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BioFocus

Applications Manual

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Applications

This section of the manual outlines only a few of the many different applications that are possible with the BioFocus® 2000 and the BioFocus 3000 systems. For a more complete list, please contact your local Bio-Rad office and ask for the CE Application Notes Index. Please consult this literature for more information on CE separation techniques for your compound of interest. Also hundreds of CE journal articles as well as CE textbooks are currently available in many libraries.

This section of the manual assumes that the user has basic familiarity with at least one of the BioFocus CE systems.

Capillary Zone Electrophoresis (CZE) at Low pH

CZE (also known as free zone capillary electrophoresis) at low pH is used for peptides, some proteins, and small molecules such as drugs and inorganic acids. This section gives specific instructions for running the Peptide Calibrator that comes with each BioFocus CE system. It is recommended that the user run the Peptide Calibrator to become familiar with the system and to check the functionality of the instrument and capillary cartridge.

The Peptide Calibrator contains a mixture of 25 µg each of the following nine peptides in lyophilized form: bradykinin, angiotensin II, alpha-melanocyte stimulating hormone, thyrotropin releasing hormone, luteinizing hormone-releasing hormone, leucine enkephalin, bombesin, methionine enkephalin, and oxytocin. After proper reconstitution (see “Instructions for Separations with the Acidic Phosphate Buffer (low pH CZE) on page 4”), the final concentration of each peptide is 50 µg/ml.

The best peptide separations are usually achieved under acidic conditions, typically below pH 3, and the electrolyte supplied with this system is 0.1 M phosphate buffer, pH 2.5. A protocol for achieving a separation is given below.

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Supplies Needed

- Phosphate Buffer, 0.1 M, pH 2.5, Catalog #148-5010
- Peptide Calibration Set, Catalog #148-2012
- BioFocus Capillary Cartridge, 24 cm x 25 µm, Coated, Catalog #148-3031
- Microcentrifuge tubes, 500 µl , Catalog #223-9503
- Distilled or deionized water, filtered
- Pipettes or syringes for reconstituting the peptide calibrator and transferring buffer

Instructions for Separations with the Acidic Phosphate Buffer (low pH CZE)

1. Turn on the system and allow a few minutes for the detector to stabilize. Carousel compartment temperature may be left at ambient (Not Set).
2. For optimal results, the salt concentration of the sample solution should be one tenth that of the buffer solution. This causes a stacking effect that produces a sharper, more concentrated sample band. The Peptide Calibrator is reconstituted by adding 50 µl of the phosphate buffer (0.1 M phosphate buffer, pH 2.5, Catalog #148-5010) and 450 µl of distilled water. After reconstitution, refrigeration at 4_ C is recommended.
   If you wish to use Multi Inject for injection of an internal standard with CZE, we recommend that you prepare the internal standard in the run buffer (0.1 M phosphate buffer, pH 2.5, Catalog #148-5010). Sample should be prepared in 1:10 (v:v) diluted run buffer. For best separation efficiency, inject the internal standard using a low-pressure Pre Inject cycle (5-10 psi*sec) before electrophoretic injection of the sample.
3. For the Peptide Calibrator, program a Single Run Group CZE Method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. The recommended conditions for running the Peptide Calibrator are listed below.

Inject time	20 psi*sec pressure or 8 sec at 10 kV
Mode	Constant Voltage
Polarity	+ to –
Running Voltage	10 kV
Running Current	12 µA
Current Limit	50 µA
Wavelength	200 nm

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Run time	15 minutes
Range	0.02 AUFS
Rise Time	1 second
Prep cycle(s)	1 high pressure pre-inject cycle: 60 sec with Phosphate Buffer, pH 2.5

4. Load your peptide sample, waste, and phosphate buffer vials into the correct inlet and outlet positions.
5. Place a Bio-Rad 24 cm x 25 µm cartridge (Catalog #148-3031) in the cartridge holder and push the levers down to lock it firmly in place.
6. Make sure the coolant reservoir (right side) is filled with water and the helium or nitrogen gas tank valve is open.
7. Start the program.

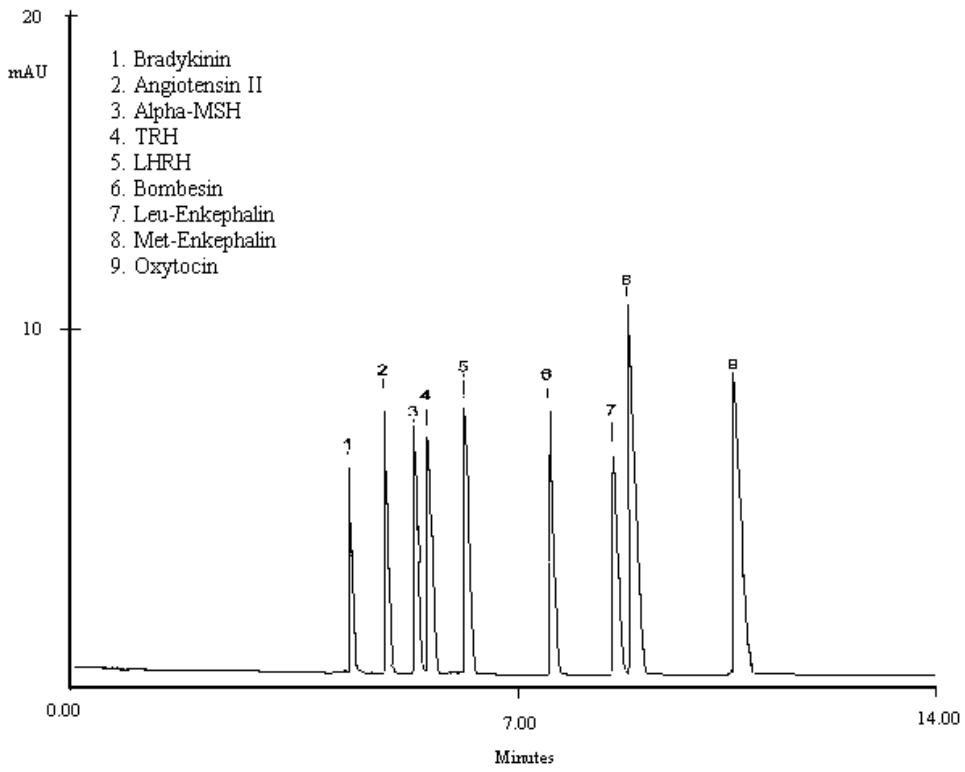


Figure 1 Separation of the Bio-Rad Peptide Calibration Set in a Bio-Rad 24 cm x 25 µm coated capillary cartridge at pH 2.5 and 10 kV.

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Capillary Zone Electrophoresis (CZE) at High pH

Many proteins require a high pH buffer for optimal separations. A coated capillary greatly enhances the reproducibility of high pH separations. CZE at high pH uses the same procedure used for low pH analysis, with the addition of an extra capillary purge and rinse between each run and usually with a reversal of run polarity (or as dictated by the protein pI). A protocol with a detailed description of the prep cycles is given below.

Supplies Needed

- Basic Protein Analysis Buffer, 0.3 M sodium borate with additives, pH 8.5, Catalog #148-5023
- Capillary Wash Solution, 60 ml, Catalog #148-5022
- BioFocus Capillary Cartridge, 24 cm x 25 µm, coated, Catalog #148-3031
- Distilled or deionized water, filtered
- Pipettes or syringes for transferring buffer and sample
- Microcentrifuge tubes, 500 µl, Catalog #223-9503
- Protein Calibration Set, Catalog #148-2013

Instructions for Separations with the Basic Protein Analysis Buffer (high pH CZE)

1. Turn on the system and allow a few minutes for the detector to stabilize. Carousel compartment temperature may be left at ambient (Not Set) or cooled for labile samples.
2. The Basic Protein Analysis Buffer (pH 8.5) is recommended for samples with pI's of less than 8 or greater than 9. In the former case, the sample ions are negatively charged and the polarity should be - to +. In the latter case, sample ions are positively charged and the polarity should be + to –.
   For optimal results, the salt concentration in the sample solution should be one tenth that of the buffer solution. This causes a stacking effect that produces a sharper, more concentrated sample band. With the Basic Protein Analysis Buffer, the sample solution should have a salt concentration of 30 mM or less.
   If you wish to use Multi Inject for injection of an internal standard with CZE, we recommend that you prepare the internal standard in the run buffer (Basic Protein Analysis Buffer, pH 8.5, Catalog #148-5023). Sample should be prepared in 1:10 (v:v) diluted run buffer. For best separation efficiency, inject the internal standard using a

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low-pressure Pre Inject cycle (5-10 psi*sec) before electrophoretic injection of the sample.

3. Use either a Single Run Group CZE Method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. The following can be used as general guidelines. Make adjustments where necessary.

Inject time	20 psi*sec pressure or 8 sec electrophoretic (8 kV)
Mode	Constant Voltage
Polarity	- to + or + to – as dictated by sample proteins
Running Voltage	10 kV
Running Current	9 µA
Current Limit	50 µA
Wavelength	200 nm
Run time	20 minutes
Range	0.02 AUFS
Rise Time	1 second
Prep cycles	3 high pressure pre-inject cycles: 60 sec Capillary Wash Solution, 30 sec Water, 90 sec Basic Protein Analysis Buffer

4. Load your sample, wash, waste, and buffer vials into the correct inlet and outlet positions for the Configuration.
5. Insert a Bio-Rad 24 cm x 25 µm coated capillary cartridge (Catalog #148-3031) in the cartridge holder and lock firmly in place by pushing the levers down.
6. Make sure the coolant reservoir (right side) is filled with water or other coolant and the helium or nitrogen gas tank valve is open, and start the program.

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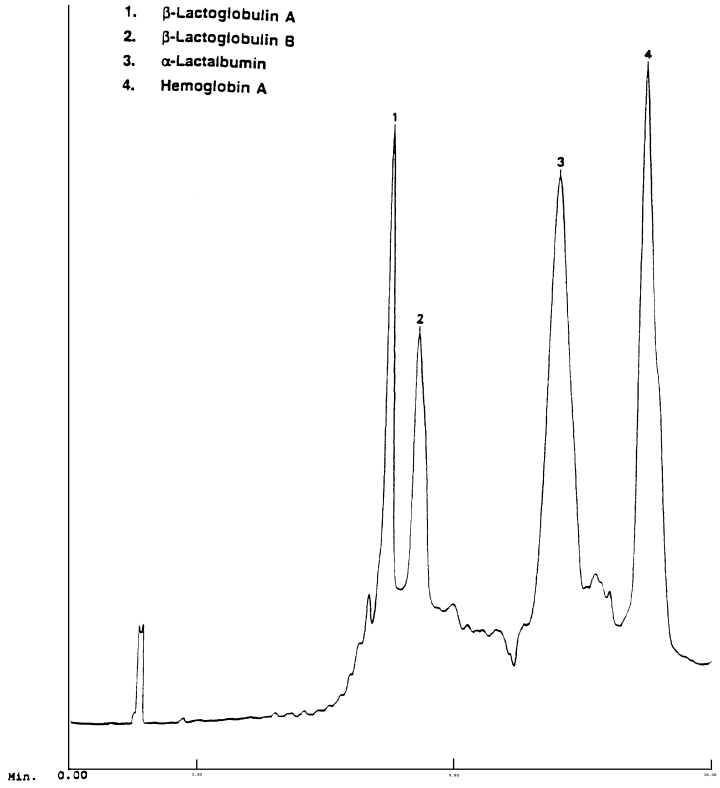


Figure 2 Separation of the Bio-Rad Protein Calibration Set in a Bio-Rad coated 24 cm x 25 µm capillary cartridge at pH 8.5 and 10 kV.

