One-step oligonucleotide purifications using anion-exchange chromatography resins

Phu T. Duong, Shigeru Nakatani and J. Kevin O'Donnell
Tosoh Bioscience LLC, 156 Keystone Drive, Montgomeryville, PA 18936

Abstract

The use of synthetic oligonucleotides as biotherapeutic agents continues to grow. Our experiments focused on a one-step purification using anion exchange chromatography resins where the best selectivity was found at pH=9.0. Not surprisingly, longer columns (up to 20cm) gave better resolution between the product peak, the N-1 and N+1 moieties. A crude (<50% pure) 20-mer oligonucleotide was purified to greater than 98% with >85% recovery in a single step. Purity was confirmed by HPLC and gel electrophoresis. Larger particle-size resins exhibited more flexibility when applying simple gradients, whereas, smaller particle-size chromatography resins worked best when the gradient was modified. At higher loading conditions, TSK-GEL SuperQ-5PW and Toyopearl GigaCap Q-650M resins (20µm and 75µm) gave the best resolution and recovery of the resins tested. The 1000Å pore size of these resins offered excellent binding kinetics and less peak tailing. A comparison between DNA and RNA based oligonucleotide separations will also be discussed.

Introduction

Oligonucleotides are short, linear sequences of deoxyribonucleic acid or ribonucleic acid that are generally made through chemical synthesis. The interest in using oligonucleotides as therapeutic agents continues to grow each year. Many oligonucleotide products produced by biopharmaceutical companies are working their way through clinical trails.

Because of the unique structure of these molecules and the way they are synthesized, oligonucleotides require special considerations during chromatographic purification. During the synthesis of the oligonucleotide there are a small percentage of "failure" sequences (N-1 is the common nomenclature) that taken collectively may produce measurable amounts of impurity. The similarity in the impurities requires high resolution techniques to adequately purify the final product. Typically reversed phase and high resolution anion exchange chromatography are the two modes used. This poster describes the use of high resolution anion exchange chromatography for the purification of synthetic DNA- and RNA-based oligonucleotides.

Experimental

Oligonucleotide:

The phosphodiester deoxyoligonucleotide (20-mer) used in this study had the following sequence:

5' - GAA TTC ATC GGT TCA GAG AC - 3'

and was purchased unpurified (estimated at 54% purity by HPLC) in lyophilized form from Trilink Biotechnology, San Diego, CA. The extinction coefficient was 199.9 OD units/µmol and the molecular weight of the free acid was 6140.9 Da. This sequence was chosen to minimize the amount of secondary structure effects during the purification experiments.

RNA sense sequence (H03B) with Na+ ions which has a FW=6400.0Da used in this study was synthesized and provided by Chemgenes Corporation, Willington, MA.

Prior to purification the oligonucleotide was reconstituted in 20mmol/L Tris-HCl at pH=7.0 containing 10mmol/L EDTA

Experimental

Analytical Analysis

Representative crude samples and chromatographic fractions were analyzed using a TSKgel DNA-NPR column (4.6mm ID x 7.5cm L) run on an Agilent 1100 HPLC system. The TSKgel DNA-NPR is a 2.5µm non- porous anion exchange resin for the rapid separation of large biomolecules like oligonucleotides and DNA fragments. Oligonucleotide fractions from each of the columns were injected (15µL) onto the analytical column untreated. After injection, a linear gradient from Buffer A (20mmol/l Tris-HCl pH=8.0) to B (buffer A + 1.0mol/L NaCl) was run over 23 minutes. All samples were eluted in the first 10 minutes of the gradient. Quantitative analysis was obtained using Agilent's ChemStation software.

Gel electrophoresis

Both crude and purified oligonucleotides were analyzed by gel electrophoresis for further confirmation of purity. Approximately 5µg from the purified oligonucleotide fractions were loaded onto a 20% PAGE gel run in TBE (from Invitrogen) and separated at a constant voltage (150V) until the tracking dye migrated to the bottom of the gel. The gel was then fixed and visualized using silver stain according to the manufacturer's directions.

