TOSOH

One-step 5’-DMT-protected Oligonucleotide Purification

Your Challenge

- You need separate steps for DMT cleavage & removal which increases process time, complexity, and risk of degradation
- RPC for DMT-protected/off uses excess solvents, limiting sustainable production.

Our Solution

TSKgel Phenyl-3PW (20) HIC resin

- One-step on-column purification

What was done?

- One-step on-column purification process optimization & scale-up using TSKgel Phenyl-3PW (20)

What was the result?

- A safe & robust process which produced > 99% DMT-off, 90% pure, chemically intact 20-mer oligo with 97% yield

Efficient one-step purification and on-column 5’ DMT cleavage at pH 4.0 using TSKgel Phenyl-3PW (20) resin, preserving oligo integrity and enabling scalable ssDNA purification

Your Benefit

Streamlined, scalable & sustainable oligo purification with high yield and product integrity.

https://separations.us.tosohbioscience.com/Process_Media/id-8943/TSKgel_Phenyl-3PW_20

Oligonucleotide Purification

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SEPARATION & PURIFICATION
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Application Note

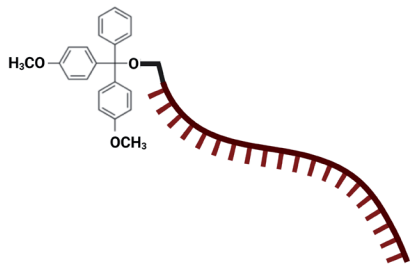
One-step 5′-DMT-protected Oligonucleotide Purification Using a Novel Hydrophobic Interaction Chromatography Resin

Introduction

Synthetic oligonucleotides, such as antisense oligonucleotides (ASOs), siRNAs, aptamers, and DNA/RNA probes, are short, single-stranded DNA or RNA molecules that bind to mRNA, blocking translation and preventing unwanted protein synthesis. These oligonucleotides are synthesized via phosphoramidite-based solid-phase synthesis, during which the 5′-hydroxyl group is protected by an acid-labile 5′-dimethoxytrityl (DMT) group. DMT prevents side reactions during chain elongation and can be retained after synthesis to aid purification. However, DMT must be removed from the final product.

DMT is hydrophobic in structure, which enables purification by hydrophobic interaction chromatography (HIC). Notably, shortmer impurities lack the 5′-DMT group because failed couplings are not capped. As a result, shortmers are more hydrophilic and separate efficiently from full-length products during HIC. The HIC step can also be coupled with on-column acid-mediated DMT cleavage. In this study, we demonstrate the one-step purification and on-column DMT removal of a 20-mer ssDNA oligonucleotide *(Figure 1)* using a novel HIC resin, TSKgel® Phenyl-3PW (20).

Figure 1. Graphic representation of DMT-protected oligonucleotide for this study.



Sample

The oligonucleotide used consisted of a 20-mer single-stranded DNA (ssDNA) containing a 5′-DMT cap with a randomly selected nucleotide sequence of 5′-GAA TTC ATC GGT TCA GAG AC-3′ (TriLink BioTechnologies, San Diego, CA, USA).

HIC Resin

The HIC resin selected for this study was TSKgel Phenyl-3PW (20). TSKgel Phenyl-3PW (20) is a novel, phenyl-substituted HIC resin based on polymethacrylate beads. See *Table 1* for additional characteristics.

Table 1. Characteristics of TSKgel Phenyl-3PW (20) resin.

Property	TSKgel Phenyl-3PW (20)
Bead material	polymethacrylate
Average bead diameter	20 µm
Average pore diameter	25 nm
Maximum pressure	2.0 MPa
Dynamic binding capacity*	25 mg/mL (at 1.5 mol/L, >45 mg/mL)

*Crude DMT-on 20-mer oligonucleotide; 10 mmol/L NaOH, 10 mol/L $(NH_4)_2SO_4$; 4 min residence time

Results and Discussion

Purification in an ammonium sulfate gradient

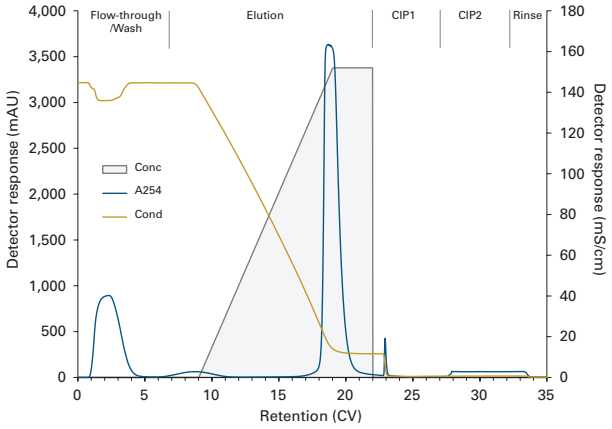
The 20-mer DMT-protected oligonucleotide was first purified in a linearly decreasing salt gradient from 1 mol/L to 0.05 mol/L ammonium sulfate *(Table 2, Figure 2)*.

