

GEN-PAK FAX COLUMNS

CONTENTS

I. INTRODUCTION

II. INSTALLATION

III. GENERAL USAGE CONSIDERATIONS

IV. GENERAL METHOD DEVELOPMENT GUIDELINES

V. EXAMPLE USING HAE III DIGESTED DNA

VI. TROUBLESHOOTING

Waters Gen-Pak™ FAX columns offer the highest resolution available in anion-exchange HPLC of nucleic acids. The Gen-Pak FAX column contains a weak anion exchanger based on DEAE functionalized non-porous resin. It contains 2.5 µm particles and is well suited for analytical and micro-preparative applications.

I. INTRODUCTION

This manual covers the use of the Waters Gen-Pak FAX column. The Gen-Pak FAX column is a 4.6 x 100 mm steel column containing a polymer-based high-performance anion-exchange packing. The column is designed to perform high-resolution analysis and purification on various nucleic acid species (up to at least 5,000 base pairs) such as DNA restriction fragments, polymerase chain reaction (PCR) products, plasmids and synthetic oligonucleotides.

II. INSTALLATION

a. Column

Before you attach a new column, attach a union in place of the column and flush the HPLC system free of previously used solvent.

Note: To achieve optimum performance from each column, use tubing of 0.010 inch i.d. or smaller to connect the injector to the column and the column to the detector.

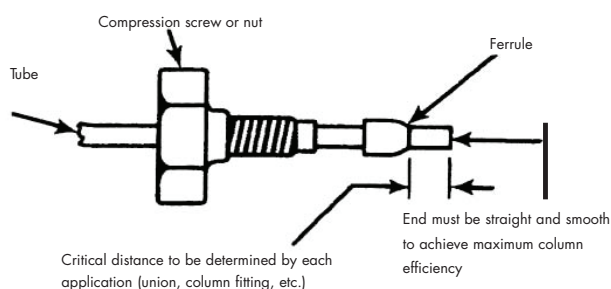
Procedure to install the column:

1. Remove the end plugs from the column. (Be sure to save the end plugs for use when the column is removed from the system for storage.) The column outlet is indicated by an arrow on the label showing the direction solvent should flow.
2. Finger-tighten the tubing compression screws and then wrench-tighten them 1/4 to 1/2 turn.

Note: Do not over-tighten; this damages the connection.

3. Make sure that the compression fitting is in good condition and properly prepared as shown in Figure 1. Because fittings may vary, it is important to verify that the tubing in your system bottoms in the column end nuts.

Figure 1: Ferrule and Compression Screw Assembly



b. Tubing

Follow the next three steps to cut tubing to connect a new steel column or to improve the end connections on existing fittings:

1. Using a three-cornered file with a cutting edge, scribe the circumference of the tubing at the desired break.
2. Grasp the tubing on both sides of the scribe mark with cloth-covered pliers (to prevent marring the tube surface) and gently work the tube back and forth until it separates.

3. Slide the compression screw, followed by the ferrule (large end of the taper first) over the tube. Be certain to bottom the tube in the fitting seat to assure a leak-free connection.

c. Testing

To validate system and column performance, test each new column with a standard sample. This provides a basis for detecting system component changes and troubleshooting.

Suggested samples include:

- Hae III digest of Φ X 174 RF DNA
- BstNI digest of pBR322 DNA

III. GENERAL USAGE CONSIDERATIONS

This section presents guidelines that can extend the life of your column and help you achieve the best possible chromatographic results.

a. Samples

When preparing and using samples:

- Centrifuge or filter the sample before injection.
- Do not inject samples containing microparticulates.

b. Solvents

When preparing and using solvents:

- Use pure buffer salts.
- Use of high quality reagents, water, and solvents is critical in preparing chromatography eluents. Fouling of the Gen-Pak FAX resin, leading to a loss in retention and / or separation efficiency, occurs faster on this column chemistry due to the small surface area of the non-porous resin particles. As such, all prepared HPLC eluents should be filtered through a solvent compatible 0.45 μ m or 0.22 μ m depth filter.
- Solvents should be degassed (vacuum filtration, sonication or helium sparged) prior to use.
- Use of organic solvents other than methanol or acetonitrile at greater than 10% in water is not recommended.

c. Operating Pressure

Do not exceed 4,000 psi operating pressure or about 1 mL/min at 25 °C.

d. pH range

Stay within a pH range of about 1.5 to 12 (do not use concentrated acids or bases).

e. Flow

Make flow rate changes in a gradual manner (less than 1 mL/min) to avoid column voiding. Never reverse flow in the column.

f. Cleaning

Clean the column between nucleic acid injections with 3 to 5 mL of with 3 to 5 mL of 20 - 40% acetic acid.

g. Storage

When you store a column for less than 24 hours, you typically do not need to follow special storage procedures. However, be sure that the column never dries out; this can degrade chromatographic performance.

