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Application Note 100

High-Resolution Analysis and Purification of Oligonucleotides with the DNAPac™ PA-100 Column

INTRODUCTION

The DNAPac PA-100 anion-exchange column is specifically engineered to provide unit-base resolution of synthetic oligonucleotides to 60 bases and beyond. This column is packed with a pellicular anion-exchange resin in which quaternary amine-functionalized microbeads are bound to a 13-µm diameter nonporous polymeric substrate (see Figure 1). The rapid mass transport characteristics of this resin result in higher resolution oligonucleotide separations than are possible with traditional macroporous resins or reversed-phase columns. Additionally, the DNAPac PA-100 can be operated routinely under strongly denaturing conditions, including high temperature or high pH. Anion-exchange separations under denaturing conditions are particularly useful for the resolution of oligonucleotides with high guanine (G) content or with regions of complementary sequence.

In this application note, methods for the separation of various synthetic oligonucleotides on the 4-mm diameter DNAPac PA-100 analytical column are presented. Included are pH 8.0 and pH 12.4 gradient programs. Also presented are strategies for the scale up of analytical separations to semipreparative purifications using a 22-mm diameter column.

EQUIPMENT

Dionex DX 500 HPLC system consisting of:

- GP40 Gradient Pump
- AD20 UV/Visible Absorbance Detector
- LC20 Chromatography Enclosure

Dionex PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Deionized water, 17.8 MΩ-cm resistance or better

Sodium hydroxide solution, 50% w/w, low carbonate

Tris(hydroxymethyl)aminomethane (molecular biology grade)

Sodium chloride

Sodium perchlorate monohydrate

Disodium ethylenediaminetetraacetate (EDTA), dihydrate (molecular biology grade)

$pd(A)_{12–18,}$ (Pharmacia Biotech Inc., Piscataway, New Jersey, USA)

$pd(A)_{40–60,}$ (Pharmacia Biotech Inc.)

$pd(G)_{12–18,}$ (Pharmacia Biotech Inc.)

-20 sequencing primer, crude 17-mer, (Genosys Biotechnologies, Inc. Woodlands, Texas, USA)

CONDITIONS

Columns:	DNAPac PA-100 analytical, 4 x 250 mm DNAPac PA-100 guard, 4 x 50 mm DNAPac PA-100 semipreparative, 22 x 250 mm
Eluent A:	Deionized water
Eluent B:	0.20 M NaOH
Eluent C:	0.25 M Tris-Cl, pH 8.0
Eluent D:	Option 1: 1.0 M NaCl Option 2: 2.0 M NaCl Option 3: 0.375 M $NaClO_4$
Flow Rate:	1.5 mL/min, except where noted
Inj. Volume:	200 µL
Detection:	UV, 260 nm

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent A: Deionized H_2O

Vacuum degas 1 L of deionized water.

Eluent B: 0.20 M NaOH

Dilute 10.4 mL of low carbonate 50% w/w sodium hydroxide to 1 L with degassed, deionized water. Pellets of NaOH are coated with a layer of sodium carbonate and are not suitable for eluent preparation.

Eluent C: 0.25 M Tris-Cl, pH 8.0

Dissolve 30.28 g of tris(hydroxymethyl)amino-methane in 800 mL of deionized water. Adjust the pH to 8.0 by the addition of approximately 10 mL of concentrated HCl. Add deionized water to a final volume of 1 L and vacuum degas the solution.

Eluent D, Option 1: 1.0 M NaCl

Dissolve 58.45 g of sodium chloride in 800 mL of deionized water. Add deionized water to a final volume of 1 L and vacuum degas the solution.

Eluent D, Option 2: 2.0 M NaCl

Dissolve 116.9 g of sodium chloride in 800 mL of deionized water. Add deionized water to a final volume of 1 L and vacuum degas the solution.

Eluent D, Option 3: 0.375 M $NaClO_4$

Dissolve 52.67 g of sodium perchlorate monohydrate in 800 mL of deionized water. Add deionized water to a final volume of 1 L and vacuum degas the solution.