Table 2. HIC purification in an ammonium sulfate gradient.

Media: TSKgel Phenyl-3PW (20)
Column: 5 mm ID × 5 cm (1 mL)
Flow rate: 1.0 mL/min (300 cm/hr)
Detection: UV @ 254 nm (mAU), conductivity (mS/cm)
Temperature: ambient
Instrument: ÄKTA avant™ 25 (Unicorn 7.3)

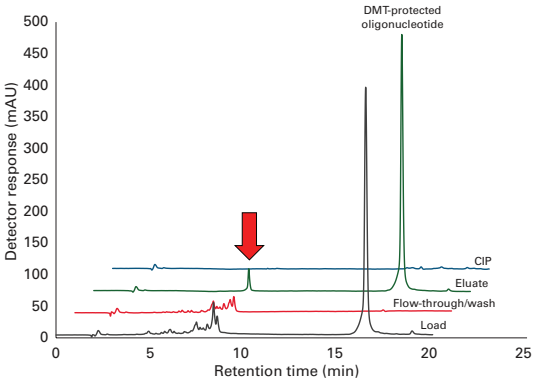
Phase	Mobile phase	CV
Equilibration	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	10
Load	DMT-protected 20-mer, 2 mL @ 0.5 mg/mL (1 mg-oligo/mL-resin)	2
Wash	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	5
Gradient Elution	10 mmol/L NaOH, 1.0 – 0.05 mol/L $(NH_4)_2SO_4$	10
Gradient Delay	10 mmol/L NaOH, 0.05 mol/L $(NH_4)_2SO_4$	5
CIP1	water	5
CIP2	0.5 mol/L arginine	5

Figure 2. FPLC elution profile for DMT-protected oligonucleotide purification in a linearly decreasing ammonium sulfate gradient.



Fractions were collected and analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) using the TSKgel OligoDNA-RP column *(Figure 3)*.

Figure 3. Elution profile for RP-HPLC analysis of oligonucleotide load and fractions.



Column:	TSKgel OligoDNA-RP, 4.6 ID mm × 15 cm
Mobile phase:	A: 100 mmol/L TEAA, pH 7.0
	B: acetonitrile
Gradient:	5 – 35% B, 20 min
Flow rate:	1.0 mL/min
Detection:	UV @ 254 nm (mAU)
Temperature:	45 °C
Injection vol.:	5 – 10 L (ca. 2.5 g)
Sample:	HIC fractions
Instrument:	Agilent 1100
	(Chromatograms are baseline corrected based on a water blank)

Analysis of these fractions demonstrated a large improvement in purity. While the starting material was 75% pure, the eluate was 94% pure. The recovery was 97%. As expected, only a minimal amount of DMT cleavage occurred (indicated by the red arrow in *Figure 3*).

Optimization of the one-step on-column purification with simultaneous DMT cleavage at pH 4.0

The purification process was modified to include on-column DMT cleavage *(Table 3)*.

Table 3. HIC purification with on-column cleavage at pH 4.0.

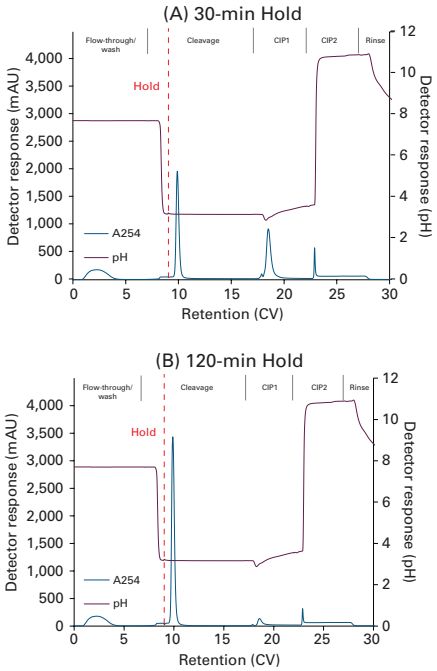
Media:	TSKgel Phenyl-3PW (20)
Column:	5 mm ID × 5 cm (1 mL)
Flow rate:	1.0 mL/min (300 cm/hr)
Detection:	UV @ 254 nm (mAU), pH
Temperature:	ambient
Instrument:	ÄKTA avant 25 (Unicorn 7.3)

Phase	Mobile phase	CV
Equilibration	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	10
Load	DMT-protected 20-mer, 2 mL @ 0.1 mg/mL (0.2 mg-oligo/mL-resin)	2
Wash	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	5
Cleavage	200 mmol/L acetic acid, 1.0 mol/L $(NH_4)_2SO_4$	10*
CIP1	water	5
CIP2	0.5 mol/L arginine	5

*A pH 4.0 hold for 120 min was included after 2 CVs.

