Protocol for pH-gradient chromatofocusing of the native and desialylated human apo-transferrin

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Abstract

This protocol describes the method used for the separation of the native human apo-transferrin from the desialylated apo-transferrin using low-pressure pH gradient ion exchange chromatography. The separation is performed using specialized pH gradient ion exchange chromatography buffers, pISep (CryoBioPhysica, Inc.). The mixture of desialylated apo-transferrin and native apo-transferrin is dissolved in the start buffer (pISep A, pH = 8) and injected onto two HiTrap Q HP (GE Healthcare) anion exchange chromatography column connected in a series. Elution is done by a single step linear gradient (0 – 100 % pIsep B, pH = 4) using ÄKTA Start FPLC system (GE Healthcare). Protein concentration in the eluate is monitored by measuring absorbance at 280 nm and the protein fraction recovery can be calculated by integration over the chromatogram surface area. After separation, the pH value of each fraction containing eluted protein is measured, corresponding to the approximate protein pI value. The observed pI values for the native and desialylated apo-transferrin sialoforms differ significantly and hence can be fully separated.

Keywords

transferrin, sialoforms, FPLC, chromatofocusing, pISep buffers

1.1 Materials and instrumentation

1) pISep Buffer Kit - Concentrate A and Concentrate B (CryoBioPhysica, Inc., cat.no. 20055) [1]
2) HiTrap™ Q HP columns, 1 mL (GE Healthcare, cat. no. 17115301)
3) Äkta Start FPLC system (GE Healthcare)
4) Bubble Trap for Microfluidics Kit, 97 µL internal volume (Darwin Microfluidics, product SKU: LVF-KBT-M)
5) pISep Gradient Maker software v2.0 (CryoBioPhysica, Inc.)
6) Unicorn™ Start software v1.1 (GE Healthcare)
7) Ethanol, 96 % (Merck, cat. no. 100974)
8) Sodium chloride, cryst. (Kemika, cat. no. 14175)
9) Hydrogen chloride, 37 % (Carlo Erba Reagents, cat. no. 403871)
10) Sodium hydroxide, pellets 2 – 5 mm (Kemika, cat. no. 1452506)
11) Water, double distilled in an all-glass apparatus

1.2 pH gradient chromatofocusing

1.2.1 Prepare pISep stock buffers.

a) **Buffer A:** Add 5 mL pIsep concentrate B and 1 mL pISep concentrate A to approximately 400 mL of water and set pH to 2.4. Fill the volumetric flask almost to the mark, readjust the pH to 2.4 if needed. Add the remaining volume of water to the volumetric flask.
b) **Buffer B:** Add 5 mL pIsep concentrate B to approximately 400 mL of water and set pH to 10.8. Fill the volumetric flask almost to the mark, readjust the pH to 10.8 if needed. Add the remaining volume of water to the volumetric flask.

If different volumes of buffers are needed, readjust the starting volumes of pISep concentrates and final volume of buffer accordingly. Use 5 M and 1 M HCl or 5 M and 1 M NaOH for pH adjusting.

1.2.2 Generate pISep gradient.

Gradient is generated by using pISep pH Gradient Maker program (Figure 2.1). First step after starting the pISep pH Gradient Maker version 2.0. is to input data file name into the text box. Program will automatically set data file name extension to .csv. Initial and final pH values should be imported into their respective text boxes. This software is used only for pISep buffers and pH values should not exceed: 2.4 to 10.9 range. Step slope of linear gradient (pH units per column volume) should be set. In our simulations we usually used the value of 0.1 pH / column volume units for linear gradient step slope parameter. We did not calculate and use in simulation expected pH / column volume value for our ÄKTA Start FPLC system since this parameter should not have an effect on the composition of the calculated elution and gradient buffers. For a set of buffers it is possible to vary pH / column volume ratio in the experiment by changing initial parameters such as: number and volume of fractions and slope of the gradient (ratios of mixing buffers). Points per linear step parameter

