Application Overview

Purification of Nucleic Acids

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Introduction

Nucleic acids have become increasingly important in molecular biology, especially with the advent of antisense and gene therapies. To help meet this growing need, Tosoh provides a wide selection of prepacked columns and bulk media for nucleic acid analysis and purification.

Oligonucleotides

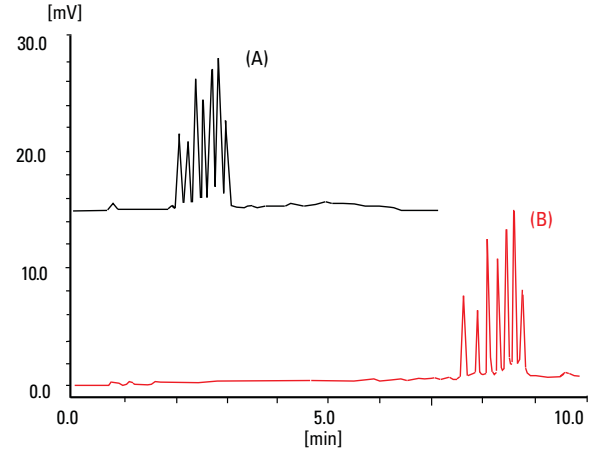
The use of oligonucleotides as primers in sequencing techniques, site-specific mutagenesis, hybridization probes, and possible therapeutic agents, like antisense, has increased the need for the synthesis, analysis and purification of high quality oligonucleotides.

A. Reversed phase separation of oligonucleotides

Most synthesis protocols for oligonucleotides incorporate the use of a protective group on the 5' terminal. Typically this protective group is dimethoxytrityl (DMT), which is a hydrophobic compound. One strategy for separating DMT-on final products from DMT failures is the use of reversed phase chromatography.

The effect of gradient conditions on the separation of 12-18mer polyadenylic oligonucleotides is shown in *Figure 1*. With the TSKgel Super-ODS column, this separation can be performed in less than five minutes under the conditions listed in *Figure 1*[1].

Figure 1. Separation of oligonucleotides on TSKgel Super-ODS



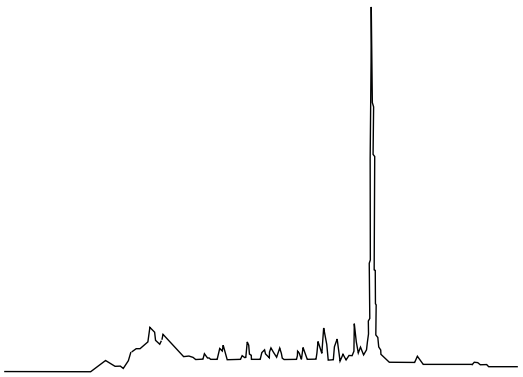
Sample:	12-18mer polyadenylic oligonucleotides
Column:	TSKgel Super-ODS, 4.6mm I.D. x 100cm
Elution:	20mM P.B.+5mM TBAP (pH 6.0)/CH_3CN
Gradient	(A) linear, 32-49% ACN in 5 minutes
	(B) linear, 20-40% ACN in 10 minutes
Flow rate:	1.5ml min
Detection:	UV @ 260nm
Temperature:	40˚C

B. Anion exchange

Because the charge density of oligonucleotides is uniform, the net negative charge generally is a function of the length of the oligonucleotide. Thus, anion exchange separation of oligonucleotides is usually based on length. Sequence failures, therefore, can be resolved from full length products.

