



Purification of Oligonucleotides Using High Resolution Strong Anion Exchange Chromatography Resins

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Introduction

Oligonucleotides are short, linear sequences of deoxyribonucleic acid or ribonucleic acid that are generally manufactured by chemical synthesis. Because of the unique structure of these molecules and the way they are synthesized, oligonucleotides require special considerations during chromatographic purification.

TSKgel[®] SuperQ-5PW (20), TOYOPEARL[®] SuperQ-650S and experimental TOYOPEARL GigaCap[®] Q-650S (not yet commercially available) resins are strong anion exchange chromatographic resins for biomolecule purification. They are intended for use in intermediate purification and polishing process steps; however their use as a primary purification resin for synthetic oligonucleotides has been established.

By comparing the performance of the TSKgel SuperQ-5PW (20), TOYOPEARL SuperQ-650S and the experimental TOYOPEARL GigaCap Q-650S resins at similar loading levels relative to their dynamic binding capacity; the ability of these particular resins to purify oligonucleotides from their impurities can be demonstrated.



Experimental Information

The phosphorothioate deoxyoligonucleotide (24-mer) used in this study was supplied unpurified (estimated at 66.5% purity by AEX HPLC) in liquid form from Girindus America Inc. The oligonucleotide is single stranded with a concentration of 981 OD/mL (39.2 mg/mL) by UV at 260 nm and a molecular weight of 7698 amu as a free acid.

TSKgel SuperQ-5PW (20), TOYOPEARL SuperQ-650S (Tosoh Bioscience LLC) and experimental TOYOPEARL GigaCap Q-650S resin (Tosoh Corporation) resins were packed in 0.66 cm ID × 18.0 ± 0.5 cm columns.

A TSKgel DNA-STAT HPLC column (4.6 mm ID × 10.0 cm) was used for analytical analysis of fractions taken during purification and to determine crude and final oligonucleotide purity. Oligonucleotide fractions that were >85% pure were selected for pooling.

Reversed Phase UPLC analysis was completed using a Waters Acquity® OST C18 Column 1.7 μm (2.1 mm ID × 10.0 cm).