Capillary Isoelectric Focusing (CIEF)

Separation of proteins based on their isoelectric points can be achieved using isoelectric focusing. Capillary IEF consists of three phases: loading the capillary with sample and ampholytes, focusing sample components into bands, and mobilization of the sample bands past the detector. A coated capillary permits a pH gradient to be established without interference from electroendosmosis.

In the loading phase, the sample and ampholyte mixture is injected by pressure into the capillary. Focusing occurs when high voltage is applied and the sample ions migrate to their neutral point in the pH gradient formed by the ampholytes. Mobilization is achieved by changing the composition of the solution in one of the electrolyte vials, resulting in a pH shift which causes the focused sample bands to move past the detector.

In the IEF procedure that follows, the inlet electrode is the anode and the inlet vial contains anolyte; the outlet electrode is the cathode and the outlet vial contains catholyte or mobilizer. With this setup, the samples with the highest pI's focus closest to the outlet electrode and samples migrate past the detector in order of decreasing isoelectric points.

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Supplies Needed

- CE-IEF Catholyte, Sodium Hydroxide, Catalog #148-5028
- CE-IEF Anolyte, Phosphoric Acid, Catalog #148-5029
- CE-IEF Mobilizer, Catalog #148-5030
- CE-IEF Bio-Lyte® 3-10 Ampholytes, 2%, with 0.5% TEMED and 0.2% methyl cellulose, Catalog #148-5031
- Bio-Lyte 3-10 Ampholytes, 40% solution without TEMED or methyl cellulose, Catalog # 163-1112 (for proteins with pI's below 8.0).
- Bio-Rad TEMED (N,N,N',N'-Tetramethylethylenediamine), Catalog #161-0801.
- Methyl Cellulose (Catalog #M-0512, Sigma Chemical Co., St. Louis, MO) or Hydroxypropylmethylcellulose (Sigma Catalog #H-7509), viscosity of 2% aqueous solution at 25°C = 4000 centipoises. (For ease of use while preparing ampholyte solutions, it is convenient to prepare a 2% stock solution of the cellulose polymer in water.)
- Bio-Rad IEF Standards (pI 4.65-9.60), Catalog #161-0310
- BioFocus Capillary Cartridge, 17 cm x 25 µm, coated, Catalog #148-3030 **or**
- BioFocus Capillary Cartridge, 24 cm x 50 µm, coated, Catalog #148-3035 **or**
- BioCAP LPA Coated Capillary (50 µm x 375 µm OD x 1 m, Catalog #148-3070) installed in a Bio-Rad User Assembled Cartridge. Total capillary length, 24 cm.
- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Distilled or deionized water, filtered
- Pipettes or syringes for transferring reagents and sample
- Microcentrifuge tubes, 500 µl , Catalog #223-9503
- Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter, Catalog #343-0012

Sample Preparation for Isoelectric Focusing

For many protein samples, the only preparation required prior to isoelectric focusing is addition of ampholytes. The sample should be mixed with Bio-Lyte

3-10 Ampholytes (2% aqueous solution) to yield a final concentration of 1% ampholytes. This Bio-Lyte solution is specifically formulated for use with a BioFocus 17 cm x 25 µm cartridge to generate a pH 3-10

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gradient between the capillary inlet and detector window. For best detection sensitivity, the final protein concentration of the protein-ampholyte mixture should be approximately 200 - 500 µg/ml per peak. It is strongly recommended that the sample be filtered through a 0.45 µm micropore filter prior to analysis to minimize risk of plugging the capillary. The Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter (Catalog #343-0012) can be used for filtering small-volume samples.

The Bio-Lyte 3/10 ampholyte mixture (Catalog #148-5031) supplied for capillary IEF contains 0.2% methyl cellulose and 0.5% TEMED. TEMED focuses at the very basic end of the pH gradient, and occupies the portion between the detection point and the outlet of a 17 cm x 25 µm capillary. The pH 3-10 ampholyte gradient therefore occupies the portion between the inlet and the detection point of the 17 cm x 25 µm capillary. Therefore, all proteins with isoelectric points between 4-9.5 will be detected during cathodic mobilization. However, if the sample does not contain proteins with pI's above 8, improved resolution can be obtained by eliminating the TEMED. The following guidelines can be used:

If using a 17 cm x 25 µm capillary for Bio-Rad IEF Standards (Catalog #161-0310) and samples containing proteins with pI's above 8, dilute in Bio-Lytes 3/10, a 2% aqueous solution containing 0.5% TEMED and 0.2% methyl cellulose (Catalog # 148-5031), for a final protein concentration of 200-500 µg/ml and an ampholyte concentration of 1-2%. For best results, mix 10 µl of the Bio-Rad IEF Standards with 490 µl ampholytes in a 500 µl vial and centrifuge for 2 min. in a microcentrifuge.

If using a 24 cm x 50 µm IEF capillary, use Bio-Lytes 3/10, a 40% aqueous solution without TEMED or methyl cellulose (Catalog # 163-1112), then add TEMED (Bio-Rad, Catalog #161-0801) and methyl cellulose (Sigma, Catalog #M-0512) for a final ampholyte concentration of 2% and final TEMED and methyl cellulose concentrations of 0.354% and 0.2%, respectively.

For samples containing only proteins with pI's below 8, dilute to a protein concentration of 200-500 µg/ml with water or dilute buffer (< 5 mM), then add Bio-Lytes 3/10, a 40% solution (Catalog # 163-1112), for a final ampholyte concentration of 1-2%, then add methyl cellulose for a final concentration of 0.2%. Centrifuge for 2 min. in a microcentrifuge.

If working with narrow range ampholytes, be sure to mix them with wide range ampholytes (pH 3-10). Begin by adding narrow range ampholytes in increments of 10% to wide range ampholytes and testing different concentrations in the sample solution until peak resolution is optimized. As stated earlier, the final ampholyte concentration in the sample solution should be around 1%.

The presence of salt in a sample will interfere with the IEF process, causing a loss in resolution, long focusing and mobilization times, and increased risk of protein precipitation. For this reason, final sample salt

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concentration should be no greater than 5 mM. If the protein concentration of the sample is high, dilution of the sample in Bio-Lytes may be sufficient to reduce the salt concentration. If the protein concentration is less than 1 mg/ml, the sample should be dialyzed against 1% Bio-Lytes prior to analysis.

Protein precipitation is the most frequently encountered problem in capillary isoelectric focusing. In this technique, proteins become highly concentrated in narrow zones at their isoelectric points, with reduced solubility and an increased tendency to aggregate. Protein precipitation can be minimized by addition of a detergent to the protein-ampholyte mixture. The detergent should have good protein solubilization properties, but not interfere with the IEF process or with detection. Reduced Triton® X-100 (available from Aldrich Chemical Co., Milwaukee, WI, Catalog #28,210-3) has been shown to be effective in reducing protein precipitation and has minimal absorbance at 280 nm. Triton concentration in the sample/ ampholyte solution should be in the range of 0.1-1.0%.

Shutdown Procedure for IEF

After an IEF separation, it is usually not necessary to rinse the capillary to prepare it for the next separation. However, if the cartridge is to be stored, we recommend that you program the Shutdown method as the last run group in your automation sequence.

Instructions for IEF Separations

1. Turn on the system and allow a few minutes for the detector to stabilize. Carousel compartment temperature may be left at ambient (Not Set) or cooled for labile samples.
2. The proteins in solution should be present at a concentration of approximately 500 µg/ml. This solution is then mixed with an equal volume of ampholyte solution. If you wish to use Multi Inject for injection of internal pI markers with IEF, we recommend that you inject the internal pI markers after the sample using a Post Inject cycle at 20 psi*sec. Samples and markers should be prepared in 2% 3-10 Bio-Lyte ampholytes.
3. Program a Single Run Group IEF method, or define a new Configuration, Method and Autosequence using the IEF templates in the database. Enter the operating parameters appropriate to the sample's characteristics. The following can be used as general guidelines. Make adjustments where necessary. If using a 17 cm x 25 µm capillary, make sure to increase the inject time to 60 sec at 100 psi.

Inject time	20 sec at 100 psi (for 50 µm x 24 cm capillary)
Mode	Constant voltage
Polarity	+ to -

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Focus endpoint	240 seconds or 0.3 µA
Focusing voltage	15 kV
Mobilization voltage	15 kV
Current limit	100 µA (for all phases)
Detection	280 nm
Run time	30 minutes (focus & mobilization time)
Range	0.1 AUFS
Rise time	1 second
Capillary temp.	27°C
Autosampler temp.	20°C
Prep cycle(s)	none

4. Load your sample, waste, and reagent vials into the correct inlet and outlet positions for your programmed Configuration.
5. Insert a Bio-Rad 17 cm x 25 µm or 24 cm x 50 µm coated capillary cartridge into the cartridge holder and lock firmly in place by pushing the levers down.
6. Make sure the coolant reservoir (right side) is filled with water or other coolant (Fluorinert) and the helium or nitrogen gas tank valve is open.
7. Start the program.

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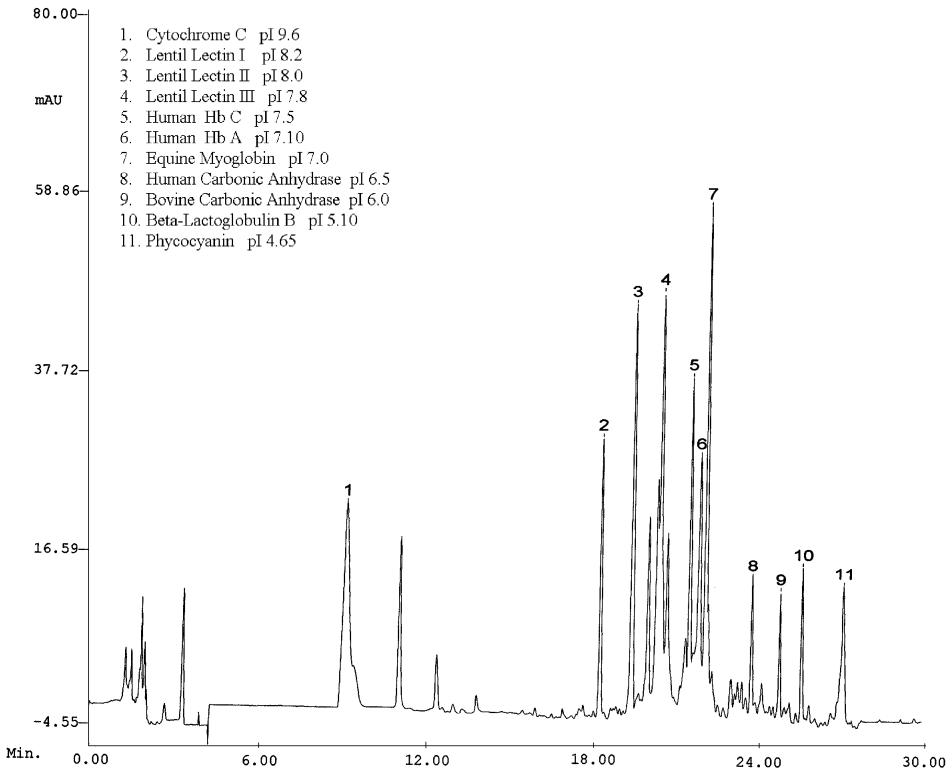


Figure 3 Separation of Bio-Rad IEF standards by CIEF using 3-10 ampholytes, 50 µm x 24 cm coated capillary and cathodic mobilization.

