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[7] High-Resolution Nucleic Acid Separations by High-Performance Liquid Chromatography

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Introduction

The central role of nucleic acids in genetic engineering has spawned the development of numerous techniques for isolating, characterizing, and separating these compounds. Workers in this field have enjoyed the tolerance of genetic techniques for minimal purity of the components they manipulate. However, the realization of the therapeutic, agricultural, and medical diagnostic utility of various forms of nucleic acids has created a need, sometimes imposed by regulatory agencies, for higher purity components. Examples of nucleic acids for which high purity is now expected or required include the following: plasmids and restriction fragments used for DNA sequencing of master cell banks and genetic mapping, oligonucleotide probes and polymerase chain reaction (PCR) products to be probed for forensic and diagnostic applications, primers for PCR reactions, modified oligonucleotides in therapeutic applications, and synthetic ribozymes. Industrial research and development has responded to these demands by providing methods that are faster and offer greater purity or capacity than traditional electrophoretic and ultracentrifugal methods for purifying nucleic acids. For some applications, the new methods must also be amenable to scaleup.

A comparison of high-performance liquid chromatography (HPLC) with traditional electrophoretic and ultracentrifugal techniques reveals that

the weakest aspects of the traditional techniques are actually the strengths of HPLC. Automation of HPLC sampling, data reduction, fraction collection, and reporting functions enhances quantification and promotes greater throughput and recovery. HPLC also offers the opportunity for scaleup. In comparison, gel electrophoretic and ultracentrifugal methods are difficult to automate and quantify, present problems with recovery, are not readily scaleable, and for some applications, offer little practical advantage over current HPLC techniques (e.g., synthetic oligonucleotide separation[1]).

This chapter is organized in sections on applications and procedures for a wide variety of nucleic acids. Single-stranded (ss) nucleic acid separations include: (1) synthetic oligonucleotides (e.g., PCR and sequencing primers, oligonucleotide probes, and ribozymes), and (2) phosphorothioate (antisense) oligonucleotides. Double-stranded (ds) nucleic acid separations include: (1) restriction fragments (RFs), (2) PCR products, and (3) plasmids.

Numerous informative reviews on the use of HPLC for separations of nucleic acids have been published.[2-10] However, some new anion-exchange HPLC columns are now available that were not evaluated in prior reviews. Among these are columns packed with nonporous resins containing diethylaminoethyl (DEAE) or quaternary alkylamines as anion-exchange sites. Examples of these columns, which are capable of resolution far superior to their predecessors, are DEAE-NPR (Toyo Soda, Tokyo, Japan), Gen-Pak Fax (Millipore, Bedford, MA), and NucleoPac PA100 (Dionex, Sunnyvale, CA). An evaluation of three of these columns for fast restriction fragment separation (less than 20 min) indicated little practical advantage of one column over the others.[11] Figure 1 shows the separation of restriction fragments produced by *Hae*III digestion of pBR322 on these three columns. In Fig. 1, the DEAE-NPR and Gen-Pak Fax are compared with a NucleoPac

[1] P. J. Oefner, G. K. Bonn, C. G. Huber, and S. Nathakarnkitkool, *J. Chromatogr.* **625,** 331 (1992).
[2] J. A. Thompson, *BioChromatography* **1**(1), 16 (1986).
[3] G. Zon and J. A. Thompson, *BioChromatography* **1**(1), 22 (1986).
[4] J. A. Thompson, *BioChromatography* **1**(2), 68 (1986).
[5] J. A. Thompson, *BioChromatography* **2**(1), 4 (1987).
[6] J. A. Thompson, *BioChromatography* **2**(2), 68 (1987).
[7] J. A. Thompson, *BioChromatography* **2**(4), 166 (1987).
[8] G. Zon, Purification of synthetic oligonucleotides. *In* "High Performance Liquid Chromatography in Biotechnology" (W. S. Hancock, ed.), pp. 307–397. Wiley, New York, 1990.
[9] L. W. McLaughlin and R. Bischoff, *J. Chromatogr.* **418,** 51 (1987).
[10] D. Riesner, HPLC of plasmids, DNA restriction fragments and RNA transcripts. *In* "HPLC of Proteins, Peptides, and Polynucleotides" (M. T. W. Hearn, ed.), pp. 689–736. Verlag Chemie, Weinheim, New York, 1987.
[11] E. Katz, Comparison of Anion Exchange HPLC Columns for DNA Analysis. The Pittsburgh Conference, March 9–12, 1992, Book of Abstracts, Paper 1200.

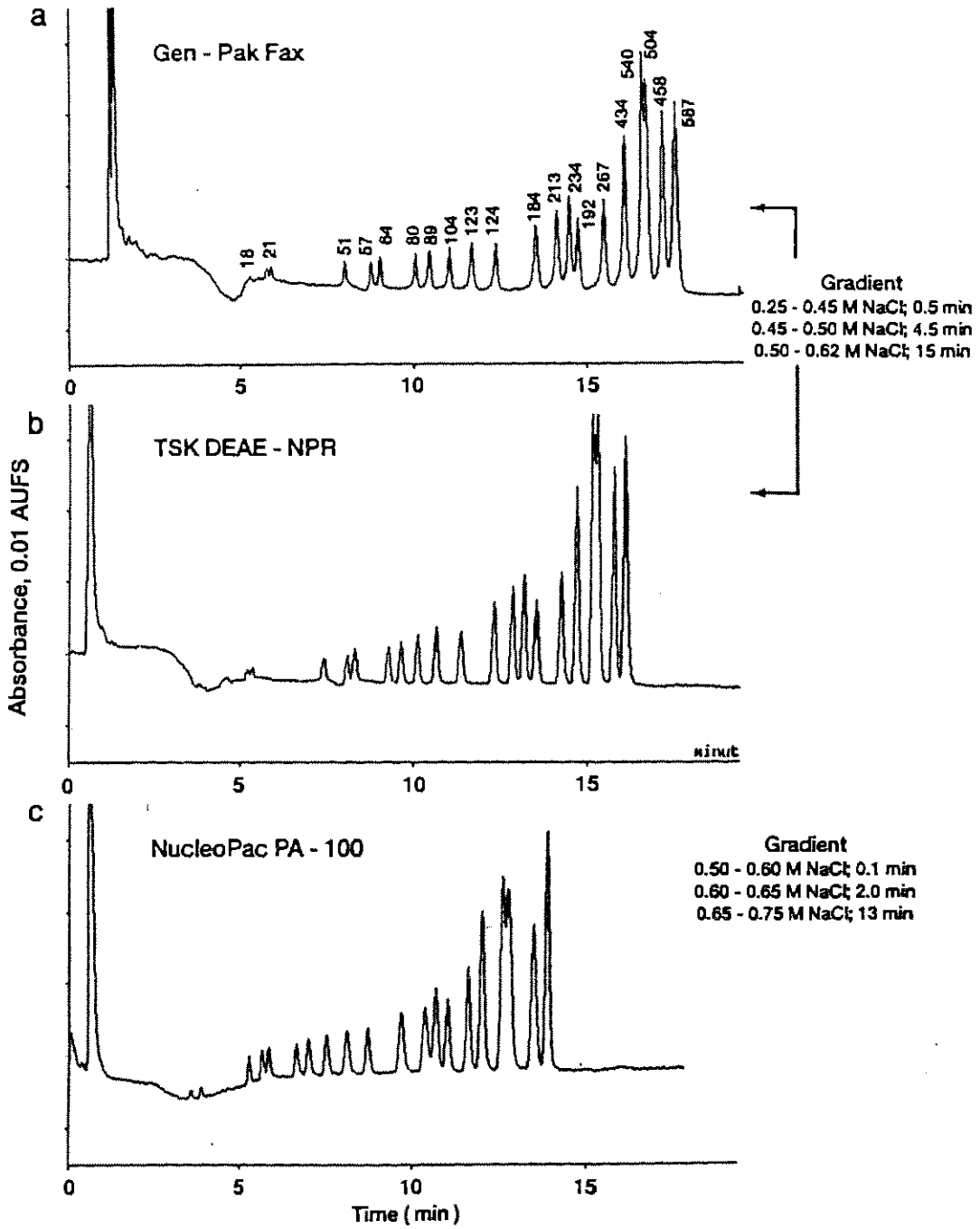

FIG. 1. Separation of the components of a *Hae*III digest of pBR322 on three different anion-exchange columns: (a) TSK DEAE-NPR (4.6-mm i.d. × 35 mm long, middle trace); (b) Millipore Gen-Pak Fax (4.6-mm i.d. × 100 mm long, top trace); (c) Dionex NucleoPac PA100 (4.0-mm i.d. × 50 mm long, bottom trace). Flow and gradient conditions as indicated. [Prepared by E. Katz (Perkin-Elmer Corporation, Norwalk, CT).]

PA100 *guard* column. The DEAE-NPR, Gen-Pak Fax, and similar nonporous products contain media with 2- to 3-μm diameter particles in stainless steel columns. These products contain weak anion-exchange functions (DEAE), precluding use at pH 12.4, where hydrogen bonding is abolished (use of high-pH eluents prohibits formation, during chromatography, of double-stranded structures by oligonucleotides having internally complementary sequences). These DEAE columns are short (3.5–10 cm × 0.46-cm diameter) and exhibit substantial back-pressure (1500–2250 psi at 0.5 ml/min, 20°, 4.6-mm i.d. × 10 cm[12]). We describe the use of the NucleoPac PA100 column (Dionex) that contains a strong anion-exchange function (quaternary amine, allowing the use of eluents in pH range from pH 0.7 to pH 12.5), and is available in columns of polyetheretherketone (PEEK) (inert to halide salts) and in a variety of column dimensions (4 × 50 mm to 22 × 250 mm). This column exhibits low back-pressure (800–1200 psi at 1.0 ml/min, 24°, 4.0-mm i.d. × 25 cm with a 4.0-mm i.d. × 5-cm long guard column), indicating its utility for scaleup.

Equipment

The Dionex DX-300 liquid chromatograph employed in this work consists of a gradient pump (AGP), an eluent degas module (EDM-II, used to degas the eluent before use and to apply a 5- to 10-psi helium overpressure to the eluent bottles during chromatography), a variable-wavelength absorbance detector (VDM-II), an AS3500 autosampler equipped with sample cooler and column oven, and a biocompatible injection system. These components are controlled by Dionex AI450 software running on a Dell 316SX computer. Fractions are collected with a Gilson (Middleton, WI) FC-80 fraction collector. Ethanol precipitates are sedimented in a Fisher (Pittsburgh, PA) 235C microcentrifuge placed in a 4° chromatography refrigerator (Revco, Asheville, NC) or by using an Eppendorf (Hamburg, Germany) 5402 refrigerated microcentrifuge. Samples are dried in a Virtis (Gardiner, NY) model 12SL lyophilizer or a Savant (Hicksville, NY) SpeedVac (SVC100 equipped with a VP100 vacuum pump and RT490 refrigerated trap).