For longer term storage, follow the procedure described below:

1. Flush the column with approximately 25 mL of Milli-Q® water to remove salts.
2. Flush the column with approximately 4 mL of a mixture of 10 percent methanol and 90% Milli-Q water.
3. Disconnect the column.
4. Screw end plugs firmly in place and return the column to its box.
5. Store the column at 4 °C.

Note: Before reusing the column, flush 4 with 25 mL of Milli-Q water to remove any methanol prior to introducing buffers.

IV. GENERAL METHOD DEVELOPMENT GUIDELINES

This section discusses the use of Waters Gen-Pak FAX columns for purifying DNA restriction fragments, polymerase chain reaction (PCR) products, plasmids, and synthetic oligonucleotides.

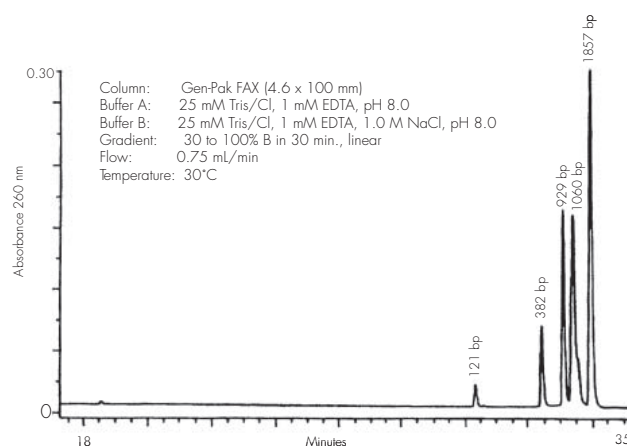
a. Separating Double Stranded DNA Fragments

DNA fragments are usually isolated using gel electrophoresis. Although resolution is good, the technique has limited mass capacity, often gives low yields of extracted fragments, and is time consuming.

The Gen-Pak FAX column is a useful alternative for the rapid purification and analysis of such nucleic acid species. Separations are often accomplished in about 30 minutes. Recoveries of biologically active material directly from the column are usually greater than 95%. Direct UV monitoring of the column effluent provides subnanogram sensitivity without the need for indirect visualization via ethidium bromide staining or autoradiography.

Depending upon sample complexity, as much as 50 to 100 µg of DNA can be separated in a single run. Since the separation is based primarily upon the overall charge of each fragment, smaller fragments elute prior to larger ones using an ionic strength gradient as shown in Figure 2.

Figure 2: Separation of 3.0 µg BstN I Digest of pBR322 DNA

**b. Buffers**

The preferred chromatographic buffer is 25 mM Tris/Cl, 1mM EDTA, pH 8.0. The recommended buffers for DNA fragmet separations are:

- Buffer A: 25 mM Tris/Cl, 1 mM EDTA, pH 8.0
- Buffer B: 25 mM Tris/Cl, mM EDTA, 1.0 M NaCl, pH 8.0

The inclusion of EDTA in the buffers is recommended to protect the DNA from nucleases during and after the chromatography¹. Filter and vacuum degas the buffers before use.

Useful chromatography can be obtained with sodium phosphate buffer, but the phosphate coprecipitates with the DNA in the common ethanol-precipitation procedures.

c. Equilibration and flow

Rigorous control of column equilibration is essential for predictable and reproducible chromatography. Before injection, equilibrate the column with 10 column volumes of buffer at initial chromatographic conditions. The column can be used at flow rates from below 0.5 to 1 mL/min, but 0.75 mL/min is recommended for general use.

d. Temperature Effects

The conformation and intermolecular hydrogen bonding of DNA fragments, and therefore the Gen-Pak FAX separation of those fragments, are affected by temperature. At higher temperatures, the structure of DNA is more open or relaxed so more ionic groups can interact with the column. In general, higher ionic strength is required as temperature is elevated.

Since conformation is dependent on base sequence, variations in temperature over the range of 30 to 60 °C can be used to optimize a particular separation. Where elevated temperatures are not required, the column should still be operated at a controlled temperature, typically 5 °C above ambient, to ensure reproducibility.

e. Detection

The separation of subnanogram quantities of DNA can be monitored at 260 nm, provided that the buffers are pure and free of UV absorbing contaminants. Assess blank gradients on a properly equilibrated column prior to performing actual sample chromatography.

f. Sample Size

Depending upon the size differences among the fragments to be isolated, as much as 50 to 100 µg of DNA can be applied to the column. Injection volume is essentially unlimited as long as the ionic strength of the sample is at least 0.1 M less than that required to elute the fragment of interest. For injection volumes above 100 µL, the sample should be at pH 8.

