Chromatofocusing with Polybuffer™ and Polybuffer exchanger

Chromatofocusing is a powerful, yet easy-to-use, column chromatography method for separating proteins (1). This technique is particularly useful for the final polishing steps of protein purification. Proteins with the same isoelectric point (pI) are focused in high concentration and resolved from other proteins that have different pI in a pH gradient formed without using any external gradient formers or electric current. By contrast to isoelectric focusing, chromatofocusing can separate proteins of similar net charge but different net surface charge distribution. Various applications (2 to 4) that chromatofocusing can be used for include:

- Preparative separation of proteins based on pI differences
- Separating isoforms of a protein that are difficult to resolve based on other physico-chemical characteristics such as size, net charge, hydrophobicity or ligand affinity.

The basic requirement for high resolution chromatofocusing is a separation media that can reproducibly form a linear pH gradient over a broad pH range as well as exhibit even binding capacities. Polybuffer exchanger PBE 94 is based on Sepharose CL-6B, a cross-linked agarose matrix. Charged amine groups are coupled to monosaccharide units in the 90 µm Sepharose CL-6B particles by ether linkages.

Polybuffer

The charged groups have been selected to give an even buffering capacity across a broad pH range. PBE 94 is designed for separating proteins with pI ranging from pH 4 to 9.

Polybuffer 74 and 96 are amphoteric buffers with a wide and even buffering capacity. The buffers are designed to form pH gradients with intervals of up to 3 pH units for optimal protein separation on PBE 94.

Table 1. Medium characteristics

Product	Functional group	pH stability*	Mean particle size
PBE 94	Secondary, tertiary and quarternary amines	Long term: 2–12 Short term: 1–14	90 µm

* Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.

Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within GE Healthcare.

Contents

1. Selection of pH gradient and buffers

No gradient-forming equipment is required for chromatofocusing since the gradient is generated on the column as buffer and medium interact. The upper limit of the gradient is defined by the pH of the start buffer and the lower limit of the gradient is defined by the pH of the elution buffer. Polybuffer perform best in pH intervals of 3 pH units or less and the narrowest pH intervals are likely to give the highest resolution.

The gradient volume is defined by the strength of the elution buffer (low buffer concentrations give gradual pH changes and good separations between peaks). The optimal gradient volume may need to be determined by experimentation. Recommended gradient volumes and buffers are indicated in Table 2. The pre-gradient volume is the volume of elution buffer that passes through the column before the pH begins to decrease, hence the total volume of buffer required is always greater than the gradient volume.

1.1 Gradients for proteins of known pI

Choose a pH interval so that the protein of interest is eluted after running 30% to 50% of the pH gradient. Narrow pH intervals will give optimal resolution.

1.2 Gradients for proteins of unknown pI

Since most proteins have isoelectric points in the range of 7 to 4, start with pH interval 7 to 4 on PBE 94. Proteins with a pI above 7 will pass through the column and can be collected and re-run at a higher pH if desired. Once a suitable pH range is established, select the most suitable pH gradient and compatible medium to achieve the optimal resolution.

Table 2. Recommended buffer systems for gradients with PBE 94.

pH	Start buffer	Elution buffer	Dilution factor	Approx. volume (in column volumes) Total volume	Pre-gradient volume
9–8	0.025 M ethanolamine, pH 9.4, HCl	Pharmalyte 8–10.5, pH 8.0, HCl	1:45	12	1.5
9–7	0.025 M ethanolamine, pH 9.4, HCl	Polybuffer 96, pH 7.0, HCl	1:10	14	2
9–6	0.025 M ethanolamine, pH 9.4, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:10	12	1.5
8–7	0.025 M Tris, pH 8.3, HCl	Polybuffer 96, pH 7.0, HCl	1:13	10.5	1.5
8–6	0.025 M Tris, pH 8.3, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:13	12	3
8–5	0.025 M Tris, pH 8.3, CH_3COOH	Polybuffer 74 (70%) + Polybuffer 96 (30%) pH 5.0, CH_3COOH	1:10	10.5	2
7–6	0.025 M imidazole, pH 7.4, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:13	10	3
7–5	0.025 M imidazole, pH 7.4, HCl	Polybuffer 74 pH 5.0, HCl	1:8	14	2.5
7–4	0.025 M imidazole, pH 7.4, HCl	Polybuffer 74, pH 4.0, HCl	1:8	14	2.5
6–5	0.025 M histidine, pH 6.2, HCl	Polybuffer 74, pH 5.0, HCl	1:10	10	2
6–4	0.025 M histidine, pH 6.2, HCl	Polybuffer 74, pH 4.0, HCl	1:8	9	2
5–4	0.025 M piperazine, pH 5.5, HCl	Polybuffer 74, pH 4.0, HCl	1:10	12	3

