Essentials in biore

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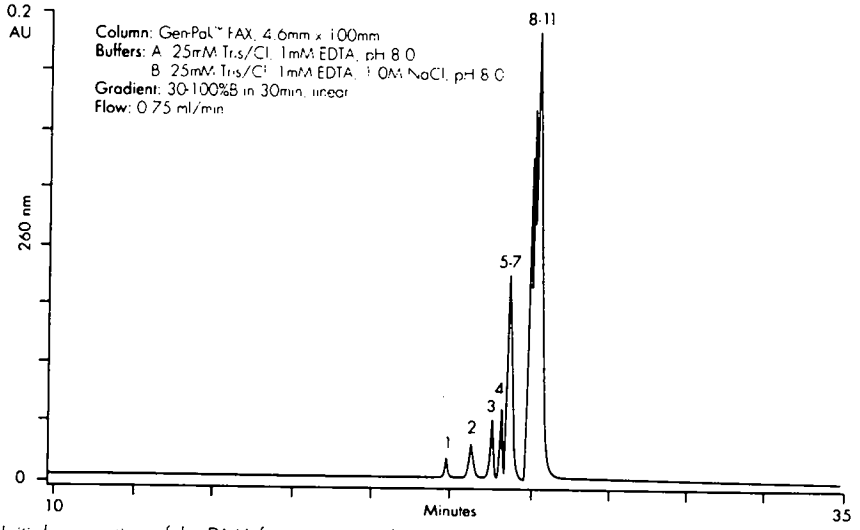
Waters Gen-Pak™ FAX Column
HPLC Separation of DNA Restriction Fragments and PCR Products

Part II: Guidelines for Separation Development

Chromatographic alternative to gel electrophoresis

The DNA fragments generated by restriction enzyme digestion or the polymerase chain reaction are classically 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[1]. The Waters Gen-Pak™ FAX column, a polymer based anion exchanger, is a useful alternative for the rapid purification and analysis of such nucleic acid species. Separations are often accomplished in about 30 minutes with excellent recoveries (>95%) of biologically active material. Direct UV monitoring of the column effluent provides subnanogram sensitivity without the need for indirect visualization via ethidium bromide staining or autoradiography. Depending on sample complexity, as much as 50 to 100µg of DNA can be separated in a single run. Part I of this application brief series (Lit. Code T74) describes general principles for the successful use of Waters Gen-Pak FAX chemistry. In this brief, guidelines for separation development are described.

Figure 1: Initial Separation of 2µg HaeIII Digest of ØX 174 DNA at 30°C Using a Steep Salt Gradient



Initial separation of the DNA fragment sample using a steep ionic strength gradient provides the information required to select a narrower salt concentration range for subsequent separations.

Systematic approach to separation development

Optimizing a DNA fragment separation on Gen-Pak FAX requires a systematic evaluation of the elution profile for a particular sample. This process can be illustrated using the HaeIII digest of ØX 174 which contains 11 fragments ranging in size from 72 to 1353 base pairs. Chromatography is initially performed using a relatively steep ionic strength gradient. As shown in Figure 1, these conditions resolve neither the 234, 271, 281 and 310 (peaks 4-7) nor the 603, 872, 1078 and 1353 (peaks 8-11) base-pair fragments. Resolution is easily modified by systematic adjustment of gradient conditions. Most frequently, use of a shallower ionic strength gradient improves resolution of closely related fragments. The HPLC system used for this series of experiments was determined to have a system delay of 10 minutes, which corresponds to a system volume of 7.5ml at 0.75ml/min. (see inset). Using this information, the NaCl concentration required to elute the 72 base pair fragment at a retention time of 22 minutes can be calculated as 0.56M. Similarly, the ionic strength required to elute the 1353 base pair fragment is determined to be 0.65M. Utilizing a shallower gradient profile, from 0.54M to 0.67M over the same 30 minute time period results in significant improvement in the resolution of the digest (Figure 2).