Experimental

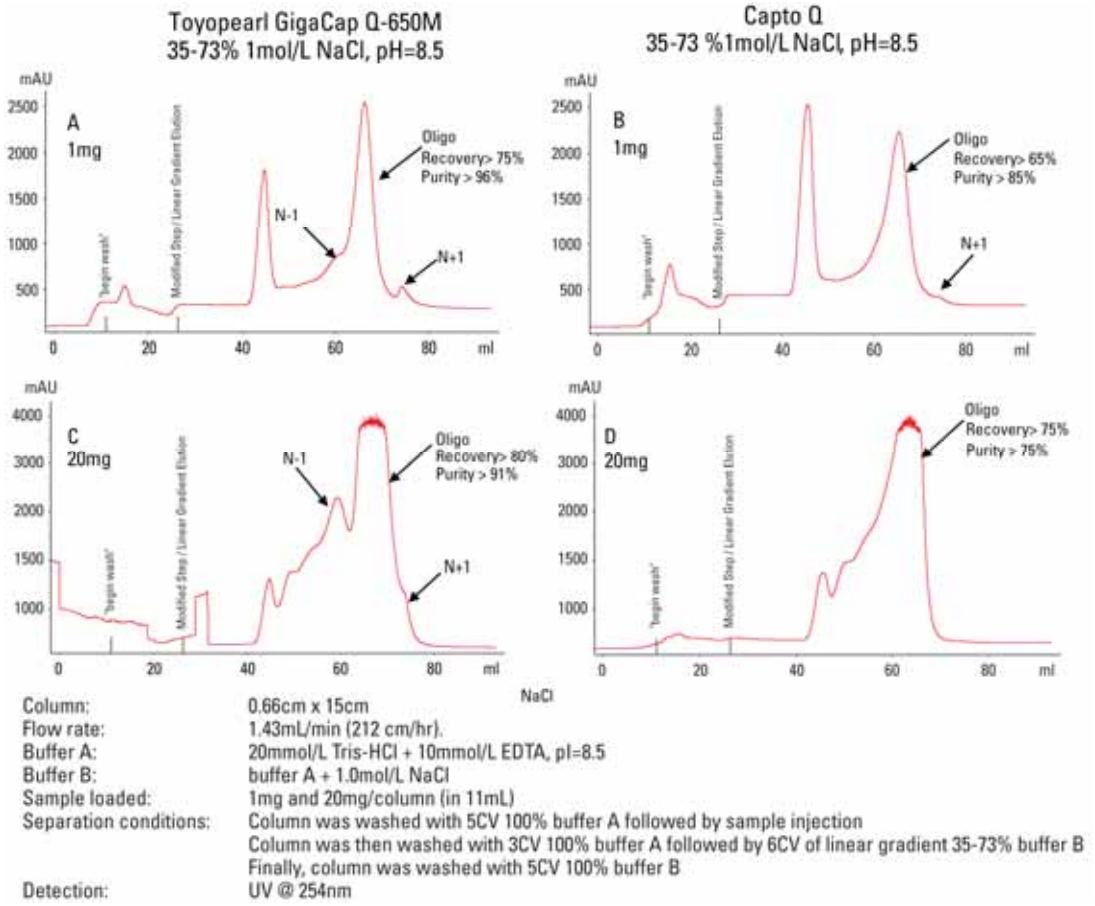
Chromatographic Resins

Strong anion exchange resins (Toyopearl Gigacap Q-650M, Capto Q, TSKgel SuperQ-5PW (20) ,Source 15Q, Toyopearl SuperQ-650M and Q Sepharose Fast Flow) were used in this study. All resins were packed into 0.66cm Omnifit columns at the desired length and evaluated for packing efficiency prior to use.