Preparation for Anion-Exchange High-Performance Liquid Chromatography of DNA

Preparation of Eluents

The eluent system we use contains four components: E1, deionized distilled H_2O; E2, 0.2 *M* NaOH; E3, 0.25 *M* Tris-HCl, pH 8; and E4, 0.375

[12] D. J. Stowers, J. M. B. Keim, P. S. Paul, Y. S. Lyoo, M. Merion, and R. M. Benbow, *J. Chromatogr.* **444**, 47 (1988).

M $NaClO_4$. See the Appendix for specific gradient conditions used for each application.

E1: Deionized Distilled H_2O

Water is deionized to <5 μS, glass distilled, and stored in a glass carboy fitted with a vapor trap to minimize absorption of CO_2. This water is used for the preparation of all eluents.

E2: 0.2 M NaOH

Carbonate, at high pH, is a divalent anion that adversely affects resolution of nucleic acids eluted by monovalent anions (e.g., CH_3CO^-, Cl^-, ClO_4^-). Commercial NaOH pellets are coated with a layer of sodium carbonate produced by adsorption of CO_2 from the air. Hence, use of NaOH pellets for eluent preparation is counterproductive. Prepare NaOH eluents by diluting a 50% (w/w) solution of NaOH solution (e.g., Fisher) that is low in carbonate. Pipette 20.8 ml of 50% NaOH into 1980 ml of distilled, deionized H_2O in a 2-liter graduated cylinder, using a wide-bore 25-ml plastic pipette. Mix the solution for 2 to 3 min with a magnetic stirrer, then transfer the entire solution to a plastic 2-liter eluent reservoir connected to the HPLC system. Degas with helium sparging for ~5 min, seal the reservoir, and maintain 4- to 8-psi overpressure until the reservoir is empty or refilled.

E3: 0.25 M Tris-Cl–HCl

Dissolve 30.28 g of Tris–OH [e.g., Sigma (St. Louis, MO) Trizma base] in 800 ml of distilled, deionized H_2O while stirring with a magnetic stirrer, and bring to pH 8 with 2 *N* HCl (e.g., Fisher, ~60 to 65 ml) using a calibrated pH meter. Bring to 1 liter with distilled, deionized H_2O after removing the stirring bar, replace the stirring bar, and stir for 2 to 5 min. Transfer the entire solution to a glass 1-liter reservoir connected to the HPLC system. Degas with helium sparging for ~5 min, seal the reservoir, and maintain 4- to 8-psi overpressure until the reservoir is empty or refilled.

E4: 0.375 M $NaClO_4$

Under the conditions used, the E4 solution has exhibited no capacity for oxidation of nucleic acid samples or for the components of common HPLC instrumentation.

Both anhydrous $NaClO_4$ (Aldrich, Milwaukee, WI) and the monohydrate (Fluka, Ronkonkoma, NY) have yielded acceptable results. Dissolve 45.92 g (anhydrous) or 52.67 g (monohydrate) of $NaClO_4$ in 800 ml of

distilled, deionized H_2O in a 1-liter graduated cylinder, using a magnetic stirrer. Remove the stir bar, bring to 1 liter with distilled, deionized H_2O, replace the stirrer, and mix for 2 to 5 min. Transfer the entire solution to a 1-liter reservoir connected to the HPLC. Degas with helium for ~5 min, seal the reservoir, and maintain 4- to 8-psi overpressure until the reservoir is empty, or refilled.

Single-Stranded Nucleic Acids

Synthetic Oligonucleotides

Oligonucleotides are used as probes for specific sequences in diagnostic and forensic tests, primers for DNA sequencing and PCR amplification, therapeutics that inhibit the retrovirus replication cycle when containing modified bases or backbone, catalytically active ribozymes, and a wide variety of related applications.

A detailed discussion of the synthesis of these compounds is outside the scope of this chapter. Useful discussions of this process are available.[8,13,14] Synthetic oligonucleotides are typically 10 to 30 bases long, and are usually constructed using automated DNA synthesizers. The products of these synthesizers contain several components that must be removed prior to use. The oligonucleotides are simultaneously released from the support, phosphate deprotected in the instrument, and delivered in ammonia. The delivered product typically includes truncated sequences, some partially depurinated oligomers, deprotected oligomers, and the target, full-length fully dimethoxytrityl (DMT)-protected oligonucleotide. For forensic and diagnostic probes, integrity and purity of the oligonucleotide are paramount.

Purification from "Machine-Grade" Single-Stranded DNA. Historically, purification has been accomplished by a combination of reversed-phase extraction (i.e., sample-preparation cartridges) and denaturing polyacrylamide gel electrophoresis (PAGE). PAGE is labor intensive and not amenable to scaleup. Reversed-phase HPLC is also useful,[7] but is usually also accompanied by PAGE as an orthogonal method to confirm the product purity. In this section, we discuss the use of strong anion-exchange HPLC on a nonporous resin for purification and analysis of oligonucleotides.

The ammonia solution delivered by the synthesizer is brought to 4%

[13] S. Agrawal and J.-Y. Tang, *Tetrahedron Lett.* **31**(52), 7541 (1990).

[14] S. Gryaznov and R. Letsinger, *Nucleic Acids Res.* **20**(8), 1979 (1992); see also **20**(13), 3403 (1992).

(v/v) triethylamine to keep the solution slightly basic during initial processing or storage at −20° (prevents unintentional deprotection).

Removing the contaminants prior to anion-exchange chromatography can be advantageous, if a large fraction of the oligomers in the solution are truncated or depurinated, and especially when column capacity overload is approached. Prepurification can be accomplished with any of the numerous reversed-phase cartridges designed for that purpose. The following procedure was developed on a C_{18} Sep-Pak (Millipore).[15]

Reversed-Phase Extraction. The dried synthetic oligonucleotide mixture is resuspended in an appropriate volume (~2.0 ml for a 0.2-μmol synthesis) of distilled, deionized H_2O containing 30 m*M* triethylammonium bicarbonate (TEABC). The hydrophobic character of the dimethoxytrityl (DMT)-protecting group affords a simple method for separating the protected oligonucleotide from partially deprotected contaminants. The aqueous sample is applied to the Sep-Pak cartridge that has been preconditioned with 10 ml of acetonitrile (CH_3CN), 5 ml of 100 m*M* TEABC in 30% CH_3CN, and 5 ml of 25 m*M* TEABC. After application, the 5′-OH-terminated ("truncated") oligomers are eluted with 15 ml of 25 m*M* TEABC in 10% CH_3CN. The 5′-DMT-terminated oligonucleotides are then eluted with 10 ml of 0.1 *M* TEABC with 30% CH_3CN. The DMT-oligonucleotide fraction is evaporated to complete dryness (or to a gum) in a centrifugal concentrator [e.g., Savant (Hicksville, NY) SpeedVac], and stored at −20°. The capacity of the Sep-Pak is ~50 A_{260} (1 to 2 mg) of total oligonucleotide.

Deprotection of the "trityl-on" enriched oligonucleotides is accomplished by complete resuspension in 1 ml of 3% (v/v) acetic acid (for a 0.2-μmol synthesis) at 24°, followed by incubation for 10 min. After incubation, the solution is neutralized with a few drops of concentrated NH_4OH. The released "trityl-on" residues may be removed by passing the neutralized solution through a Sep-Pak, as described above, or by extraction into an equal volume of ethyl acetate (vortex, allow to separate by spinning in a microcentrifuge, and discard the upper layer). A small aliquot (e.g., 10 μl) may be sampled and diluted for quantification of enriched oligonucleotide by measuring absorbance at 260 nm.

Anion-Exchange Separations of Oligonucleotides

Anion-exchange chromatography at different pH values affords somewhat orthogonal or complementary separations of oligonucleotides. Elution by $NaClO_4$ at pH 8 usually results in the target oligonucleotide being

[15] K. M. Lo, S. S. Jones, N. R. Hackett, and H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2285 (1984).

separated in a single peak that elutes after truncated or other undesirable components. However, oligonucleotides with palindromic or other forms of self-complementary sequences may form loops or hairpins that alter their chromatographic behavior. Also, truncated oligonucleotides containing residual DMT residues may coelute with full-length detritylated oligonucleotides.

Another condition that may interfere with the resolution of synthetic oligonucleotides is the presence of several consecutive guanine residues, which are capable of hydrogen-bonding associations atypical of Watson–Crick base pairing. Such oligonucleotides may exhibit broad peaks with low absorbance at 260 nm. Solutions with pH values above 12.4 (25 m*M* NaOH) are incapable of maintaining hydrogen bonding. Chromatography in 25 m*M* NaOH is thus recommended for oligonucleotides suspected of containing poly(G) runs or self-complementary sequences. This feature of high-pH chromatography and the potential problems accompanying pH 8 chromatography indicate that both purity assessment and purification should be done at pH 12.4.

Programs useful for the separation of oligonucleotides 8 to 30 bases long at pH 8 and pH 12.4 are listed in the Appendix as programs 1 and 2. These gradients will adequately separate standard phosphodiester oligonucleotides from 8 to 30 bases long. Each guanine and thymine base will contribute an extra negative charge at pH 12.4, thus oligonucleotides high in these bases will tend to elute later, relative to oligomers containing less G and T, at the higher pH.

Recovery of oligonucleotides (except phosphorothioates) after collection may employ ethanol precipitation, or by conversion of buffer to a volatile salt with a desalting column [e.g., Pharmacia (Piscataway, NJ) NAP converted to the ammonium acetate form], followed by vacuum evaporation of the volatile salt (e.g., SpeedVac). For ethanol precipitation, a 0.1 vol of 2.5 *M* ammonium acetate is added to the collected fractions, the solution gently mixed, 2.5 vol of ice-cold ethanol added, the solution mixed again, and the resulting suspension (precipitate not visible) centrifuged at 12,000 *g* for 7 min in a refrigerated microcentrifuge (Eppendorf 5402) or in a standard microcentrifuge (Fisher 235C) in a 4° chromatography refrigerator. Phosphorothioates should be concentrated by vacuum evaporation after buffer exchange on Pharmacia NAP columns (Sephadex G-25) using 10 m*M* ammonium acetate as eluent because of their low recovery from ethanol precipitates.

Sample Preparation

Ten to 500 μl of suspension buffer (TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH8) as a control, or 0.1 to 1000 μg of sample in TE, are placed in autosam-

pler vials [Sun Brokers (Wilmington, NC) vials, caps, and septa]. They are then capped and placed into chilled (8°) vial racks in the autosampler.

System Equilibration

The first run in a schedule should contain a blank run of Tris-EDTA (TE) or H_2O to elute any compounds left stored in or adsorbed onto the column since its last use. Following the blank run, samples containing 50 ng or more can be analyzed without further system equilibration.

Analytical Oligonucleotide Separations

Under the conditions described above, injections (~0.05–10 μg/peak) yield symmetric peaks. An example of a 2.7-μg injection of the Cetus PCR01 primer (base composition, G8 C5 T7 A5; Perkin-Elmer Cetus, Norwalk, CT) is shown in Fig. 2. These traces clearly show the presence of numerous truncated sequences typical of crude or "machine-grade" oligonucleotides at both pH 8 and pH 12.4.