Excellent recoveries, in most cases more than 95 percent, of biologically active material have been obtained purifying samples containing fragments of 5000 base pairs and less.

g. Sample Treatments

DNA fragments generated by restriction enzyme digestion or from the polymerase chain reaction may be extracted with phenol/chloroform prior to chromatography. However, the reaction may be injected directly since most proteins are completely unretained under conditions used for the separation (i.e., 0.30 M NaCl).

In either case perform these steps before injection:

1. Remove particulate material from samples by centrifugation or filtration through a 0.45 µm filter.
2. Heat treated samples at 37 °C for 10 min prior to injection to disrupt any reannealed termini of the DNA fragments.

h. Gradients

Typical DNA fragments elute from the Gen-Pak FAX column between 0.4 and 0.75 M NaCl at 30°C. Equilibrate the column to 0.1 M below the ionic strength required to elute the smallest fragment of interest. Useful gradients are usually 5 to 10 mM/min at 0.75 mL/min.

i. Column Washing

Upon completion of each separation, wash the column with a small volume of 20 - 40% acetic acid to maintain its chromatographic characteristics as well as to eliminate sample carry-over from injection to injection.

Procedure

After the last fragment elutes, perform the following procedure:

1. Flush the column with 5 mL of buffer B.
2. Pump or inject 3 to 5 mL of 20 - 40% acetic acid onto the column. This may be performed with the column at 60 °C.
3. Flush the column with about 5 mL of buffer B.
4. Reequilibrate the column for the next sample injection.

Note: Washing the column with sodium hydroxide does not perform as well as the above acetic acid procedure and is not recommended.

V. EXAMPLE USING HAE III PLASMID DIGESTED DNA

This section shows an example of how to develop a method using Gen-Pak FAX columns. The example in this chapter can serve as a model for adapting this technique to the requirements of a particular sample.

The example describes the evolution of the separation of a restriction enzyme digest, the Hae III digest of ϕ X 174 RF DNA. This DNA sample contains 11 fragments ranging in size from 72 to 1353 base pairs.

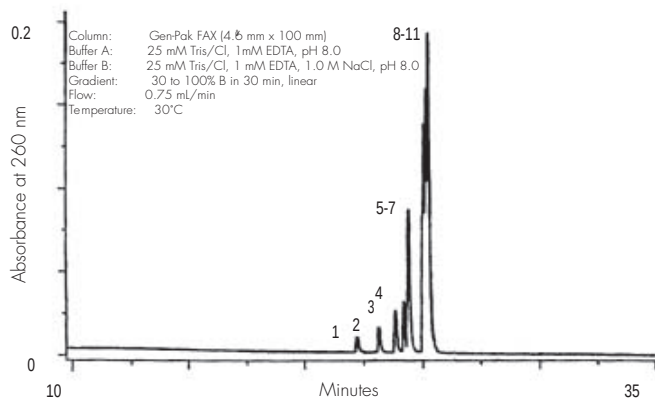
Table 1: Number of Base Pairs in Each Fragment

Fragment	Base Pairs
1	72
2	118
3	194
4	234
5	271
6	281
7	310
8	603
9	872
10	1078
11	1353

a. Initial Gradient

Chromatography is initially performed using a relatively steep ionic strength gradient. As Figure 3 shows, these conditions do not completely resolve the 234, 271, 281, and 310 (peaks 4-7) nor the 603, 872, 1078, and 1353 (peaks 8-11) base pair fragments.

Figure 3: Initial Separation of 2 μ g of Hae III Digest of ϕ X 174 RF DNA at 30°C using a Steep Salt Gradient



b. Gradient Optimization

Resolution can often be improved by systematic adjustment of gradient conditions. In the current example, examination of the initial chromatogram indicates a more shallow gradient is required.

c. Calculating NaCl Concentration

It is possible to calculate the approximate NaCl concentration required to elute the first (72bp) and last (1353 bp) restriction fragments from the data obtained in the initial chromatography. First, determine the system delay volume from the point of gradient formation to the detector cell.

d. Calculating System Delay Volume

To calculate the system delay volume from point of gradient formation to detector cell:

1. Remove the column from the system, insert a union, and set the detector to 260 nm.
2. Flush the pump and solvent lines with eluent A (Milli-Q water)
3. Start the flow of eluent B (0.01% acetone in Milli-Q water)
4. Monitor the detector output until it rises to the new, higher value of about 0.032 absorbance units if a 1 cm detector cell is used.

