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Analysis and Purification of Synthetic Oligonucleotides, DNA Restriction Fragments, PCR Products and Plasmids By HPLC

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Introduction

The purification and analysis of synthetic oligonucleotides, DNA restriction fragments, polymerase chain reaction (PCR*) products and plasmids are of growing importance in both academic and industrial settings. Traditionally, techniques such as gel electrophoresis and ultracentrifugation have been used for the isolation and analysis of these molecules. Unfortunately, these techniques are time consuming (i.e. hours to days) and labor intensive. In addition, product recoveries are often low and may contain interfering substances (e.g. cesium chloride, ethidium bromide or polyacrylamide) derived from the separation matrix.

High performance liquid chromatography (HPLC) provides a single step alternative to gel electrophoresis and ultracentrigugation for this application and has been proven effective in the analysis and purification of a variety of biomolecules including proteins, peptides and amino acids. Furthermore, because the separation and detection techniques are nondestructive, direct isolation of biologically active material is easily accomplished. This poster will describe the principles involved with this chromatographic technique and will detail its application to the purification and analysis of synthetic oligonucleotides, DNA restriction fragments, PCR products and plasmids.

* See U.S. Pate to No. 4683202 to Cetus Corporation

Synthesis of Oligonucleotides

The advent of automated, solid-phase DNA synthesizers of improved coupling efficiency and multi-strand synthesis capability has eased the previously time-consuming and complicated process of constructing biologically active synthetic oligonucleotides. In brief, synthesis involves the systematic stepwise addition of specific nucleotides [protected at their 5' hydroxy end with a dimethoxytrityl group (DMT)] to a solid phase support containing the previously attached nucleotides. Yet despite numerous technological advancements, coupling efficiency in each synthesis cycle remains below 100% resulting in contamination of the desired synthetic oligomer product by sequences of shorter length. The amount of product contaminants is proportional to the length of the synthesis product as shown in Figure 1.

Figure 1: Synthetic Oligonucleotide Length Compared to Theoretical Yield at Various Coupling Efficiencies





Rapid Oligonucleotide Purification Using Disposable Columns

Purification of full length oligonucleotide product from failure sequences can be performed within 30 minutes on an Oligo-PakTM column and syringe. Between 10 and 50 O.D. 260nm units (1 O.D. 260nm unit = 33 μ g oligonucleotide) of DMT-protected crude material can be purified in a single run. In addition, on-column product detritylation can also be performed on this acid and alkali stable chemistry. The final product is of suitable purity for use as primers for sequencing or PCR or as hybridization probes for southern blot analysis.

These single use, disposable columns separate via the principle of reverse phase chromatography. Separations are based upon the relative hydrophobicities of the different species present in the mixture. The least hydrophobic molecules elute prior to the more hydrophobic species. For this method, the oligomer must be produced such that the 5' DMT group is left on the oligomer upon completion of the synthesis and removal from the synthesis support. The DMT group renders the product more hydrophobic, allowing it to be separated from the failure sequences which have less hydropholes, 5' hydroxy groups present. Since these failure sequences are less retained on the cartridge they can be easily removed from the DMT protected product. Figure 2 compares a reversed-phase HPLC separation from a 20mer synthesis (DMT on) before and after purification on an Oligo-Pak Column showing the effectiveness of this technique.



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Reverse Phase HPLC Purification of Synthetic Oligonucleotides

Reverse phase HPLC of DMT-protected synthetic oligonucleotides operates on the same principle as that employed using Oligo-Pak columns. However, the increased resolving power obtained with HPLC gradient separations can also be effectively used to separate smaller DMT containing sequences which can arise via product depurination or incomplete end-capping from the DMTprotected full length material.

Quantities up to 100 O.D. 260nm units of DMT-protected reaction mixture can be chromatographed in a single 40 minute run using the high-resolution Delta-Pak™ C18, 300Å chemistry (Figure 3). For purification of multimilligram quantities, larger column configurations are available and have been successfully used to purity up to 3000 O.D. 260nm units of a phosphorothioated 21mer using a single 25mm x 100mm PrepPak™ cartridge column (Figure 4).

Figure 3: Reverse Phase HPLC of Tritylated 50mer



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Figure 7: Autoradiogram of Fractions Collected from Gen-Pak FAX Purification of Detritylated 20mer



Analysis and Purification of Restriction Fragments by Anion Exchange HPLC

High-resolution, anion exchange HPLC on the Gen-Pak FAX column is well for the analysis and purification of restriction fragments. Using a simple gradient of increasing salt concentration, DNA fragments elute from this chemistry in order of increasing negative charge which is proportional to increasing DNA length. The separation of a <u>Hae</u> III digest of PhiX 174 DNA is shown in Figure 8. As noted, the separation is monitored at 260nm which provides a high level of detection sensitivity without requiring the use of toxic additives such as ethidium bromide. In addition, useful preparative applications involve the isolation of small insert DNA (e.g. <1000 base pairs) from the larger vector fragments (e.g. >3000 base pairs) or in purifying various sized restriction fragments less than 1000 base pairs in length.