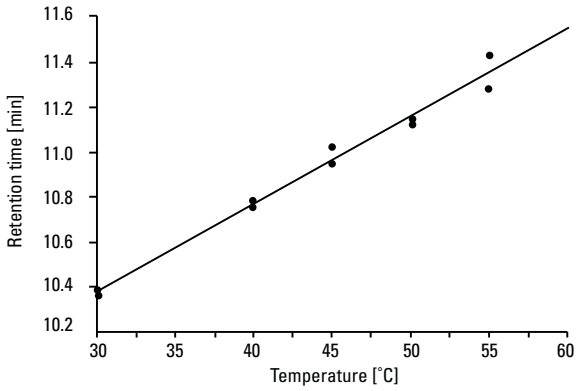
Figure 2 demonstrates the separation of a synthetic 20mer oligonucleotide on a TSKgel DNA-NPR column. This separation was effected using a fifteen minute linear sodium chloride gradient. As shown in *Figure 3*, retention of the oligonucleotide increased linearly with increasing column temperature[2]. Work done with oligonucleotides on the TSKgel DEAE-5PW column demonstrated the effect of salt type and temperature on the retention of various length oligonucleotides[3]. *Figure 4* shows the separation of oligonucleotides on the TSKgel DEAE-5PW with an ammonium formate gradient.

Figure 2. Separation of 20mer oligonucleotide on TSKgel DNA-NPR



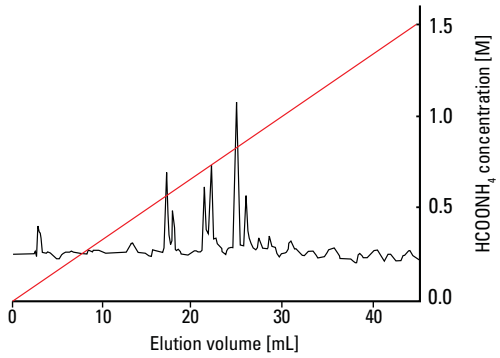
Column:	TSKgel DNA-NPR, 4.6mm ID x 7.5cm
Sample:	5'-GTG-TGG-GCA-CTC-GAA-GAT-AC-3'
Load:	10µl of 2.5 units/mL
Flow rate:	1ml/min
Buffer A:	10mM NAOH
Buffer B:	10mM NaOH with 1M NaCl
Gradient:	0-100% B in 15 minutes
Detection:	UV @ 260 nm
Temperature:	ambient

Figure 3. Retention time vs. Temperature



Column:	TSKgel DNA-NPR, 4.6mm ID x 7.5cm
Sample:	20mer phosphodiester oligonucleotide
Flow rate:	1mL/min
Buffer A:	10mM NaOH
Buffer B:	10mM NaOH + 1M NaCl
Gradient:	0-100% B in 15 minutes
Detection:	UV @ 260nm

Figure 4. Separation of Oligonucleotide on TSKgel DEAE-5PW with an Ammonium Formate Gradient



Column:	TSKgel DEAE-5PW, 7.5mm ID x 7.5cm
Sample:	6, 7, 9, 10, 12, 16, 23, and 26 base phosphodiester oligonucleotides
Load:	20mL
Flow rate:	1mL/min
Buffer A:	0.01M ammonium formate
Buffer B:	1.5M ammonium formate
Gradient:	Linear, 0.01M to 1.5M ammonium formate in 45 minutes
Detection:	UV @ 260nm
Temperature:	50˚C

Backbone-modified oligonucleotides are increasingly used for antisense therapy. These novel oligos have the benefit of longer half-lives due to resistance to endogenous nucleases. One common type of backbone-modified oligonucleotides is phosphorothioates where one of the two nonbridged oxygen atoms in the phosphate linkage has been replaced by a sulfur atom. The separation of several phosphorothioates on a TSKgel DEAE-2SW column is shown in *Figure 5*[4].

Restriction fragments

Double-stranded DNA and single-stranded RNA fragments are generated when larger nucleic acid strands are treated with restriction endonucleases. The restriction endonucleases cleave the longer nucleic acids at specific sites and produce a discernible pattern of fragments. This process is used to help determine the sequence of the parent strand of DNA or RNA.