The system delay volume corresponds to the volume that has flowed from the start of eluent B to the midpoint of the absorbance rise plus the excluded column volume of 0.6 mL.

e. Using a More Shallow Gradient

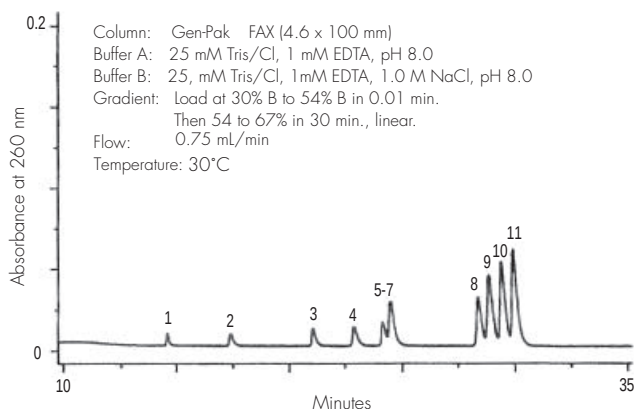
The system delay volume for this instrument was 7.5 mL, corresponding to 10 minutes at 0.75 mL/minute.

Under initial conditions, elution occurs as follows:

- The 72 base-pair fragment elutes at 22 minutes, reflecting the NaCl concentration at 12 minutes in the gradient table of 0.56 M NaCl
- The 1353 base-pair fragment elutes at 0.65 M NaCl

Figure 4 shows the results of changing to a more shallow NaCl gradient, from 0.54 to 0.67 NaCl, over the same 30 minute time interval. As this figure shows, the change significantly improved the resolution.

Figure 4: Separation of 2 µg of Hae III Digest of ØX 174 RF DNA at 30 °C using a Shallow Salt Gradient

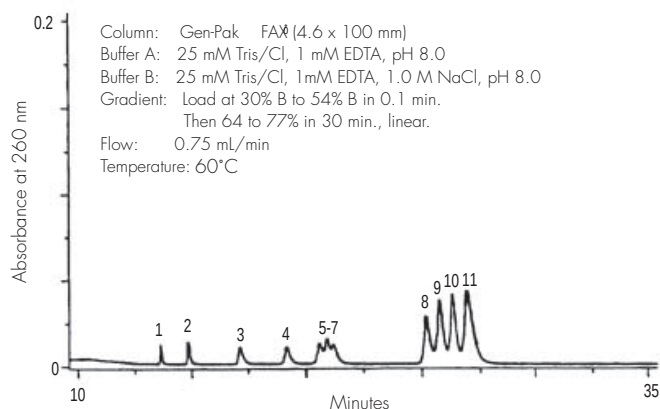


f. Temperature Optimization

Adjustments of gradient slope may not produce adequate resolution of DNA fragments in every case. However, changes in the temperature at which the separation is performed can alter the relative retention of the fragments. As Figure 5 shows, chromatography of the Hae III digest of ØX 174 at 60 °C yields slightly less resolution of the 72 and 118 base-pair fragments.

Since it is not possible to predict the best separation temperature for a particular sample, it is often useful to compare separations at two temperatures, such as 30 °C and 60 °C. Although increased NaCl concentration is required to elute DNA fragments from the column at elevated temperatures, chromatography at such temperatures does not shorten the column life nor affect the biological activity of the collected samples.

Figure 5: Separation of 2 µg of Hae III Digest of ØX 174 RF DNA at 60 °C using a Shallow Gradient



VI. TROUBLESHOOTING

Liquid chromatography columns have a finite life that is directly related to the care and use they receive. Column life is influenced by the number of injections, cleanliness of sample and solvent, frequency of solvent changeover, and procedures for handling and storage.

If you observe a change in the following areas, take immediate steps to determine the reason for the changes.

- Retention of a particular compound
- Resolution between two compounds
- Peak shape

Until the cause of the change is determined, you should question the validity of the results of any separation using the column

a. Pressure Buildup/Loss of Resolution

With continued use, it is possible that excessive pressure buildup (more than 4,000 psi at 1.0 mL/min at 25°C) or loss of resolution may be experienced. These may both be caused by the buildup of particulates from samples or eluents on the inlet filter. Filtering samples and eluents greatly extends inlet filter and column life.

b. Possible Corrective Actions

If pressure buildup or loss of resolution occurs, any of the following corrective actions, listed in order preference, may help:

- Perform a 20 - 40% acetic acid wash as described in Column Washing Section. The length of the wash can be extended to 10 to 15 minutes.
- Replace the inlet filter with a new one (Waters Part Number WAT015715)

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