Semipreparative Purifications. Semipreparative purifications (>100 μg of oligonucleotide per peak) can be performed by scaling up the column diameter (9 × 250 mm = 5-fold scaleup, 22 × 250 mm column = 30-fold scaleup), or by overloading the column. To scale up directly by increasing

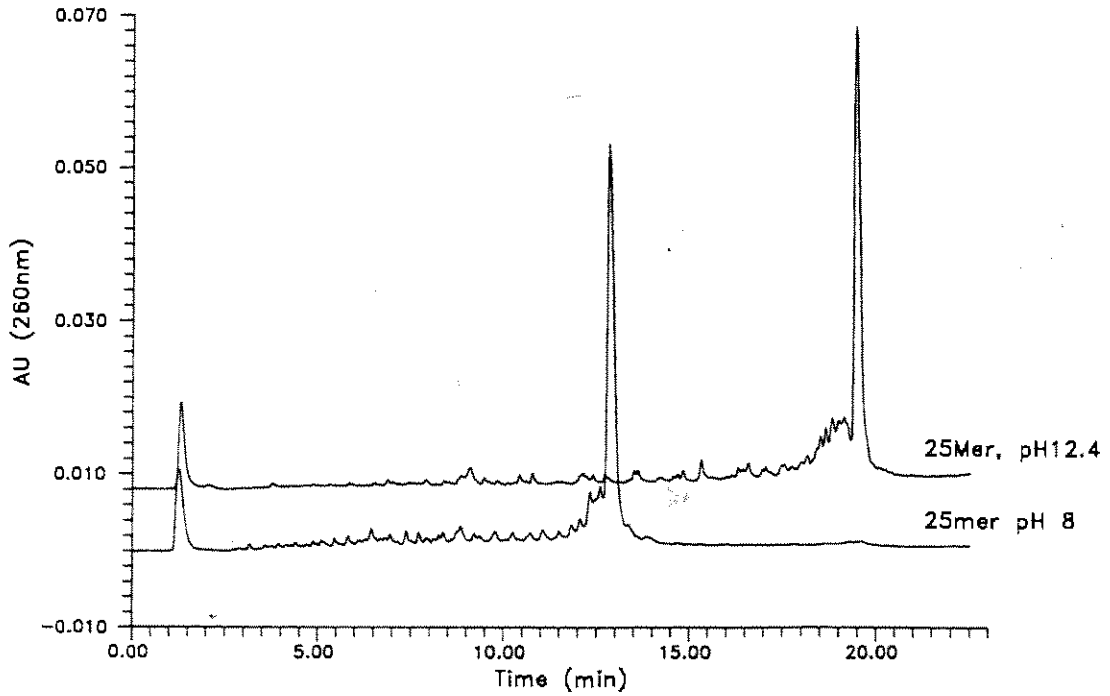

FIG. 2. Analytical-scale separation of a detritylated, crude 25-base oligonucleotide (G8, C5, T7, A5) from truncated contaminants on the NucleoPac PA100 column at 30°. Gradient conditions given in Appendix, gradients 1 and 2.

the column diameter, the flow rate should be raised by the same factor as the column capacity. Hence, a 50-μg purification on a 4 × 250 mm column at 1 ml/min could be scaled to 250 μg at 5 ml/min (9 × 250 mm column), or 1.5 mg at 30 ml/min (22 × 250 mm column). Under these conditions, the chromatograms would appear essentially identical.

Larger scale purifications can also be accomplished by overloading the analytical capacity of the NucleoPac column. This approximates the process of displacement chromatography. Under these conditions, the target analyte (i.e., the full-length oligonucleotide) can act as an eluent, displacing shorter length oligomers ahead of itself as it migrates through the column. The resulting trace does not indicate resolution of the full-length from smaller oligomers, because the latter are immediately replaced within the detector by the "pushing" full-length oligonucleotide. Figure 3 shows a trace typical of this chromatographic mode. A 1-mg sample of machine-grade Cetus PCR-01 primer was applied to the column, here in 0.15 *M* ammonium acetate. The smaller oligonucleotides were eluted with a step change to

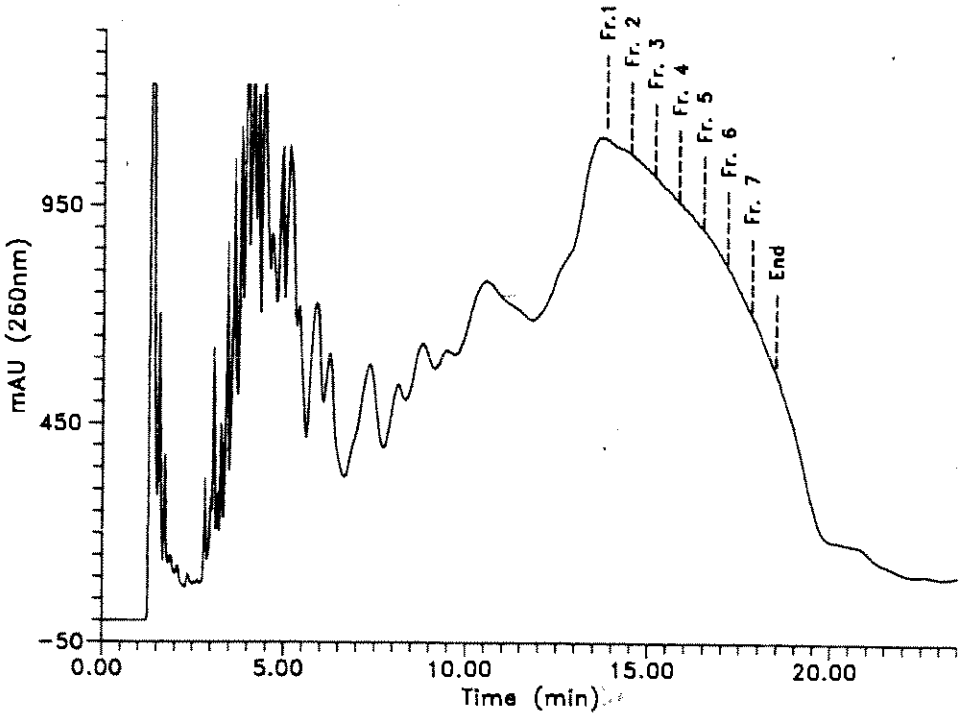

FIG. 3. Preparative loading of a 4 × 250 mm NucleoPac PA100, using a volatile eluent (ammonium acetate). One milligram of the same 25-base oligomer shown in Fig. 1 was purified by application in 0.15 *M* ammonium acetate. A step to 0.9 *M* in 1 min elutes most of the smaller truncated oligomers. The target detritylated 25-mer is eluted using quasidisplacement mode chromatography by a gradient from 0.9 to 1.1 *M* ammonium acetate in 20 min (1.5 ml/min). Each of the indicated fractions (1–7) exhibited the presence of a single 25-base oligonucleotide. The yield was 60% (24 of 40 A_{260}) at a final purity of >97%.

0.9 *M* ammonium acetate in 1 min. Elution of the longer oligonucleotides was accomplished with a shallow gradient (0.9 to 1.1 *M* ammonium acetate over 20 min). The resulting trace exhibits peaks of increasing width as the last (target) peak pushes the smaller oligomers ahead of it. Finally, the target oligomer elutes in a broad peak between 13.5 and 19.5 min. In this experiment, seven 1.0-ml fractions were collected between 13.5 and 18.5 min. Rechromatography of aliquots of the collected fractions revealed an essentially pure oligonucleotide component in each of these fractions. The collected fractions comprised 60% of the total A_{260}-absorbing material applied to the column [24 of 40 optical density units] at a purity greater than 97%.

Extended Length Oligonucleotides

The $NaClO_4$ gradient programs devised for normal-length oligonucleotides can, with minor modification, be applied to the separation of longer oligomers. Programs 3 and 4 in the Appendix describe the modified gradient programs.

Under these conditions, analytical-scale injections (~0.05–20 μg) of oligonucleotides of 30 to >70 bases yield sharp symmetric peaks. Examples of purity checks of gel-purified oligomers with 44 and 76 bases are shown in Fig. 4. Although less prominent in these gel-purified samples, the traces

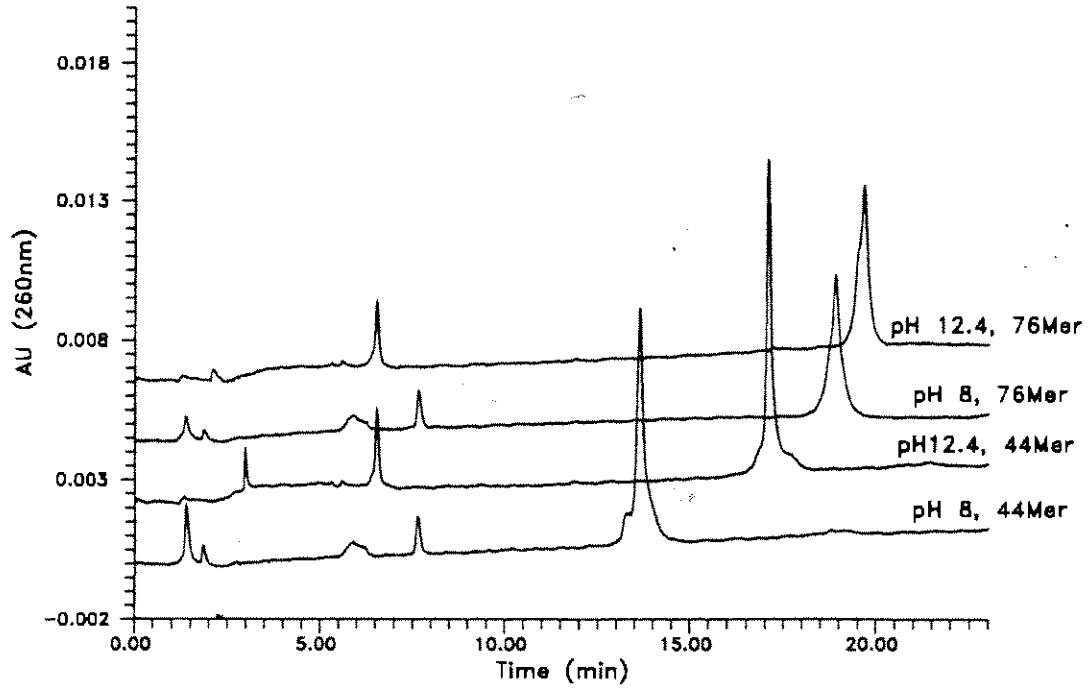

FIG. 4. Purity test of gel-purified 44- and 76-base oligonucleotides using the NucleoPac PA100 at 30°. Gradient methods 3 and 4 of the Appendix were used.

reveal the presence in contaminants of both oligonucleotide preparations at each pH. The pH 8 traces exhibit the presence of some "$n - 1$"-mer in both the 44-mer and 77-mer traces. The pH 12.4 traces demonstrate some "$n + 1$"-mer in the 44-mer, as well as some "$n - 1$"-mer in both oligonucleotide preparations. Injections represent 20 pmol (44-mer) or 8.5 pmol (76-mer).