Experimental Conditions

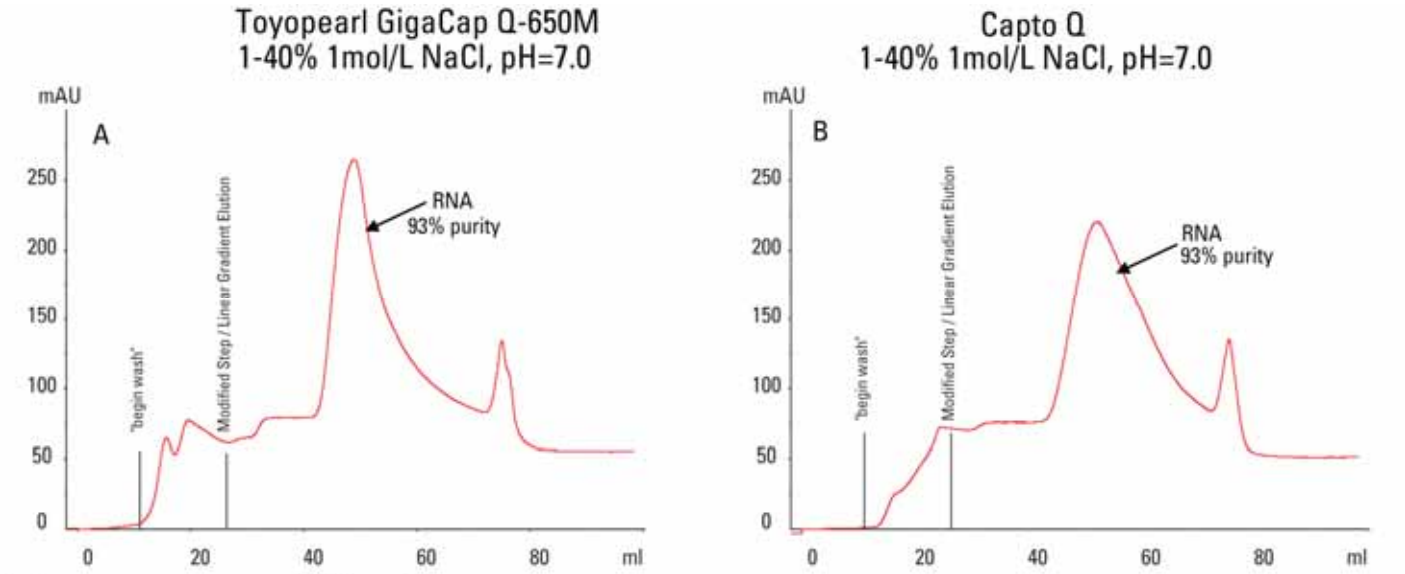
The conditions for running the chromatographic columns are listed in each figure.

Figure 1: Purification of DNA oligonucleotide using Toyopearl GigaCap Q-650M and Capto Q under optimal linear gradients and pH conditions



Toyopearl GigaCap Q-650M and Capto Q were run at different pH and gradient conditions. In all cases, good selectivity was observed. The pH and gradient conditions were adjusted to give the best apparent resolution between the desired product, N-1 and N+1 oligonucleotides. The optimal gradient and pH for the DNA oligonucleotide are shown for both resins. At 1mg load, Toyopearl GigaCap Q-650M showed good selectivity and resolution but Capto Q had less recovery and resolution. Increasing amounts of oligonucleotide were injected onto the columns. Although the peak quickly went off-scale at 254nm, Toyopearl GigaCap Q-650M resin showed that the highest load (20mg) (Fig. 1, panel B) still exhibited good resolution, whereas, the resolution of Capto Q resin deteriorated rapidly. The identification of the N-1 and N+1 peaks/shoulders were confirmed by analytical HPLC analysis.

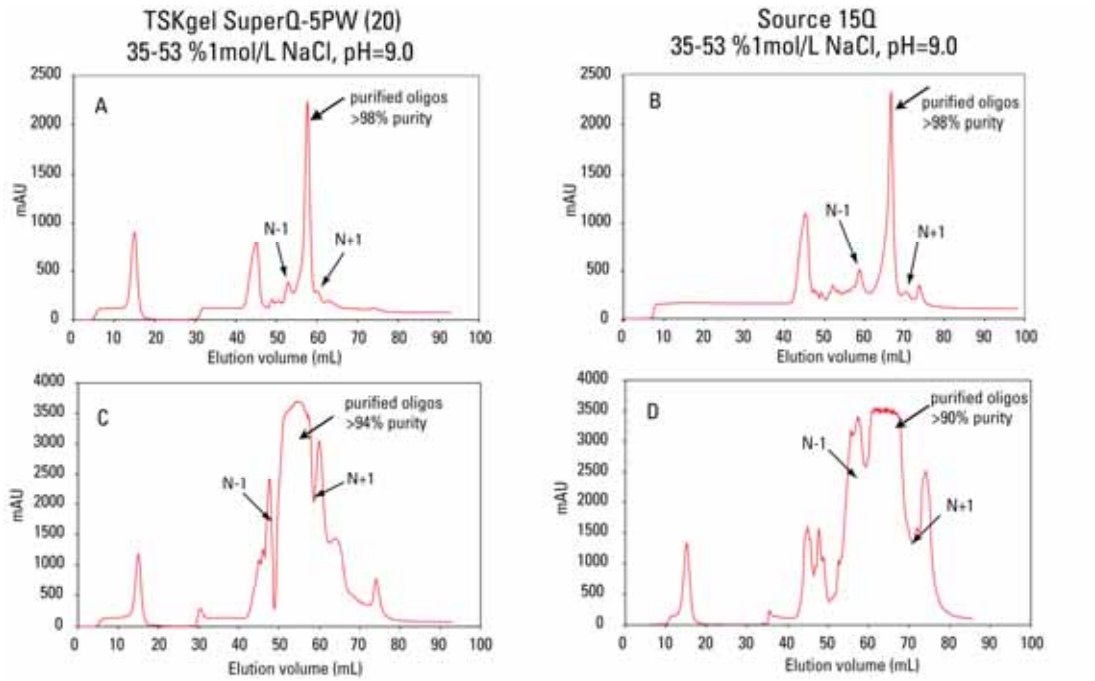
Figure 2: Purification of an RNA oligonuceotide using Toyopearl GigaCap Q-650M and Capto Q under optimal linear gradients and pH conditions