Capillary Dynamic Sieving (CDS) of DNA

Capillary Dynamic Sieving (CDS) is a specialized form of CZE performed with buffers containing hydrophilic polymer additives. CDS allows the simple and rapid size separation of analytes with identical mass-to-charge ratios with direct detection and without polymerizing a gel within the capillary. Analytes which can be analyzed by CDS include single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), oligonucleotides, and SDS-denatured proteins.

The following supplies and instructions are recommended for a dynamic sieving separation of dsDNA restriction fragments and PCR* products.

Supplies Needed

- PCR Analysis Buffer, pH 8.3, Tris-Borate-EDTA plus sieving polymer, Catalog #148-5024

* The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffman-LaRouche. Use of the PCR process requires a license.

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- Low Range DNA Size Standards (88 bp-1,746 bp), Catalog #170-3465
- BioFocus Capillary Cartridge, 24 cm x 50 µm, coated, Catalog #148-3035 **or**
- BioCAP LPA Coated Capillary (50 µm x 375 µm OD x 1 m), Catalog #148-3070
- installed in a Bio-Rad User Assembled Cartridge. Total capillary length, 24 cm. **or**
- BioCAP LPA Coated Capillary (75 µm x 375 µm OD x 1 m), Catalog #148-3071 installed in a Bio-Rad User Assembled Cartridge. Total capillary length, 24 cm.
- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Distilled or deionized water, filtered
- Pipettes or syringes for transferring buffer and sample
- Microcentrifuge tubes, 500 µl, Catalog #223-9503
- Microcon™-30 and -50 Microconcentrators, Amicon Catalog # 42409 and 42415
- MF-Millipore® Membrane Filters, 0.025 µm pore size, 25 mm diameter, Millipore Catalog #VSWP 025 00

Desalting Procedure

The electrophoretic sample loading method is very sensitive to sample ionic strength. For efficient sample loading, the salt concentration of the sample should be no more than 30 mM. Since the standard reaction buffer for the polymerase chain reaction contains 10 mM Tris + 50 mM KCl, a desalting step before analysis is recommended.

You may desalt or concentrate your PCR samples most efficiently by ultrafiltration using centrifugal concentrators. Microcon™ microconcentrators, supplied by Amicon in a full range of membrane cut-offs, provide a fast and efficient means of desalting/concentrating either single- or double-stranded DNA/RNA samples. They can be used in any centrifuge with a fixed-angle rotor that can accept 1.5 ml tubes. Typically, a 500 µl dsDNA sample (>50 bp) can be reduced to 10 µl using a Microcon-30 unit in about 8 minutes.

Alternatively, you may desalt your DNA sample by float dialysis using membrane filters. During dialysis, other small unwanted molecules such as mononucleotides are also removed from the sample matrix. The MF-Millipore Membrane Filters (Millipore Catalog #VSWP 025 00) can be used for dialysis of PCR products with the following procedure:

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1. In a petri dish filled with deionized water, float an MF-Millipore Membrane Filter (0.025 µm pore size, 25 mm diameter), shiny side up.
2. Using a pipette, apply a 5-25 µL (a droplet) of sample to the top of the filter. Dialyze sample for 15 to 30 minutes. To avoid sample degradation, dialysis should be performed at 4°C. The petri dish should be covered to avoid sample evaporation.
3. Transfer dialyzed sample to a vial and centrifuge for 2 minutes before injection.

Preconcentration Procedure

Polymerase chain reactions (PCR) which have gone through many amplification cycles usually have sufficient product DNA for detection without sample preconcentration. If not, ultrafiltration (see above) or ethanol precipitation and reconstitution of the sample in a small volume may be required, using the procedure outlined below. This procedure is recommended since the final aqueous ethanol wash will desalt the sample.

Note that other concentration methods such as lyophilization may result in high salt levels upon sample reconstitution, which will interfere with electrophoretic sample loading.

1. Add 1/10 volume of 3 M sodium acetate (pH 5.2) to the sample.
2. Add 2 volumes of cold 95% ethanol.
3. Hold in a dry ice + ethanol bath for 2 hours.
4. Centrifuge in a microcentrifuge for 15 minutes.
5. Draw off the supernatant and add 250 µl 70% aqueous ethanol.
6. Centrifuge for 4 minutes.
7. Draw off the supernatant and reconstitute the sample in the desired volume of water.

Instructions for Separations with the PCR Product Analysis Buffer

The run conditions used for dynamic sieving are basically the same as for CZE at high pH, but vary for detection wavelength, buffers, and cartridges. Larger diameter capillaries (50 µm or 75 µm) and a longer run buffer prep cycle (80 sec for a 75 µm x 24 cm capillary and 180 sec for a 50 µm x 24 cm capillary) are needed because of the increased viscosity of the buffer solutions that contain the polymer additives. To prevent carryover of the viscous run buffer into the sample, it is desirable to program a zero-second prep cycle after the run buffer prep cycle using a prep vial filled with deionized water. This additional prep cycle serves to rinse the viscous buffer from the electrode and capillary surfaces.

Use of predefined configuration, method, and autosequence templates provided in the BioFocus operating software is recommended for performing dsDNA separations with the PCR Product Analysis buffer. If you wish, you can also define a new configuration, method, and autose-

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quence from the predefined templates using different parameters. If the reference PCR method template has been previously deleted from your database, define a new run method with the following values:

Inject time	4 sec electrophoretic (10 kV) or 20 psi*sec (pressure)
Mode	Constant Voltage
Polarity	- to +
Running Voltage	2.5 kV
Running Current	6 µA
Current Limit	50 µA
Wavelength	260 nm
Run time	30 minutes
Range	0.01 AUFS
Rise Time	1 second
Capillary Temp.	40°C
Autosampler Temp.	20°C
Prep cycle	2 high pressure pre-inject cycles: 180 sec PCR Analysis Buffer, 0 sec water

If you use a Single Run Group method, immediately after the last run, be sure to program a SRG Shutdown method to rinse the capillary with water for 300 sec to prevent the capillary from clogging. If you use the Main screen menus, then program a Shutdown method as the last run group in your automation sequence and select the Vials UP option from the toolbar.

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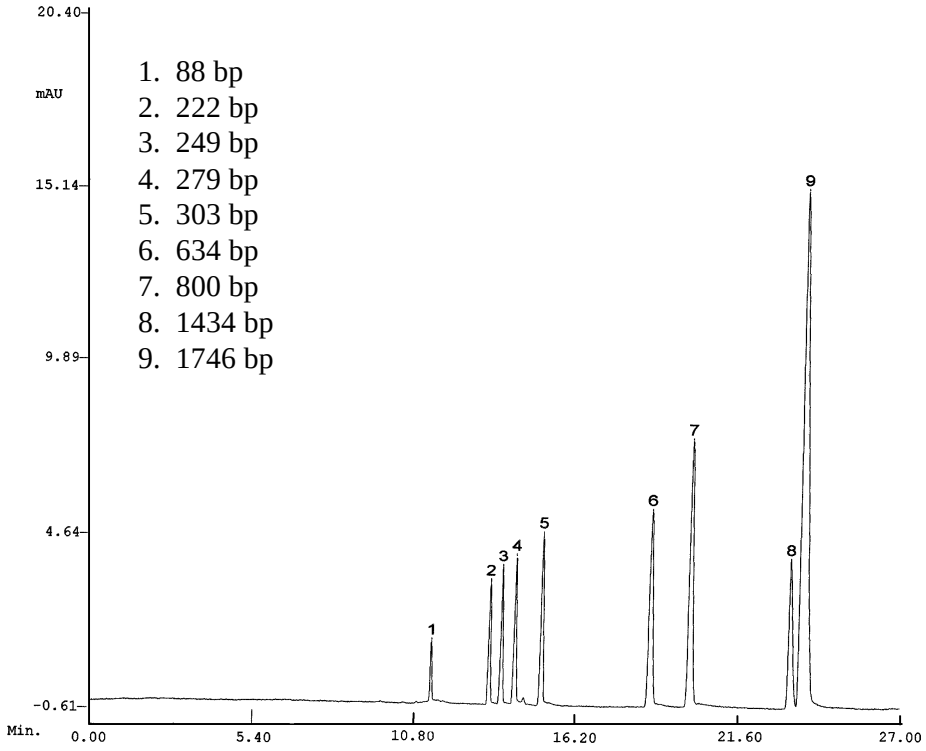


Figure 4 Capillary dynamic sieving of Low Range DNA Size Standards (88 - 1746 bp)

Dynamic Sieving of SDS-Protein Complexes

Capillary dynamic sieving a-

weight and for purity assessment of protein samples.

y-

Supplies Needed

- CE-SDS Protein Kit, Catalog # 148-4160, which includes the following 7 items:

  CE-SDS Protein Run Buffer, 50 ml, Catalog # 148-

  2.

  148-5033

  3. CE-SDS Protein Size Standards, 200 µl (20 mg/ml), Catalog # 148-2015 (supplied in reduced form, with

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4. CE-SDS Internal Reference, 3 ml (1 mg/ml benzoic acid), Catalog # 148-2016
5. CE-SDS Basic Wash Solution, 0.1 N NaOH, 30 ml
6. CE-SDS Acidic Wash Solution, 0.1 N HCl, 30 ml
7. Two BioCAP Bare Silica Capillaries, 50 µm ID x 375 µm OD x 40 cm, Catalog #148-3060 (should be installed in a Bio-Rad User Assembled Cartridge). Total capillary length 24 cm after installation.

- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Reducing agent, 2-mercaptoethanol, Catalog #161-0710 or
- dithiothreitol, Catalog #161-0613
- Microcentrifuge vials, 500 µl, Catalog #223-9503
- Large capacity microcentrifuge tubes for purge buffer and waste, 1500 µl, Catalog #223-9480
- Distilled or deionized water, filtered
- Pipettes or syringes for preparation and transfer of samples and buffers
- Microcentrifuge for degassing buffers and samples
- Water bath, 95-100°C.

Preparation of Standards

The sample preparation procedure is designed to quantitatively convert proteins to SDS-protein complexes. Samples and standard mixtures should be prepared fresh daily.

1.

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The maximum tolerable salt concentration in the prepared sample

pressure injec

The potassium salt of SDS has low solubility. If the sample is known to contain potassium salts, it should be dialyzed against 25 mM Tris-HCl

NOTE: Use of a contact heater block may not pr - vide sufficient heat for

1.

CE SDS Internal Reference. If samples are to be analyzed in reduced form, 2-mercaptoethanol (Bio-Rad Catalog #161-0710) or dithiothreitol

Use of a water bath for sample and standard m-

2.5% or 15 mM, respectively. Add deionized water to bring total sample volume to 200 µl.

water bath for 10 minutes. Cool the mixture, centrifuge for 2 min. in a microcentrifuge, then hold at 4 °C prior to analysis.