0.5 M EDTA, pH 8.0

Add 93.1 g of disodium ethylenediaminetetraacetate dihydrate to 400 mL of deionized water. While stirring, add NaOH (approximately 10 g of pellets) until the EDTA is completely dissolved and the pH is stable at 8.0. Add deionized water to a final volume of 500 mL and autoclave to sterilize.

TE Buffer, pH 8.0

10 mM Tris-Cl, pH 8.0
1 mM EDTA, pH 8.0

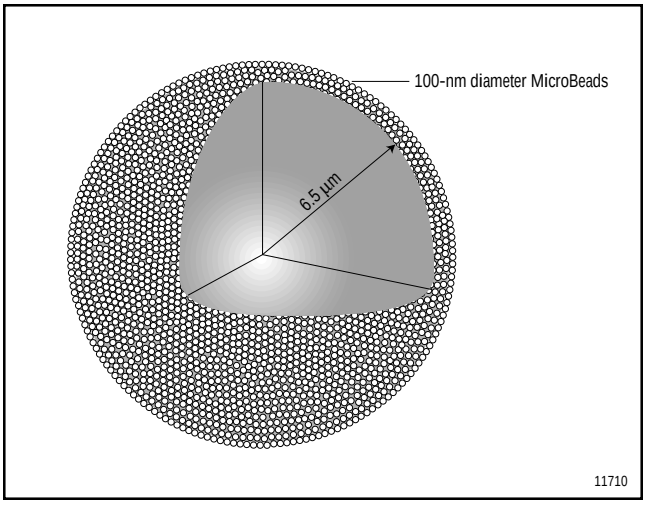

Figure 1 *Pellicular structure of the DNAPac PA-100 resin.*

SAMPLE PREPARATION

$pd(A)_{12–18}$, $pd(A)_{40–60}$, and $pd(G)_{12–18}$

Each lyophilized DNA homopolymer sample was dissolved in TE buffer to produce a 1-mg/mL solution. Before injection, DNA was diluted to the appropriate concentration with deionized water. The "p" in the chemical symbol indicates that the oligomers are phosphorylated.

-20 Sequencing Primer

The lyophilized, unpurified synthetic oligonucleotide was suspended in TE buffer. To remove particulate matter, the sample was centrifuged at 12,000 x *g* for 5 minutes and the supernatant was transferred to a fresh tube. The DNA concentration was determined by measuring the absorbance at 260 nm and applying the manufacturer's suggested conversion factor of 1.0 A_{260} = 30 µg/mL. The oligonucleotide was diluted with deionized water to the indicated concentration before injection.

RESULTS AND DISCUSSION

High-Resolution Separations at pH 8.0

The high resolving power of the DNAPac PA-100 can be demonstrated by the analysis of synthetic DNA homopolymers. Typical results for the analysis of 1 µg of $pd(A)_{12–18}$ are shown in Figure 2. A gradient of 100 to 450 mM NaCl in the presence of 25 mM Tris-Cl pH 8.0 was used to elute the pd(A) oligonucleotides.

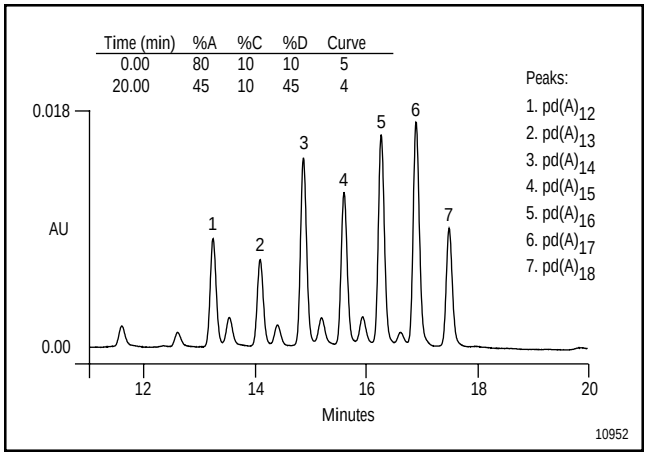

Figure 2 *Separation of 1 µg of pd(A)$_{12–18}$ at pH 8.0 with a 100 to 450 mM NaCl gradient. Small peaks represent dephosphorylated impurites (see text). Eluent D, Option 1 was used: 1.0 M NaCl.*