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (212 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA, pH=7.0
Buffer B:	buffer A + 1.0mol/L NaCl
Sample loaded:	1mg/column (in 11mL)
Separation conditions:	Column was washed with 5CV 100% buffer A followed by sample injection Column was then washed with 3CV 100% buffer A followed by 6CV of linear gradient 1-40% buffer B Finally, the column was washed with 5CV 100% buffer B
Detection:	UV @ 254nm
Fractions:	0.5ml fractions were taken from the peak of interest and analyzed on TSKgel DNA-NPR column

The pH and gradient conditions were adjusted to give the best apparent resolution between the desired product. In this case, Tirs-HCL buffer with pH =5.5, 7.0, 9.0 and 10.0 were applied and the optimal pH for both resins to separate RNA is pH=7.0. Toyopearl GigaCap Q-650M showed a smaller elution volume but slight tailing compared to Capto Q. Both resins produced > 93% purified RNA.

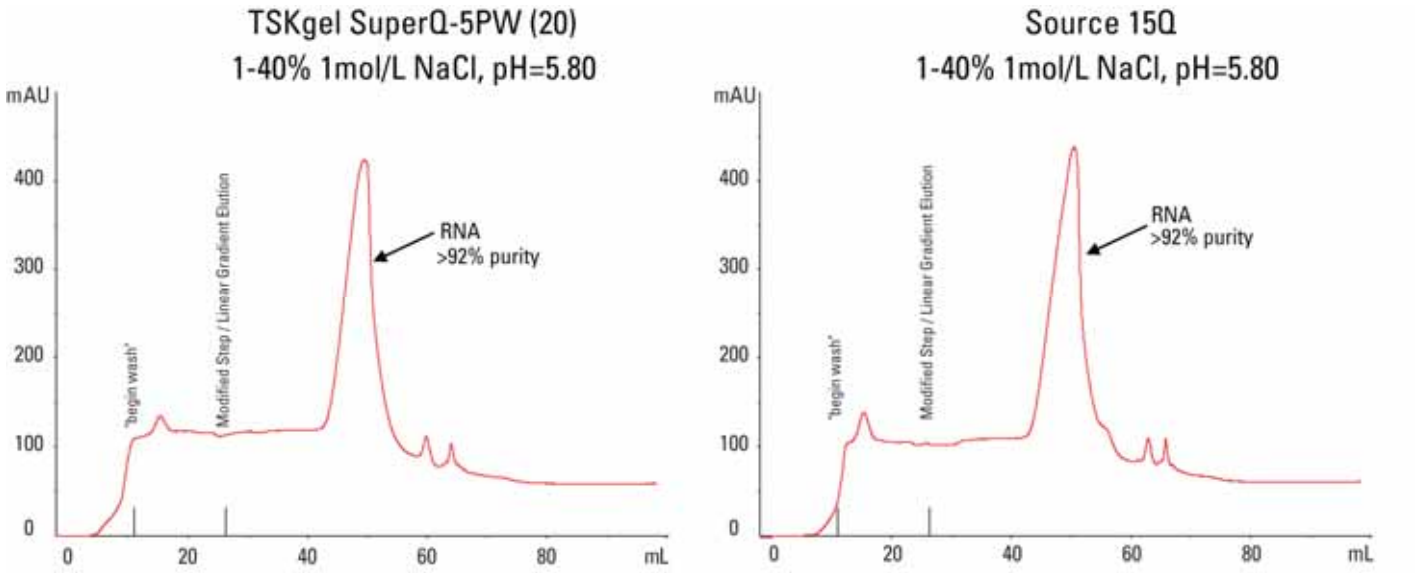
Figure 3: Purification of DNA oligonucleotide using TSKgel SuperQ-5PW and Source 15Q under optimal linear gradients and pH conditions



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	buffer A + 1.0mol/L NaCl pH 9.0
Sample loaded:	DNA-based oligonucleotides were loaded as followed: 1mg/column panels A & B, 20mg/column panels C &D.
Separation conditions:	Column was washed with 5CV 100% buffer A followed by 11mL injection Column was then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B Finally, column was washed with 5CV 100% buffer B
Detection:	UV @ 254nm
Fractions:	0.5ml fractions were taken from peak of interest and analyzed on TSKgel DNA-NPR column

At low loading conditions, the Source 15Q gave similar selectivity when compared to the TSKgel Super Q-5PW (20) resin. However, as the load was increased to at least 20mg per column the TSKgel SuperQ-5PW (20) surpassed the Source 15Q material for peak recovery and purity.