Complexes by Dynamic Sieving

Use of predefined configuration, method, and autosequence templates r-

buffer. If you wish, you can also define a new configuration, method, and autosequence from the predefined templates using different p - rameters. If the reference SDS-Protein method template has been pr - viously deleted from your database, define a new run method with the

Inject time	10 kV for 5 sec (electrophoretic) or
Mode	Constant Voltage
	- to +
	15 kV
Running Current	
Current Limit	50 µA
	220 nm
Run time	
Range	0.01-0.05 AUFS
	1 sec
Capillary temp.	
Autosampler	20°C
	5 high pressure pre-inject cycles: 90 sec 0.1 N NaOH,

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60 sec 0.1 N HCl,
120 sec CE-SDS Protein Run Buffer,
0 sec with 1:1 diluted CE-SDS Protein Sample Buffer
0 sec with 1:1diluted CE-SDS Protein Sample Buffer

When analyzing multiple samples, best run-to-run reproducibility is achieved by using a fresh reagent set (inlet and outlet run buffers and prep reagents) for every ten runs.

If you wish to use Multi Inject to inject the internal reference standard with the SDS-Protein system, add the standard in the reagent database and use a Pre Inject cycle at 120 psi*sec before the electrophoretic injection (at 10 kV, 5 sec) of the sample. If you are using a compound other than the supplied benzoic acid as an internal standard, we recommend that you prepare it in water and use the same injection parameters. The sample can also be introduced by pressure injection; however, this results in loss of resolution.

When using sieving buffers containing polymers, it is strongly recommended that the capillary tips not be exposed to air for extended lengths of time. Exposure will cause drying, leading to capillary plugging. To minimize the risk of capillary plugging, the following precautions are recommended:

1. Place deionized water in the waste vial so the capillary outlet is immersed during prepping (use 200 µl water for 500 µl waste vial or 500 µl water for 1.5 ml waste vial).



2. Program the BioFocus system to leave the buffer vials in the elevated po sition following analysis (click on the "Vials up at end of run" button on the toolbar).

sec and water for 120 sec. This sequence may be automatically pro grammed at the end of an automation sequence using a Shutdown Method in the BioFocus software.
method, immediately after the last run, be sure to program an SRG Shutdown method to prevent the capillary from plugging.

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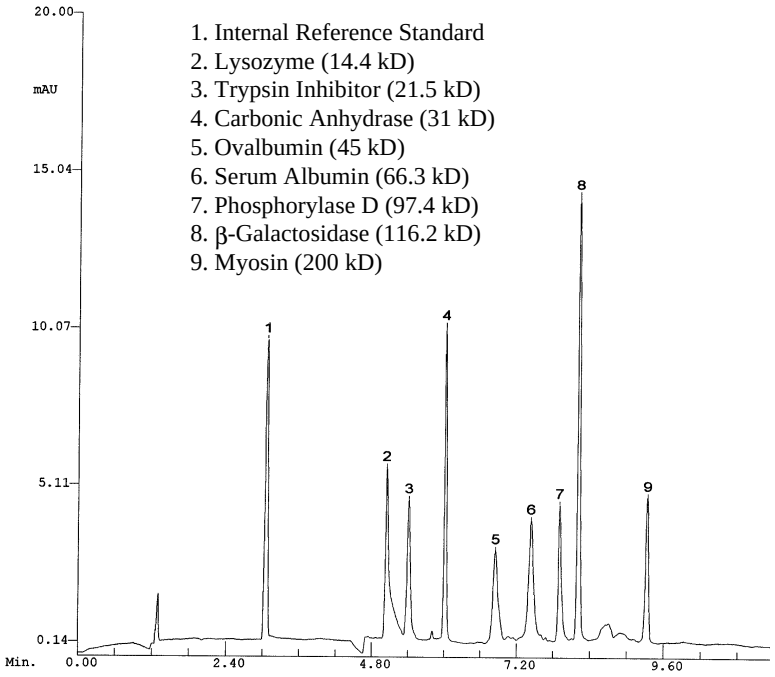


Figure 5 Rapid separation of Bio-Rad's CE SDS-Protein Size Standards (14-200 kD) by dynamic sieving using the CE SDS-Protein Run Buffer and a 50 µm x 24 cm uncoated capillary.

Capillary Gel Electrophoresis (CGE)

Capillary Gel Electrophoresis (CGE) allows automated analysis of oligonucleotide primers and probes, antisense therapeutic agents, and DNA sequencing reaction products with speed and convenience not possible with slab-gel techniques.

The following supplies and instructions are recommended for rapid analysis of synthetic oligonucleotides using denaturing polyacrylamide gel-filled capillaries on the BioFocus CE systems.

Supplies Needed

- µPAGE™-5 (5%T, 5%C) polyacrylamide gel-filled capillary, 75 µm x 75 cm long (50 cm effective length) with 100 mM Tris-borate, 7 M urea buffer, pH 8.3. J&W Catalog # 193-5211, installed in a Bio-Rad User Assembled Cartridge
  or
- µPAGE-3 (3%T, 3%C) Polyacrylamide gel-filled capillary, 75 µm x 75 cm long (50 cm effective length) with 100 mM Tris-borate, 7 M urea buffer, pH 8.3. J&W Catalog # 193-3211, installed in a Bio-Rad User Assembled Cartridge
- BioFocus User Assembled Capillary Cartridge Assembly Kit , Catalog #148-3050
- µPAGE pd$(A)_{25\text{-}30,\ 40\text{-}60}$ Oligonucleotide Standard.

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- µPAGE Buffer, 100 mM Tris-borate, 7 M Urea, pH 8.3. J &W Catalog #590-4001
- Pipettes and syringes for transferring buffer and sample
- Microcentrifuge tubes, 500 µl, Catalog #223-9503

Sample Preparation

1. Under vacuum, dry the HPLC-purified and ethanol-precipitated synthetic oligonucleotide to a residue.
2. For oligonucleotides with <30 bases, resuspend in deionized water and analyze.
3. For oligonucleotides with >30 bases, or to eliminate secondary structure of oligonucleotides with high G content and self complementary regions, resuspend in 66.6% formamide, 33.3% water and heat to >90°C for 5 minutes prior to analysis.

Instructions for CGE Separation of Synthetic Oligonucleotides

1. Turn on the system and allow a few minutes for the detector to stabilize. Set carousel compartment temperature at 20°C.
2. For optimal results, prepare oligonucleotide samples as described above.
3. Load your standards, samples, waste and run buffer vials into the correct inlet and outlet positions. For best results, refresh buffer vials after every other injection.
4. Program your Configuration and Methods before installing the gel-filled capillary. New gel-filled capillaries or capillaries which have been idle for 4 or more hours should be preconditioned before use. Program a main screen menu Configuration, three CZE Methods (at 5 kV, 8 kV and 10 kV) with no injection for capillary conditioning, and one CZE Method at 10 kV with electrophoretic injection for running standards and samples. Make sure no prep cycles are defined. Then, link all methods in an Automation Sequence.
5. The following values should be used for the $pd(A)_{25\text{-}30,\ 40\text{-}60}$ Oligonucleotide Standard. Values for capillary conditioning steps are given in parenthesis.

   Inject time 5 kV for 3 sec (no injection for conditioning steps)

   Mode Constant Voltage

   Polarity - to +

   Running Voltage 10 kV (5 kV, 8 kV or 10 kV for conditioning steps)

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Current Limit	50 µA
Wavelength	260 nm
Run time	24 min. (5-10 min. for each conditioning step)
Range	0.01 AUFS
Rise Time	1 sec
Capillary	20°C
Autosampler	20°C
Prep cycles	none



Vials up button

6. Program the BioFocus system to leave the buffer vials in the elevated position following each analysis (click on the "Vials up at end of run" button on the toolbar).

7. Gel-filled capillaries should be protected from thermal shock, voltage shock, and dehydration. Please read J&W's µPAGE Column Storage, Installation and Operating Instructions before installing the gel-filled capillaries in a Bio-Rad User Assembled Cartridge.

8. Install your capillary in a User Assembled Cartridge according to instructions supplied by Bio-Rad Laboratories. To avoid gel dehydration and consequent bubble formation, it is very important that the gel capillary tips be immersed in run buffer solution not more than a few seconds after cutting and remain in this solution at all times. Immediately after cutting the ends of the capillary, place the cartridge in the cartridge holder and push the levers down to lock it firmly in place.

9. Make sure the coolant reservoir (right side) is filled with water and the helium or nitrogen gas tank valve is open.

10. Start the program.

11. After the capillary conditioning procedure, the current should stabilize at approximately 4-5 µA. The gel capillary is now ready for use with your sample. If the current fluctuates, abort the run and inspect the capillary inlet for voids (bubbles).

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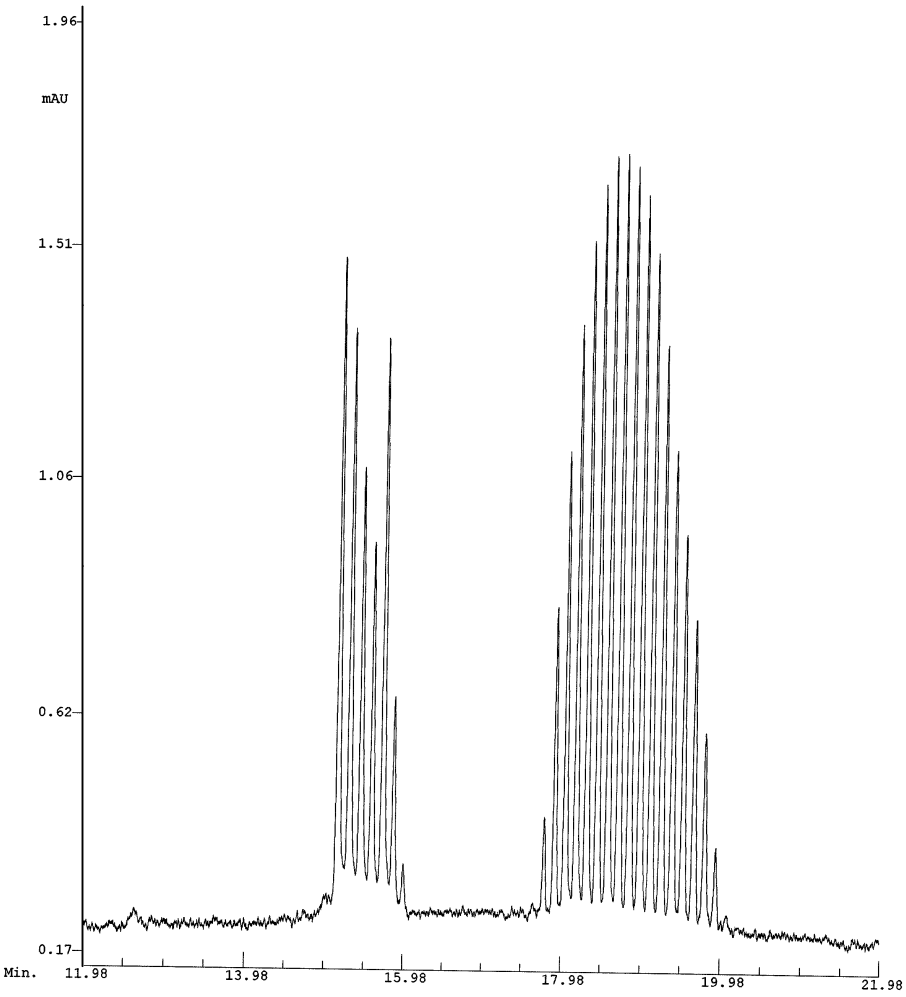


Figure 6 Separation of pd(A)$_{25\text{-}30,\ 40\text{-}60}$ Oligonucleotide Standard in a 5%T, 5%C polyacrylamide gel-filled capillary (75 µm x 50 cm) using the BioFocus 3000 CE system.