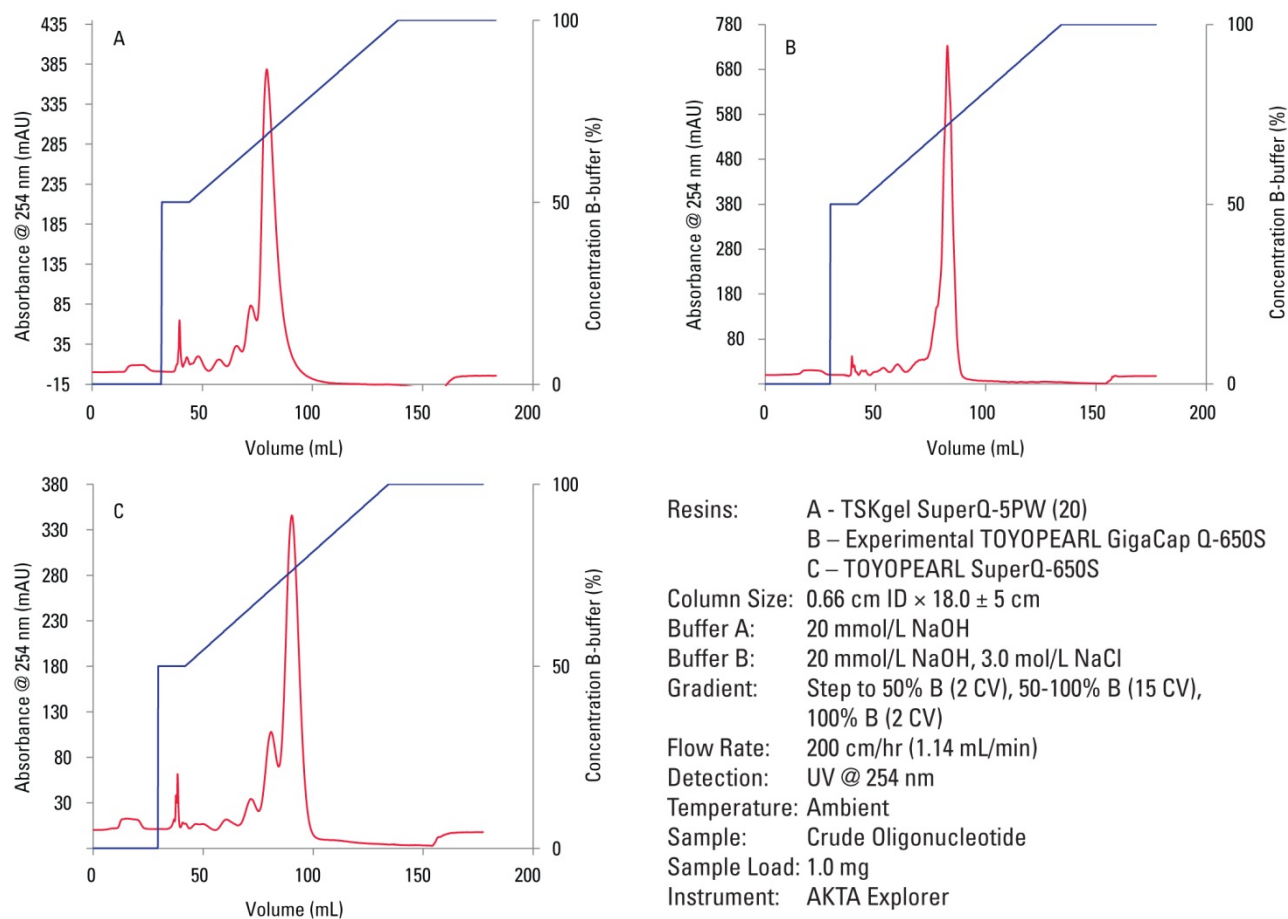


Table 1: Properties of the Anion Exchange Resins Used in this Study

	TSKgel SuperQ-5PW (20)	TOYOPEARL SuperQ-650S	Experimental TOYOPEARL GigaCap Q-650S
Lot#	5QA05RM	65QASB01M	65GQSC503R
Particle size	20 μm	35 μm	30 μm
Pore diameter	1000 \AA	1000 \AA	1000 \AA
Ion exchange capacity	0.14 meq/mL gel	0.26 meq/mL gel	0.17 meq/mL gel
Absorption capacity (BSA)	60 g/L gel	129 g/L gel	189 g/L gel
Recommended Max Pressure	20 bar	3 bar	3 bar



Figure 1: Initial Resin Screening with 1.0 mg Crude Oligonucleotide Load



The TSKgel SuperQ-5PW (20) and TOYOPEARL SuperQ-650S resins resolved the putative N-1 peak from the main oligonucleotide peak. With the experimental TOYOPEARL GigaCap Q-650S resin the N-1 peak was visible as a shoulder to the full length oligonucleotide peak. There was no visible N+1 peak at this load concentration. However, HPLC analysis of the crude oligonucleotide confirmed that the N+1 impurity was minimal in this sample.



Table 2: Dynamic Binding Capacity of Crude Oligonucleotide

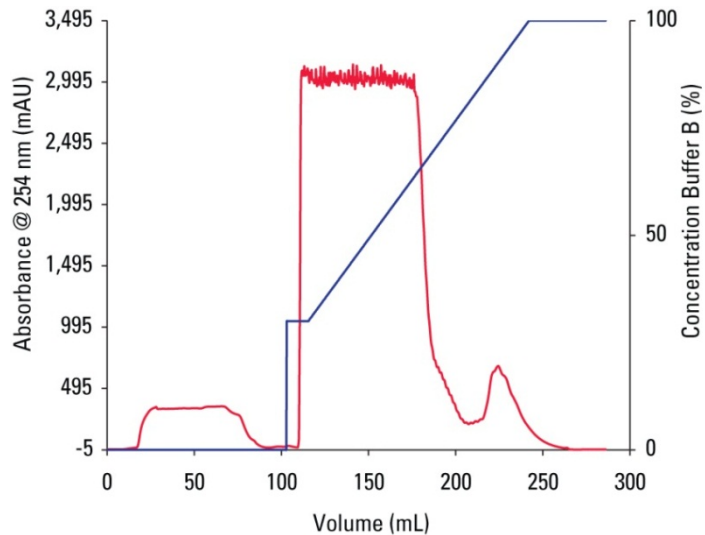
	TSKgel SuperQ-5PW (20)	TOYOPEARL SuperQ-650S	Experimental TOYOPEARL GigaCap Q-650S
DBC @ 10% Breakthrough 200 cm/hr	46.4 mg/mL	53.6 mg/mL	36.8 mg/mL