Phosphorothioate (Antisense) DNA

Phosphorothioate DNA differs from normal (phosphodiester) DNA by a substitution of ionized sulfur for ionized oxygen on the phosphate backbone. These modified oligonucleotides can be synthesized as phosphodiesters using hydrogen phosphonate chemistry, and treated postsynthetically with sulfur in carbon disulfide. Alternatively, phosphorothioates can be synthesized by phosphoramidate chemistry, with the exception of the oxidation step. Where iodine is normally used to oxidize the phosphoramidate to phosphate, replacement of iodine with tetraethylthiuram disulfide, or other appropriate thiol reagent, results in a phosphorothioate product. The latter approach offers greater repetitive yield (efficiency) and allows for the synthesis of mixed phosphodiester/phosphorothioate oligonucleotides.

The sulfur contributes a subtantially greater hydrophobicity to the DNA, thus altering some of the characteristics affecting its manipulation. The sulfur also contributes to a much greater affinity for the NucleoPac anion-exchange media. Hence, the gradient methods that elute phosphodiester DNA will not adequately elute phosphorothioate DNA of similar length. Another alteration in the procedure for working with these modified oligonucleotides involves the conditions used to deprotect. These oligonucleotides are deprotected by incubation in 80% acetic acid for 3 hr at 24°. Ethyl acetate extraction of these modified oligonucleotides may yield poor recoveries. Gradient methods for elution of phosphorothioate oligonucleotides 10–30 bases in length are provided as methods 5 (pH 8) and 6 (pH 12.4) in the Appendix.

Chromatograms of phosphorothioate oligonucleotides also exhibit peaks that are fairly broad but often well separated, because conversion of phosphoramidate to phosphorothioate during the oxidation step, described above, results in chiral phosphorus atoms between each pair of bases. The phosphoramidate oxidation occasionally results in a residual phosphodiester bond. Even a single residual phosphodiester linkage in a 27-base phosphorothioate oligonucleotide will subtantially decrease the affinity of the oligonucleotide for the anion-exchange phase. This allows the separation of fully phosphorothioated oligonucleotides from those harboring residual phosphodiesters. For example, an oligonucleotide with 15 bases that con-

tains 14 phosphorothioate linkages is separable from the same sequence 15-mer containing 13 phosphorothioate and 1 phosphodiester bond. If both truncated and incompletely phosphorothioated but full-length sequences are present, overlapping retention of the component oligonucleotides and their chiral counterparts will contribute to broad peaks and apparently incomplete resolution when compared to standard phosphodiester DNA.

Figure 5 compares the chromatography of a prepurified 15-mer phosphorothioate at pH 8 and pH 12.4. At pH 8, the full-length, fully phosphorothioated oligomer appears as a broad peak indistinguishable from sequences containing truncated sequences or residual phosphodiester linkages. At pH 12.4, the full-length, fully phosphorothioated oligomer is better resolved (blank traces are included to reveal "system" peaks that elute under these conditions).

The complexity of this sample matrix and the hydrophobicity of the phosphorothioates suggest that a reversed-phase sample cleanup step would be effective. Figure 6 compares the pH 8 and pH 12.4 chromatography of a phosphorothioate oligomer containing 15 thymidine residues. The sample was prepurified by the trityl-on method, using a polymer reversed-phase column (Hamilton, Reno, NV, PRP-1). The prepurification procedure should result in the presence of only full-length oligomers. However, anion-exchange chromatography on the NucleoPac column reveals the presence

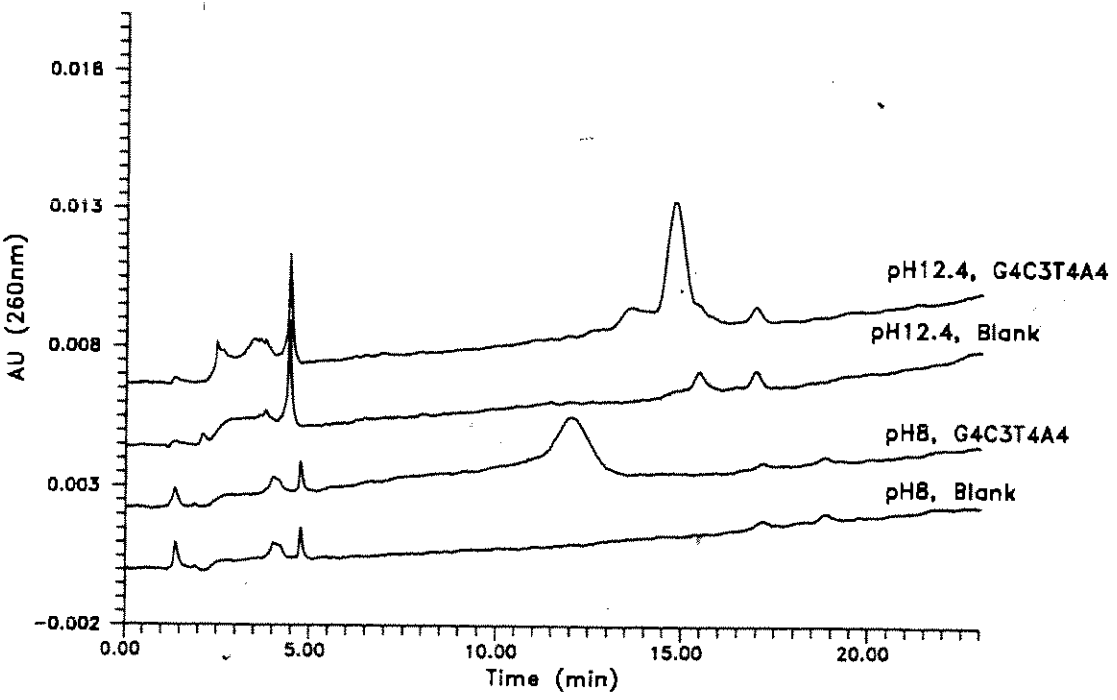

FIG. 5. Purity analysis of a mixed-sequence 15-base phosphorothioate oligonucleotide at pH 8 and 12.4 and at 30°. Gradient methods 5 and 6 of the Appendix were used. Traces from the blanks are included to identify "eluent system" peaks.

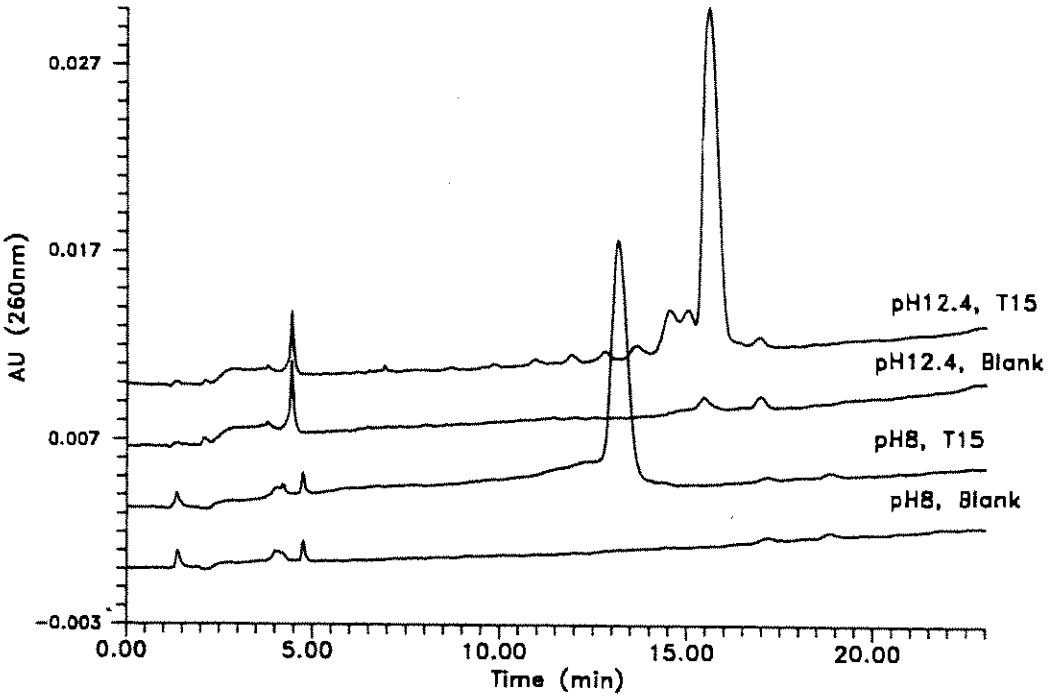

FIG. 6. Purity test of a "reversed-phase prepurified" T15 phosphorothioate oligonucleotide at pH 8 and 12.4 using the NucleoPac PA100 at 30°. Gradient methods 5 and 6 of the Appendix were used. Traces from the blanks are included to identify "eluent system" peaks.

of at least eight earlier-eluting oligomers. Bergot and Egan[16] and Bergot and Zon[17] described the separation of the full-length oligonucleotides containing differing numbers of residual phosphodiester linkages by anion-exchange chromatography. Subsequently, isolated oligomers were characterized by nuclear magnetic resonance (NMR) spectroscopy. The authors have now confirmed the separation of these species on the NucleoPac column (B. J. Bergot, personal communication, 1990).

Recovery by ethanol precipitation is substantially poorer for phosphorothioates than for phosphodiesters. When the target oligonucleotide contains more than 20 bases, ultrafilters with nominal molecular weight cutoff (NMWC) values of 5000 or less can be used to concentrate and desalt phosphorothioates. Alternatively, gel-permeation media (e.g., Sephadex G-25, as in Pharmacia NAP desalting columns) can be used to exchange salt from the purification step with a volatile buffer (e.g., 10 m*M* ammonium acetate) that is removed by vacuum evaporation (SpeedVac or lyophilizer). This desalting approach typically results in >80% recovery.

[16] B. J. Bergot and W. Egan, *J. Chromatogr.* **599,** 35 (1992).
[17] B. J. Bergot and G. Zon, *Ann. N.Y. Acad. Sci.* **30,** 310 (1991).