Micellar Electrokinetic Capillary Chromatography (MECC)

Micellar Electrokinetic Capillary Chromatography (MEKC or MECC), a hybrid of electrophoresis and chromatography, uses ionic micelles (anionic or cationic) as the separation carrier and electroosmotic flow (EOF) as the mobile phase pump. MECC is the only electrophoretic technique that can be used for the separation of both neutral and charged analytes.

MECC is most commonly performed using uncoated capillaries with high pH buffers to generate high rates of EOF. The separation of neutral species by MECC is accomplished by the use of ionic surfactants, such as sodium dodecyl sulfate (SDS), in the run buffer. At concentrations above the critical micelle concentration (CMC), aggregates of in-

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dividual SDS molecules (micelles) are formed. In the micelle, the hydrophobic dodecyl tails associate at the micelle interior, and the anionic sulfate heads lie on the micelle surface. Sample molecules, distributed between the mobile phase and the surfactant micelles, are separated by differences in their mobile phase:micelle partition ratios. The direction of EOF is towards the cathode, while the negatively-charged micelles migrate in the opposite direction, towards the anode.

Sample molecules can exhibit the following behavior in an SDS micellar system:

Neutral molecules: Very hydrophilic molecules will be swept through the capillary at the rate of osmotic flow, passing the detector at a time t_{eof} (chromatographically, having a k' = 0). Very hydrophobic molecules will remain totally inside the micelle and move past the detector at the time it takes the micelle to traverse this distance, t_{mc} (chromatographically, having a k' = infinity). Sample molecules of intermediate hydrophobicity will be distributed between t_{eof} and t_{mc}.

Anionic molecules: These will have an electrophoretic mobility in the same direction as the micelle. If an anionic analyte has a very high electrophoretic mobility, it could migrate after t_{mc}. If anionic sample molecules have hydrophobic groups, their migration will be modified by interaction with the micelle.

Cationic molecules: These should move in the direction of EOF, and (if they are very polar) can migrate ahead of t_{eof}. However, cations can be retarded by ion-exchange and hydrophobic interactions with the micelle surface and display long migration times.

To determine the value of t_{eof}, a very hydrophilic neutral species such as nicotinamide or methanol can be injected. For anionic SDS micellar systems, timepidium bromide or quinine hydrochloride are good markers for the micelle (t_{mc}).

The following supplies and instructions are recommended for an SDS micellar separation of water-soluble vitamins.

Supplies Needed

- MECC Run Buffer, 100 mM sodium borate (pH 8.5) with 50 mM SDS, (adjust a 100 mM boric acid solution to pH 8.5 with NaOH, then make it up 50 mM in SDS)
- Electroosmotic flow marker, t_{eof} (nicotinamide or methanol)
- Marker for the micelle, t_{mc} (timepidium bromide or quinine hydrochloride)
- Relative mobility marker, t_{is} (internal standard, ethyl p-aminobenzoate for vitamins)
- Basic Wash Solution, 0.1 N NaOH
- Acidic Wash Solution, 1 N HCl

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- BioFocus Capillary Cartridge, 50 cm x 50 µm, uncoated, Catalog #148-3040, **or**
- BioCAP Bare Silica Capillary, 50 µm ID x 375 µm OD x 1 m, Catalog #148-3062 installed in a Bio-Rad User Assembled Cartridge. Total capillary length 50 cm.
- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Large capacity microcentrifuge tubes for purge buffer and waste, 1500 µl, Catalog #223-9480
- Distilled or deionized water, filtered
- Pipettes or syringes for preparation and transfer of samples and buffers
- Microcentrifuge for degassing buffers and samples

Sample Preparation

For good reproducibility, run buffer and sample pH should be the same and tightly controlled (<0.02 pH units). For best results, take sample up in 10X diluted MECC run buffer without SDS (10 mM sodium borate, pH 8.5), adjust sample pH to 8.5, and then bring the SDS concentration up to 50 mM. Filter and degas samples by spinning them in a microcentrifuge for about 2 minutes.

Use of two internal standards is recommended: one as a neutral hydrophilic *electroosmotic flow marker*, another as a relative *mobility marker*. The neutral EOF marker is used to determine effective mobilities. The effective mobility of each analyte peak is then normalized to that of internal standard by calculating relative mobility (μ_{rel}) as:

$$\mu_{rel} = \frac{\mu_{eff}^{an}}{\mu_{eff}^{is}}$$

where μ_{eff}^{an} = effective mobility of the analyte

μ_{eff}^{is} = effective mobility of the internal standard

Instructions for MECC Separations with the BioFocus

1. Use Pressure Diagnostics to precondition a new uncoated capillary by using the following steps:
   a. Purge for 5 minutes with 1 N HCl. Let the capillary sit for 1 hour.

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b. Purge for 5 minutes with deionized water.

c. Purge for 5 minutes with 0.1 N NaOH. Let the capillary sit for 1 hour.

d. Purge for 5 minutes with deionized water.

e. Purge for 5 minutes with MECC run buffer.

Plan to discard the first 5-7 runs. The capillary should then be stable for hundreds of runs.

2. Program a Single Run Group (SRG) method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. Program the following method values:

Inject time	3 psi*sec (pressure)
Mode	Constant Voltage
Polarity	+ to –
Running Voltage	15 kV
Running Current	20 µA
Current Limit	50 µA
Wavelength	200 nm
Run time	30 min.
Range	0.05 AUFS
Rise Time	1 sec
Capillary temp.	20°C
Autosampler temp.	27°C
Prep cycle(s)	1 high pressure pre-inject cycle: 60 sec with run buffer

If you wish to use Multi Inject to inject internal standards with MECC, we recommend that you inject the internal standards before the sample using a Pre Inject cycle. Samples and internal standards should be prepared in 1:10 (v:v) diluted MECC run buffer containing full-strength surfactant (e.g., 50 mM SDS).

3. If you plan to run MECC separations with the same buffer system for extended periods, create a Shutdown Method which purges the capillary for 3 minutes, only with the run buffer (eliminate the prep cycle with Nitrogen).



Vials up button

4. Program the BioFocus to leave vials in the up position at the end of an automation sequence (click on the "Vials up at end of run" button on the toolbar). This will minimize stabilization time when starting the next automation sequence.

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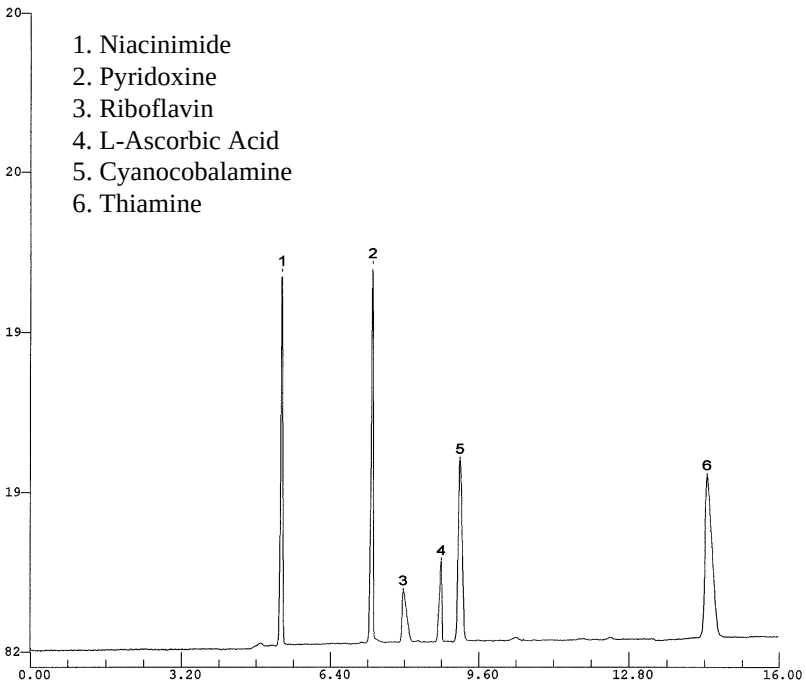


Figure 7 MECC separation of six water-soluble vitamins (100-200 µg/ml each) using the BioFocus CE system and a 50 µm x 50 cm uncoated capillary.

Calculation of Effective Mobility

In capillary zone electrophoresis, migration time or apparent mobility should not be used for the identification of solute peaks when electro-osmotic flow (EOF) is present, because it varies considerably with the state of the capillary. The effective mobility of a solute is more characteristic and is a better parameter for peak identification (see J. Beckers *et al.*., *J. Chromatogr.* **537**, 407 (1991)). Effective mobility (μ_{eff}) is calculated as:

$$\mu_{eff} = \mu_{app} - \mu_{eof}$$

where μ_{app} = apparent mobility

μ_{eof} = EOF coefficient

Apparent mobility (μ_{app}) is calculated as:

$$\mu_{app} = \frac{L_{eff}}{(T_m)(E)}$$

where

L_{eff} = effective length of the capillary in cm (distance from capillary inlet to the detection point)

T_m = migration time of the analyte in seconds

E = field strength in volts/cm

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EOF coefficient (μ_{eof}) is calculated as:

$$\mu_{eof} = \frac{L_{eff}}{(T_{eof})(E)}$$

where

T_{eof} = migration time of neutral marker

Thus,

$$\mu_{eff} = \mu_{app} - \mu_{eof} = \frac{L_{eff}}{(T_m)(E)} - \frac{L_{eff}}{(T_{eof})(E)}$$

Since

$$\frac{V}{L_c}$$

where L_c = capillary length

V = voltage

The equation for the calculation of μ_{eff} for any analyte peak or the internal standard can be written as:

$$\mu_{eff} = \frac{(Lc)(L_{eff})}{(V)(T_m)} - \frac{(Lc)(L_{eff})}{(V)(T_{eof})}$$

In micellar electrokinetic capillary chromatography, the equivalent parameter is "pseudo-effective mobility", μ_{eff}^{ps}, and it is calculated in the identical manner for μ_{eff} as defined above for capillary zone electrophoresis (Ackermans *et al., J. Chromatogr.* **585**, 123 (1991)).

Chiral Analysis by Capillary Zone Electrophoresis (CZE)

Chiral analysis by CZE usually involves the addition of a chiral selector to a low-pH run buffer. Chiral selectors include native and derivatized cyclodextrins, crown ethers, bile salts, proteins, and carbohydrates. Selectivity can be altered by adjusting the type and concentration of the chiral additive, and also by the use of other additives such as alcohols, surfactants, urea, and metal ions.

Cyclodextrins (CDs) are the most widely used chiral selectors. They are nonionic cyclic oligosaccharides consisting of six, seven, eight, or nine glucose units, designated as α, β, γ and δ-CDs, respectively. Their shape is similar to a truncated cone, with a relatively apolar cavity, and two relatively hydrophilic rims containing the hydroxyl groups.