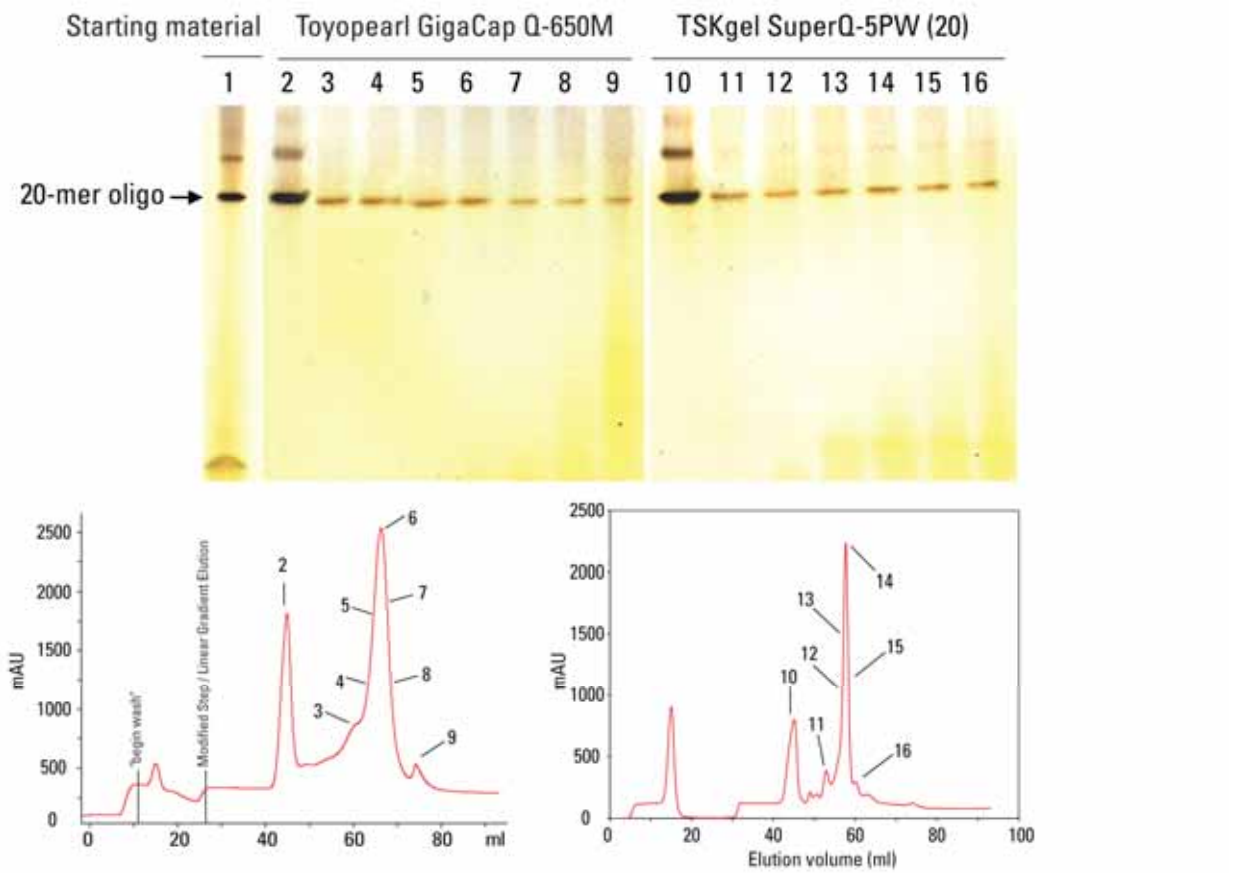
Figure 4: Purification of RNA using TSKgel SuperQ-5PW and Source 15Q under optimal linear gradients and pH conditions



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	buffer A + 1.0mol/L NaCl pH 9.0
Sample loaded:	RNA-based oligonucleotide was loaded as followed: 1mg/column
Separation conditions:	Column was washed with 5CV 100% buffer A followed by 11mL injection Column was then washed with 3CV 100% buffer A followed by 6CV of linear gradient 1-40% buffer B Finally, column was washed with 5CV 100% buffer B
Detection:	UV @ 254nm
Fractions:	0.5ml fractions were taken from peak of interest and analyzed on TSKgel DNA-NPR column

The pH and gradient conditions were adjusted to give the best apparent resolution between the desired product. In this case, Tris-HCL buffer at pH =5.5, 7.0, 9.0 and 10.0 were applied. The optimal pH for both resins to separate RNA was pH=5.80. Both resins generate very similar results and both can produce >92% purified RNA.