should be added, it represents number of points used for linear gradient step simulations. We mostly used 99 points as it is a maximum number of points allowed for the simulation. Calculation of the linear gradient is performed by pressing the “CALCULATE pH STEP” button. Program exports result to a named .csv file. By pressing “PLOT LINEAR GRAPH” button program will display plotted data. Black curve represents the ideal pH buffer titration curve for the experiment. Blue curve represents expected titration curve of the two buffers (Figure 2.2.). In our experience this single step titration curve yields good results (only slight deviation from the ideal linear model) for our relatively narrow pH gradient values in our experiments. Composition of the 2 buffer solutions (starting and elution buffer) can be obtained by either hovering over first and last point on the graph or by opening and inspecting the .csv file. Gradient maker software also allows for several linear steps in simulations. If one would perform a single linear step simulation in a wider pH range, in order to make the final gradient more linear one could add multiple linear steps in the ÄKTA Start FPLC software experiment setup. Scaling the slopes of multiple linear substeps should be done so that the resulting function is linear in the whole pH range. More information about the usage of the pISep software (including video tutorial) is available online.[1]

1.2.3 Prepare starting elution buffer 1.

Mix 39.33% of pIsep stock buffer A and 60.67% of pIsep stock buffer B (ratios are obtained using pISep pH Gradient Maker Software version 2.0, Figure 2.1). Adjust the pH value to 8.0 with 1 M HCl or NaOH.

1.2.4 Prepare gradient buffer 2.

Mix 81.37% of pISep stock buffer A and 18.63% of pISep stock buffer B (ratios are obtained using pISep pH Gradient Maker Software version 2.0). Adjust the pH value to 4.0 with 1 M HCl or NaOH.