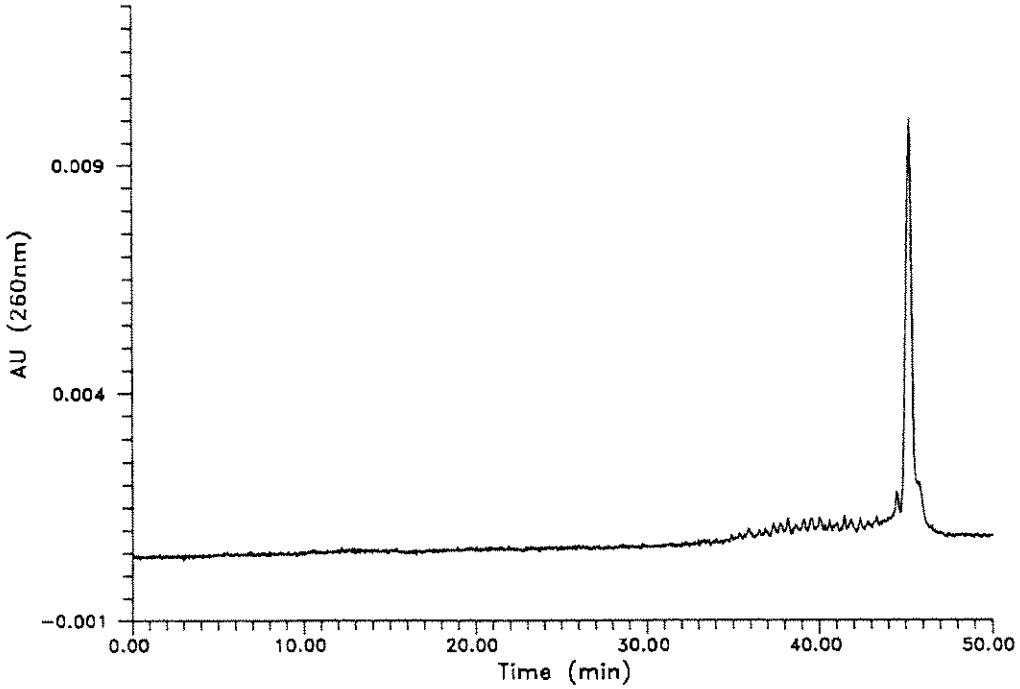

FIG. 7. Analysis of "purified" synthetic oligonucleotides. Gel-filled capillaries are also useful for determining the purity of synthetic oligonucleotides. This RP-HPLC-purified oligonucleotide was found to be only 70% pure, with the presence of at least 20 failure sequences detected. Separation conditions: capillary length, 50 cm total, 45 cm effective; capillary inner diameter, 75 μm; gel, polyacrylamide; injection, 20 sec at 0.5 kV, cathode electroinjection; separation field strength, 225 V/cm, 5 μA, 55 mW; capillary cooling, forced air cooling, ambient temperature; detection, 260-nm absorbance; sample, RP-HPLC-purified 32-mer, proprietary sequence.

High-Performance Liquid Chromatography versus Capillary Electrophoresis

For the purposes of analytical high-resolution separation, one can efficiently use gel-filled capillaries in 7.5 *M* urea and capillary electrophoresis equipment (e.g., Dionex CES1) to separate single-stranded nucleic acids. Figure 7 shows an example from the unpublished work of R. McCormick, H. Kumar, and N. Avdalovic (1993). It shows an HPLC (trityl on)-purified 32-mer profile run on a gel-filled capillary (6T/3.33C). This purified oligomer is found to be only 70% pure with at least 20 failure sequences detected.

The choice of the system (HPLC versus gel-filled capillary electrophoresis) to be used for the analysis of single-stranded DNA samples will depend on many factors, not the least of which is the availability of stable, gel-filled capillaries giving reproducible migration times. For oligonucleotides

of 8–30 bases, HPLC has been characterized as superior, in speed of analysis, and practically equivalent in resolution, compared to gel-filled capillary electrophoresis.[1]

Double-Stranded DNA

Restriction Fragments and Polymerase Chain Reaction Products

Restriction enzymes are nucleases that reproducibly cut both strands of duplex DNA at sequences that are unique for each restriction enzyme. One use for these enzymes is to generate specific dsDNA sequences for insertion into and excision from plasmid or phage vectors during cloning. The size of cloned sequences varies widely and depends on the vectors used. Most of the plasmid and phage vectors used for cloning are substantially larger than the sequences inserted into them. Restriction nucleases recognize sequences from 4 to 14 bases long, and their activity results in cuts with or without overlapping (complementary) ends.

The polymerase chain reaction (PCR) process allows prodigious amplification of specific DNA sequences in a relatively short time. This is accomplished by polymerase-mediated extension of oligonucleotide primers complementary to, and bracketing, the desired sequence. With each cycle of extension, the concentration of the target sequence is doubled, until the primer, polymerase, or nucleoside triphosphate (NTP) concentration becomes limiting. The resulting dsDNA sequences are usually in the 50- to 2000-bp range and do not harbor "sticky" ends. For subsequent use, these sequences may require purification from the reaction matrix, which often contains high concentrations of glycerol, protein, and either oligonucleotide primers or NTPs.

Polyacrylamide and agarose gel electrophoresis are proven methods for separating and recovering such sequences, but they are labor-intensive techniques. Hence, a rapid method for automatic and unattended purification/recovery of restriction fragments and PCR products is valuable. The method should resolve nanogram to tens of microgram quantities of dsDNA in the 50- to 2000-bp size range, be relatively insensitive to sequence and base composition, and retain components primarily according to length. The method should also be fairly rapid, allowing high throughput, but be inexpensive enough to accommodate only occasional use.

The eluent system employed for the separation of ssDNA species can also be used to resolve many restriction DNA fragments. Gradient method 7 (see the Appendix) allows the separation of linear DNA species in the range of 72 to more than 1300 bp when restriction nuclease *Hae*III is used

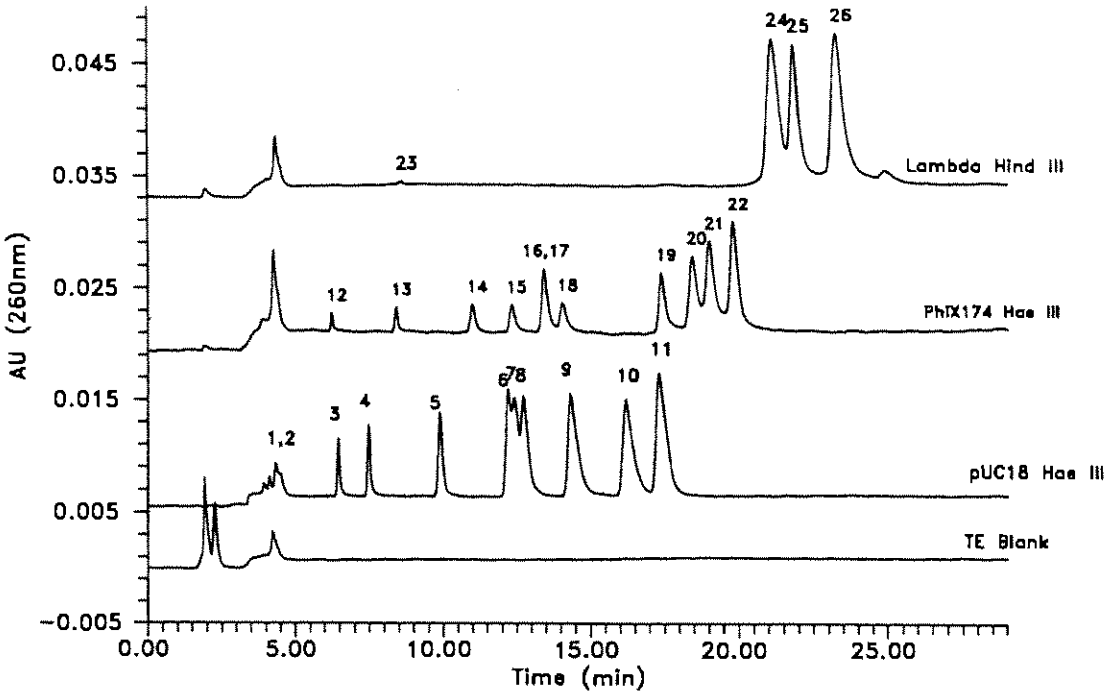

FIG. 8. Separation of DNA restriction fragments by $NaClO_4$ elution with a NucleoPac PA100 column. The bottom trace is a blank to show "system peaks." The middle traces show the separation of blunt-ended DNA fragments generated by treatment of pUC18 (lower middle trace) and ϕX174 (upper middle trace) with *Hae*III. Peaks 1–11 from pUC18: 1 (11 bp), 2 (18 bp), 3 (80 bp), 4 (102 bp), 5 (174 bp), 6 (267 bp), 7 (257 bp), 8 (298 bp), 9 (434 bp), 10 (458 bp), 11 (587 bp). Peaks 12–22 from ϕX 174: 12 (72 bp), 13 (118 bp), 14 (194 bp), 15 (234 bp), 16 and 17 (271 and 281 bp), 18 (310 bp), 19 (603 bp), 20 (872 bp), 21 (1078 bp), 22 (1353 bp). Peaks 23–26 from phage λ: 23 (125 bp), 24 (564 + 2027 + 2322 + 4361 + 6557 bp), 25 (9416 bp), 26 (23100 bp). Top trace: Fragments from the digestion of λ DNA by *Hin*dIII. This nuclease generates "sticky ends." The 564-, 2027-, 2322-, 4361-, and 6557-bp segments coelute if not denatured prior to injection (peak 24).

(Fig. 8). This nuclease cleaves GGCC sequences, leaving blunt ends. The restriction nuclease cleavage of λ DNA with *Hin*dIII (A ↓ AGCTT) results in fragments with "sticky" ends and lengths from 125 to 23,100 bp. The top trace in Fig. 8 shows that the sticky ends will limit resolution of some fragments. In this trace, peaks at 125, 9416, and 23,100 bp are observed; however, five fragments (564, 2027, 2322, 4361, and 6557 bp) are observed to coelute. This can be minimized by exposing the solution to elevated temperatures (e.g., 65° for 10 min) and quick cooling just prior to injection. The heat dissociates the 4-base hydrogen bonding, and quick cooling helps prevent reannealing before analysis. This task can be automated using the heating function of the system autosampler.