Resin: Multiple (listed above)
Column Size: 0.30 cm ID × 10.0 cm (0.707 mL)
Buffer A: 20 mmol/L NaOH
Buffer B: 20 mmol/L NaOH, 3.0 mol/L NaCl
Gradient: No Gradient
Flow Rate: 200 cm/hr (0.24 mL/min)
Detection: UV @ 254 nm
Temperature: Ambient
Sample: Crude Oligonucleotide (0.5 mg/mL)
Sample Load: Stop loading at 10% breakthrough
Instrument: AKTA Explorer

The dynamic binding capacities were determined using crude oligonucleotide under the conditions listed above. The data indicate that the TOYOPEARL SuperQ-650S resin has a greater capacity for crude oligonucleotide than the TSKgel SuperQ-5PW (20) and the Experimental TOYOPEARL GigaCap Q-650S resins under the conditions tested. The resins were then loaded at approximately 80% of the dynamic binding capacity to assess the purity and yield.



Figure 2: TSKgel SuperQ-5PW (20) Resin – 80% Dynamic Binding Capacity Load

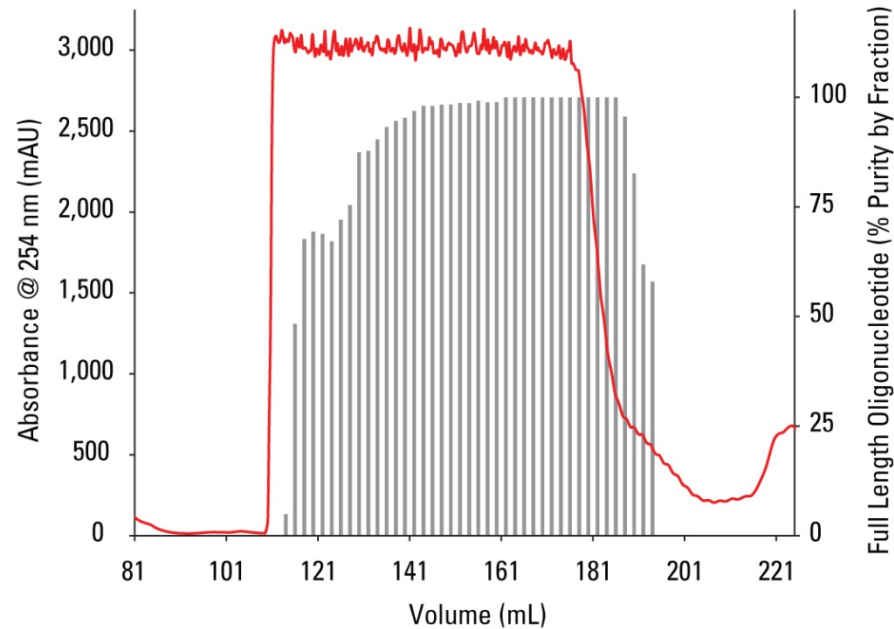


Resin: TSKgel SuperQ-5PW (20)
Column Size: 0.66 cm ID × 18.5 cm (6.30 mL)
Buffer A: 20 mmol/L NaOH
Buffer B: 20 mmol/L NaOH, 3.0 mol/L NaCl
Gradient: Step to 30% B (2 CV), 30-100% B (15 CV), 100% B (2 CV)
Flow Rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Temperature: Ambient
Sample: Crude Oligonucleotide
Sample Load: 80% of Dynamic Binding Capacity (37.1 mg/mL)
Instrument: AKTA Explorer

The elution profile for TSKgel SuperQ-5PW (20) resin went off scale for UV at 254 nm. At this resin loading concentration, there is a visible putative N+1 peak that was not previously visible when 1 mg of crude material was loaded onto the column. The N+1 peak was visibly resolved from the main peak at this higher loading level while the N-1 peak is not visibly discernable. HPLC analysis of individual fractions (data not shown) indicates that the N-1 peak is separated from the full length oligonucleotide.