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Cyclodextrins interact with analytes mainly by inclusion complexation mechanisms. Chiral selectivity results from inclusion of an hydrophobic portion of the analyte in the CD cavity and also from hydrogen bonding to the chiral hydroxyl moieties. At low pH, electroosmotic flow is minimal and the uncharged CD does not migrate. However, basic compounds become protonated and migrate towards the detector. When the analyte becomes included into the CD cavity, its mobility is greatly reduced. The extent of the inclusion depends on the stability of the complex formed. If the two enantiomers have different stability constants, one enantiomer migrates more slowly than the other and chiral resolution is achieved.

The following supplies and instructions are recommended for the chiral separation of the (-) and (+) enantiomers of epinephrine and norepinephrine by CZE.

Supplies Needed

- Run Buffer, 10 mM Tris (adjusted to pH 2.4 with H_3PO_4) plus 18 mM Heptakis (2,6-di-O-methyl-β-cyclodextrin)
- BioFocus Capillary Cartridge, 24 cm x 25 µm, coated, Catalog #148-3031
- Microcentrifuge tubes, 500 µl, Catalog #223-9503
- Large capacity microcentrifuge tubes for purge buffer and waste, 1500 µl, Catalog #223-9480
- Distilled or deionized water, filtered
- Pipettes or syringes for preparation and transfer of samples and buffers
- Microcentrifuge for degassing buffers and samples

Instructions for Chiral Separations with the BioFocus

Program a Single Run Group CZE Method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. Program the following method values:

Inject time	6 kV for 6 sec
Mode	Constant Voltage
Polarity	+ to −
Running Voltage	9.6 kV
Running Current	4 µA
Current Limit	50 µA
Wavelength	206 nm

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Run time	8 min.
Range	0.01 AUFS
Rise Time	1 sec
Capillary temp.	20°C
Autosampler temp.	20°C
Prep cycle(s) buffer	1 high pressure pre-inject: 120 sec with run

For a more extensive discussion of chiral separations by CZE or MEKC, refer to *"An Introduction to Chiral Analysis by Capillary Electrophoresis"*, by Salvatore Fanali (Bio-Rad Bulletin No. 1973).

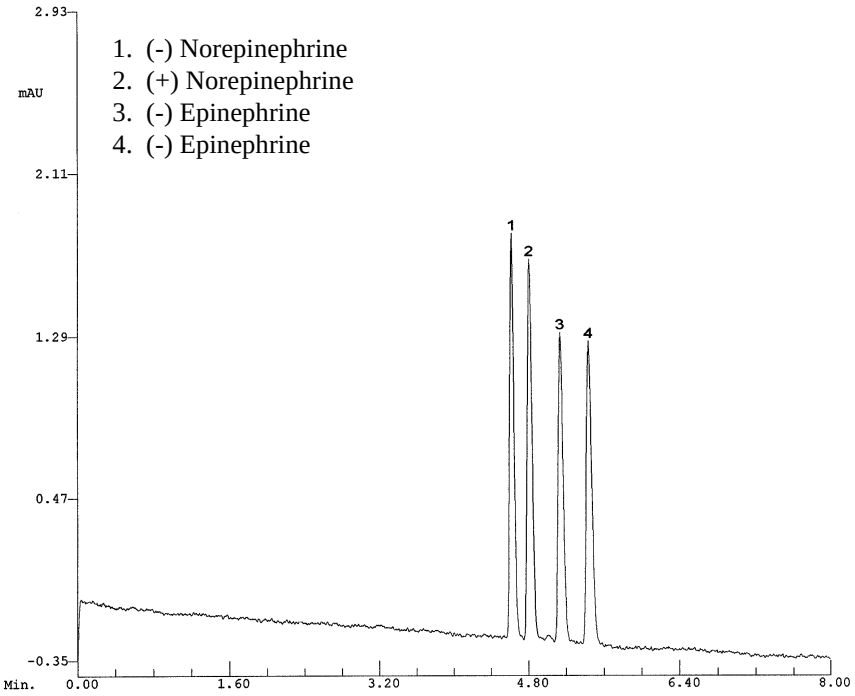


Figure 8 Chiral separation of the (-) and (+) enantiomers of epinephrine and norepinephrine using the BioFocus CE system and a 25 µm x 24 cm coated capillary.

Capillary Ion Analysis (CIA) of Inorganic Anions by Indirect Detection

Capillary ion analysis (CIA) is a capillary electrophoretic technique designed for the simple and rapid determination of inorganic and organic ions.

Since the majority of the ions do not absorb in the UV, capillary ion analysis is performed predominantly by indirect UV detection. This is

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accomplished by adding a UV-absorbing salt (co-ion) into the background electrolyte. The co-ion must possess at least one unique UV-absorbing maximum in a region that is not shared by the analytes. In this application, pyromellitic acid (PMA) is used as the buffer co-ion, since it has strong absorbance between 250-260 nm where there is no interference from analyte anions.

To achieve good peak symmetry and high sensitivity, it is important that the UV-absorbing co-ion have a mobility similar to that of the analyte anion. If the mobility of the co-ion is significantly higher or lower than that of the analyte, the analyte peak will display tailing or fronting, respectively. PMA is well-matched for the analysis of high-mobility inorganic anions such as chloride, sulfate, and nitrite. To analyze anions with mobilities significantly different from PMA, one can select from a family of co-ions. Typical co-ions for the intermediate- and low-mobility electrolytes are phthalate and *p*-hydroxybenzoate, respectively. For example, phthalic acid is suitable for the analysis of organic acids.

Capillary ion analysis separations require that the electroosmotic flow (EOF) moves in the same direction as the analytes, negative to positive. This is accomplished by adding an EOF modifier consisting of an aliphatic quaternary ammonium compound to the electrolyte. The EOF modifier dynamically coats the inner wall of the uncoated fused-silica capillary through electrostatic attraction to the silanol groups and forms a bilayer that exposes the positively charged quaternary ammonium groups to the electrolyte. This reverses the direction of EOF, and independent of pH, a stable anodic EOF is established. The electrolyte pH can then be varied to change selectivity of anions without affecting the magnitude of EOF. In this application, hexamethonium hydroxide is added to the running electrolyte (pyromellitic acid) as the osmotic flow modifier.

For quantitative analysis, the negative peaks produced during the separation can be inverted during integration by selecting the Invert box in the Integrator File Open window before starting the integration process. When performing integration automatically during execution of an automation sequence, an integration method should be used in which the "Invert Negative Peaks" on the integration parameters screen is checked. This will invert the electropherogram so the negative ion peaks will appear in the integration database as positive peaks. See the integration manual for more information.

The following supplies and instructions are recommended for the analysis of inorganic anions by indirect detection using the BioFocus CE system. For best results, we strongly recommend use of large capacity vials (1.5 ml) for buffers.

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Supplies Needed

- Background Electrolyte, 2.25 mM Pyromellitic acid, 6.5 mM NaOH, 0.75 mM Hexamethonium hydroxide, 1.6 mM Triethanolamine, pH 7.5-7.9.
- Pyromellitic Acid, Sigma Chemical Co. Catalog #P-8544
- Triethanolamine, Sigma Chemical Co. Catalog #T-1377
- Hexamethonium Bromide (osmotic flow modifier), Sigma Chemical Co. Catalog #H-0879.
- AG 1-X8 Anion Exchange resin, hydroxide form, Bio-Rad Catalog # 143-2445 (to convert hexamethonium bromide to the hydroxide form, see below).
- Basic Wash Solution, 0.1 N NaOH
- Distilled or deionized water, filtered
- BioFocus Capillary Cartridge, 50 cm x 50 µm, uncoated, Catalog #148-3040 **or**
- BioCAP Bare Silica Capillary, 50 µm ID x 375 µm OD x 1 m, Catalog #148-3062 installed in a Bio-Rad User Assembled Cartridge. Total capillary length 50 cm.
- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter, Catalog #343-0012
- Microcentrifuge tubes, 500 µl, Catalog #223-9503
- Large capacity microcentrifuge tubes for run buffer, purge buffer and waste, 1.5 ml, Catalog #223-9480
- Pipettes or syringes for preparation and transfer of samples and buffers
- Microcentrifuge for degassing buffers and samples

Preparation of Hexamethonium Hydroxide Stock Solution (25 mM)

1. Add 0.95 g hexamethonium bromide to a 100 ml volumetric flask containing 50 ml deionized water. Mix thoroughly, and then dilute to 100 ml with deionized water (25 mM stock solution).
2. Pack a 5- or 10-ml column with AG 1-8X resin. Wash the column with 10 ml 1 M NaOH followed by 10 ml deionized water. Add 10 ml 25 mM hexamethonium bromide stock solution to the column and discard the first 2 ml eluate and retain the remaining 8 ml.

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Sample Preparation

Depending on the analyte, the quantitative range is from about 100 parts per billion (ppb) to approximately 10 parts per million (ppm), using a 50 µm I.D. fused silica capillary. To bring analyte concentrations into the linear calibration range and to enhance stacking, samples with concentrations exceeding 10 ppm should be diluted in water instead of the running electrolyte. If the analyte of interest is in trace amounts with respect to a major constituent, select the optimum mobility electrolyte that best matches the analyte of interest and if possible, increase the ionic strength of the running electrolyte.

Instructions for Analyzing Inorganic Anions by Indirect Detection Using the BioFocus System

1. Program a Single Run Group CZE Method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. Program the following method values:

Inject time	5 psi*sec (pressure)
Mode	Constant Voltage
Polarity	- to +
Running Voltage	20 kV
Running Current	7 µA
Current Limit	50 µA
Wavelength	250 nm
Run time	8 min.
Range	0.01 AUFS
Rise Time	1 sec
Capillary temp.	20°C
Autosampler temp.	20°C
Prep cycle(s)	1 high pressure pre-inject cycle: 240 sec with background electrolyte

2. Prepare fresh background electrolyte daily. Filter through a Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter (Catalog #343-0012) that is prerinsed with deionized water prior to use and spin for 2 min. using a microcentrifuge.
3. Use Pressure Diagnostics to precondition a new uncoated capillary by using the following purge sequence:
   a. Purge for 5 minutes with deionized water.
   b. Purge for 10 minutes with 0.1 N NaOH.
   c. Purge for 5 minutes with deionized water.
   d. Purge for 4 minutes with background electrolyte.

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Vials up button

4. For best results, replace running electrolyte every five runs.
5. When using the same capillary on consecutive days, create a Shutdown Method which purges the capillary with deionized water (120 sec), 0.1 N NaOH (300 sec), deionized water (120 sec) and finally with the background electrolyte (240 sec). Program the BioFocus to leave vials in the up position at the end of the automation sequence (click on the "Vials up at end of run" button on the toolbar). This will ensure day-to-day reproducibility and minimize stabilization time when starting runs the next day.