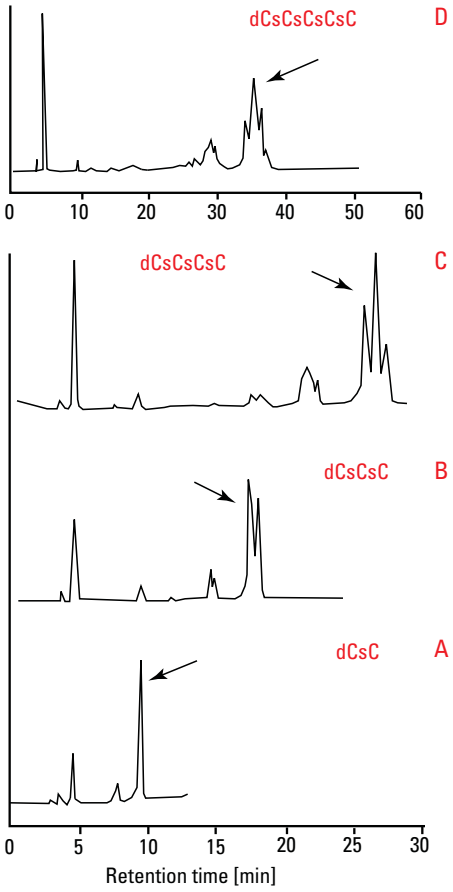
A. Ion exchange

Figure 6 demonstrates a comparison of an a-DNA/Hind III digest separated on either a TSKgel DNA-NPR or DEAE-NPR column[5]. Both columns are weak ion exchangers containing diethylaminoethyl groups. However, the TSKgel DNA-NPR is longer, 7.5cm vs. 3.5cm, and has a lower dead volume than the TSKgel DEAE-NPR column. These features allow the TSKgel DNA-NPR to separate the digest more effectively. With either column, the separation is performed very rapidly.

B. Size exclusion

Size exclusion chromatography (SEC) with silica or polymer packings can be used to separate DNA and RNA fragments based on their size. DNA fragments smaller than 300 bases have been separated into discrete peaks using the TSKgel G3000SW and G4000SW columns. Recovery of the fragments from these columns was greater than 90%. A plot of chain length versus elution volume for double-stranded DNA is shown in *Figure 7*[6].

Figure 5. Separation of phosphorothioates using a TSKgel DEAE-2SW



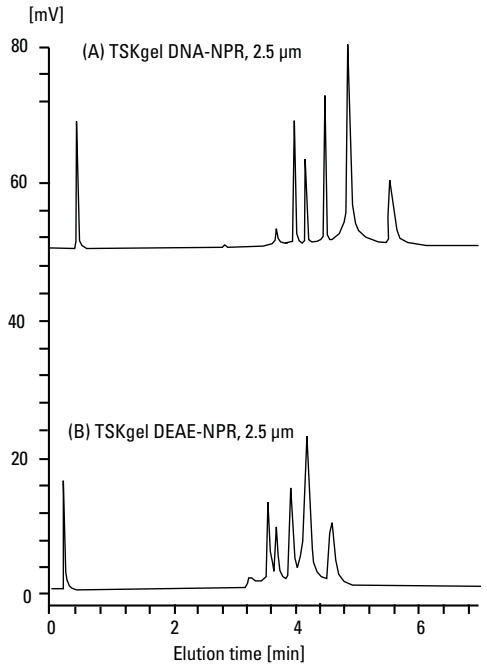
Column:	TSKgel DEAE-2SW, 4.6mm x 25cm
Sample:	A. 2 base phosphorothioate oligonucleotide B. 3 base phosphorothioate oligonucleotide C. 4 base phosphorothioate oligonucleotide D. 5 base phosphorothioate oligonucleotide
Flow rate:	1ml/min
Buffer A:	50mM ammonium acetate
Buffer B:	1.5M ammonium acetate
Gradient:	linear, 0-100% B in 60 minutes
Detection:	UV @ 254nm
Temperature:	ambient