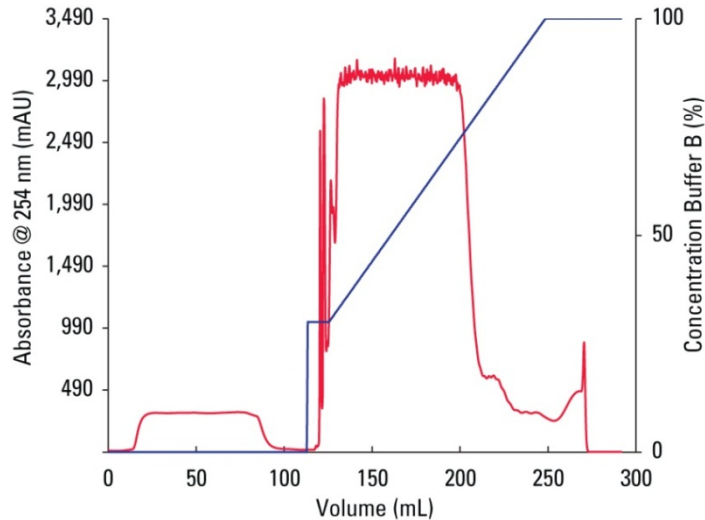
Figure 3. TSKgel SuperQ-5PW (20) Resin – Fraction Purity Histogram Overlaid



The elution profile for the TSKgel SuperQ-5PW (20) resin went off scale at 254 nm. The enlarged image of the main oligonucleotide peak, when overlaid with a histogram showing the AEX HPLC results for fraction purity, highlights the chromatographic separation of the full length oligonucleotide that could not be determined by examining the elution profile alone. Note: This figure highlights just the area of Figure 2 where the full length oligonucleotide elutes.



Figure 4. TOYOPEARL SuperQ-650S Resin – 80% Dynamic Binding Capacity Load

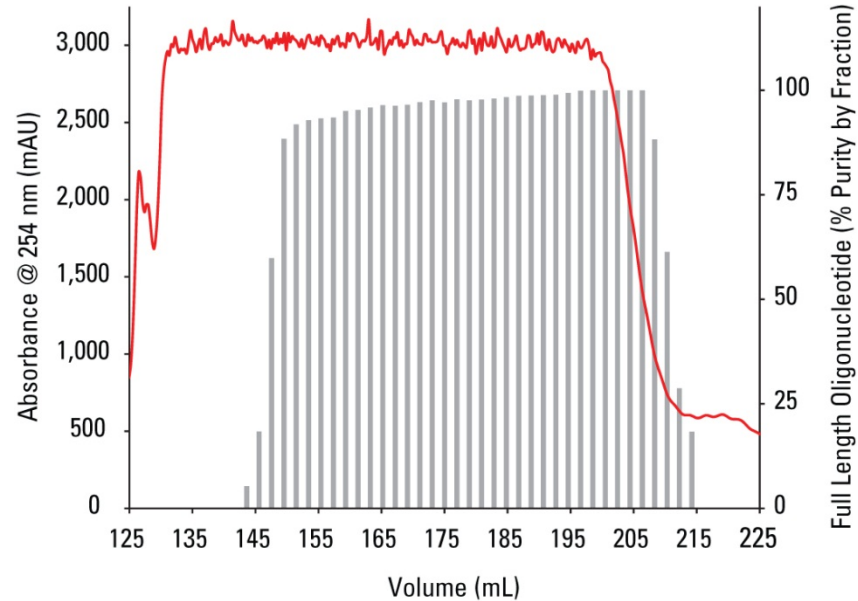


Resin: TOYOPEARL SuperQ-650S
Column Size: 0.66 cm ID × 18.0 cm (6.16 mL)
Buffer A: 20 mmol/L NaOH
Buffer B: 20 mmol/L NaOH, 3.0 mol/L NaCl
Gradient: Step to 30% B (2 CV), 30-100% B (15 CV),
100% B (2 CV)
Flow Rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Temperature: Ambient
Sample: Crude Oligonucleotide
Sample Load: 80% of Dynamic Binding Capacity (45.0 mg/mL)
Instrument: AKTA Explorer

The elution profile for TOYOPEARL SuperQ-650S went off scale for UV at 254 nm. At this resin load concentration, there was a visible N+1 peak that was resolved from the main oligonucleotide peak. The N-1 peak was partially visible in the chromatogram, though HPLC analysis of the individual fractions verifies that the N-1 peak was separated from the whole oligonucleotide. The peak that is visible prior to the step gradient in the flow-through and wash was identified as residual blocking groups from the synthesis reaction and does not indicate product breakthrough.