1.3 Selection of counter-ions

As can be seen in Table 2, the most commonly used counter-ion is chloride. Other monovalent counter-ions can be used, but it is essential that these anions have a pKa at least two pH units below the lowest point of the chosen gradient.

Acetate is not recommended as a counter-ion for Polybuffer 74 because it has a high pKa. Multivalent counter-ions with a net charge below -1 are not recommended. Note that iminodiacetic acid is a multivalent ion.

Bicarbonate ions can result from the presence of atmospheric CO_2 or badly stored buffers. All high pH, amine-containing buffers adsorb atmospheric CO_2 and so generate bicarbonate ions which can disrupt a pH gradient by causing a fluctuation or plateau in the region of pH 5.5 to 6.5, depending on conditions.

These effects are most marked with Polybuffer 96 in pH gradients ending at pH 6, and can be minimized by using acetate as the counter-ion or setting the lower limit to pH 6.5. Adsorption of CO_2 can be minimized by storing solutions under nitrogen in tightly sealed bottles and at 3°C to 8°C in the dark.

2. Buffer preparation

Use high quality water and chemicals. Filter buffers through 0.45 µm or 0.22 µm filters under vacuum to ensure that the solutions are thoroughly degassed. The presence of air bubbles in the column can significantly interfere with the resolution.

Ensure that all buffer components are stored correctly to avoid adsorption of CO_2 (especially those containing amines). Use fresh Polybuffer from a previously unopened bottle whenever possible. Store previously opened solutions under nitrogen in tightly sealed bottles at 4°C and in the dark to minimize adsorption of CO_2.

Prepare and use all buffers carefully at the same temperature to ensure correct pH and ionic strength. Use fresh buffers whenever possible. Buffers stored under refrigeration must reach the temperature at which they were prepared before running a separation.

The concentration of the start buffer is especially important when working at low ionic strength where microenvironments can vary by as much as 1 pH unit. Alterations in ionic strength may occur if readjustments to pH are made when buffers have been overtitrated with acid. Ensure that the start and elution buffers are at the same low ionic strength to avoid large pH changes at the beginning or end of a pH gradient.

1. Select start and elution buffers from Table 2 according to the pH gradient required and the medium to be used.
2. Calculate the required buffer volumes according to the column volume used. For the elution buffer, dilute Polybuffer and/or Pharmalyte™ in distilled water to approximately 95% of the final volume required.
3. Titrate buffers to the correct pH with the listed acid (e.g. 1 to 2 M or saturated iminodiacetic acid).
4. When the final pH is reached, add distilled water to obtain the total final volume.

For shallower gradients within the same pH interval, prepare the elution buffer as normal, but dilute to a larger volume. Note that proteins elute with increased volumes when using diluted eluents so pre-gradient and total volumes also need to be increased.

3. Sample preparation

Correct sample preparation is extremely important. Simple steps to clarify a sample before applying it to a column will avoid the risk of blockage, reduce the need for stringent washing procedures and extend the life of the packed medium.

Samples must be clear and free from particulate matter. For small sample volumes, a syringe-tip filter of cellulose acetate or PVDF can be sufficient for sample filtration.