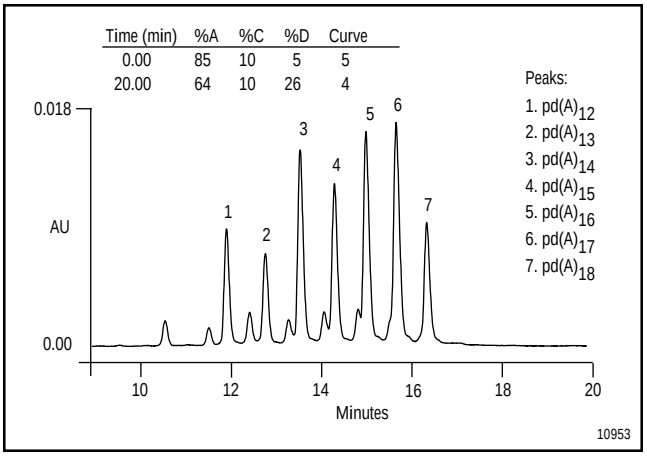

Figure 3 *Separation of 1 µg of pd(A)$_{12–18}$ at pH 8.0 with a 19 to 98 mM $NaClO_4$ gradient. Slightly different selectivity relative to NaCl elution is observed (see Figure 2). Eluent D, Option 3 was used: 0.375 M $NaClO_4$.*

The sample was injected at a relatively low NaCl concentration of 100 mM to ensure efficient binding of the oligonucleotides to the column. The pd(A) homopolymer mixture was resolved into seven main peaks, which represent the seven phosphorylated oligonucleotides described by the manufacturer. Also detected were seven minor peaks, which coeluted with the d(A)$_{12–18}$ oligonucleotides produced by dephosphorylation of the pd(A)$_{12–18}$ sample with alkaline phosphatase (data not shown).

DNA can be eluted from the DNAPac PA-100 by a variety of anions other than chloride, including acetate,[1] bromide,[2] and perchlorate.[1] The use of $NaClO_4$ as an eluent has been thoroughly investigated.[1] Perchlorate is particularly useful for the analysis of phosphorothioate DNA, in which sulfur has been substituted for non-bridging oxygen on the phosphate backbone. Since this sulfur substitution results in an increased affinity of DNA for the DNAPac resin, conventional salt gradients such as 0–2 M sodium chloride often will not elute phosphorothioates.[1,2] For the pd(A)$_{12–18}$ sample, gradient elution with $NaClO_4$ rather than NaCl produced similar chromatographic results (see Figure 3). Relative to NaCl, much lower concentrations of $NaClO_4$ are needed for DNA elution.

A somewhat different NaCl gradient was used for analysis of a pd(A)$_{40-60}$ oligonucleotide mixture (see Figure 4). In addition to the 21 expected peaks, at least one additional minor peak was detected. Since these

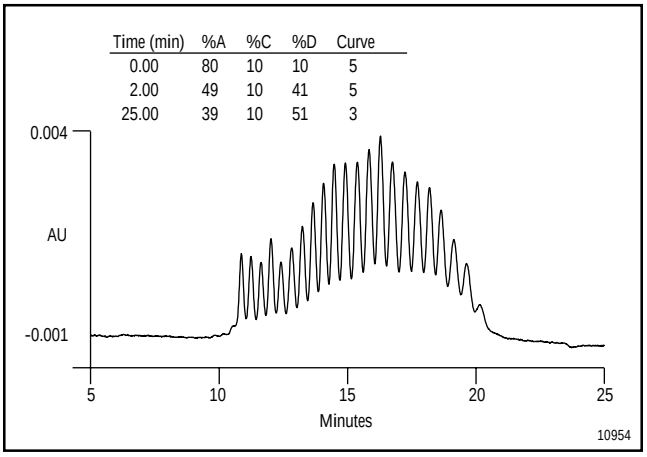

Figure 4 *Separation of 1.5 µg of pd(A)$_{40-60}$ at pH 8.0 with a shallow 410 to 510 mM NaCl gradient. The sample was injected at 100 mM NaCl to ensure efficient binding of the DNA to the column. Eluent D, Option 1 was used: 1.0 M NaCl.*