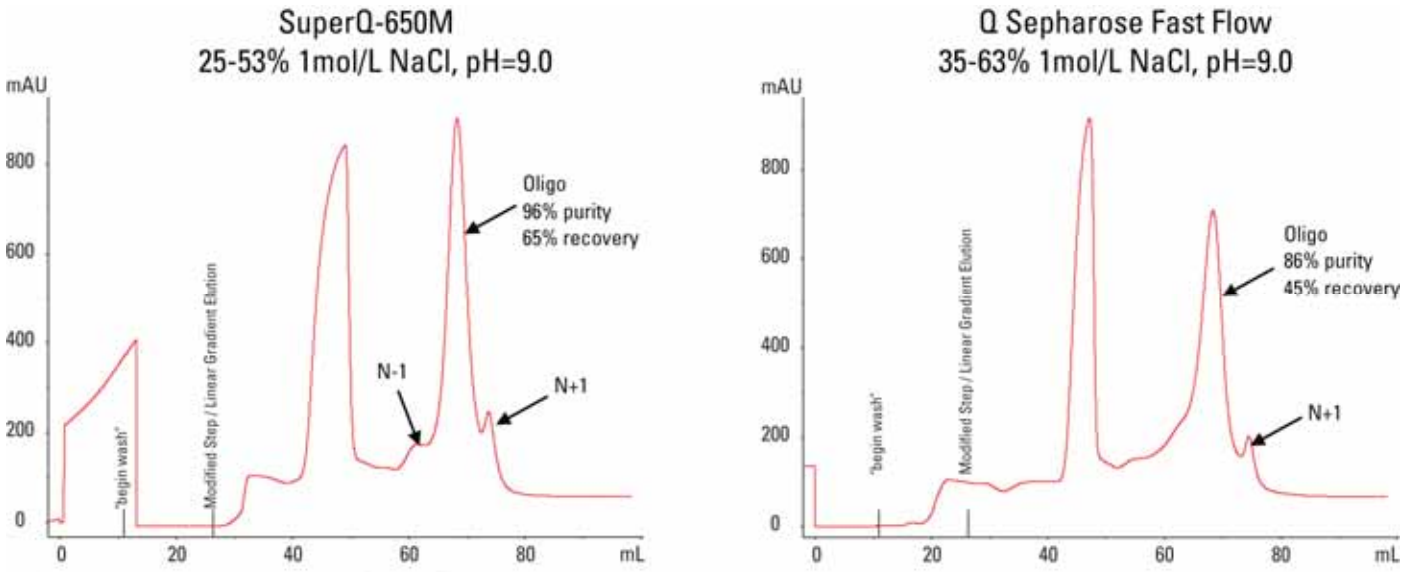
Figure 5: Gel electrophoresis analysis of purified oligonucleotides from Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW resin



*20% PAGE-TBE gel 1.0mm with 12 wells. Oligonucleotide was stained using manufacturer's silver staining method.

Purity of oligonucleotide from both resins were confirmed by gel electrophoresis. Results showed that the purity obtained from both resins was very high. Numbers represent the indicated lane on the PAGE gel.