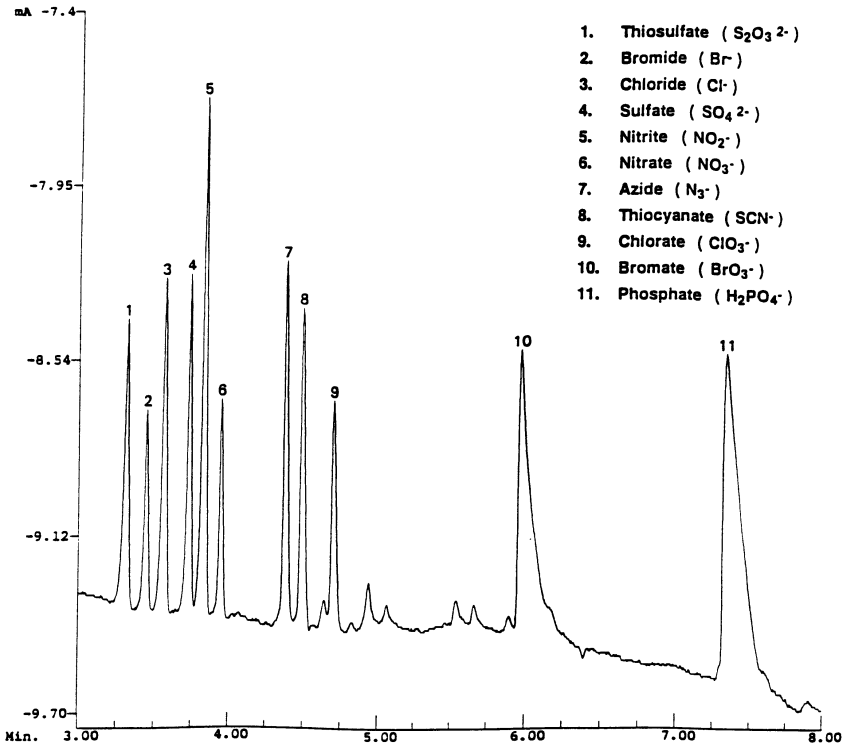


Figure 9 Capillary ion analysis of inorganic anions by indirect detection in a 50 µm x 50 cm uncoated capillary at pH 7.5 and 20 kV.

Analysis of Organic Acids by Capillary Electrophoresis with Indirect Detection

The following supplies and instructions are recommended for the analysis of organic acids by indirect detection using the BioFocus CE system.

Supplies Needed

- Background Electrolyte, 5 mM sodium phthalate (pH 5.6) with 0.5 mM CTAB (Cetyltrimethylammonium Bromide)

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- Phthalic acid, Sigma Chemical Co. Catalog #P 8657
- Cetyltrimethylammonium bromide (CTAB), as osmotic flow modifier,
- Sigma Chemical Co. Catalog #H 5882
- Basic Wash Solution, 0.1 N NaOH
- Distilled or deionized water, filtered
- Bare Silica Capillary, 75 µm ID x 375 µm OD, installed in a Bio-Rad User Assembled Cartridge. Total capillary length 100 cm.
- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter, Catalog #343-0012
- Microcentrifuge tubes, 500 µl, Catalog #223-9503
- Large capacity microcentrifuge tubes for run buffer, purge buffer and waste, 1500 µl, Catalog #223-9480
- Pipettes or syringes for preparation and transfer of samples and buffers
- Microcentrifuge for degassing buffers and samples

Sample Preparation

To bring analyte concentrations into the linear calibration range and to enhance stacking, samples should be diluted in water instead of the running electrolyte.

Instructions for Analyzing Organic Acids by Indirect Detection Using the BioFocus System

1. Program a Single Run Group CZE Method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. Program the following method values:

Injection	10 kV for 5 sec
Mode	Constant Voltage
Polarity	- to +
Running Voltage	20 kV
Running Current	12 µA
Current Limit	50 µA
Wavelength	254 nm
Run time	20 min.

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Range	0.01 AUFS
Rise Time	1 sec
Capillary temp.	20°C
Autosampler temp.	4°C
Prep cycles	1 high pressure pre-inject cycle; 120 sec with background electrolyte

2. Prepare fresh background electrolyte daily. Filter through a Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter (Catalog #343-0012) that is prerinsed with deionized water prior to use and spin for 2 min. using a microcentrifuge.
3. Use Pressure Diagnostics to precondition a new uncoated capillary by using the following purge sequence:
    a. Purge for 2 minutes with deionized water.
    b. Purge for 10 minutes with 0.1 N NaOH.
    c. Purge for 5 minutes with deionized water.
    d. Purge for 10 minutes with background electrolyte.
4. For best results, replace running electrolyte every five runs.
5. When using the same capillary on consecutive days, create a Shut-down Method which purges the capillary with deionized water (120 sec), 0.1 N NaOH (300 sec), deionized water (120 sec) and finally with the background electrolyte (240 sec). Program the BioFocus to leave vials in the up position at the end of the automation sequence (click on the "Vials up at end of run" on the toolbar). This will ensure day-to-day reproducibility and minimize stabilization time when starting runs the next day.

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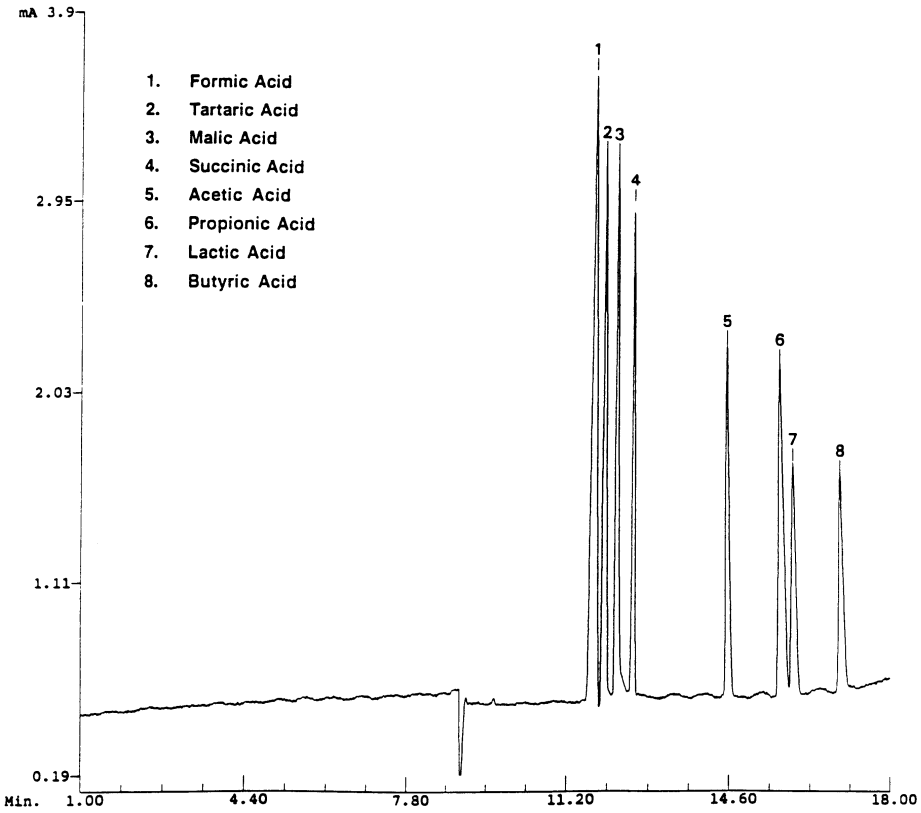


Figure 10 Analysis of organic acids by capillary electrophoresis with indirect detection using the BioFocus system.

Analysis of Inorganic Cations by Capillary Electrophoresis with Indirect Detection

Many mono and divalent metal cations can be analyzed by CZE with indirect UV detection using 4-methylbenzylamine as the background electrolyte and lactic acid as the complexing agent. Lactic acid makes possible the separation of metal ions with almost identical mobilities by complexing the individual ions to varying degrees. However, NH_4^+ and K^+ cations which also have virtually identical mobilities are not complexed by lactic acid. Incorporation of 18-crown-6 ether into the electrolyte containing lactic acid allows selective complexation of K^+ and reduces its mobility to permit a good separation. The crown ether also increases the migration time for Sr^{2+}, Pb^{2+}, and Ba^{2+} by complexation to form a bulkier, less mobile species. Addition of methanol to the electrolyte allows trace analysis of some metal ions (e.g., Ca^{2+} and Mg^{2+}) in the presence of large concentration of another metal ion (e.g., Na^+).

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The following supplies and instructions are recommended for the analysis of inorganic cations by indirect detection using the BioFocus CE system.

Supplies Needed

- Background Electrolyte, 11 mM Lactic acid, 7.5 mM 4-Methylbenzylamine, mM 18-crown-6 ether (pH 4.3) plus 8% MeOH.
- L (+)-Lactic acid (FLUKA, Catalog #69771)
- 4-Methylbenzylamine (ALDRICH, Milwaukee, WI, Catalog #M3,120-1)
- 18-crown-6 ether (ALDRICH, Milwaukee WI, Catalog #18,665-1)
- Methanol, HPLC grade (EM Science)
- Cation standards, analytical grade or better (FLUKA)
- Distilled or deionized water, filtered
- BioCAP Bare Silica Capillary, 75 µm ID x 375 µm OD, Catalog #148-3061 installed in a Bio-Rad User Assembled Cartridge. Total capillary length 65 cm.
- BioFocus User Assembled Capillary Cartridge Assembly Kit, Catalog #148-3050
- Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter, Catalog #343-0012
- Microcentrifuge tubes, 500 µl, Catalog #223-9503
- Large capacity microcentrifuge tubes for purge and waste, 1.5 ml, Catalog #223-9480
- Pipettes or syringes for preparation and transfer of samples and buffers
- Microcentrifuge for degassing buffers and samples

Sample Preparation

Prepare stock solutions (~1000 ppm) of chloride salts of metal cations (analytical or better grade) in deionized water. Standard mixtures at ~1-3 ppm level should be prepared in polyethylene vials by stepwise dilution of the stock solutions using deionized water. Concentrations are expressed in ppm of free metal cation.

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Instructions for Analyzing Inorganic Cations by Indirect Detection Using the BioFocus System

1. Program a Single Run Group CZE Method, or define a new Configuration, Method and Autosequence using the CZE templates in the database. Program the following method values:

Injection	1 psi*sec
Mode	Constant Voltage
Polarity	+ to –
Running Voltage	18 kV
Running Current	5 µA
Current Limit	50 µA
Wavelength	214 nm
Run time	12 min.
Range	0.01 AUFS
Rise Time	1 sec
Capillary temperature	20°C
Autosampler temp.	20°C
Prep cycle(s)	1 high pressure pre-inject cycle; 40 sec with electrolyte

2. Filter the background electrolyte through a Bio-Rad 0.45 µm Micro Prep-Disc Membrane Filter (Catalog #343-0012) that is prerinsed with deionized water prior to use and spin for 2 min. using a microcentrifuge.
3. Use Pressure Diagnostics to precondition a new uncoated capillary by using the following purge sequence:
   a. Purge for 2 minutes with deionized water.
   b. Purge for 10 minutes with 0.1 N NaOH.
   c. Purge for 5 minutes with deionized water.
   d. Purge for 2 minutes with background electrolyte.
4. For best results, replace running electrolyte every five runs and use large capacity microcentrifuge tubes (1500 µl, Catalog #223-9480) for buffers and waste.
5. When using the same capillary on consecutive days, create a Shutdown Method which purges the capillary with deionized water (120 sec), 0.1 N NaOH (300 sec), deionized water (120 sec) and finally with the background electrolyte (240 sec). Program the BioFocus to leave vials in the up position at the end of the automation sequence (click on the "Vials up at end of run" on the toolbar). This will ensure day-to-day reproducibility and minimize stabilization time when starting runs the next day.