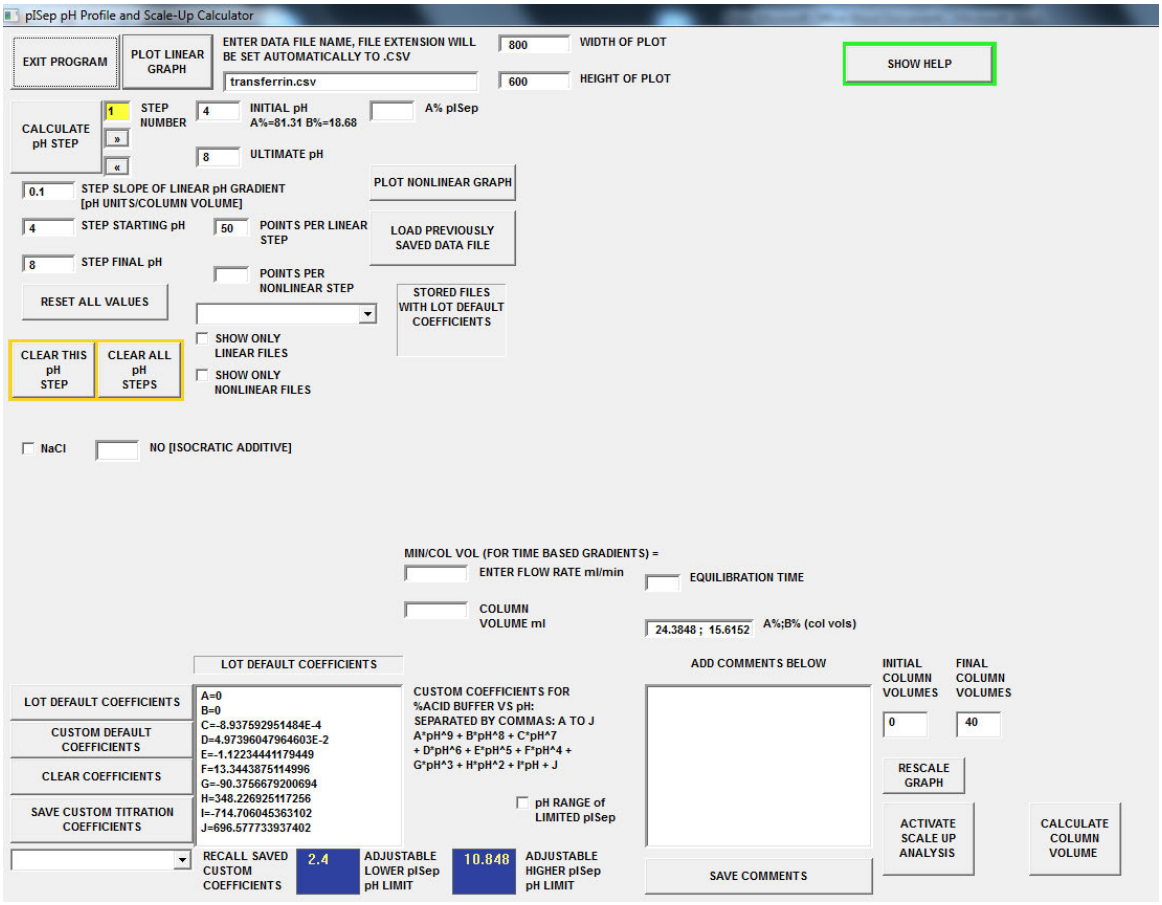

Figure 2.1. Example of pISep gradient generation process by using pISep pH Gradient Maker Software version 2.0.

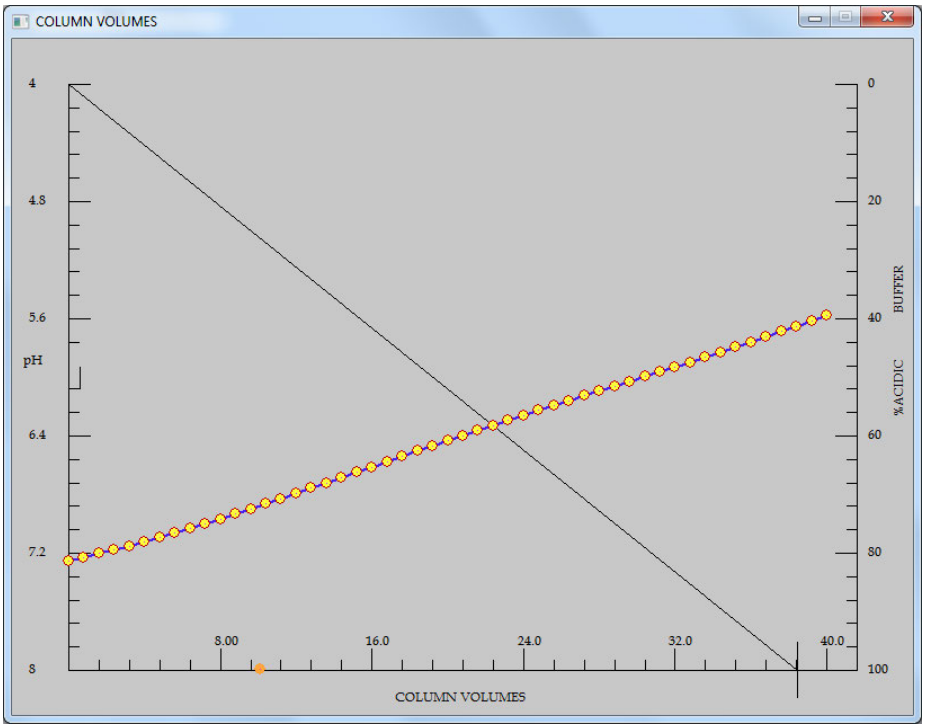

Figure 2.2. Linear gradient plot generated with pISep pH Gradient Maker Software version 2.0.