Table 1. TSKgel SEC column selection guide for DNA and RNA fragments

Molecular weight range	Base length	Recommended column
Double-stranded DNA	**Base pairs**	
<40,000	<55	G2000SW/SW_{XL} or G3000SW/SW_{XL}
40,000-80,000	55-110	G3000SW/SW_{XL}
80,000-250,000	110-375	G4000SW/SW_{XL}
250,000-1,000,000	375-1,500	G5000PW/PW_{XL}
RNA fragments	**Base length**	
<60,000	<165	G2000SW/SW_{XL} or G3000SW/SW_{XL}
60,000-120,000	165-330	G3000SW/SW_{XL}
120,000 - 1,200,000	330-3,300	G4000SW/SW_{XL}
1,200,000-10,000,000	3,300-27,500	G5000PW/PW_{XL}

Table 1 lists the SEC columns, along with the recommended molecular weight and base pair ranges, for DNA and RNA fragments[7].

Figure 6. λDNA/Hind III Digest Separation on TSKgel DNA-NPR and DEAE-NPR

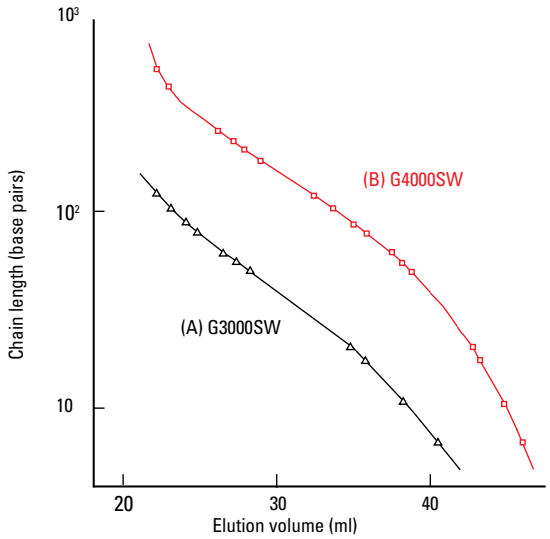


Column: (A) TSKgel DNA-NPR, 2.5µm, 4.6mm ID x 7.5cm
(B) TSKgel DEAE-NPR, 4.6mm ID x 3.5cm
Sample: λDNA/Hind III digest, 1µg in 4µL
Elution: 10-min linear gradient from 0.5 to 1.0M NaCl in 20 mM Tris-HCl buffer, pH 9.0
Flow rate: 1.0mL/min
Detection: UV @ 260nm

PCR

The polymerase chain reaction (PCR) is a valuable biochemical tool which can amplify a specific piece of DNA of up to 6kb. It is used for diagnosis of disease, mutation detection and site-directed mutagenesis. Liquid chromatography is a rapid and sensitive post-PCR technique. In particular, ion exchange chromatography can be used to analyze PCR generated fragments. *Figure 8* shows the detection of a 130bp target derived from HIV using a nonporous TSKgel DEAE-NPR column[8].

Figure 7. Chain Length vs. Elution Volume for Double-Stranded DNA Fragments on TSKgel G3000SW and G4000SW Columns



Column: (A) TSKgel G3000SW, two 7.5mm ID x 60cm columns in series
(B) TSKgel G4000SW, two 7.5mm ID x 60cm columns in series
Sample: Hae III-cleaved pBR322 DNA
Load: 13µg in 50µL
Flow rate: (A) 1mL/min, (B) 0.33mL/min
Buffer: 0.05M Tris-HCl, 0.2M NaCl, 1mM EDTA, pH 7.5
Detection: UV @ 260nm
Temperature: 25˚C