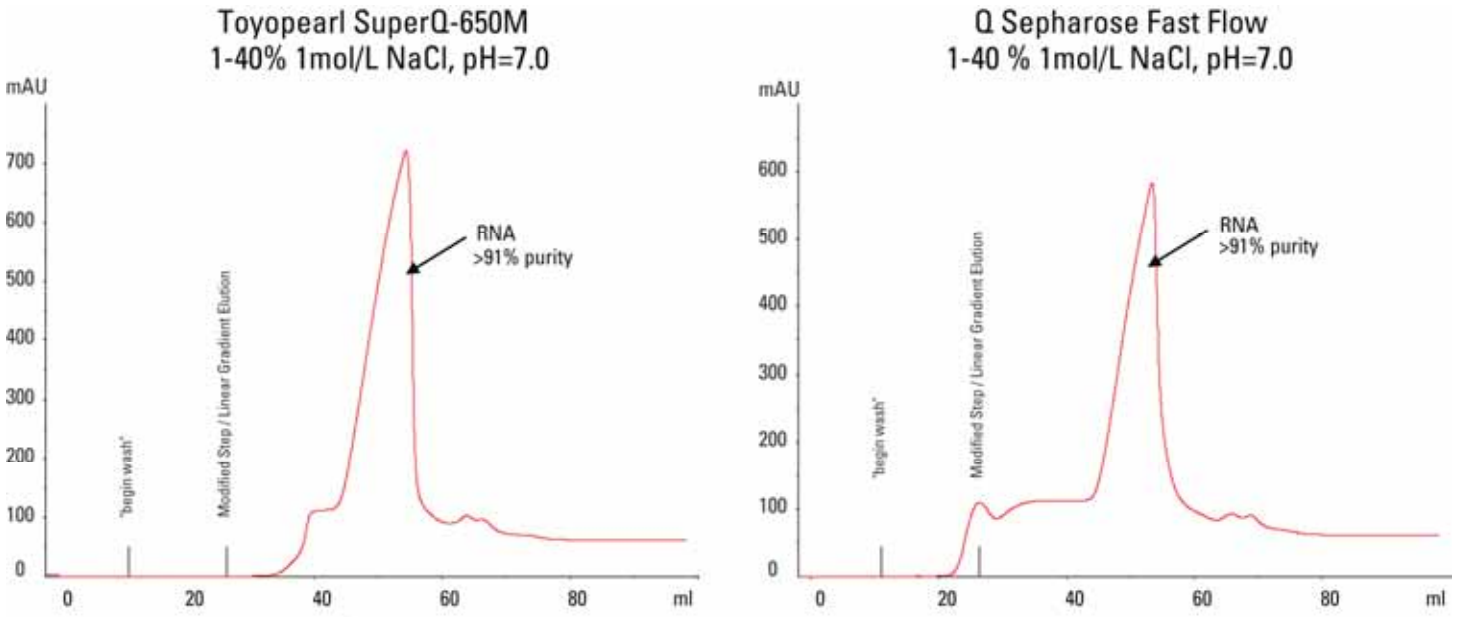
Figure 6: Purification of DNA oligonucleotide using Toyopearl SuperQ-650M and Q Sepharose Fast Flow under optimal linear gradients and pH conditions



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	buffer A + 1.0mol/L NaCl pH 9.0
Sample loaded:	DNA based oligonucleotides were loaded as followed: 1mg/column
Separation conditions:	Column was washed with 5CV 100% buffer A followed by 11mL injection Column was then washed with 3CV 100% buffer A followed by 6CV of linear gradient of buffer B Finally, column was washed with 5CV 100% buffer B
Detection:	UV @ 254nm
Fractions:	0.5ml fractions were taken from peak of interest and analyzed on TSKgel DNA-NPR column

Toyopearl SuperQ-650M and Q Sepharose Fast Flow were run at different pH and gradient conditions. The pH and gradient conditions were adjusted to give the best apparent resolution between the desired product, N-1 and N+1 oligonucleotides. In this case, Tris-HCl buffer pH=9.0 was the optimal pH for both resins. At 1mg load, Toyopearl SuperQ-650M gave better selectivity, peak recovery, resolution and purity when compared to the Q Sepharose Fast Flow resin.

Figure 7: Purification of RNA using Toyopearl SuperQ-650M and Q Sepharose Fast Flow under optimal linear gradients and pH conditions



Column:	0.66cm x 15cm
Flow rate:	1.43ml/min (250 cm/hr).
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	buffer A + 1.0mol/L NaCl pH 9.0
Sample loaded:	RNA based oligonucleotide was loaded as followed: 1mg/column
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11ml injection Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 1-40% buffer B Finally, column is washed with 5CV 100% buffer B
Detection:	UV @ 254nm
Fractions:	0.5ml fractions were taken from peak of interest and analyzed on TSKgel DNA-NPR column

The pH and gradient conditions were adjusted to give the best apparent resolution between the desired product. In this case, Tirs-HCL buffer at pH =5.5, 7.0, 9.0 and 10.0 were applied. The optimal gradient and pH are shown for both resins. At 1mg RNA per column with buffer at pH=7.0, Toyopearl SuperQ-650M and Q Sepharose Fast Flow resins produce similar selectivity and peak purity. However, Toyopearl SuperQ-650M resins had a higher peak recovery.