1.2.5 Prepare ÄKTA FPLC assay.

Connect two Hitrap Q HP 1 mL chromatographic columns in series (using „drop to drop“ method, as described in the manual and Note 1.2.8 in this protocol) to the ÄKTA Start system.[2] Wash columns with water (5 column volumes (CV) or more if needed; 1 CV = 0.962 mL). If there is an observable change in either absorbance at 280 nm or conductivity in time you should continue washing the columns until there is no change in either absorbance or conductivity. Before every column wash make sure that there are no bubbles in the tubes connecting buffers to the ÄKTA Start system. It is good practice to do a pump wash with 5 mL or more of the solution before use. Striking or shaking tubes gently with your fingers while performing pump wash can help to eliminate residual bubbles. If possible, use a debubbler device to ensure no bubbles enter the columns during the experiment. After columns have been washed with water, wash them with at least 5 CV of buffer A.

1.2.6 Load sample into the sample loop.

Prepare 0.5 – 1 mL of the protein sample by dissolving needed amount of protein in starting buffer 1. Satisfactory signals can be obtained with as little as 0.15 mg of transferrin per single chromatofocusing run. It is good practice to check the pH value of the protein solution and readjust the pH value if needed. For this experiment pH value should ideally be 8.0 (it can be slightly above 8.0 but should not be below that value). Transfer the protein solution into a 1 mL syringe and load into the sample loop (default ÄKTA Start sample loop size is 1 mL).

Rinse the sample loop first with water and then with buffer 1 before injecting the sample. Note that minimum sample waste results from loading 50% or less loop volume (Figure 2.3) and adjust the loop and/or sample volume accordingly.

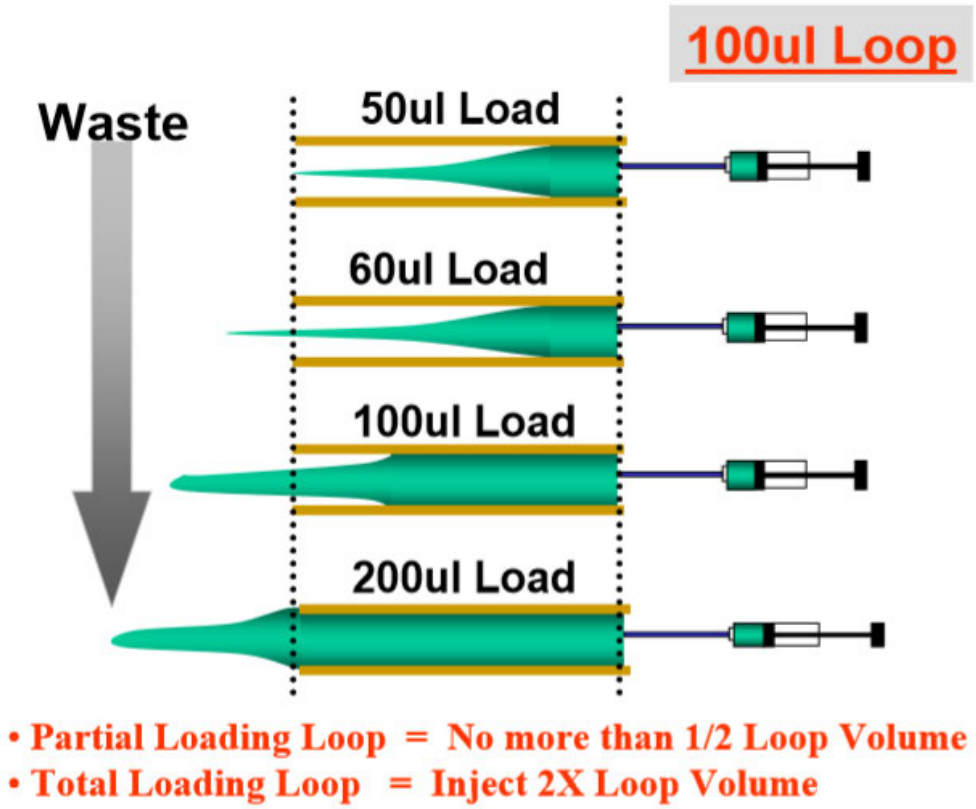

Figure 2.3. Sample loop loading scheme.[3] Note that total loop loading requires approximately double volume of sample (i.e. 50% waste). Minimum waste results from loading 50% or less loop volume.