If the pH of the sample is too low or different buffers are used, both the gradient and resolution may be affected. Depending on the sample volume, use a HiTrap™ Desalting or HiPrep™ 26/10 Desalting column to remove high salt concentrations and/or transfer the sample into the start buffer. The pH of the sample is not critical if the buffer concentration of the sample is very

low. It may be possible to simply dilute the sample if the pH and final volume are not critical.

Since chromatofocusing is a binding technique, the volume of the sample is not significant as long as all of the sample can be applied and focused before the proteins of interest begin to elute. As a general rule, do not load a sample volume that is greater than half of the total column volume. For more information about sample preparatioin see the handbook Ion Exchange Chromatography & Chromatofocusing: Principles and Methods.

4. Performing a separation

Recommended flow: 30 to 40 cm/h.

Start and elution buffers: see Table 2.

Monitor the eluent at 280 nm since Polybuffer absorb at wavelengths below 280 nm. Monitor the pH throughout the run as fluctuations can occur that may affect the separation, for example, due to the presence of CO_2 in the buffer.

Using a column for the first time or after long term storage:

1. Inject 0.5 column volumes of 5 M NaOH
2. Equilibrate with start buffer until the buffer leaving the column is at the same pH as the start buffer.
3. Apply 0.5 column volumes of 2 M salt solution containing the same anion as used in the acid for pH titration of the start buffer.
4. Re-equilibrate the column with start buffer until eluent leaving the column is at the start pH.

To check for pH fluctuations or other disturbances caused by incorrect buffer conditions or contaminants, consider performing a blank run using elution buffer (total volume as indicated in Table 2).

Using a column for repeated runs:

1. Equilibrate with start buffer until buffer leaving the column is at the same pH as the start buffer. See Table 2 for recommended pre-gradient volumes.

2. Adjust the sample to a pH of the start buffer or exchange sample into the start buffer.
3. Apply elution buffer. Note that there is a pre-gradient volume that will be eluted before the pH gradient begins (see Table 2 for typical pre-gradient volumes).
4. Apply sample.
5. Apply elution buffer using gradient volumes as indicated in Table 2. Check for pH fluctuations or other disturbances during elution.
6. Wash with 2 column volumes of 2 M NaCl to elute any material still bound to the column.
7. Re-equilibrate with 5 column volumes of start buffer until UV absorbance and pH/conductivity values are stable.

To maintain a clean column, inject 0.5 column volumes of 1 M NaOH every tenth run (or more frequently if required). The injection of NaOH can be followed by 0.5 column volumes of 75% acetic acid.

If the fractions are to be analyzed by reversed phase chromatography, Polybuffer may interact with the pairing ions used during the run. If the pairing ions are non-hydrophobic, Polybuffer will elute in the void volume and the retained samples can be eluted with a gradient of organic solvent. However, if the pairing ions are very hydrophobic, Polybuffer will be retained on the column and give an absorption peak at wavelengths below 280 nm during an elution.

If organic solvents must be used, note the following:

- Before running a column, check the solubility of the sample and all buffers.
- The pH interval required may be altered since the pKa values of the charged groups in the buffers, Polybuffer and chromatofocusing medium will be increased.
- Perform a blank gradient to ensure maintenance of a linear pH gradient. Linearity is likely to vary more at the high and low end of a pH interval 9 to 4. Adjust to the highest pH in the useful buffering range of the start buffer substance and adjust the elution buffer to approximately 0.5 pH units higher than recommended in Table 2.

5. Optimization

- If results suggest that there may be a problem of sample solubility during the separation, include additives such as betaine (10% w/v), taurine (4% w/v) or glycerol (1% to 2%) in the start and elution buffers to improve solubility. These additives should not affect the separation, but may need to be removed at a later stage thus adding an extra step to the purification strategy.
- To improve resolution:
  - dilute Polybuffer (up to 1:20), but note that this will increase the pre-gradient and total gradient volumes so the time at which components elute will change and the volume of an eluted peak may increase
  - decrease sample load
  - decrease flow
- To improve selectivity, use a shallower gradient (increase gradient volume), but note that this will result in broader peaks because each pH interval will occupy a larger space on the column. However, the resolution within a pH interval will increase.
- To change selectivity, use alternative buffers, salts or counter-ions.