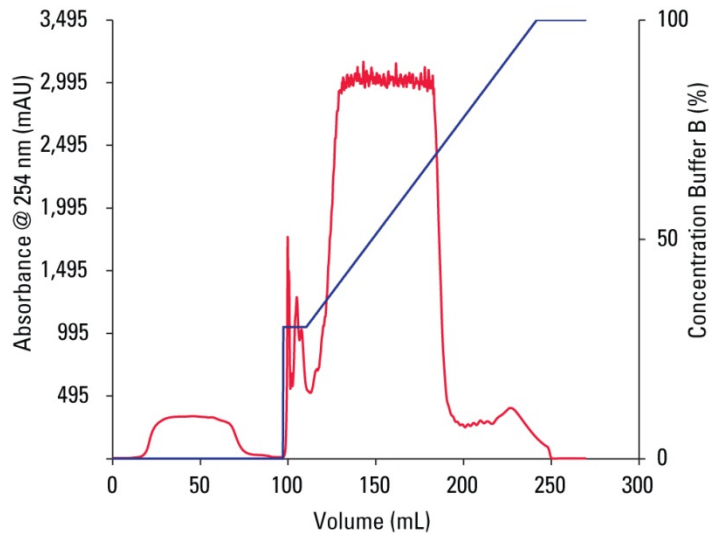
Figure 5. TOYOPEARL SuperQ-650S Resin – Fraction Purity Histogram Overlaid



The elution profile for the TOYOPEARL SuperQ-650S resin went off scale at 254 nm. The enlarged image of the main oligonucleotide peak, overlaid with the fraction purity histogram, also indicates that the separation of the full length oligonucleotide from the N-1 and other impurities is well defined. This is indicated by the sharp increase in purity for the first three fractions. Note: This figure highlights just the area of Figure 4 where the full length oligonucleotide elutes.



Figure 6. Experimental TOYOPEARL GigaCap Q-650S Resin – 80% Dynamic Binding Capacity Load

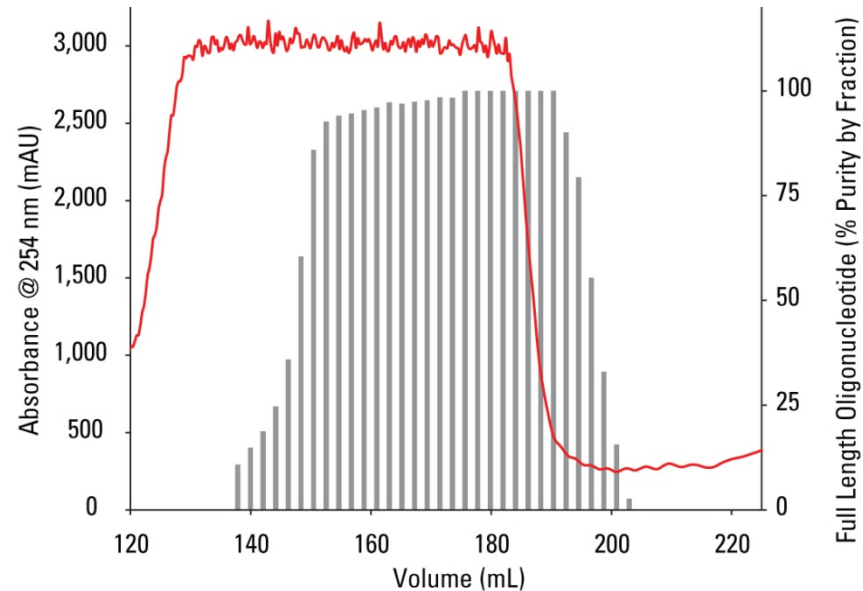


Resin: Experimental TOYOPEARL GigaCap Q-650S
Column Size: 0.66 cm ID × 18.0 cm (6.16 mL)
Buffer A: 20 mmol/L NaOH
Buffer B: 20 mmol/L NaOH, 3.0 mol/L NaCl
Gradient: Step to 30% B (2 CV), 30-100% B (15 CV), 100% B (2 CV)
Flow Rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Temperature: Ambient
Sample: Crude Oligonucleotide
Sample Load: 80% of Dynamic Binding Capacity (30.0 mg/mL)
Instrument: AKTA Explorer

The elution profile for the experimental TOYOPEARL GigaCap Q-650S resin went off scale for UV at 254 nm. At this load concentration, both the N-1 shoulder and the N+1 peak are visible with the N+1 peak being visibly resolved. While the N-1 shoulder is only partly discernable in this chromatogram, HPLC analysis of individual fractions shows that the N-1 impurity was effectively separated from the full length oligonucleotide.