oligonucleotides are longer and possess a stronger negative charge than the pd(A)$_{12–18}$ species, higher NaCl concentrations are needed for elution. After sample injection at 100 mM of NaCl, the NaCl concentration was stepped to 410 mM over 2 minutes. A shallow (410 to 510 mM) NaCl gradient over 23 minutes was used for elution of the DNA. This gradient strategy couples the high resolving power of shallow gradients with short run times.

High pH Separations

Self-complementary sequences or poly-G stretches can result in intra- and intermolecular associations of oligonucleotides. These associations can prevent efficient oligonucleotide separation under non-denaturing conditions. The DNAPac PA-100 can be operated at high temperature (to 90 °C) or at high pH (to pH 12.4) for the separation of samples that contain such sequences. As an example, the attempted separation of $pd(G)_{12–18}$ at room temperature with a 100 to 900 mM NaCl gradient at pH 8.0 is shown in Figure 5. The poly-G sequence composition causes the sample to elute as one broad, diffused band. At pH 12.4, hydrogen bonding is abolished. As a consequence, the seven primary pdG homopolymers are resolved at pH 12.4, as are a number of minor contaminating species (see Figure 6). Relative to pH 8.0, an additional negative charge is present on the bases G and T at pH 12.4. This extra charge provides additional separation selectivity because it causes oligonucleotides with high levels of G and T to elute later than sequences low in G and T.

Analytical to Semipreparative Scale Up

DNAPac PA-100 columns are available in diameters of 9 mm and 22 mm for semipreparative applications. Analytical separations on the 4-mm diameter column can be scaled directly to the larger columns, so that methods development for semipreparative applications can be performed rapidly on the analytical column with small amounts of sample. For direct scale up of analytical gradient chromatography, a constant column volume scaling rule should be observed: *The number of column-volumes of eluent delivered over the duration of the gradient should remain constant*. To demonstrate scale up of a method from the 4-mm diameter to the 22-mm diameter column, an analytical separation was developed for the "-20 primer," a crude synthetic 17-mer with the sequence 5'-GTAAAACGACGGCCAGT-3'. A 7.5 to 124 mM $NaClO_4$ gradient over 20 minutes at pH 8.0 was used for separation of the primer from the various failure sequences in the sample. Figure 7a shows an analytical separation of 1.0 µg of this sample on the 4-mm diameter column.

The objective of the scale up exercise was the transfer of this analytical method from the 4-mm diameter column at a flow rate of 1.5 mL/min to the 22-mm diameter column at 10.0 mL/min.

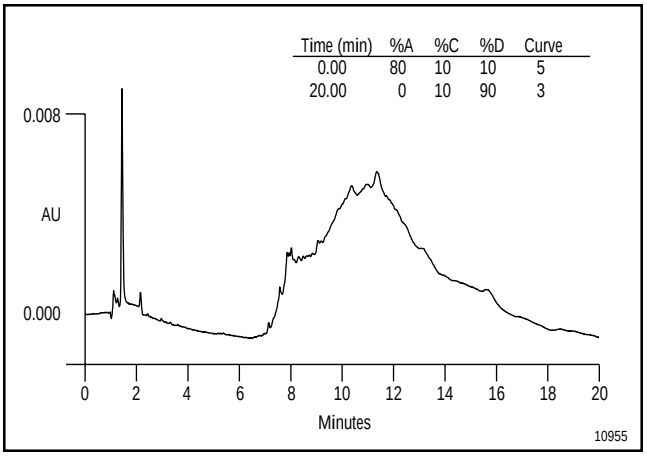

Figure 5 *Attempted separation of 3 µg of $pd(G)_{12–18}$ with a NaCl gradient at pH 8.0. Self-complementary sequences or poly-G regions in DNA can prevent oligonucleotide separation under non-denaturing conditions. Eluent D, Option 1 was used: 1.0 M NaCl.*