Optional: In order to eliminate bubbles when loading the sample loop using the built-in sample port on the ÄKTA Start system, we use a custom bubble trap sample applicator (Figure 2.4). Transfer the protein solution into a 1 mL syringe and connect the syringe to the sample applicator. The initial smaller part of the sample volume should be directed to waste since it is very likely to contain bubbles. Try to direct as little sample as possible to waste; only enough to ensure no bubbles enter the chromatographic column. After loading the sample, some protein will remain in the tube connecting the sample applicator and sample loop port (approximately 100 µL). In order to minimize protein waste, connect another 1 mL syringe filled with elution buffer 1 to the sample applicator, remove bubbles as described and inject 100 µL in order to push the remaining protein sample to the sample loop.

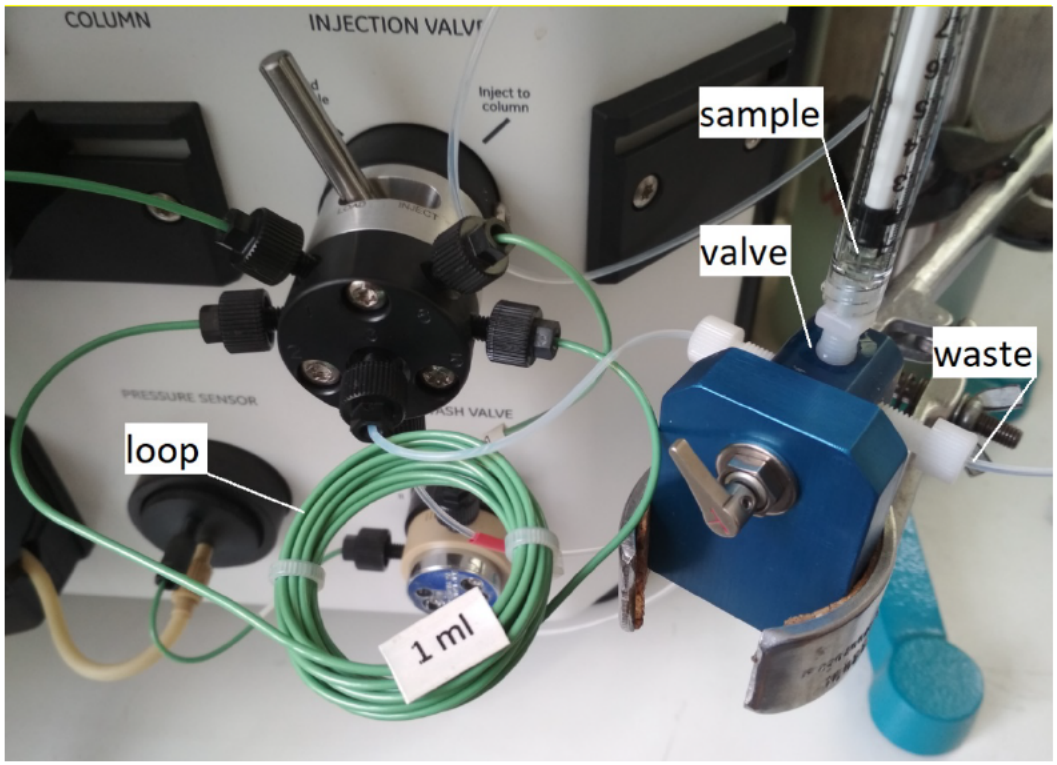

Figure 2.4. Custom bubble trap sample applicator for ÄKTA Start system, using Hamilton HVP3-2 valve (cat. no. 86778). After connecting the syringe, direct the initial part of the sample containing bubbles into waste and load only bubble-free sample. Note the 45° angle of the valve when in closed position.

1.2.7 Perform chromatofocusing.

Start the single step gradient chromatofocusing experiment with initial parameters as described in Table 2.5. Collect the fractions in 2 mL centrifuge tubes. It is recommended to measure the pH values of collected fractions. The measured pH corresponds to the approximate protein pI value. Fractions that contain significant amount of protein can be determined by inspecting the absorbance curve (280 nm). Store the collected protein fractions at 2-8°C if not processed further immediately.