If resolution is satisfactory, it may be possible to increase flow rate in order to speed up the separation. For more information about troubleshooting, see the handbook Ion Exchange Chromatography & Chromatofocusing: Principles and Methods.

6. Packing a column

Prepacked columns are likely to give the highest resolution and the most reproducible results, particularly with the MonoBead matrix used in Mono P™ 5/50 GL and Mono P 5/200 GL.

Pack columns in the start buffer to be used for the separation (see Table 2) using long, narrow columns such as Tricorn™ 10/300. The amount of medium required will depend on the amount of sample, the nature of the sample and contaminants and the degree of resolution required. For

most separations, 20 to 30 ml of Polybuffer exchanger will be sufficient to separate from 1 to 200 mg protein/pH unit in the gradient.

Degas the start buffer and the slurry to avoid air bubbles which can interfere within the separation.

A step-by-step demonstration of column packing can be seen in "Column Packing – The Movie".

1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the recommended buffer. Ensure no air is trapped under the column net. Close the column outlet leaving 1 to 2 cm of buffer in the column.
3. Gently resuspend the medium.

Avoid using magnetic stirrers since they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) depending on the required bed volume. The slurry concentration should be approx. 50% of the settled bed volume.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.

   When slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see Ordering information for details). This ensures that the slurry has a constant diameter during packing, minimizing turbulence and improving column packing conditions.
6. Immediately fill the column with buffer.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate. For a narrow, long column the flow rate should be about 100 cm/h, which corresponds to 1.3 ml/min for a Tricorn 10/300 column.

If the recommended flow rate cannot be obtained, use the maximum flow rate the pump can deliver.

Do not exceed the maximum operating pressure of the medium or column.

9. Maintain the packing flow rate for at least 3 column volumes after a constant bed height is obtained. Mark the bed height on the column.

Do not exceed 75% of the packing flow rate during any purification.

10. Stop the pump and close the column outlet. Remove the top piece and carefully fill the rest of the column with buffer to form an upward meniscus at the top.
11. Insert the adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the adapter should be open) until the mark is reached. Lock the adapter in position.
13. Connect the column to the pump and begin equilibration at a lower flow rate. Re-position the adapter if necessary.

A well packed column is essential for high performance, so it is worth checking the column performance by injecting acetone to determine column efficiency and peak symmetry. An alternative may be checking the packing using a colored marker. Use bovine cytochrome c which is strongly repelled from the medium due to its high pI (10.5). The progress of the protein band through the column can be visualized in order to check for any distortion caused by poor column packing or air bubbles.

7. Removing Polybuffer

For most practical applications it is not necessary to remove Polybuffer since the amount that elutes with any sample is extremely low. Polybuffer do not interfere with enzyme assays or amino acid analysis, but they may interfere with certain protein assays such as Lowry.

Polybuffer can be removed from protein samples using a gel filtration medium with a suitable fractionation range. It is recommended to follow any separation by monitoring the absorbance of Polybuffer (A_{215} nm) as well as the absorbance of the eluting protein (A_{280} nm, A_{254} nm or A_{215} nm) in order

to optimize the running conditions and ensure effective separation. For practical and theoretical information on gel filtration chromatography, refer to the handbook *Gel Filtration–Principles and Methods* available from GE Healthcare.

8. Cleaning

Since certain proteins may precipitate at or near their pI, blockage of the top filter on a chromatofocusing column is often the most common reason for an increase in back pressure. Reverse the flow direction and run through 2 column volumes of elution buffer at 30 cm/h. Return to normal flow direction, run through 5 column volumes of elution buffer.

To remove severe contamination (often indicated by an increase in column back pressure) proceed as follows:

Reverse flow direction and run the following sequence of solutions at a flow rate of 15 to 30 cm/h.