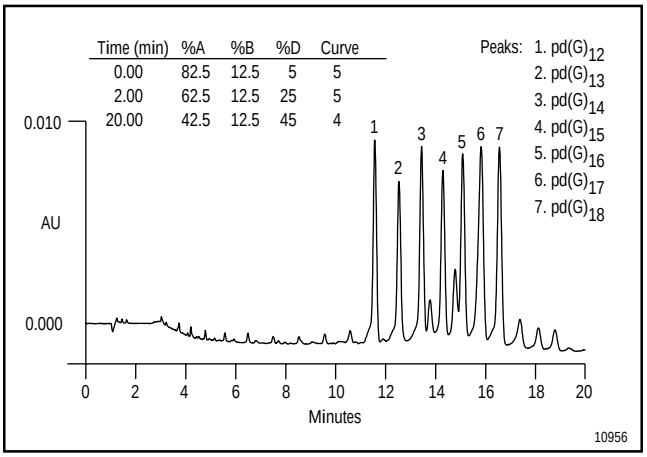

Figure 6 *Resolution of $pd(G)_{12–18}$ homopolymers at pH 12.4 with a 500 to 900 mM NaCl gradient. The sample was injected at 100 mM of NaCl. At high pH, hydrogen bonding between poly-dG sequences is eliminated. Eluent D, Option 2 was used: 2.0 M NaCl.*

The analytical method was modified in the following three ways to accomplish the transfer:

1) The flow rate of the analytical method was decreased from 1.5 mL/min to 0.33 mL/min. A 10.0-mL/min flow rate on the 22-mm diameter column is equivalent to a 0.33-mL/min flow rate on the 4-mm column because flow rate scales with the cross-sectional area of the column:

$$10.0 \text{ mL/min} \times \frac{\pi(2 \text{ mm})^2}{\pi(11 \text{ mm})^2} = 0.33 \text{ mL/min} \quad \{1\}$$

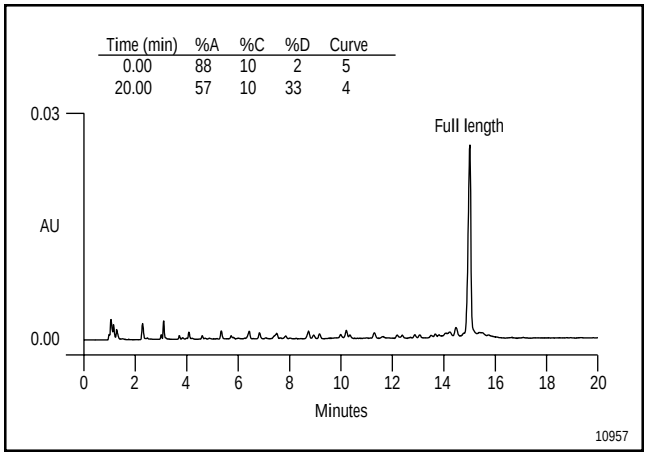

Figure 7a *Separation of the -20 primer from synthesis failure sequences. A 7.5 to 124 mM $NaClO_4$ gradient was used for analysis of the 1-µg sample. Eluent D, Option 3 was used: 0.375 M $NaClO_4$.*

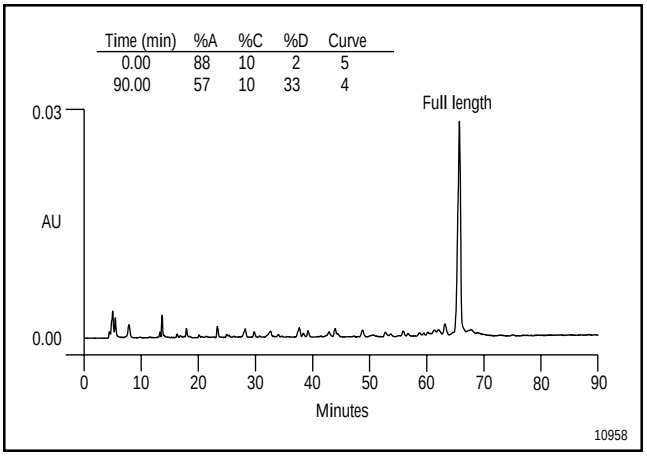

Figure 7b *Separation of 1 µg of the -20 primer with a 90-minute $NaClO_4$ gradient delivered at 0.33 mL/min. Note the similarity between this separartion and the 20-minute separation shown in Figure 7a. Eluent D, Option 3 was used: 0.375 M $NaClO_4$.*