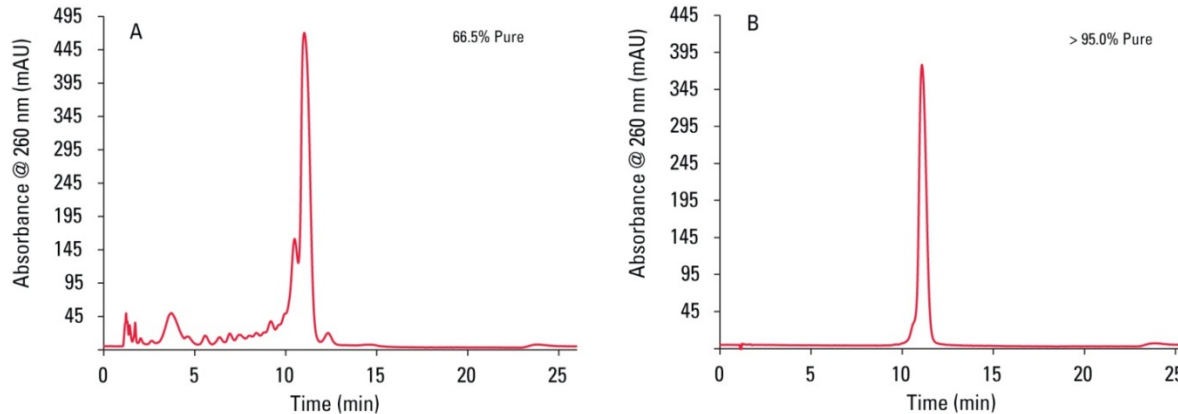
Figure 7. Experimental TOYOPEARL GigaCap Q-650S Resin – Fraction Purity Histogram Overlaid



The elution profile for the experimental TOYOPEARL GigaCap Q-650S resin went off scale at 254 nm. The enlarged image of the main oligonucleotide peak, again overlaid with a histogram showing the AEX HPLC results for fraction purity, highlights the chromatographic separation of the full length oligonucleotide. Note the gradual increase in fraction purity for this resin. Note: This figure highlights just the area of Figure 6 where the full length oligonucleotide elutes.



Figure 8. HPLC Analysis of Crude and Purified Oligonucleotide Using a TSKgel DNA-STAT Column

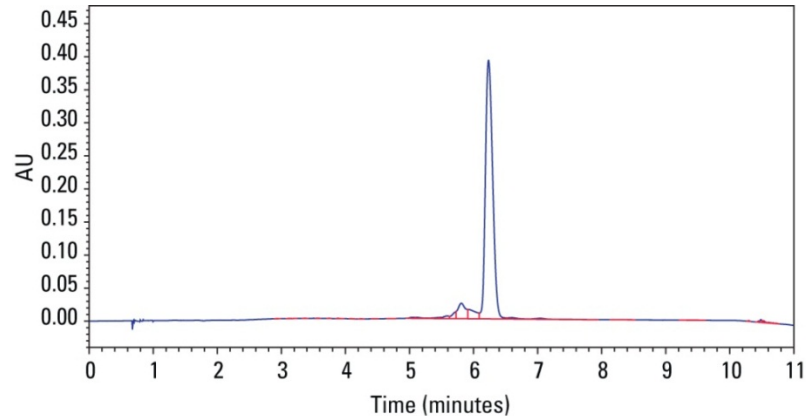


Column: TSKgel DNA-STAT column,
4.6 mm ID × 10.0 cm
Buffer A: 100 mmol/L Tris, 10% ACN pH 8.0
Buffer B: 100 mmol/L Tris, 2.0 mol/L NaCl,
10% ACN pH 8.0
Gradient: 30-100% B (20 minutes)
Flow Rate: 180 cm/hr (0.5 mL/min)
Detection: UV @ 260 nm
Temperature: 60° C
Injection Vol.: 10 µL
Sample: A - crude oligonucleotide
B - pure oligonucleotide Pool
Instrument: Agilent 1100 HPLC

Both crude oligonucleotide and purified individual fractions from the columns were analyzed on a TSKgel DNA-STAT column to assess purity. Purified fractions that were $\geq 85\%$ pure were pooled together and analyzed for overall purity. The data indicated that the oligonucleotide was purified to a minimum of $>95\%$ for all three resins. The purified oligonucleotide chromatogram is representative of the purified oligonucleotide samples throughout this study. See Table 3 for purity and recovery information for all three resins.