1. Wash with 4 column volumes of 1 M NaCl.
2. Rinse with 2 column volumes of distilled water.
3. Wash with 4 column volumes of 1 M NaOH.
4. Rinse with 2 column volumes of distilled water.
5. Wash alternately with 0.5 column volumes of 0.1 M HCl and 2 column volumes of distilled water until the elution profile is constant.
6. Wash with 4 column volumes of 1 M NaCl.
7. Reverse flow direction and re-equilibrate column in the start buffer. If back pressure remains high, change the top filter.

Save time by monitoring any cleaning procedure to check for elution of contaminants. Depending on the nature of the contaminants, the following cleaning solutions can also be used: 100% isopropanol, 20% acetonitrile, 2 M NaOH, 75% acetic acid, 20% ethanol, 100% methanol or up to 6 M guanidine hydrochloride, cationic or non-ionic detergents. Always rinse with at least 2 column volumes of distilled water after using any of these cleaning solutions. When using organic solvents, wash the column using

sawtooth gradients e.g. run from 0% to 100% solvent in 5 column volumes, then from 100% to 0% in 5 column volumes, including 1% trifluoroacetic acid in the water and organic solvent.

If column performance is still not restored, inject a solution of 1 mg/ml pepsin in 0.1 M acetic acid containing 0.5 M NaCl and leave overnight at room temperature or one hour at 37°C. Depending on the contaminant, other enzymes may also be used, e.g. DNase. After any enzymatic treatment, repeat the steps to remove severe contamination described previously.

9. Chemical stability

Polybuffer exchangers are stable in all commonly used, aqueous buffers in the range of pH 3 to 12 and compatible with urea and other strong dissociating agents.

Avoid oxidizing agents and anionic detergents.

10. Storage

PBE 94

PBE 94 is delivered in 20% ethanol. If the packed column is to be stored for more than two days after use, wash with 5 column volumes of distilled water and 5 column volumes of 20% ethanol.

Polybuffer 96, Polybuffer 74

Store at 3°C to 8°C in the dark, preferably under nitrogen. Avoid microbial contamination. To minimize adsorption of CO_2, store solutions under nitrogen in tightly sealed bottles after use.

References

1. **Chromatofocusing: A preparative protein separation method.** *Trends in Biochemical Sciences* (1982) **7**:168-170. Sluyterman L.A.A.E.
2. **Chromatofocusing nonporous reversed-phase high-performance liquid chromatography/ electrospray ionization time-of-flight mass spectrometry of proteins from human breast cancer whole cell lysates: a novel two dimensional liquid chromatography/mass spectrometry method.** *Rapid Communications in Mass Spectrometry* (2001) **15**: 291-296. Chong, B.E., Yan, F., Lubman, D. and Miller, F.R.
3. **Purification and Characterization from Rat Kidney Membranes of a Novel Plateletactivating Factor (PAF)-dependent Transacetylase That Catalyzes the Hydrolysis of PAF, Formation of PAF Analogs, and C2-ceramide.** *Journal of Biological Chemistry* (1999) 274: 8655-8661. Karasawa, K., Qiu, X. and Lee, T.
4. **Chromatofocusing. Protein Purification** (2nd Edition) (1998), 207-238. Eds: Jansson, J and Rydén. L. Author: Hutchens, T.W.

11. Ordering Information

Product	Quantity	Code No.
PBE 94	200 ml	17-0712-01
Polybuffer 74	250 ml	17-0713-01
Polybuffer 96	250 ml	17-0714-01
Pharmalyte pH 8–10.5	25 ml	17-0455-01
Related products		
Mono P 5/50 GL	1	17-5170-01
Mono P 5/200 GL	1	17-5171-01
Glass Tube 10/300 (used as a reservoir)	1	18-1153-18
Tricorn 10/300 column	1	18-1163-18
Packing Connector 10-10	1	18-1153-23
Column Packing – The Movie	1	18-1165-33
Ion Exchange Chromatography & Chromatofocusing: Principles and Methods	1	11-0004-21
HiTrap Desalting, 5 ml	5	17-1408-01
HiPrep Desalting	1	17-5087-01

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