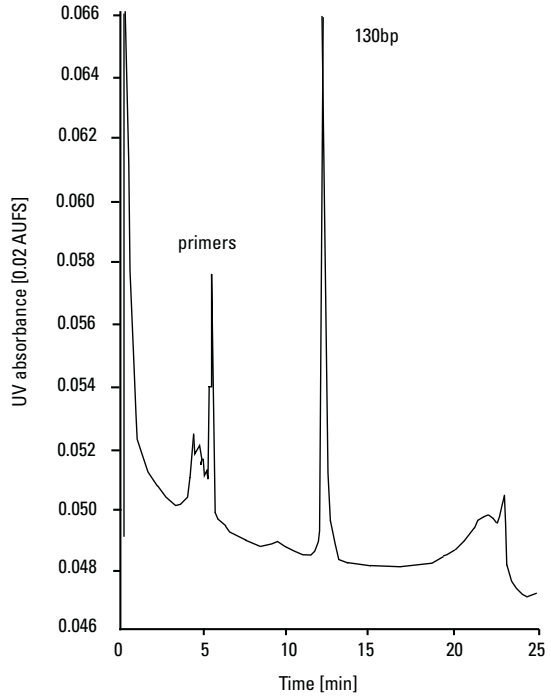
Plasmids

Plasmids are extrachromosomal DNA found in bacteria and yeast which carry genetic information and replicate independently of the bacterial or yeast chromosome. They are circular molecules of duplex DNA ranging in size from 1 to 200kb. Plasmids are useful for molecular cloning. With the advent of gene therapy, there is an increased need for purified plasmids.

A. Ion Exchange

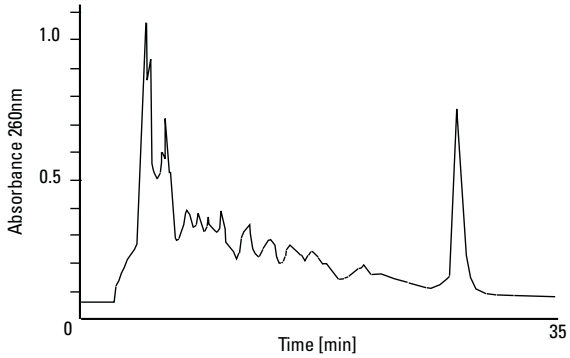
Figure 9 illustrates the separation of crude pBR322 plasmid on a TSKgel DEAE-5PW column[9]. This chromatographic separation provides purified plasmid in one hour, as opposed to a conventional Cs-Cl density gradient ultracentrifugation which can take up to three days.

Figure 8. Detection of HIV-1 PCR-Amplified 130-bp Target using a TSKgel DEAE-NPR Column



Column:	TSKgel DEAE-NPR, 4.6mm ID x 3.5cm
Sample:	HIV-1 PCR-amplified 130bp target
Load:	20µL
Flow rate:	1mL/min
Buffer A:	20mM Tris-HCl with 0.25M NaCl, pH 7.7
Buffer B:	20mM Tris-HCl with 1M NaCl, pH 7.7
Detection:	UV @ 260nm
Temperature:	ambient

Figure 9. Detection of HIV-1 PCR-Amplified 130-bp target using a TSKgel DEAE-NPR Column



Column:	TSKgel DEAE-5PW, 7.5mm ID x 7.5 cm
Sample:	pBR322 plasmid
Load:	2.5mg in 1mL
Flow rate:	1mL/min
Buffer A:	25mM Tris-HCl, 1mM EDTA, pH 8.0
Buffer B:	25mM Tris-HCl, 1mM EDTA, pH8.0, with 1M NaCl
Gradient:	25-60% B in 30 minutes
Detection:	UV @ 260 nm
Temperature:	ambient

Purification of a 13-mer DNA Phosphorothioated Crude Deprotected Oligonucleotide by Strong Anion Exchange Chromatography Using TSKgel DNA- NPR

TSKgel
APPLICATION NOTE

Introduction

The TSKgel DNA-NPR column provides excellent chromatography and superior durability against the harsh HPLC method conditions required for oligonucleotide separations.

Due to the unique conditions involved with oligonucleotide separations including elevated pH and aggressive temperatures, adequate separations of the product from the N -1 peak and other impurities can be challenging. Additionally, column degradation as a function of the harsh conditions is common. Therefore, careful consideration of the composition and durability of the stationary phase is required. Recently, a customer focused on the synthesis of oligonucleotides, evaluated the TSKgel DNA-NPR column for use as an analytical tool in the development and quality control tests of a crude deprotected 13-mer oligonucleotide.