In order to calculate the amount of protein in the individual fractions, it is possible to integrate the absorbance curve (absorbance values on y-axis, volume values on x-axis) using Unicorn™ Start software. Absorbance can be expressed as:

$$A = \varepsilon \cdot l \cdot c;$$

where A is the absorbance, l is the optical path and c is the molar concentration.

Molar concentration can be substituted as $c = n / V$ and the amount of protein n can be written as $n = m / M$:

$$A = \frac{\varepsilon \cdot l}{V \cdot M} \cdot m;$$

multiplying the expression by M gives values of the integral on the left hand side of the equation:

$$A \cdot V = \frac{\varepsilon \cdot l}{M} \cdot m.$$

By inspecting the upper equation it can be seen that the proportionality coefficient between the surface of the integral ($A \cdot V$) and protein mass m is the ($\varepsilon \cdot l$ / M). We used: M (Transferrin) = 81000 g mol^{-1} and l = 1 cm. We also experimentally determined native human transferrin molar absorption coefficient.[4] Calculating the mass of the protein can easily be done by dividing the integral values ($A \cdot V$) by coefficient ($\varepsilon \cdot l$ / M). Unicorn™ software will display integral values in dimensions of 10^{-6} L (mAu · mL).

Protein recovery can be calculated form the amount of protein in individual fractions and the initial amount. Keep in mind that amount of protein that has been actually loaded onto the column is probably lower than the amount of protein in the initial sample. Additionally, some amount of the protein may remain in the column and/or tubes after elution.

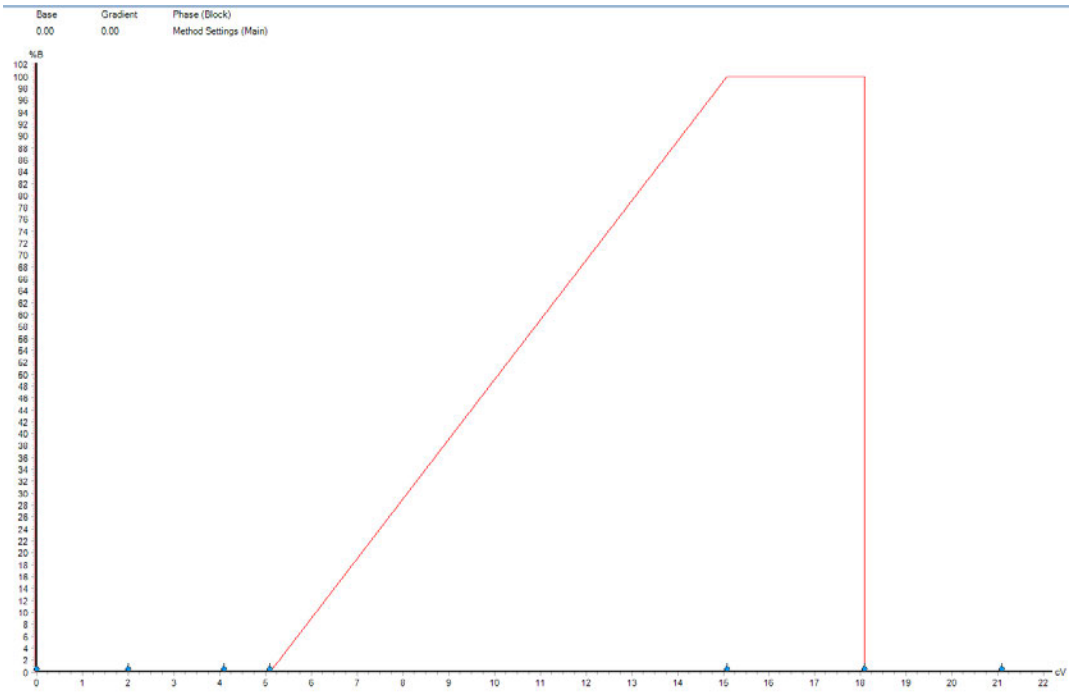

Figure 2.4. Single-step linear gradient for apo-transferrin sialoforms separation. Initially after loading the sample from the sample loop (4 mL, to allow quantitative loop rinsing), 100% buffer 1 is applied to the columns for equilibration (2 CV), followed by the linear gradient (0 – 100% buffer B, 10 CV). After the gradient, 100% buffer 2 is applied final wash out (3 CV), followed by 100% buffer 1 for equilibration (3 CV). Total run time is approximately 30 min.