2) The duration of the analytical method was increased to 90 minutes. The constant column volume scaling rule dictates that the 20-minute gradient at 1.5 mL/min is equivalent to a 90.9-min gradient at 0.33 mL/min:

$$20 \text{ min} \times \frac{(1.5 \text{ mL/min})}{(0.33 \text{ mL/min})} = 90.9 \text{ min} \quad \{2\}$$

Separation of 1 µg of DNA on the 4-mm diameter column with a 90-minute gradient at a flow rate of 0.33 mL/min is shown in Figure 7b. Nearly identical chromatograms were obtained in Figures 7a and 7b, which demonstrates the scaling fidelity of separations on the DNAPac PA-100.

3) The final step in the scale up was to transfer this 90-minute separation from the 4-mm diameter to the 22-mm diameter column. For this transfer, the gradient used for Figure 7b was delivered to the 22-mm diameter column at a flow rate of 10.0 mL/min. Figure 7c shows the separation of 30 µg of DNA by this method. The chromatography scales in a highly predictable manner, so that analytical methods can be transferred easily to preparative applications.

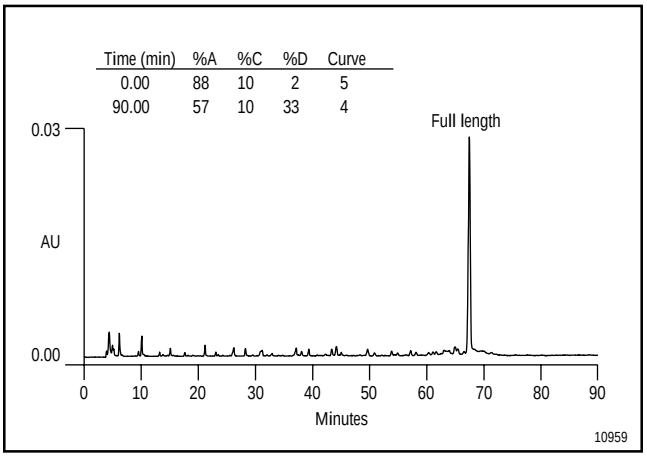

Figure 7c *Scale up of the -20 primer analytical separation to the 22-mm diameter DNAPac column. A 30-µg sample was analyzed at a flow rate of 10.0 mL/min. Eluent D, Option 3 was used: 0.375 M $NaClO_4$.*

Oligonucleotide Purification

Injections up to approximately 10 µg of crude synthetic DNA yield sharp, symmetric peaks on the analytical 4-mm diameter column. On the 9-mm and 22-mm diameter columns, upper load limits for analytical chromatography are approximately 50 µg and 300 µg, respectively. Purification of larger samples is possible by overloading the analytical capacity of the DNAPac column. The full-length oligonucleotide can elute as a very broad peak under overload conditions. However, a highly pure full-length oligonucleotide typically is present throughout this peak. Figure 8a shows an example of overload purification of 150 µg of the -20 primer on the 4-mm diameter analytical column. The separation was performed with a 90-minute $NaClO_4$ gradient at a flow rate of 0.33 mL/min, exactly as in the analytical separation in Figure 7b. The target oligonucleotide appears as an intense 3-minute peak beginning at about 61 minutes. Three 1-minute fractions were collected as shown in Figure 8b and

reanalyzed (see inset for fraction #2). The overall purity of the oligonucleotide collected from 61 to 64 minutes was > 98%. For another example of overload purification, a 1-mg purification of crude primer DNA to > 97% purity on the 4-mm diameter DNAPac has been demonstrated in reference 1.

