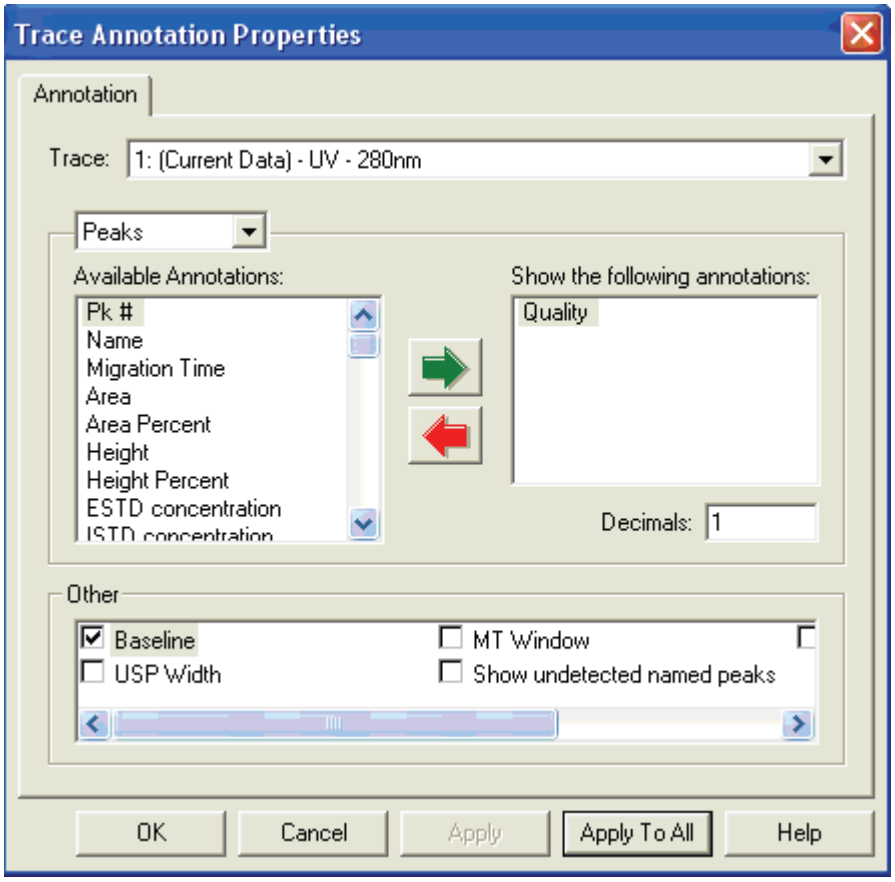


3 Select **File > Method > Save** to save the method.

4 Select **Analysis > Analyze**.

5 To display the calculated pI values in the cIEF separation (UV trace), right-click inside the UV trace and select **Annotations**. In **Available Annotations** select **Quality** and then select **Add**, see Figure 2.17. In this analysis, quality corresponds to calculated pI value.

Figure 2.17 Trace Annotation Window



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

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 electro-osmotic 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 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 light passes through both the aperture and capillary window (shine a flashlight on the back of the aperture).
	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 in the incorrect position.	Check the method and vial positions.
	The capillary is filled with air.	Fill the sample vial with 200 µL of cIEF sample. Ensure 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 new urea-cIEF gel solution. Store this solution at 2° 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.

Problem	Possible Cause	Corrective Action
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.

APPENDIX A

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 module to use this feature.

1. Start by closing all the PA 800 application windows except for the 32 Karat main window.
2. In the 32 Karat main window, select **Tools > Enterprise Login**.
3. Enter the user name and the password. Select **OK**. The default user name is **PA800**, and the default password is **Plus**.
4. Right-click on the cIEF icon and select **Configure > Instrument**.
5. Select **Configure**. The PA 800 System Configuration dialog will display.
6. Select **Options**.
7. On the General tab, select **System Suitability**, **Qualitative Analysis**, and **Caesar Integration**.