Table 2.5. ÄKTA Start FPLC system method summary (method name: HiTrap Q HP 2COL Tf gradient v1.2).

UNICORN start 1.1 Variable list

Phase	Block	Variable	Value	Unit
Method Settings	Main	ColumnVolume	1.924	{mL}
Method Settings	METHOD SETTINGS	HighPressureValue	0.3	{MPa}
Method Settings	METHOD SETTINGS	FlowRate	1	{mL/min}
Prime and Equilibration	Equilibration	B Concentration	0	{%B}
Prime and Equilibration	Equilibrate	Equilibration Volume	2	{CV}
Sample Application	Sample Application Manual Injection Volume	Loop Sample Volume	4	{mL}
Elution and Fractionation	Elution Start at B concentration	Elution Start at B Concentration	0	{%B}
Elution and Fractionation	Start frac (Elution)	Last tube filled action	Pause	{}
		Elution Fixed Fractionation Volume	1.5	{mL}
Elution and Fractionation	Elution Linear gradient	Elution Linear gradient B concentration	100	{%B}
		Elution Linear gradient Volume	18	{mL}
Elution and Fractionation	Elution gradient Delay Volume	Elution gradient Delay Volume	1.5	{mL}
Wash out unbound	WashOutUnbound B Concentration	WashOutUnbound B Concentration	0	{%B}
Wash out unbound	Start Fractionation (WashOutUnbound)	Fractionation Volume	1.5	{mL}
Wash out unbound	WashOutUnbound Wash Volume	Wash Column With	3	{CV}

1.2.8 Washing and storing the column

After each consecutive experiment it is important to ensure that the buffer in columns is the buffer 1 (starting buffer) before loading the protein. If columns contain mixture of the buffer 1 and buffer 2 at the start of the experiment, it is possible that protein fractions will elute immediately due to inappropriate initial pH values. It is good practice to **rinse the column with small amount of buffer 1 before each experiment**. If no change in either absorbance or conductivity is detected during rinsing, it is safe to assume that columns are filled with pure buffer 1.

After performing multiple experiments it is good practice to rinse the columns first with 0.01 M HCl and then with water. **Do not leave columns filled with 0.01 M HCl longer than needed for washing purposes.** When columns are not to be used for extended periods, they should first be thoroughly washed with 20% ethanol solution and stored at 2-8°C. Additionally, it is recommended to label the columns and **keep separate columns for separate proteins to avoid contamination.**[5]

Note: Columns should be replaced by running the built-in „Column preparation“ method on the ÄKTA Start system (0.5 mL / min, 20% ethanol) while setting a large volume in order to ensure that protocol will not end during the time needed to remove and seal the columns. After the method is started, first loosen the bottom connector on the column to disconnect the tube and then tightly seal the bottom of the column with an appropriate cap (usually supplied with the columns). One should ensure no air is trapped between the cap and the column (“drop-to-drop” method). Once the bottom of the column is sealed, **the pressure in the column will start to rise quickly** and it is recommended to loosen the top connector on the column as quickly as possible. If the pressure rises too much, method will automatically halt and the software will display an error message on screen. In this event, disconnect the column and resume the method (click continue). Once the top of the column is disconnected, seal it with an appropriate plug (also usually supplied with the column) while ensuring no air remains trapped (“drop-to-drop” method). During the column exchange process usage of laboratory gloves and paper towels is advised due to rinsing solution leakage.

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