Figure 8: Separation of DNA Restriction Fragments on the Gen-Pak FAX Anion Exchange HPLC Column



Qualitative and Quantitative Analysis of PCR Reaction Mixtures by Anion Exchange HPLC

The analysis of PCR amplification mixtures can also be rapidly obtained on the Gen-Pak FAX column as illustrated in Figure 9. As noted, ample component resolution is afforded by this method. As such, this rapid qualitative analysis can be used in the optimization of reaction conditions for PCR amplifications. In addition, quantitative analysis is also easily obtained. Based upon peak area determinations and calculations against an external standard, it is possible to determine the amount of a specific PCR product contained in an amplification mixture. In this example, it was determined that 323ngs and 6.6ngs of the desired 465 base pair PCR product were contained in amplification mixtures containing 3ng (Figure 9:Top) and 1 fg (Figure 9:Bottom) of Hepatitis B Virus (HBV) S-gene template.





Figure 10: Purification of a 344 bp PCR Product From Gen-Pak FAX Column Prior to Sequencing





Anion Exchange HPLC Purification of Plasmids

The purification of plasmids from bacterial proteins, chromosomal DNA and RNA is generally accomplished using cesium chloride gradient centrifugation. Typical run times range from 5 to 48 hours, depending upon rotor design, and must frequently be repeated to affect complete plasmid separation from the bacterial cell contaminants.

HPLC separations on the Waters Protein-Pak™DEAE 8HR chemistry are performed within 60 minutes using a simple NaCl gradient in a Tris/EDTA, pH 8.0 buffer. This chemistry is capable of purifying microgram to milligram quantities of plasmid DNA starting from an alkaline lysed, RNase and Proteinase K digested, polyethylene glycol precipitated sample. As indicated in the chromatogram of the purification of 2.4 mgs of a 2939 base pair plasmid (pRNH124), contaminating RNA is well resolved from the plasmid (Figure 12). Agarose gel electrophoresis of the HPLC purified plasmid confirmed that it was free of contaminating RNA. Furthermore, the isolated plasmid was biologically active as indicated by its ability to be cut with restriction enzymes as well as to transform competent <u>E. coli</u> cells.

Purification of PCR Products by Anion Exchange HPLC

The Gen-Pak FAX column is also ideally suited for PCR product purifications as exemplified by the isolation of a 344 base pair DNA fragment produced from an amplification of a human papilloma virus integrated within a human exocervical epithelial cell line (Figure 10). As indicated, the target DNA is easily isolated from contaminating dNTPs, primer, primer-dimers and non-specific DNA sequences using a simple NaCl gradient in a Tris/EDTA buffer system.

The quality of the HPLC purified 344 base pair PCR product was confirmed by performing standard dideoxynucleotide chain termination sequencing. A segment of the autoradiogram from the sequencing gel is shown in Figure 11 indicating excellent readability of the sequence with results comparable to those obtained using M13 subcloned DNA.







Figure 12: Autoradiogram of PCR Amplification Mixtures Where Various Concentrations of Hepatitis B Virus S Gene Templates Were Used





Autoradiogram courtesy of Dr. Peter Knudsen College of Physicians and Surgeons, Columbia University

Conclusions

The Waters Gen-Pak FAX column is useful in purifying target DNA from dNTPs, primers, primer-dimers, Taq polymerase enzyme and non-specific sequences enabling direct sequencing without subcloning.

On-Line, high-sensitivity detection and quantitation of PCR products at 260nm on the Gen-Pak FAX column is an attractive alternative to southern blot analysis for some applications.

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Anion Exchange HPLC for the Analysis of Oligonucleotides

The rapid analysis of synthetic oligonucleotides can be readily accomplished using high resolution, anion exchange HPLC of the Gen-Pak™ FAX column. Separations rely primarily upon the interaction of the negatively charged phosphate groups on the DNA backbone with the positively charged cations contained on the anion exchanger and are best performed on detritylated samples. Via the use of a gradient of increasing ionic strength, detritylated oligonucleotides elute in order of increasing chain length, often with N from N-1 resolution (Figure 5). On-line 260nm absorbance detection allows real time, high sensitivity analysis of oligonucleotide mixtures and eliminates the use of post-run visualization techniques as employed using gel electrophoresis. This rapid chromatographic technique is thus useful for monitoring the day to day efficiency performance of the DNA synthesizer used in the laboratory.