8 Select **OK** in the next three dialogs.

Making a cIEF System Suitability Method

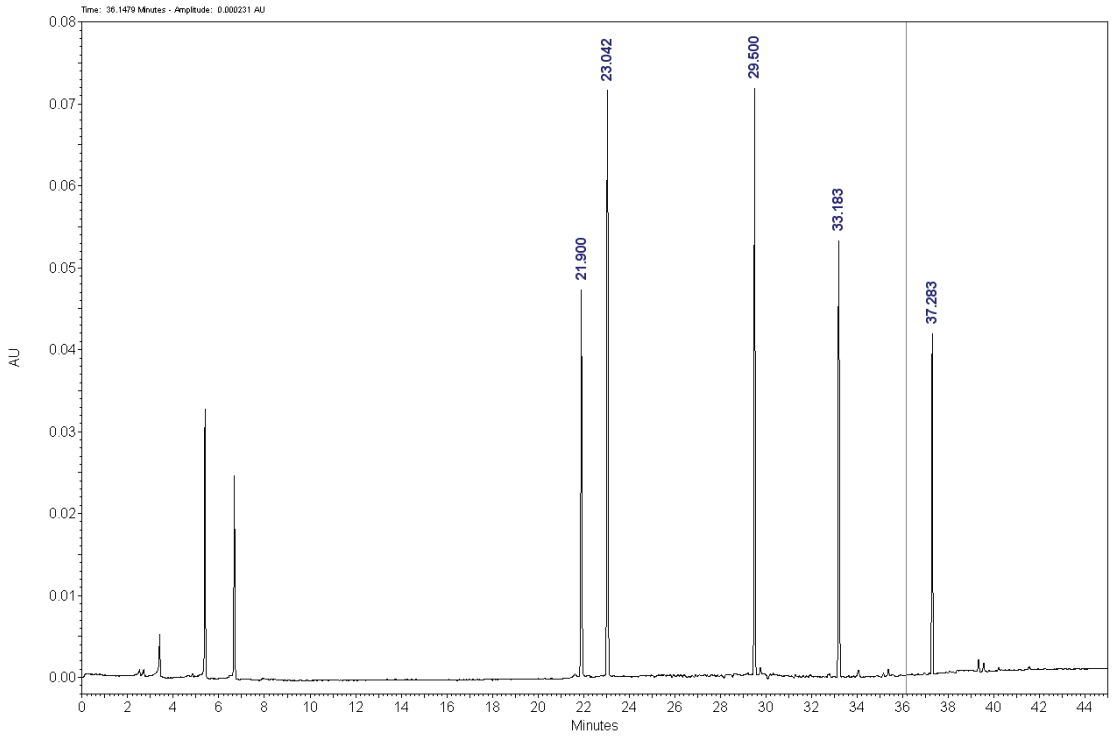
1 Select **cIEF** to open the cIEF module.

2 Open the 32 Karat method that will be converted into a System Suitability Method.

3 Open the UV trace of the separation that will be used to determine system suitability.

NOTE This example uses the separation of the five cIEF Peptide Markers, see Figure A.1.

Figure A.1 Separation with Five cIEF Peptide Markers



4 To enter the integrated peaks on the method peak table, right-click inside the UV trace and select **Graphical Programming > Define Peaks**.

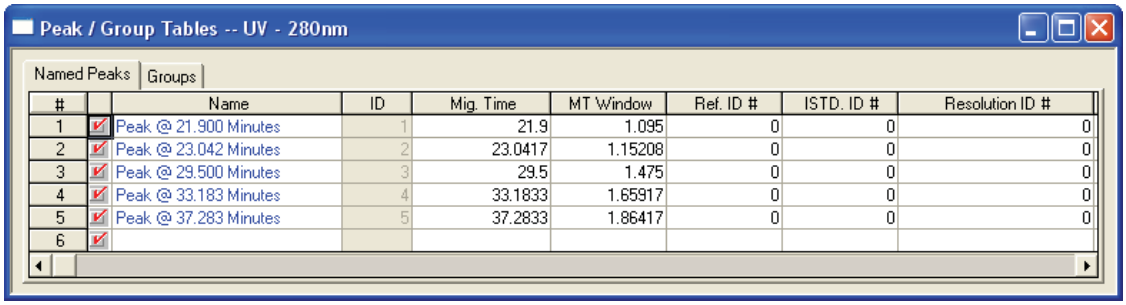
5 Select the beginning of the marker peaks and then select the end of the marker peaks.

In this example, select at 20 minutes and then at 39 minutes to include the five peptide markers.

6 Select **Method > Peaks/Groups** to open the peak table.

In this example, the table containing the Named Peaks is shown, see Figure A.2. These peaks are labeled according to their migration time.