The products of PCR reactions vary considerably in base composition. The sensitivity of anion-exchange chromatography to this parameter pre-

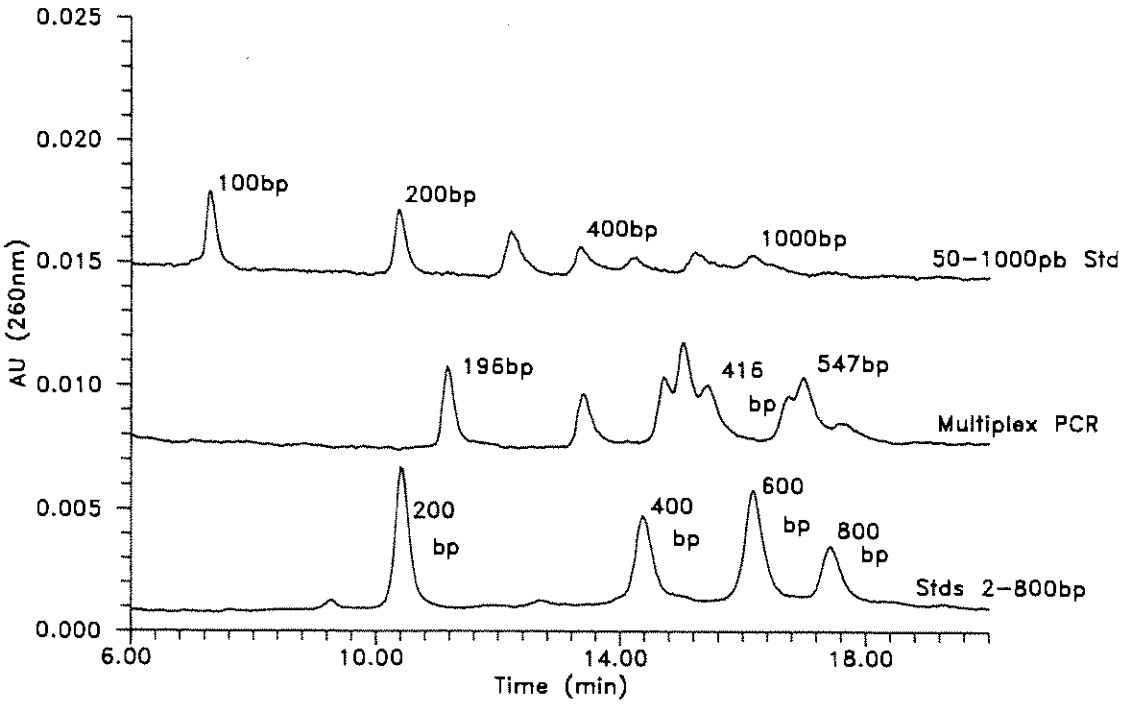

FIG. 9. Separation of the double-stranded products of PCR amplification uses the conditions in the Appendix, method 7. Two different standards (50–1000 and 200–800 bp) are shown above and below the trace from a multiplex PCR reaction employing nine sets of primers for Duchenne muscular dystrophy exons. Partial separation of eight products is demonstrated. The larger peak at ~15 min presumably contains two products.

cludes the direct assessment of product length by elution position. However, PCR products differing by more than ~10% in length are often resolved by chromatography under the conditions described for restriction fragments. Figure 9 shows a simultaneous analysis for nine different exons found in Duchenne muscular dystrophy and demonstrates resolution for eight of the nine amplified sequences known to be present in this sample.

Capillary gel electrophoresis is also a powerful tool for separating double-stranded DNA fragments of limited length. Figure 10 shows high-resolution separations performed on a low-percentage polyacrylamide gel-filled capillary (top trace), and in a sieving buffer (bottom trace). The separation of small DNA fragments up to 1 kbp in length can be resolved in gel-filled capillaries by using low-field strength. Although the separation was achieved for most of the fragments at the baseline level, more than 60 min is required to accomplish this task. The same fragments could be analyzed in half that

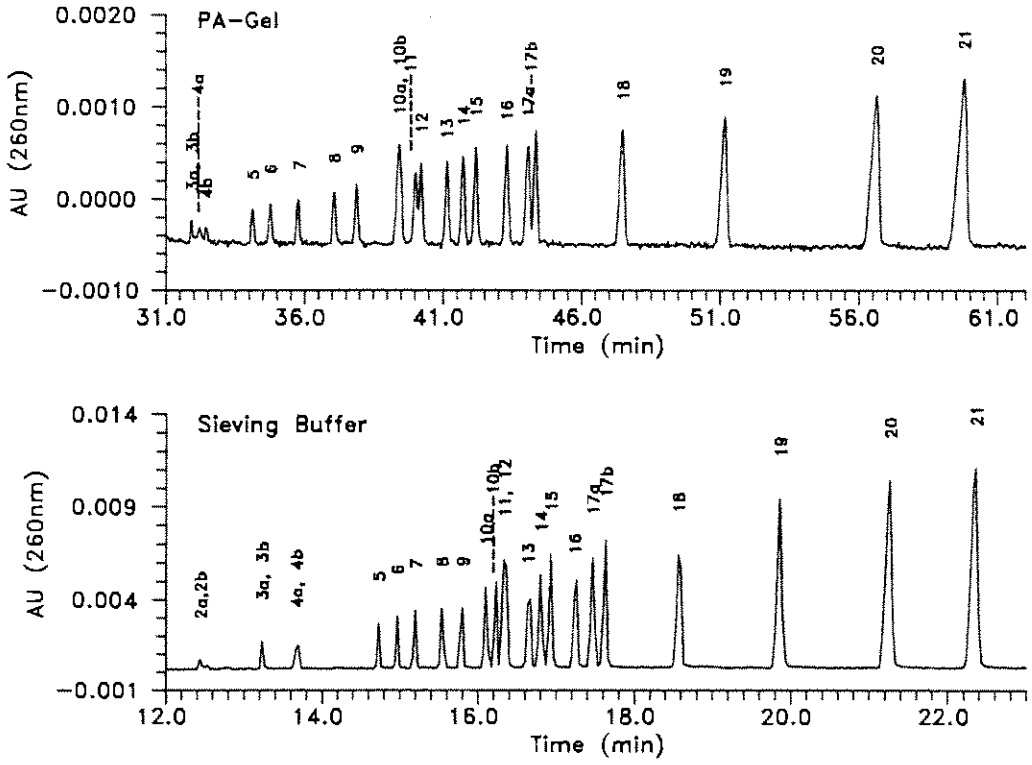

FIG. 10. Separation of low base pair DNA fragments using low-percentage acrylamide gel-filled and sieving buffer-filled capillaries. Small DNA fragments up to 1 kbp long can be separated in gel-filled capillaries at low field strengths, but analysis time is long relative to the same separation on sieving buffer capillary. The same fragments can be readily separated at higher field strengths, resulting in short analysis times using sieving buffers, because they tolerate higher field strengths without significant loss of resolution for small DNA fragments. Separation conditions for gel-filled capillary: capillary length, 45 cm total, 40 cm effective; capillary inner diameter, 75 μm; gel, polyacrylamide gel; injection, 12 sec at 0.7 kV, cathode electroinjection; separation field strength, 125 V/cm, 2 μA, 50 mW; capillary cooling, forced air cooling, ambient; detection, 260-nm absorbance; Separation conditions for sieving buffer-filled capillary: capillary length, 50 cm total, 45 cm effective; capillary inner diameter, 100 μm; sieving buffer, NucleoPhor SB1.5 kB (Dionex); running buffer, same as sieving buffer; injection, 10 sec at 1 kV, cathode electroinjection; separation field strength, 250 V/cm, 21 μA, 260 mW; capillary cooling, forced air cooling, ambient temperature; detection, 260-nm absorbance; sample, *Msp*I digest of pBR322 DNA, 500 μg/ml in TE (gel filled) or 250 μg/ml in TE (sieving buffer filled). Peaks (putative length in base pairs): 2a,b (15 + 15), 3a,b (26 + 26), 4a,b (34 + 34), 5 (67), 6 (76), 7 (90), 8 (110), 9 (123), 10a,b (147 + 147), 11 (160), 12 (169), 13 (180), 14 (190), 15 (205), 16 (217), 17a,b (238 + 238), 18 (307), 19 (404), 20 (527), 21 (622).

time using the Dionex sieving buffer. Thus, sieving buffers appear to be the better choice for dsDNA separations, and both HPLC and capillary electrophoresis are justified for assessing the purity of various single- and double-stranded DNA molecules. Perhaps the major step in determining

which technique to apply in a given situation will depend on whether or not one wants to collect the separated fractions. Other than that, these two analytical techniques are more or less equivalent.

Plasmids

Reasons for purifying plasmids are (1) for subsequent use as cloning vectors, (2) as substrates for enzyme activity (e.g., topoisomerases), (3) as templates for transcription studies, (4) to determine plasmid copy number, (5) for DNA biophysical studies (e.g., superhelical organization), (6) for hybridization analysis, and (7) for sequencing. Plasmids are usually obtained from bacterial strains by transformation, followed by growth in media allowing selection of some characteristic conferred by the plasmid (e.g., drug resistance). Plasmids currently employed are small (0.9 to ~5 kbp) to accommodate inserted sequences of a large size or number before becoming too large for efficient transformation (limiting at ~15 kbp). Most also contain readily selectable markers (e.g., drug resistance), and well-defined recognition sites for several or many restriction nucleases. Many have had the sequences controlling (restricting) the copy number inactivated, so numerous copies are present under normal growth conditions. This approach permits recovery of more than a microgram of plasmid DNA in each milliliter of bacterial culture.

Preparation of "Cleared Lysates"

The starting material used for plasmid purification is similar for many different methods. To understand the complexity of the matrix obtained before the final purification is initiated, we briefly describe a widely used method for crude plasmid preparation.

Escherichia coli-containing plasmids are incubated overnight at 37° in 10 ml of Luria–Bertani (LB) growth medium.[18] This culture is added to 990 ml of fresh, sterile LB in a 5-liter Erlenmeyer flask, with the appropriate antibiotic to select for plasmid-containing cells [e.g., chloramphenicol (170 μg/ml)]. The culture is grown to an A_{600} of 0.4 by orbital mixing (300 cycles/min) at 37°. For plasmid amplification, or to limit chromosomal DNA synthesis and hence bacterial growth, without limiting plasmid replication, 5 ml of chloramphenicol (34 mg/ml in ethanol, stored at −20°) is added to the culture, and incubation is extended for 12 to 16 hr. Cells are harvested

[18] J. Sambrook, E. F. Fritsch, and T. Maniatis, "*Molecular Cloning: A Laboratory Manual*," 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

by centrifugation at 6300 *g* for 15 min at 4°. The supernatant is discarded and the pellet resuspended in 10 ml of STE buffer (0.1 *M* NaCl, 10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0), which helps minimize release of cell wall components during later lysis. The centrifugal step is repeated (for 5 min here), and the pellet resuspended in 8 ml of ice-cold 50 m*M* Tris-HCl containing 10% sucrose, and transferred to a clean 30-ml centrifuge tube. Several methods for cell lysis can be used; here, we describe a modification of the method of Birnboim and Doly.[19]

Add 1 ml of lysozyme (10 mg/ml solution in 10 m*M* Tris-HCl, pH 8.0) to the resuspended culture, and incubate for 10 to 15 min. Add 2 ml of 0.5 *M* EDTA (pH 8.0) to chelate divalent cations and inhibit nucleases. The suspension is mixed by carefully inverting the tube several times, and is placed on ice for 10 min (vigorous mixing at this step will shear chromosomal DNA, rendering its separation from plasmid DNA difficult). Slowly add 2.75 ml of 4% (w/v) sodium dodecyl sulfate (SDS) in 0.8 *M* NaOH. Mix this solution with a glass rod to disperse the SDS completely. Add 6.9 ml of 3 *M* potassium acetate in 2 *M* acetic acid, and mix with the glass rod as before. Incubate at 4° for 5 min. A 5-min centrifugation at 12,000 *g* at 4° pellets the cell debris with the potassium precipitate of chromosomal DNA and protein. Transfer the supernatant to a fresh 50-ml centrifuge tube, add 20 ml of phenol–chloroform (1:1), and mix gently but thoroughly. Pellet the precipitated protein and transfer the supernatant to two fresh 50-ml centrifuge tubes (20 ml each). Add 12 ml of 2-propanol to each tube and let stand for 5 min to precipitate the nucleic acids. Harvest the precipitate by centrifugation at 12,000 *g* at 4° for 15 min. Carefully remove all residual solution from the sides of the tubes, and rinse with 1 ml of ice-cold 70% (v/v) ethanol. Dry in air for 15 min.