Figure 9: UPLC Analysis of TSKgel SuperQ-5PW (20) Resin Purified Oligonucleotide



Column: Waters Acquity OTS C18 1.7 μ m column
Column Size: 2.1 mm ID \times 10.0 cm
Buffer A: 100 mmol/L HFIP, 7 mmol/L TEA
Buffer B: 100% methanol
Gradient: 10-30% B (25 minutes)
Flow Rate: 0.5 mL/min
Detection: UV @ 260 nm
Temperature: 60° C
Injection Vol.: 5 μ L
Sample: Pure oligonucleotide pool
Instrument: Waters Acquity UPLC with TUV detection

The pooled fractions from the columns were analyzed on a Waters Acquity OTS C18 1.7 μ m column to assess purity. Purified fractions that were \geq 85% pure were pooled together and analyzed for overall purity. The data indicated that the oligonucleotide was purified to 86.3% for the TSKgel SuperQ-5PW (20) column. The purified oligonucleotide chromatogram is representative of the purified oligonucleotide samples throughout this study. See Table 3 for purity and recovery information for all three resins.



Table 3: Purity and Recovery for TSKgel SuperQ-5PW (20), TOYOPEARL SuperQ-650S and Experimental TOYOPEARL GigaCap Q-650S Resins

Resin	Crude Loaded	Crude Purity (AEX)	Crude Purity (RPC)	Total Recovered (OD 260)	% Full Length Oligo (AEX)	% Full Length Oligo (RPC)	% Yield of FLO (AEX)	% Yield of FLO (RPC)
TSKgel SuperQ-5PW (20)	37.1 mg/mL	66.5%	62.96%	118.8 mg	97.3%	86.3%	74.3%	69.6%
TOYOPEARL SuperQ-650S	45.0 mg/ mL	66.5%	62.96%	142.7 mg	95.6%	82.8%	74.1%	67.7%
Experimental TOYOPEARL GigaCap Q-650S	30.0 mg/ mL	66.5%	62.96%	95.3 mg	96.5%	85.5%	75.0%	70.0%

Sample purity was determined by pooling the highest purity fractions (>85% full length oligonucleotide - FLO) from each column and analyzing them on a TSKgel DNA-STAT HPLC column. The pooled fractions were also analyzed using a Waters Acquity OST C18 1.7 μm (2.1 mm ID x 10 cm) RP UPLC column. Recovery was determined by comparing the amount of full length oligonucleotide present in the crude sample loaded onto the column with the amount of full length oligonucleotide present in the pool of high purity fractions.



Conclusions:

1. Despite some minor differences in dynamic binding capacity, TSKgel SuperQ-5PW (46.4 mg/mL), TOYOPEARL SuperQ-650S (53.6 mg/mL) and experimental TOYOPEARL GigaCap Q-650S (36.8 mg/mL) resins produced oligonucleotide of similar purity and with similar yields from the same lot of crude material.
2. TOYOPEARL SuperQ-650S produced the lowest purity and yield and was eliminated from future studies currently underway.
3. The experimental TOYOPEARL GigaCap Q-650S pool volume (42 mL) was 30% less than the pool volumes for the TOYOPEARL SuperQ-650S and the TSKgel SuperQ-5PW (20) resins (60 mL).
4. The TSKgel SuperQ-5PW (20) resin and the experimental TOYOPEARL GigaCap Q-650S resins produced full length oligonucleotide of comparable purity and with similar yields. However, since the TSKgel SuperQ-5PW (20) has a greater capacity for this oligonucleotide and the experimental TOYOPEARL GigaCap Q-650S is not yet commercially available, the TSKgel SuperQ-5PW (20) would be the resin of choice to purify this oligonucleotide.
5. Differences in analytical methods can have an impact on the overall reported purity and yield. For example, IEC appeared to over estimate purity while RPC underestimated it.