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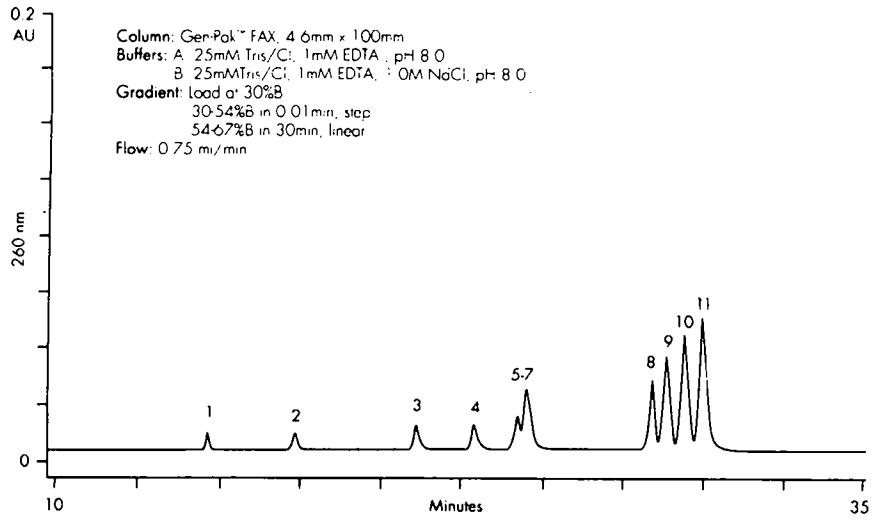
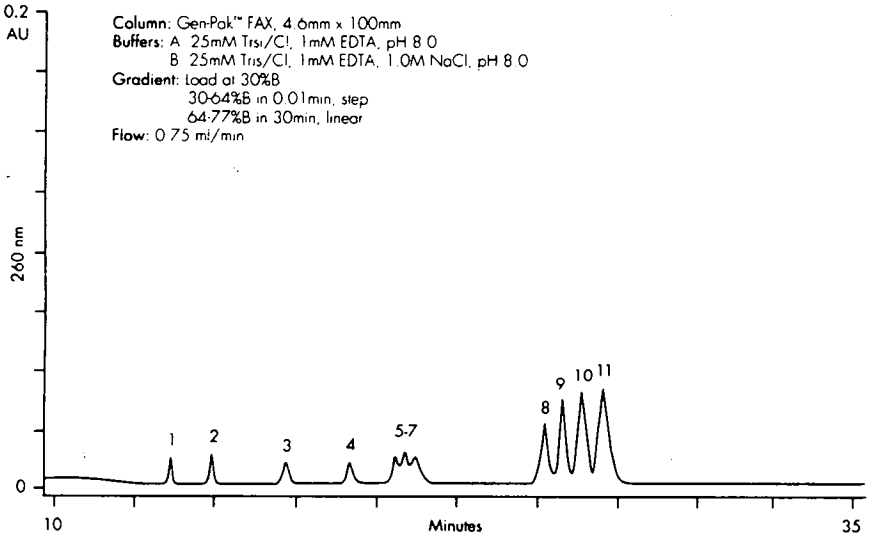
Waters Chromatography Division
34 Maple Street
Milford, MA 01757
508 478-2000

Figure 2: Modified Separation of 2µg HaeIII Digest of ØX 174 DNA at 30°C Using a Shallow Salt Gradient



A shallow salt gradient within the salt concentration range calculated from the initial chromatography results in enhanced resolution of the 11 DNA fragments.

Figure 3: Effect of Increasing Column Temperature to 60°C on the Separation of 2µg HaeIII Digest of ØX 174 DNA Using a Shallow Salt Gradient



Increasing the column temperature to 60°C changes the selectivity of the ion exchange separation by altering the conformations of the DNA fragments yielding an alternative separation from that obtained at 30°C.

Resolution enhanced by temperature control

Adjustments of gradient slope may not produce adequate resolution of DNA fragments in every case. In addition to ionic strength modifications, variations in the temperature at which the separation is performed can alter the relative retention of the fragments[2]. In the case of the HaeIII digest of ØX 174, chromatography at 60°C (Figure 3) yields slightly less resolution of the 72 and 118 (peaks 1 and 2) base pair fragments yet significantly improves the resolution of the 271, 281 and 310 (peaks 5-7) 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 several temperatures. Variations over the range of 30°C to 60°C result in no adverse effects on either the column lifetime or the biological activity of the collected fragments. Increasing ionic strength is generally required for elution as temperature is increased.

The Waters Gen-Pak FAX column is useful for the rapid purification and analysis of DNA fragments generated from restriction enzyme digestions or from the polymerase chain reaction. This guideline for separation development will help ensure successful application of this technique. For further information regarding column handling, cleaning and storage, refer to the application brief "HPLC Separation of DNA Restriction Fragments and PCR Products, Part I"; Lit. Code T74.

System Volume Calculation

Calculation of system volume is required for accurate determination of ionic strength at a particular elution time in a gradient method. Each chromatography system will have a unique volume from point of gradient formation to detector cell. This simple procedure will provide you with the necessary data to make the volume calculations

1. Remove Gen-Pak FAX column, insert union.
2. Set detector wavelength to 260nm
3. Flush inlet lines and pump with eluent A (Milli-Q™ water) and eluent B (0.01% acetone in water).
4. Pump 100% eluent A until a stable baseline is obtained.
5. Initiate step gradient to 100% eluent B.
6. Monitor detector signal until stable at higher absorbance value.
7. Measure time to mid-point of absorbance change.
8. Multiply time by flow rate to obtain delay volume.

References

1. Thompson, J. 1987, Biotech. 6(3): 246-251
2. Westman, E., et.al. 1987, Anal. Biochem. 166, 158-171

Ordering Information

Waters Gen-Pak FAX column (4.6mm x 100mm)	PN 15490
Waters Temperature Control System	PN 38039
Waters DNA Purification System	*

* Contact your local Waters technical representative to configure the optimal system for your laboratory

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