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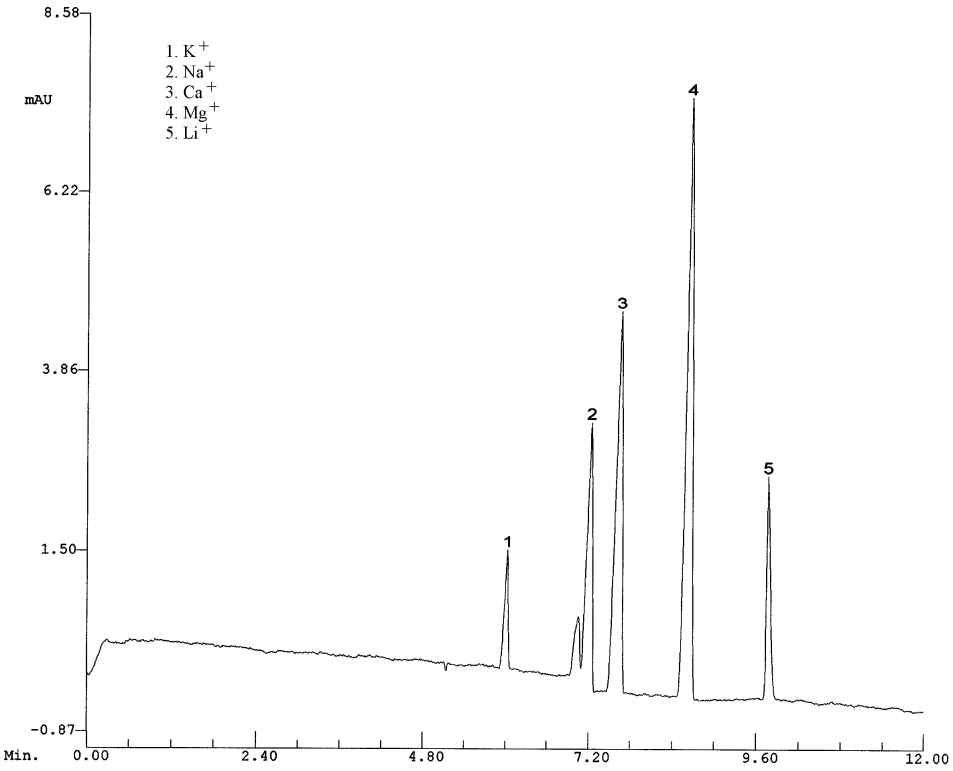


Figure 11 Analysis of inorganic cations by capillary electrophoresis with indirect detection using the BioFocus system.

Sample Injection and Sensitivity in the BioFocus 3000 System

The amount of sample loaded during injection and detection sensitivity in capillary electrophoresis depend on several variables. This section discusses the issues involved and some approximate numbers.

Pressure Injection

The amount of sample loaded during pressure injection depends upon the applied pressure and time, the viscosity of the sample, the viscosity of the buffer in the capillary, the capillary dimensions, and the temperature.

The following values show different flow rates for different size capillaries, all measured with sample injection at 20 psi*sec (5 psi * 4 sec) and using an aqueous buffer in the sample vial and capillary.

17 cm x 25 µm capillary	4.1 mm/sec
24 cm x 25 µm capillary	3.8 mm/sec
36 cm x 50 µm capillary	7.5 mm/sec

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This would correspond to injected sample volumes of 7.4 nl for the 17 cm x 25 µm capillary, 8.4 nl for the 24 cm x 25 µm capillary, and 59 nl for the 36 cm x 50 µm capillary. The effective zone widths are much smaller than these if samples are injected at low ionic strength due to the stacking effect at the sample:run buffer boundary.

The volume of sample introduced into the capillary during pressure injection can be calculated from the following equation:

$$V = \frac{\pi p t r^4}{8 L \eta}$$

where

V = volume injected in milliliters

p = gas pressure of the buffer in dynes/cm^2

t = injection time in seconds

r = capillary radius in cm (1/2 of the capillary ID)

L = capillary length in cm

η = buffer viscosity in poise or dynes x sec/cm^2

For example, using a 24 cm x 25 µm capillary filled with the Bio-Rad 0.1 M phosphate buffer, pH 2.5 (viscosity = 1.5 centipoise at 20 degrees Celsius) and an injection constant of 8 psi * sec (with 68947.6 dynes/cm^2 per psi), the injection volume can be calculated as 1.47 nanoliters of sample:

$$V = \frac{(3.1416)(8 \text{ psi x sec})(68947.6 \text{ dynes / cm}^2)(12.5 \text{ x} 10^{-4})^4}{(8)(24 \text{ cm})(0.015 \text{ poise})}$$

By the same calculation a 2 psi * sec injection into a 50 cm x 50 µm capillary filled with this phosphate buffer will introduce 2.82 nanoliters of sample.

Electrophoretic Injection

In electrophoretic injection, only ions move into the capillary. The amount of sample ions which migrate into the capillary during electrophoretic injection is strongly dependent on the ionic strength of the sample solution. A ten-fold increase of the salt concentration in a sample solution can decrease peak area by a factor of ten. Therefore, accurate quantitation of a component in multiple samples using electrophoretic injection requires that the ionic strength be the same in all sample solutions. This effect also means that sensitivity in electrophoretic injection can be dramatically increased by reducing the sample salt concentration ("sample stacking").

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Electrokinetic Injection

In electrokinetic injection, sample molecules are loaded by a combination of electrophoretic migration and electroendosmotic pumping. Electroendosmotic flow (EOF) is a function of the field strength, the buffer pH, and viscosity. Since EOF depends on the charge on the capillary wall, adsorption of sample components to the wall can change the magnitude of EOF. This in turn will affect the amount of sample loaded during electrokinetic injection. With Bio-Rad coated capillaries, electroendosmotic flow is eliminated and sample ions move into the capillary by electrophoretic migration only.

Sensitivity

"Sensitivity" best defined as the minimum detectable quantity (MDQ) or minimum detectable concentration (MDC), and is usually measured as the quantity or concentration which yields a signal-to-noise ratio of 3. For most situations, MDC is the meaningful number. From Beers Law the signal (absorbance) is dependent upon the analyte concentration, its extinction coefficient at the detection wavelength, and the pathlength of the detection window. So to compare the performance of two instruments, it is necessary that both the compound and pathlength (capillary ID) be the same.

With signals held constant (i.e., two instruments compared with the same compound, detection wavelength, and capillary ID), the critical variable is detector noise. In the BioFocus 3000 system typical noise is $< 5 \times 10^{-5}$ AU, or 50 µAU (measured for a peptide at 200 nm with a 25 µm ID capillary filled with 0.1 M phosphate buffer, pH 2.5, and an applied voltage of 10 kV).

Some calculated MDC values for 3 Bio-Rad CE calibration standards are shown in Table I. The separation conditions for these standards are given in Table II, on the next page.

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Table 1 *Minimum Detectable Concentrations (S/N = 3) for Peptides, Proteins, and Nucleic Acids*

Peptides		
bradykinin	**0.63 µg/ml**	**6 x 10^{-7} M**
luteinizing hormone		
releasing hormone	**0.34 µg/ml**	**3 x 10^{-7} M**
oxytocin	**0.45 µg/ml**	**4 x 10^{-7} M**
Proteins		
ß-lactoglobulin A	**0.56 µg/ml**	**3 x 10^{-8} M**
α –lactalbumin	**0.48 µg/ml**	**4 x 10^{-8} M**
hemoglobin A	**1.11 µg/ml**	**1.7 x 10^{-8} M**
Nucleic Acids		
249 bp fragment	**0.9 µg/ml**	**6 x 10^{-9} M**
800 bp fragment	**1.8 µg/ml**	**3.5 x 10^{-9} M**

Table 2 *Separation Conditions for Bio-Rad CE Calibration Standards*

Conditions	Peptide Calibration Set	Protein Calibration Set	Low Range DNA standard
	Cat.#148-2012	**Cat.#148-2013**	**Cat.#170-3465**
Capillary	**24 cm x 25 µm**	**24 cm x 25 µm**	**24 cm x 50 µm**
Buffer	**0.1M Phosphate, pH 2.5**	**Basic Protein Analysis, pH 8.5**	**PCR Product Analysis, pH 8.3**
Injection	**8 sec at 10 kV**	**8 sec at 10 kV**	**4 sec at 10 kV**
Run Voltage	**10 kV**	**10 kV**	**2.5 kV**
Detection Wavelength	**200 nm**	**200 nm**	**260 nm**

Determination of Capillary Purge Volume

Best reproducibility in capillary electrophoresis will be obtained when capillary purge volumes are sufficient to completely flush the capillary and fill it with a homogenous solution of the wash reagent or replenishment buffer, e.g. 5 capillary volumes. This depends upon the capillary volume and the reagent viscosity.

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Volumes for the capillary dimensions typically used in CE are given in Table 3 and viscosities for Bio-Rad CE buffers are listed in Table 4. The times required to purge five capillary volumes of Bio-Rad buffers for the common CE capillaries are listed in Table 5.

*****Buffer viscosity were determined by measuring the time required for niacinamide-doped buffer to reach the detector window when purging a capillary prefilled with undoped buffer at 100 psi using the following expression:

$$\eta = \frac{p\ t\ r^2}{8 L_e L_t}$$

where η = viscosity in poise

p = pressure in dynes/cm^2

t = time in seconds

r = capillary radius in cm

L_t = total capillary length in cm

L_e = effective capillary length in cm

Table 3 *Capillary Volumes*

Length (cm)	I.D. (µm)	Volume (µl)
17	25	0.083
24	25	0.118
24	50	0.471
36	50	0.707
50	50	0.981
24	75	1.060
36	75	1.590
50	75	2.209
100	75	4.418
50	100	3.927
100	100	7.854
50	200	15.707

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Table 4 *Viscosity of Bio-Rad Capillary Electrophoresis Buffers*

Buffer	Viscosity (cp)
100 mM Phosphate, pH 2.5	1.5
Basic Protein Analysis Buffer, pH 8.5	2.9
PCR Product Analysis Buffer, pH 8.3	12.9
PCR Product Analysis Buffer + 7 M Urea	15.8
CE SDS-Protein Run Buffer	43.0

Table 5 *Time Required to Purge 5X Capillary Volume at 100 psi*

Capillary (cm x µm)	Water	Phosphate	Basic Protein	PCR	PCR + Urea	SDS-Protein Run Buffer
17 x 25	11	16	42	138	169	459
24 x 25	21	31	84	276	338	921
24 x 50	5	8	21	69	84	230
32 x 50	10	14	28	123	150	409
36 x 50	12	18	47	155	190	517
50 x 50	23	35	90	299	366	997
24 x 75	2	4	7	31	38	102
36 x 75	5	8	21	69	84	230
50 x 75	10	15	40	133	163	444
100 x 75	41	62	161	532	652	1774
50 x 100	6	9	23	75	92	249
100 x 100	23	35	91	299	367	998
50 x 200	1.5	2	6	19	23	62
50 x 200	1	9	23	75	92	249

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