Experimental Conditions

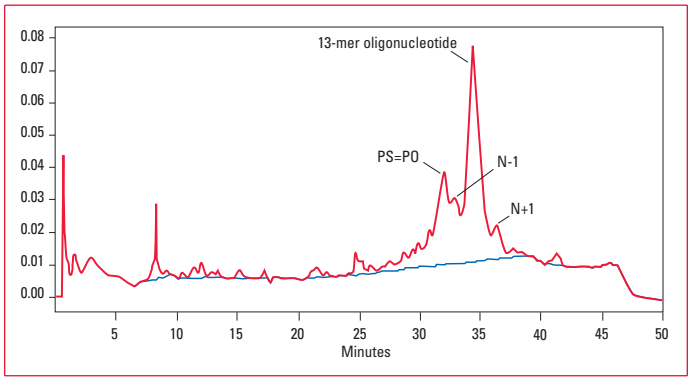
The TSKgel DNA-NPR column is packed with 2.5mm nonporous polymethacrylate beads in a 4.6mm ID x 7.5cm stainless steel housing. The buffer system is delive red at 1.0mL/min and consists of Eluent A (10mM sodium bromide, 20mM NaOH, pH 12, 1% diethylamine) and Eluent B (1 M sodium bromide, 20mM NaOH, pH 12, 1% diethylamine). Step gradients are used to administer Eluent B, starting at 20% from 3.5 min to 12 min and 55% from 12 min to 45 min. The column temperature and sample chamber temperatures are operated at 60 °C and 40 °C respectively.

The method conditions are designed to optimize resolution of all impurity peaks and inhibit any aggregation, secondary structure formation and PS=PO conversion. Specifically, sodium bromide acts as the eluting agent and diethylamine provides the buffering capacity while contributing mild chaotropic effects. The step gradient is designed to remove all the protecting groups from the column before elution the impurity analogs.

Results

Figure 1 contains the chromatographic trace of the crude deprotected 13-mer oligonucleotide. The early eluting peaks from 0–5 min exhibit a lambda max range of 220–230 nm, indicating the presence of protecting groups used in the synthesis. The N-1 peak as confirmed by mass spectrometry elutes just before the main substance peak. The PS=PO peak elutes before the N-1. Structurally, the N-1 analog is completely thioated but is missing one nucleotide. As a result, the N-1 compound is more thioated and hydrophobic than the PS=PO analog. The backside peak is an N+1 impurity verified by mass spectrometry.

Figure 1. Chromatographic trace of a crude deprotected 13-mer oligonucleotide.



Although the customers current column, widely considered an industry standard for oligonucleotide separations, provided similar chromatography; the performance declines dramatically after approximately 5 injections. Subsequent work shows the TSKgel DNA NPR column has continued to provide consistent results beyond 50 injections.

Conclusion

The method exhibits good separation of the protecting groups and impurity analogs from the 13-mer oligonucleotide product peak. The TSKgel DNA-NPR column provides superior durability and column lifetime for this application.

Selectivity studies in the analytical separation of oligonucleotides using a TSKgel® SuperQ-5PW anion exchange column

TSKgel
APPLICATION NOTE

Introduction

Synthetic oligonucleotides are becoming increasingly popular as biotherapeutic agents. When their purification moves from analytical to manufacturing scale, the selectivity is expected to remain the same. Having the same bonding chemistry is therefore extremely helpful. TSKgel SuperQ-5PW analytical columns are designed with the same backbone chemistry and selectivity as their bulk process scale resin counterparts, TSKgel SuperQ-5PW and TOYOPEARL® SuperQ-650.

This application note shows a one-step analysis of a 20-mer DNA-based oligonucleotide, using a TSKgel SuperQ-5PW, 10 µm, 7.5 mm ID × 7.5 cm column. The effect of pH, temperature, and sample load on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, are discussed.