Table 1: Summary of optimal conditions for Toyopearl GigaCap Q-650M and Capto Q resins for DNA oligonucleotide purification

Optimal conditions	Toyopearl GigaCap Q-650M	Capto Q
Sample	20-mer oligonucleotide	20-mer oligonucleotide
pH	Works well at multiple high pH 9.0 - 9.5	pH=8.0
Gradient (1.0mol/L NaCl)*	Works well at *multiple* linear gradients	Works well at *one specific* linear gradients
Binding capacity (mg/mL resin)**	>45mg	>55mg
Sample purity (%)***	Top of peak fractions >98% Whole peak >88%	Top of peak fractions >82% Whole peak >63%
Sample recovery (%)	>80%	>75%

* The linear gradient is accomplished using 1.0mol/L NaCl

** Biding capacity was established after exposing resins to 1.0mol/L NaOH for 50 cycles and using >98% purified oligonucleotide

*** Sample purity was calculated by using the peak apex only and whole peak represents pooling all fractions across the peak fractions

Table 2: Summary of optimal conditions for TSKgel SuperQ-5PW (20) and Source 15Q resins for DNA oligonucleotide purification

Optimal conditions	TSKgel SuperQ-5PW (20)	Source 15Q
Sample	20-mer oligonucleotide	20-mer oligonucleotide
pH	9.0 - 9.5	9.0 – 9.5
Gradient (1.0mol/L NaCl)*	35-53-100%	35-63-100%
Binding capacity (mg/mL resin)**	>45mg	>42mg
Sample purity (%)***	peak fractions >98%	peak fractions >96%
Sample recovery (%)	>80%	>85%

* The linear gradient is accomplished using 1.0mol/L NaCl

** Binding capacity was established after exposing resins to 1.0mol/L NaOH for 50 cycles and by using purified oligonucleotide which is >98% purity

*** Sample purity was calculated by using the peak apex fractions and whole peak represents pooling all fractions across the peak fractions

Table 3: Summary of optimal conditions for Toyopearl SuperQ-650M and Q Sepharose Fast Flow resins for DNA oligonucleotide purification

Optimal conditions	Toyopearl SuperQ-650M	Q Sepharose Fast Flow
Sample	20-mer oligonucleotide	20-mer oligonucleotide
pH	8 - 8.5	8 – 8.5
Gradient (1.0mol/L NaCl)*	25-53-100%	25-63-100%
Binding capacity (mg/mL resin)**	ND	ND
Sample purity (%)***	peak fractions >98%	peak fractions >96%
Sample recovery (%)	ND	ND

* The linear gradient is accomplished using 1.0mol/L NaCl
** Binding capacity were not determined (ND)
*** Sample purity was calculated by using the peak apex fractions and whole peak represents pooling all fractions across the peak fractions

Table 4: Binding capacity of DNA and RNA based oligonucleotides on resins after exposure to caustic

Resin	DNA (mg)
Toyopearl GigaCap Q-650M	55mg oligo/ml
Capto Q	55mg oligo/ml
TSKgel SuperQ-5PW (20)	45mg oligo/ml
Source 15Q	41mg oligo/ml

Note: CIP study: column cycled 50 times including 1.0mol/L NaOH cleaning (exposure time at least 1h/cycle)

Conclusions

- Crude DNA- and RNA-based oligonucleotides can be applied directly to all of the above resins with minimal sample preparation.
- The optimal pHs for good selectivity on all resins for DNA oligoncleotides were between pH 8.0 and 9.0. However, the optimal pHs for RNA purification using the resins were much broader at pH 5.8 to 7.0. Higher pHs can be used but RNA-based oligoncleotides will be hydrolyzed.
- Only Toyopearl GigaCap Q-650M resin can separate DNA oligonucleotides utilizing a wide-range of gradients (35-55% salt, 35-63% salt and 35-73% salt). Other resins work well under one specific gradient condition.
- All resins easily resolved the N+1 and N-1 DNA oligonucleotides, except Q Sepharose Fast Flow.
- All resins, when used in a single step purification, resulted in >96% purity. However, Q Sepharose Fast Flow resulted in only 85% purity.
- Recoveries of DNA and RNA oligonucleotides on all resins were greater than 75%, except for Toyopearl SuperQ-650M and Q Sepharose Fast Flow.
- The binding capacity for the DNA oligonucleotide used in this study on Toyopearl GigaCap Q-650M was at least 55 mg/ml resins and at least 45mg/ml resin for TSKgel SuperQ-5PW.