CONCLUSION

By using a variety of eluent salts, highly efficient separations of synthetic DNA are possible on the DNAPac PA-100. The DNAPac pellicular anion-exchange resin provides higher resolution separations of single-stranded DNA than macroporous resins or reversed-phase columns. Analytical and semipreparative chromatography additionally can be performed under denaturing conditions for the separation of difficult samples. High-resolution analytical chromatography can be transferred directly to the 9-mm and 22-mm diameter columns for semipreparative applications.

REFERENCES

1. Thayer, J.R.; McCormick, R.M.; Avdalovic, N. *Methods in Enzymology*, **1996**, *271*, 147–174.
2. Bergot, B.J.; Egan, W. *J. Chromatogr*. **1992**, *599*, 35.

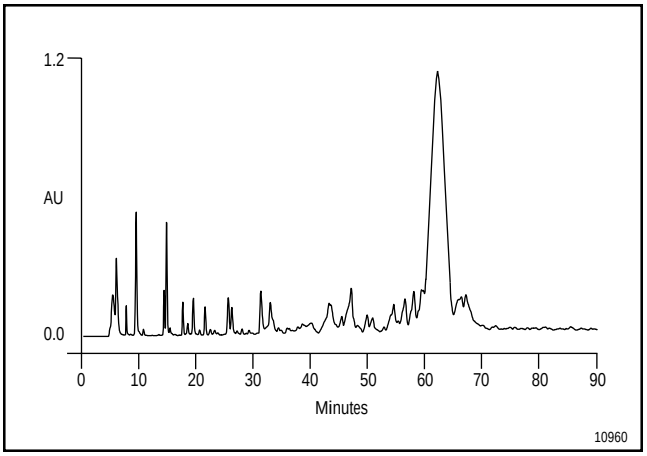

Figure 8a *Purification of 150 µg of the -20 primer on the 4-mm diameter column. The full-length primer elutes as a broad peak under these overload conditions. Chromatographic conditions are identical to those used for Figure 7b.*

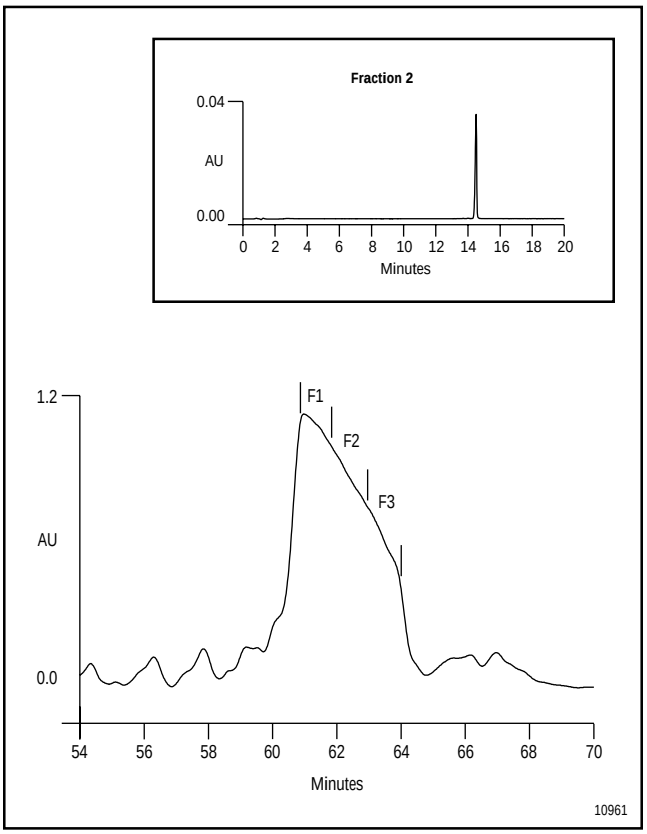

Figure 8b *Expanded view of full-length primer peak from Figure 8a. Fractions are indicated. Purity of pooled fractions was >98%. Inset: rechromatography of fraction 2.*

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