The aligned selectivity of this anion exchange column with its process scale counterparts makes the TSKgel SuperQ-5PW column extremely useful for the separation of oligonucleotides.

Experimental Conditions

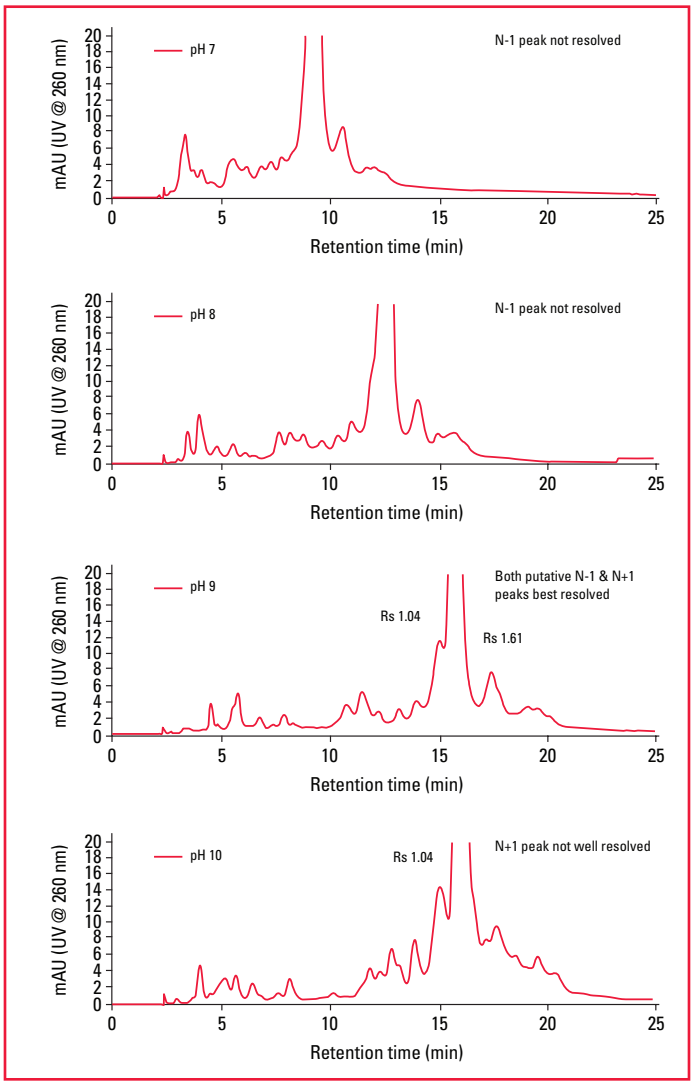
Columns:	TSKgel SuperQ-5PW, 10 µm, 7.5 mm ID × 7.5 cm (S0082-84NM)
Mobile phase:	A: 20 mmol/L Tris, pH 9.0 B: 20 mmol/L Tris, pH 9.0 + 1 mol/L NaCl
Gradient:	40-80% B over 30 minutes – an optimum gradient found to separate both N+1 and N-1 peaks of oligonucleotide
Flow rate:	0.9 mL/min
Detection:	UV @ 260 nm
Temperature:	ambient & 60 °C
Injection vol.:	15 µL
Samples:	phosphodiester deoxyoligonucleotide (20-mer) EcoRI sequence (Trilink Biotechnology, San Diego, CA): Lot# T34-C01A 5' - GAA TTC ATC GGT TCA GAG AC – 3' • purchased unpurified • extinction coefficient was 199.9 OD units/µmol • molecular weight of the free acid – 6140.9 Da • This sequence was chosen to minimize the amount of secondary structure effects during the purification experiments. • Reconstitution of oligonucleotide: for all of the experiments performed, the crude oligonucleotide was diluted into the equilibration buffer (mobile phase A) before loading onto the column. • stock concentration: 26.6 mg/mL; final dilution: 1:100 in mobile phase A • final concentration: 0.266 mg/mL = 0.266 µg/µL

Results and Discussion

Each of the three main species of oligonucleotide (N-1, N=20 and N+1) were separated under the optimum chromatographic conditions using both TSKgel SuperQ-5PW, 20 µm bulk resin and the TSKgel SuperQ-5PW column. The selectivity of the analytical column was the same as that of the bulk resin.