To induce on-column cleavage, a pH 4.0 hold was introduced. During the pH hold, the column remained fully saturated in the acidic (pH 4.0) buffer for 120 minutes before collecting the eluate. The eluate contained DMT-off oligonucleotide while the DMT stayed bound to the column. Example chromatograms show (A) 30-min and (B) 120-min hold durations with no flow *(Figure 4)*. Note that after a 30-min hold duration, cleavage is incomplete, and DMT-protected material is eluted during the CIP1 step *(Figure 4A)*.

Figure 4. FPLC elution profiles for on-column DMT cleavage with various pH 4.0 hold durations (A) 30 min (B) 120 min.



pH 4.0 hold times of 0, 30, 60, and 120 minutes were tested to identify optimal hold duration *(Figure 5)*. A 120-minute hold was sufficient to obtain almost complete (93%) conversion to DMT-off oligonucleotide, indicating successful DMT cleavage from the oligonucleotide directly on the column *(Table 4)*.

Figure 5. FPLC elution profiles for various pH 4.0 hold durations

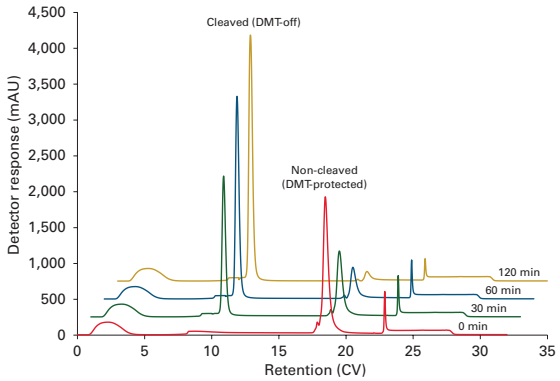


Table 4. pH 4.0 hold times evaluated and corresponding % conversion to DMT-off oligonucleotide.

Hold duration (min)	% Conversion to DMT-off
0	7
30	57
60	78
120	93

Process scale-up

The 120-minute pH 4.0 hold also proved effective when the purification process was scaled up to 10 mg-oligonucleotide/mL-resin *(Table 5, Figure 6)*.

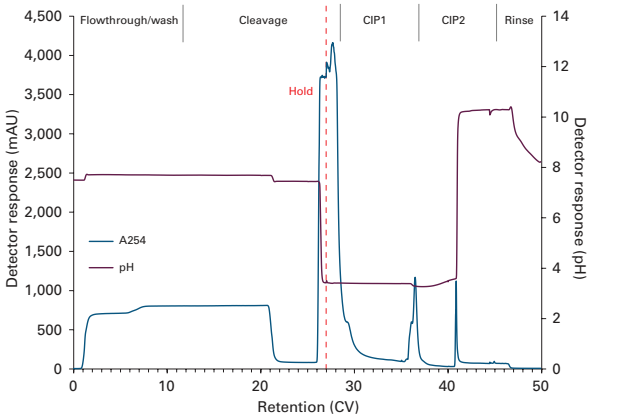
Table 5. HIC purification with simultaneous pH 4.0 on-column cleavage.

Media: TSKgel Phenyl-3PW (20)
Column: 5 mm ID × 5 cm (1 mL)
Flow rate: 1.0 mL/min (300 cm/hr)
Detection: UV @ 254 nm (mAU), pH
Temperature: ambient
Instrument: ÄKTA avant 25 (Unicorn 7.3)

Phase	Mobile phase	CV
Equilibration	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	10
Load	DMT-protected 20-mer, 20 mL @ 0.5 mg/mL (10 mg-oligo/mL-resin)	20
Wash	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	5
Cleavage	200 mmol/L acetic acid, 1.0 mol/L $(NH_4)_2SO_4$	10*
CIP1	water	5
CIP2	0.5 mol/L arginine	5

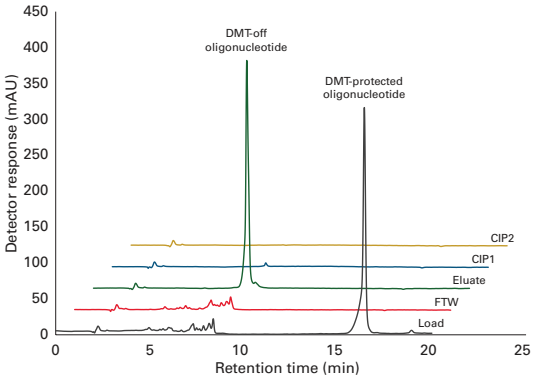
*A pH 4.0 hold for 120 min was included after 2 CVs.