The precipitate now contains supercoiled and nicked plasmid DNA, cellular RNA, and possibly some chromosomal DNA. Major contamination by high molecular weight RNA is not uncommon and can dramatically affect the resulting plasmid purification by HPLC. To eliminate coelution of RNA with plasmid DNA, treat the "cleared" lysate with DNase-free RNase. This is prepared by boiling RNase for 15 min.[15] Treatment of the cleared lysate with this preparation for 1 to 2 hr minimizes the length of residual RNA to sizes that are readily separated from plasmid DNA in the extract (Fig. 11). Resolution of supercoiled from linear and relaxed forms of this plasmid (~7.2 kbp) was confirmed by agarose gel electrophoresis. The elution of supercoiled after the relaxed and linear forms has been

[19] H. C. Birnboim and J. Doly, *Nucleic Acids Res.* **7**, 1513 (1979).

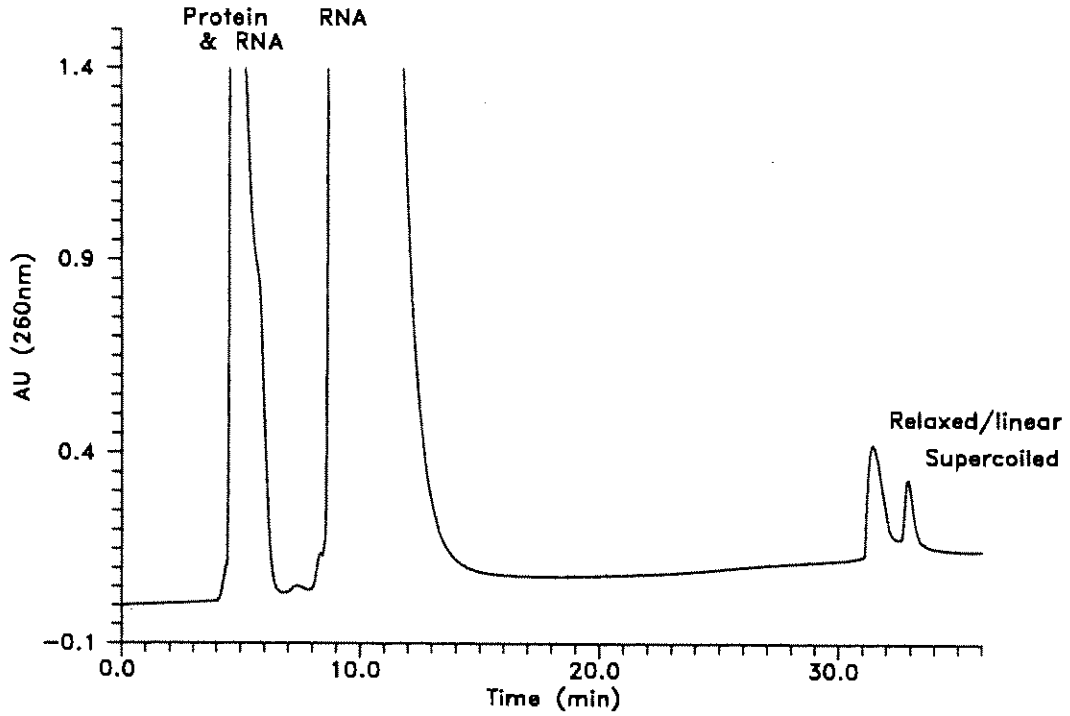

FIG. 11. Purification of a pBR322 derivative harboring an IgG insert. Gradient method 8 of the Appendix was used to separate the protein/RNA, RNA, and relaxed and supercoiled plasmids using a modified flow rate of 0.4 ml/min. This cleared lysate preparation exhibits a substantial percentage of nicked or linear plasmid. Agarose gel electrophoresis of the plasmid peaks reveals that the collected peaks remain as indicated after collection.

demonstrated for plasmids pBR322 (Fig. 12) and pUC19 (data not shown). These plasmids are 4.36 and 2.69 kbp, respectively.

The most widely accepted method for plasmid DNA isolation and purification is centrifugation in a cesium chloride gradient. Sambrook *et al.*[18] reported that it is necessary to centrifuge between 36 and 72 hr to obtain a reasonable separation between the supercoiled and linear DNA. However, major advances have been made in this field, and we briefly review the procedures available today to accomplish this task.

Isolation of Supercoiled Plasmid DNA by Isopycnic Ultracentrifugation. An excellent theoretical treatment of this subject can be found in articles by Minton[20] and Marque.[21] The base compositions, and hence densities, of plasmid and chromosomal DNAs are normally so similar that they cannot be separated by virtue of a density difference. However, it is possible to produce such a difference using the fluorescent dye ethidium bromide, which complexes with DNA by intercalation between base pairs, thus reduc-

[20] A. P. Minton, *Biophys. Chem.* **42**, 13 (1992).
[21] J. Marque, *Biophys. Chem.* **42**, 23 (1992).

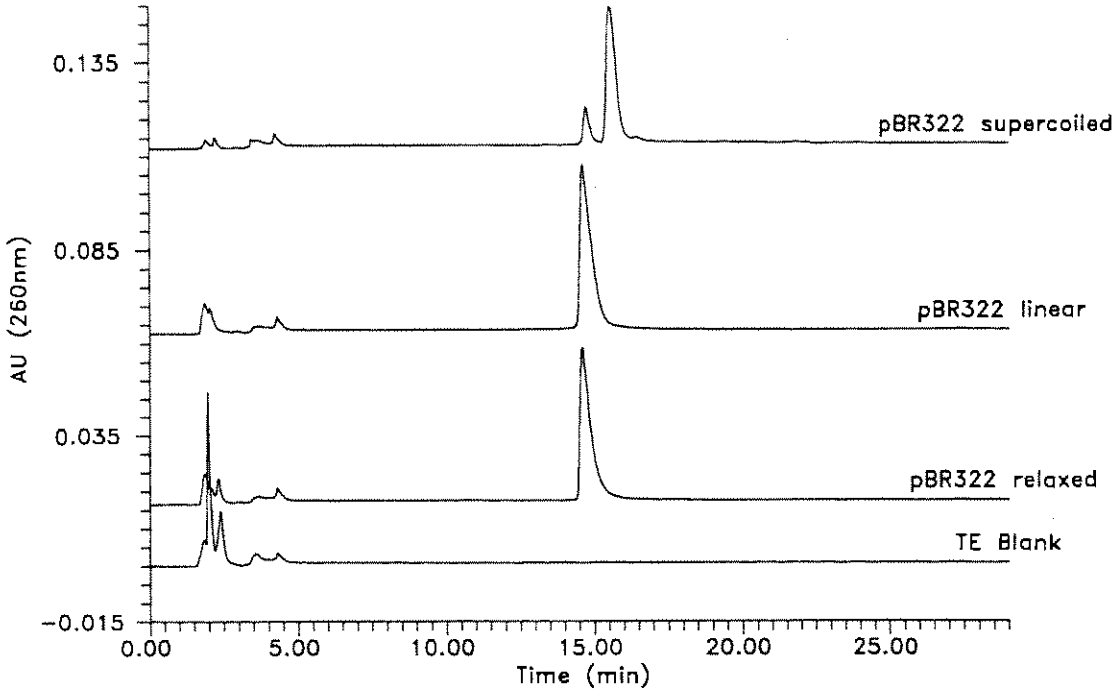

FIG. 12. Separation of supercoiled plasmid DNA (form I) from linear (form III) and nicked or relaxed (form II) DNA. A commercial preparation of supercoiled pBR322 having ~15% form II is chromatographed using the conditions described in the Appendix, gradient method 8 at 1.0 ml/min. Linear and relaxed forms were generated by treating the supercoiled stock with *Bam*HI restriction nuclease (one cleavage site on pBR322) or topoisomerase I. Agarose gels of each peak collected from these chromatograms reveal that the later eluting peak in the commercial preparation remains as form I (supercoiled) after collection, and the earlier eluting component remains as form II (relaxed).

ing its buoyant density. Intercalation of dye can occur only if the DNA double helix unwinds slightly, a process that results in strain in supercoiled plasmid DNA (SC or form I DNA), but which is unhindered in linear (form III) or nicked (form II) molecules. Consequently, in saturation concentrations of ethidium bromide, SC DNA will bind less dye per unit length, and will have a greater buoyant density than linear plasmid or chromosomal DNA as well as form II DNA of the same base composition. RNA molecules have considerably different buoyant densities and will, in principle, be easy to separate from DNA of SC, nicked, or linear DNA.

Adjustment of the CsCl concentration to 1.55 g/ml and ethidium bromide concentration to 0.6 g/ml in a plasmid preparation, followed by centrifugation to equilibrium in an appropriate rotor/ultracentrifuge (Table I), will result in the migration of SC and nicked DNA, chromosomal and linear plasmid DNA, and RNA to distinct positions (densities) in the ultracentrifuge tube. Supercoiled DNA will form a band below chromosomal DNA,

TABLE I
RECOMMENDED MINIMUM PLASMID SEPARATION TIMES[a]

Rotor	Tube size (ml)	Speed (rpm)	Time (hr)
Type 80 Ti	13.5	48,000	24
	6.3	59,000	7
	4.2	65,000	5
Type 75 Ti	13.5	49,000	24
	6.3	61,000	7
	4.2	71,000	5
Type 70.1 TI	13.5	49,000	24
	6.3	61,000	7
	4.2	70,000	5
VTi80	5.1	80,000	3
VTi65	5.1	65,000	4
VTi65.1	13.5	65,000	4.5
	6.3	65,000	4.5
VTi65.2	5.1	65,000	4

[a] Reprinted with permission from S. E. Little and D. K. McRorie, Rapid Separation of Plasmid DNA in Preparative Ultracentrifuge Rotors. Applications Data, DS-734A. Beckman Instruments, Inc., Fullerton, CA, 1989.

and RNA will migrate to near the bottom of the tube. Note that polysaccharides derived from cell wall components may also be present and will not bind ethidium bromide.