The effect of pH on selectivity and resolution was studied. The optimum pH value was found to be 9.0, as shown in the magnified view of the chromatograms in *Figure 1*.

Figure 1. Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different pH



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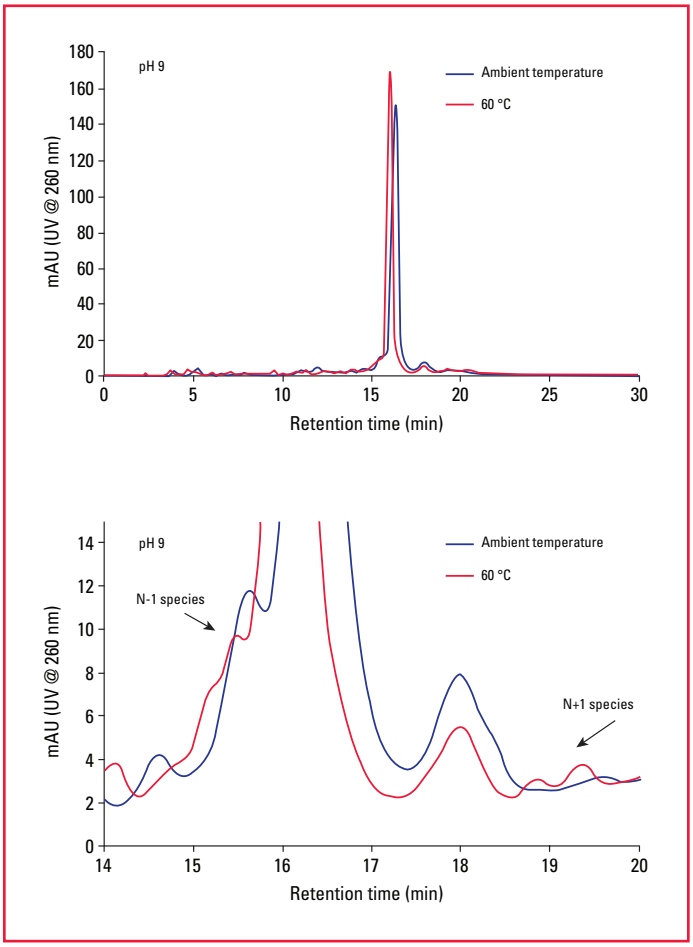
The effect of temperature on selectivity and resolution was also studied in reference to the separation of the N-1 and N+1 peaks from the main peak. As seen in *Figure 2*, better resolution could be obtained at 60 °C column temperature compared to ambient. Both the N-1 and N+1 peaks appear to be heterogeneous.

Finally, the effect of higher loading of crude oligonucleotide on the TSKgel SuperQ-PW column was measured (data not shown). Based on single injection data, the peak area analysis was linear within the experimental range of 3.98 µg to 79.68 µg. Higher loadings of crude oligonucleotide within this experimental range did not affect the peak purity percentage.

Figure 2. Analysis of Crude Oligonucleotide Using the TSKgel SuperQ-5PW Column at Different Temperature



Conclusions

These studies shows that a TSKgel SuperQ-5PW column can be used in the separation of oligonucleotides and the method can be useful for scaling up using TSKgel SuperQ-5PW bulk resin because of their shared selectivity and backbone chemistry.

Each of the three main species of oligonucleotide (N-1, N=20 and N+1) were successfully separated using the TSKgel SuperQ-5PW column at a pH of 9.0 and 60 °C, conditions determined to be optimal.

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