Figure 6. FPLC elution profile for 10 mg-oligonucleotide/mL-resin load on-column purification and DMT cleavage.



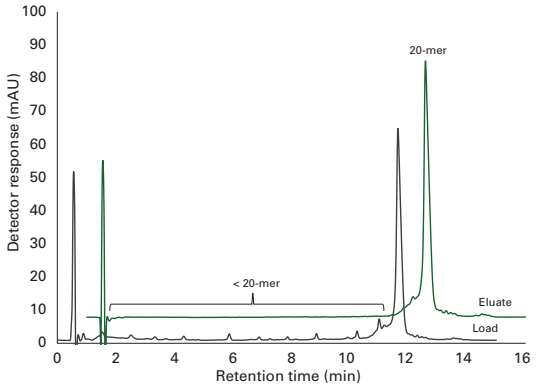
After purification and simultaneous cleavage, the load and collected fractions were analyzed to determine percent DMT-off by RP-HPLC using the TSKgel OligoDNA-RP column *(Figure 7)*. This process achieved 97% yield of DMT-off oligonucleotide, with > 99% purity.

Figure 7. Elution profile for RP-HPLC analysis using TSKgel OligoDNA-RP column.



Furthermore, the load and eluate (HIC purified product) were analyzed by AEX-HPLC using the TSKgel DNA-NPR column *(Figure 8)*. Most of the smaller (<20-mer) contaminants, commonly known as shortmers, did not bind and flowed through the HIC column. Shortmers lack the hydrophobic 5′-DMT protecting group, which is essential for interaction with the hydrophobic stationary phase. Without this hydrophobic moiety, shortmers remain hydrophilic and therefore, can effectively be removed from the full-length product.

Figure 8. Elution profile for AEX-HPLC analysis using TSKgel DNA-NPR column.



Column:	TSKgel DNA-NPR, 4.6 mm × 7.5 cm
Mobile phase:	A: 50 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 9.0 B: mobile phase A + 1.0 mol/L NaCl
Gradient:	30 – 55%B, 15 min 100%B, 2 min
Flow rate:	1.0 mL/min
Detection:	UV absorbance @ 254 nm (mAU)
Temperature:	60 °C
Injection vol.:	10 – 20 µL (ca. 1.0 µg)
Sample:	HIC fractions, diluted 1:10 in mobile phase A

(Chromatograms are baseline corrected based on a water blank)

In summary, RP-HPLC analysis showed that the (crude) oligonucleotide load contained 75% DMT-protected oligonucleotide, and the eluate (HIC purified product) contained >99% DMT-off oligonucleotide. Additionally, AEX-HPLC analysis showed that the oligonucleotide load contained 71% 20-mers, and the eluate contained 90% 20-mers. The yield of DMT-off oligonucleotide from this purification and DMT cleavage process was 97% *(Table 6)*. Additionally, a depurination assay (data not shown) to detect possible loss of adenine and guanine purine bases confirmed that the acidic cleavage and removal procedure did not damage the oligonucleotide.

Table 6. Summary of purification and simultaneous DMT cleavage with 10 mg-oligonucleotide/mL-resin load.

	% DMT-protected/off	% 20-mer	Yield (% DMT-off)
Load	75.3 (DMT-protected)	70.9	--
Eluate after 4.0 pH hold	> 99 (DMT-off)	90.3	96.9

Conclusions

The successful purification and DMT cleavage of synthetic oligonucleotides is critical for gene therapy applications. Here, we present an efficient one-step DMT-protected oligonucleotide purification process using TSKgel Phenyl-3PW (20) HIC resin. A novel on-column cleavage and removal of the 5'DMT-cap from the oligonucleotide at pH 4.0 was achieved. The low pH treatment did not damage the oligonucleotide, and the process demonstrated good scalability. In summary, this study describes an effective purification and analytical methodology for ssDNA oligonucleotides, an important modality for targeted and selective medicines.

Featured Products

Part #	Description
0045560	SkillPak 1 TSKgel Phenyl-3PW (20), 1 mL x 5 Column
0013352	TSKgel OligoDNA-RP
0018249	TSKgel DNA-NPR