Figure A.2 Named Peaks Table

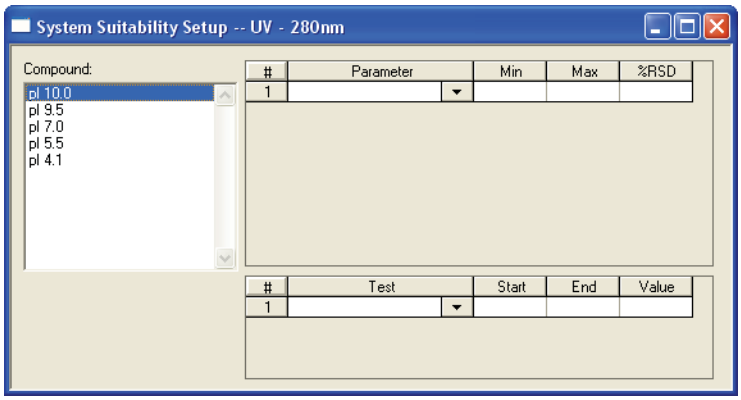


#	Name	ID	Mig. Time	MT Window	Ref. ID #	ISTD. ID #	Resolution ID #
1	Peak @ 21.900 Minutes	1	21.9	1.095	0	0	0
2	Peak @ 23.042 Minutes	2	23.0417	1.15208	0	0	0
3	Peak @ 29.500 Minutes	3	29.5	1.475	0	0	0
4	Peak @ 33.183 Minutes	4	33.1833	1.65917	0	0	0
5	Peak @ 37.283 Minutes	5	37.2833	1.86417	0	0	0
6							

7 (optional) Rename the peaks on the table. For example, name the marker peaks after their corresponding pI value.

8 Select **Method > System Suitability** to open the System Suitability dialog, see Figure A.3.

Figure A.3 System Suitability Dialog



9 Select the parameter that the data must meet to be considered a pass for each analyte.

For example, select **Quality**, which corresponds to the pI in the cIEF software module.

NOTE The %RSD column is used to test the reproducibility of multiple runs. Leave it blank if there is no test criteria.

10 Select **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 select the system suitability method.

3 In row 1 of the sequence, in the Filename column select the data to be checked by the system suitability.

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

NOTE If there is more than one data file to analyze, enter the filenames on additional rows in the sequence and highlight the files to be analyzed as a system suitability set.

5 In row 1, select **Run Type**. The Sample Run Type(s) dialog will open.

6 If only one row is marked as system suitability, both **Begin System Suitability** and **End System Suitability** will be selected.

If a set of rows are marked as system suitability:

- In the first row, select **Begin System Suitability**.
- In the last row, select **End System Suitability**.
- In all other rows, select **System Suitability Standard**.

NOTE The report template can only be selected in the row that has **Begin System Suitability** selected.

NOTE The default system suitability report is `SysSuit.brp`.

7 Select **OK** to close the dialog.

8 Select **File > Sequence > Save Sequence** to save the sequence.

9 Select **Sequence > Process**. The Process Sequence dialog will display.

10 The open sequence will display in **sequence name**.

11 Use **Run Range** to specify the rows for analysis.

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

13 For the Processing Mode, select **Reintegrate**. To see the results of each row after reintegration, select **Review**.

14 Select **Start** to reintegrate the data.

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

15 Select **Reports > View > Sequence Custom Reports** to view the system suitability report.

16 Select **System Suitability > View** to open the report.

NOTE Consult the Help file in 32 Karat Software to learn additional features for setting and performing System Suitability.

APPENDIX B

Buffer Exchange

Buffer Exchange Overview

In clEF, the presence of salts (> 50 mM) in the sample can lead to compression of the pH gradient, alter focusing conditions, and damage capillary coatings. To reduce the negative effects that sample buffer components can have on clEF separations, performing a buffer exchange prior to analysis is highly suggested.

Buffer Exchange Procedure

Load 500 µL of the protein to be desalted (5-10 mg/mL) into a Microcon* Ultracell YM 10 (PN A11530, Millipore, Billerica, MA). Centrifuge for 5 min in a Microfuge™ 18 (PN 367160, Beckman Coulter, Inc., Brea, CA) at 12,000 *g* replace the filtered solution with 20 mM Tris buffer pH 8.0. Repeat the centrifugation and buffer replacement cycles twice. Make 50 ug aliquots of and stored at -20°C or below.

Exchange Buffer

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 double-deionized water.

References

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., Yao, K. Q., *J Chromatogr*, Volume 387, pp 127, 1987.
3. Manabe, T., Miyamoto, H., 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., Vigh, G., *Electrophoresis*, Volume 30, pp 4049, 2009.

www.beckmancoulter.com and
www.CELeader.com