Anion-Exchange Chromatography. Supercoiled (form I) plasmid DNA is separated from proteins, RNA, and linear (form III) and nicked (form II) plasmid DNA using gradient method 8 (Appendix). Fractions eluting from the NucleoPac column were collected, concentrated, and desalted by ethanol precipitation, and analyzed by agarose gels in the presence of ethidium bromide. A cleared lysate, containing a pBR322-derived plasmid harboring an immunoglobulin G (IgG) insert, exhibited a large fraction (>99% of A_{260}-absorbing material) of RNA that eluted between 4 and 14 min. Two late-eluting peaks were identified as linear (eluting at ~31 min using a 0.4-ml/min flow rate; Fig. 11) and supercoiled (eluting at ~33 min from the same run). Evaluation of the shearing of pBR322 and pUC19 during chromatography at 0.4 and 1.0 ml/min by agarose gel electrophoresis in differing concentrations of ethidium bromide revealed no evidence of shearing at the higher flows (all tubing from the injector to fraction collector had an inner diameter of 0.020 in.).

Other New Microparticulate Columns

Two other microparticulate media have been described.[1] Oefner *et al.* packed columns with nonporous fused silica [2-μm particle diameter (d_p), polyethyleneimine (PEI) coated] or poly(styrene-divinylbensene (PS-DVB) grafted with poly(vinyl alcohol) (2.3-μm d_p, 0.1% PVA) and used these for anion-exchange and reversed-phase chromatography of nucleic acids (Fig. 13). These columns were compared with capillary electrophoresis, using the ability to resolve oligonucleotides and restriction fragments as benchmarks. For dsDNA, CE in the presence of hydroxyethylcellulose and ethidium bromide were judged to provide better resolution than the HPLC columns.

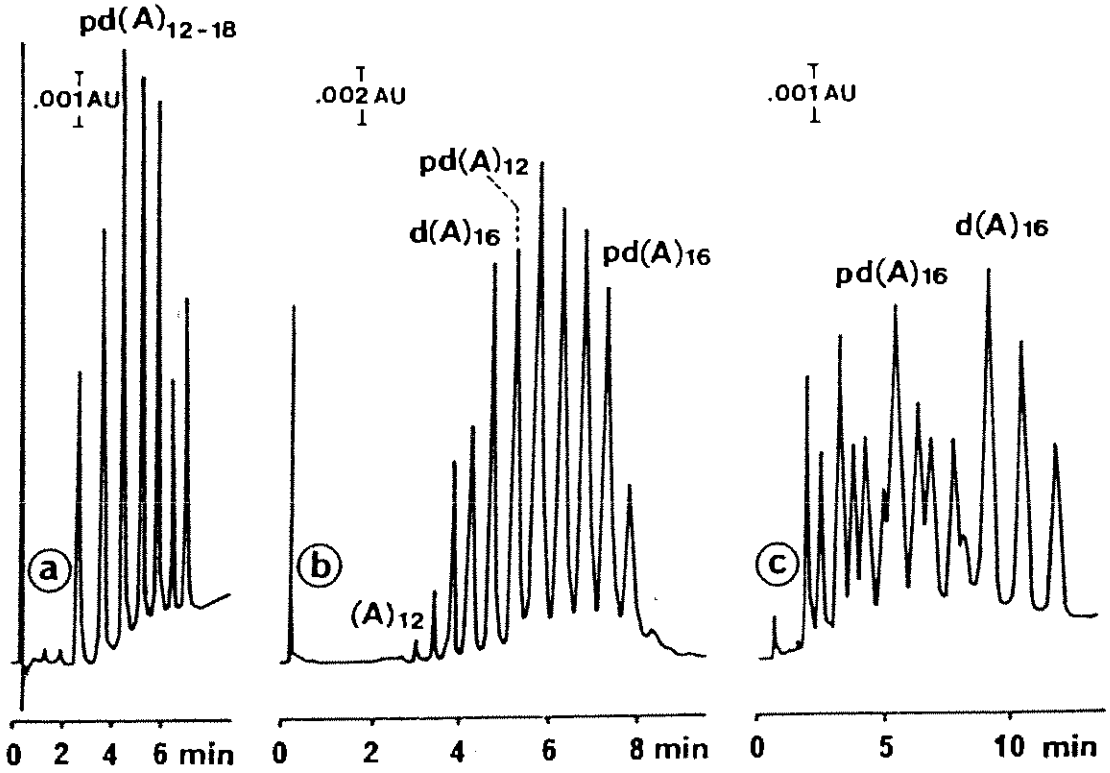

FIG. 13. HPLC separations of oligodeoxyadenylic acids. (a) Column Progel-TSK DEAE-NPR, 2.5 μm, 35 × 4.6-mm i.d.; buffer, 0.020 *M* Tris-HCl (pH 7.5); gradient, 0.15–0.5 *M* $(NH_4)_2SO_4$ in 15 min; flow rate, 1 ml/min; sample, 0.75 μg of p[d$(A)_{12-18}$]. (b) Column, PEI-silica, 2 μm, 30 × 4.6-mm i.d.; buffer, 0.05 *M* phosphate (pH 5.9)–30% (v/v) methanol; gradient, 0–0.5 *M* $(NH4)_2SO_4$ in 10 min; flow rate, 2 ml/min; sample, 1.75 μg of p[d$(A)_{12-18}$]. (c) Column, PS-DVB-PVA, 2.3 μm, 50 × 4.6-mm i.d.; buffer, 0.1 *M* TEAA (pH 7.0); gradient, 12.5–20% acetonitrile in 20 min; flow rate, 1 ml/min; sample, 0.5 μg of p[d$(A)_{12-18}$] and 0.083 μg of p[d$(A)_{16}$]. All chromatograms were obtained by means of UV detection at 254 nm at room temperature. [Reprinted by permission from P. J. Oefner *et al.*, J. Chromatogr. **625,** 334 (1992).]

Appendix: Gradient Files for Separation of Nucleic Acids Using Perchlorate Eluents

Program 1 for "Normal" Length (8- to 30-mer) Oligonucleotides at pH 8

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.50	88	0	10	2	5	Load at 7.5 m*M* ClO_4^-
20.0	1.50	52	0	10	38	3	7.5–143 m*M*/20 min convex-3
21.5	1.50	0	0	0	100	9	143–375 m*M* concave-9
25.5	1.50	0	0	0	100	5	Hold 4 min (flush column)[b]
25.6	1.50	88	0	10	2	5	Regenerate with 7.5 m*M* ClO_4^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 36 min.

[b] When different oligonucleotides are sequentially chromatographed, and carryover from one injection to another must be scrupulously avoided, several consecutive 2-ml manual injections of 0.2 *M* HCl will convert residual oligonucleotide to essentially uncharged species that are eluted by the chloride anion. When this is done, a 5-min regeneration of the stationary phase to the ClO_4 form is accomplished with 100% E4. When automated cleaning is required, a fifth eluent container may be plumbed directly into the chromatographic system under control of an electrical or pneumatic valve that converts eluent 4 from 0.375 *M* $NaClO_4$ to 0.2 *M* HCl. The Dionex system supports both electrical and pneumatic control. This allows washing and regeneration steps to be added to the method. With the automated column cleaning step, the autosampler cycle time is changed to 40 min.

Program 2 for "Normal" Length (8- to 30-mer) Oligonucleotides at pH 12.4

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.50	86	12	0	2	5	Load at 7.5 m*M* ClO_4^-
20.0	1.50	43	12	0	45	4	7.5–169 m*M*/20 min convex-5
21.5	1.50	0	0	0	100	9	169–375 m*M* concave-9
25.5	1.50	0	0	0	100	5	Hold 5 min (flush column)
25.6	1.50	86	12	0	2	5	Regenerate with 25 m*M* OH^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 36 min.

Program 3 for "Extended" Length (30- to 70-mer) Oligonucleotides at pH 8

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.50	88	0	10	2	5	Load at 7.5 m*M* ClO_4^-
0.1	1.50	86	0	10	4	5	Step to 15 m*M* ClO_4^-
20.1	1.50	47	0	10	43	3	15–161 m*M*/20 min convex-3
21.5	1.50	0	0	0	100	9	161–375 m*M* concave-9
25.5	1.50	0	0	0	100	5	Hold 5 min (flush column)
25.6	1.50	88	0	10	2	5	Regenerate with 25 m*M* OH^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 36 min.

Program 4 for "Extended" Length (30- to 70-mer) Oligonucleotides at pH 12.4

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.50	86	12	0	2	5	Load at 7.5 m*M* ClO_4^-
20.0	1.50	36	12	0	52	3	7.5–195 m*M*/20 min convex-3
21.5	1.50	0	0	0	100	9	195–375 m*M* concave-9
25.5	1.50	0	0	0	100	5	Hold 5 min (flush column)
25.6	1.50	86	12	0	2	5	Regenerate with 25 m*M* OH^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 36 min.

Program 5 for Phosphorothioates (Antisense Oligonucleotides) at pH 8

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.50	88	0	10	2	5	Load at 7.5 m*M* ClO_4^-
0.1	1.50	75	0	10	15	5	Step to 56 m*M* ClO_4^-
20.1	1.50	4	0	10	86	4	56–323 m*M*/20 min convex-4
21.5	1.50	0	0	0	100	7	323–375 m*M* concave-7
25.5	1.50	0	0	0	100	5	Hold 4 min (flush column)
25.6	1.50	88	0	10	2	5	Regenerate with 7.5 m*M* ClO_4^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 36 min.

Program 6 for Phosphorothioates (Antisense Oligonucleotides) at pH 12.4

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.50	86	12	0	2	5	Load at 7.5 m*M* ClO_4^-
0.1	1.50	73	12	0	15	5	Step to 56 m*M* ClO_4^-
20.1	1.50	0	12	0	88	4	56–330 m*M*/20 min convex-4
21.5	1.50	0	0	0	100	7	330–375 m*M* concave-7
25.5	1.50	0	0	0	100	5	Hold 5 min (flush column)
25.6	1.50	86	12	0	2	5	Regenerate with 25 m*M* OH^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 36 min.

Program 7 for Linear Double-Stranded DNA (RFs and PCR Products, pH 8)

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.00	88	0	10	2	5	Load at 7.5 m*M* ClO_4^-
0.1	1.00	47	0	10	43	5	Step to 161 m*M* ClO_4^-
26.1	1.00	32	0	10	58	3	161–218 m*M*/26 min convex-3
27.0	1.00	0	0	0	100	9	323–375 m*M* concave-9
32.0	1.00	0	0	0	100	5	Hold 5 min (flush column)
33.0	1.00	88	0	10	2	5	Regenerate with 7.5 m*M* ClO_4^-

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 45 min.

Program 8 for "Supercoiled" versus Nicked/Linear DNA (pH 8)

Time (min)	Flow (ml/min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve number	Comments[a]
0.0	1.00	88	0	10	2	5	Load at 7.5 m*M* ClO_4^-
0.1	1.00	47	0	10	43	5	Step to 161 m*M* ClO_4^-
26.1	1.00	30	0	10	60	3	161–225 m*M*/26 min convex-3
27.0	1.00	0	0	0	100	9	225–375 m*M* concave-9
32.0	1.00	0	0	0	100	5	Hold 5 min (flush column)
33.0	1.00	88	0	10	2	5	Regenerate with 7.5 m*M* ClO_4

[a] Regeneration and reequilibration of the column are completed by setting the cycle time of the autosampler to 45 min.

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