Figure 5: High Resolution Anion Exchange HPLC Analysis of Oligonucleotide Standards

Sample: 1 µg pd(A) 5, 10, 12 - 18mer Column: Gen-Pak™ FAX, 4.6mm x 100mm Eluent A: 25 mM Tris/Cl, 1 mM EDTA, pH 8.0/Acetonitrile, 90/10 Eluent B: 25 mM Tris/Cl, 1 mM EDTA, 1.0 M NaCl, pH 8.0/ Acetonitrile, 90/10 Gradient: 10-60%B in 30 min, linear, hold for 10 min Flow: 0.75 ml/min Temperature: 30°C



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Anion Exchange HPLC Purification of Oligonucleotides

Depurination as well as incomplete end-capping during the oligonucleotide synthesis reaction can result in hydrophobic DMT groups on partial length sequences. Although these DMT-containing sequences can be purified from full length tritylated product via reverse phase HPLC, anion exchange chromatography provides an alternative chromatographic technique. As indicated by the separation of a detritylated 20mer sample (Figure 6) with subsequent gel electrophoresis analysis of collected fractions (Figure 7), the Gen-Pak FAX column provides N from N-1 resolution from such synthesis mixtures. The maximum load capacity on this column is 20 O.D. 260nm units. Although larger masses of oligonucleotides can be purified in a single run on the Protein-Pak™ DEAE or Q HR anion exchange chemistries, less component resolution is obtained compared to chromatography on the Gen-Pak FAX column (Data not shown).



Figure 6: High Resolution Anion Exchange HPLC Purification of Detritylated 20mer

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Fig. 2. Chromatograms and corresponding UV spectra of (A) a necropsic blood sample from the first case and (B) a necropsic blood sample from the second case. Peaks: 1 = dextromoramide; 2 = norpropoxyphene; 3 = propoxyphene; 4 = methadone (I.S.).

Several drugs were also tested with the proposed method for possible interference (Table I). Among these compounds pethidine might inter-

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fere with norpropoxyphene, and desipramine and cyamemazine with propoxyphene. Therefore propoxyphene and/or its metabolite cannot be

TABLE III

CONCENTRATIONS OF DEXTROMORAMIDE, PROPOXYPHENE AND NORPROPOXYPHENE IN COMPARISON WITH THOSE OF PREVIOUS REPORTS (ng ml^-1)

Compound	Present study		Levels from previous reports	
	Case 1	Case 2		
Dextromoramide	194		Ther (P): 10–80 [7]	
			Tox (B): > 40 [5]	
			Post-mortem (B): 280-984 [2,5,7]	
Propoxyphene	614	4330	Ther (P): 50–570 [5]	
			Tox (B): > 2000 [5]	
			Post-mortem (B): 1000-6000 [5];	
			476–4284 or 1.4–12.6 μmol/l [9]	
Norpropoxyphene	1100	3800	Ther (P): 600–3000 [5]	
			Post-mortem (B): 1400–5900 [5]	

P = Plasma; B = blood; Ther = therapeutic; Tox = toxic.

quantitated in samples that also contain pethidine, desipramine or imipramine (a precursor of desipramine), or cyamemazine.

Application to necropsic whole-blood samples

Two cases of possible overdose were condidered. Only dextromoramide, propoxyphene and norpropoxyphene were determined by the described procedure. Nevertheless the possibly associated drugs encountered in these two cases and some of their metabolites were tested for possible interference (Table I). No interferences were observed.

Case 1. A 25-year-old man who had abused dextromoramide by intravenous injection was found dead in his home with syringes and empty phials of dextromoramide bitartrate (Palfium). He was previously treated with dextromoramide in association with propoxyphene plus acetaminophen (Diantalvic), codeine plus ethylmorphine (Neocodion), flunitrazepam (Rohypnol) and prazepam (Lysanxia). The results are presented in Table III and Fig. 2A^a. The dextromoramide

concentration in whole blood was found to be 194 ng ml^{-1} . It was about 2.5 times as great as the highest plasma therapeutic level, 10–80 ng ml⁻¹ [7], but lower than blood post-mortem range (280–984 ng ml⁻¹ [2,5,7]). The concentrations of propoxyphene (614 ng ml⁻¹) and norpropoxyphene (1100 ng ml⁻¹) were both in the therapeutic range. So this death could be attributed to the combined effect of dextromoramide and propoxyphene with possible intervention of other substances.

Case 2. An ex-abuser, 30 years old, was treated for addiction with propoxyphene (Antalvic), diazepam (Valium) and zolpidem (Stilnox). He was found dead in a hotel. At autopsy, regurgitation was observed. The results are presented in Table III and Fig. 2B. The concentrations of propoxyphene (4330 ng ml⁻¹) and norpropoxyphene (3800 ng ml⁻¹) in whole blood were greater than the highest therapeutic level and within the blood post-mortem range (Table III). This death was certainly caused by an overdose resulting from the abuse of propoxyphene.

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^a Figs. 1B and 2A seem to have different slopes. In fact, Fig. 2A was from a necropsic blood sample in which endogenous substances could affect the absorbance, whereas Fig. 1B was from a blank sample of whole blood without the same deterioration.

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