One-step DMT-protected oligonucleotide purification and simultaneous on-column detritylation using hydrophobic interaction chromatography

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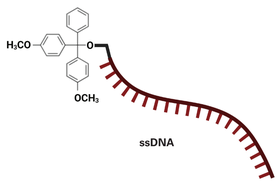
Introduction

- Synthetic antisense oligonucleotide (ASO) therapeutics continue to provide new, effective treatments for various diseases. These include debilitating neurological, metabolic, cardiovascular, and muscular conditions.
- Within the biopharmaceutical industry, the demand for purification and analytical techniques for ASOs has increased to meet expanded pipelines.
- Here, we demonstrate a one-step hydrophobic interaction chromatography (HIC) process for purification of a 5′-dimethoxytrityl (5′-DMT)-protected 20-mer single-stranded DNA (ssDNA) oligonucleotide using a TSKgel Phenyl-3PW (20) resin.
- The HIC step includes a novel on-column DMT cleavage (detritylation) method for 5′-DMT removal at pH 4.0.
- This method also removes < 20-mer ssDNA impurities that are present as DMT-off oligonucleotides in a crude starting material.
- The process was tested with a load ratio of up to 10 mg oligonucleotide per mL of resin to demonstrate good scalability.
- Analytical methods, which include reversed-phase (RP) chromatography and anion-exchange chromatography (AEX), demonstrated >95% product purity and 97% DMT-off oligonucleotide recovery for the final product.
- Additionally, a depurination assay to detect possible loss of purine bases (adenine and guanine) confirms that the acidic detritylation procedure does not cause damage to the oligonucleotide.

Oligonucleotide and HIC Resin



Graphic representation of DMT-protected 20-mer oligonucleotide for this study

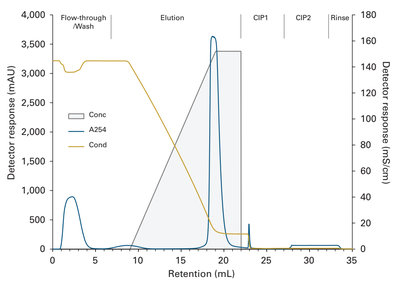
Property	TSKgel® Phenyl-3PW (20)
Bead Material	polymethacrylate
Average Bead Diameter	20 µm
Average Pore Diameter	25 nm
Maximum Pressure	2.0 MPa
Dynamic Binding Capacity*	25 mg/mL (at 1.5 mol/L, >45 mg/mL)

*Crude DMT-on 20 mer oligonucleotide; 10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$; 4 min residence time

Results 1: Purification in $(NH_4)_2SO_4$ Gradient

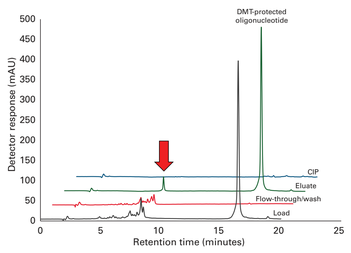
Media: TSKgel Phenyl-3PW (20)
Column: 5 mm × 5 cm (1 mL)
Flow rate: 1.0 mL/min (300 cm/hr)
Detection: UV @ 254 nm (mAU), conductivity (mS/cm)
Temperature: ambient
Instrument: ÄKTA avant™ 25 (Unicorn 7.3)

Step	Mobile phase	CV
Equilibration	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	10
Load	DMT-protected 20mer, 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
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



- The 20-mer DMT-protected oligonucleotide was purified in a linearly decreasing gradient from 1 mol/L to 0.05 mol/L $(NH_4)_2SO_4$.
- Load, flow-through/wash, and elution peaks were collected and analyzed by RP-HPLC.

RP-HPLC:



Column: TSKgel OligoDNA-RP, 4.6 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 ºC
Injection vol.: 5 – 10 µL (ca. 2.5 µg)
Sample: HIC fractions
Instrument: Agilent 1100
(Chromatograms are baseline corrected based on a water blank)

- Analysis of the fractions demonstrated that the eluate purity was 94% DMT-protected oligonucleotides with a 97% recovery as compared to the starting material which had ~75% oligonucleotide purity.
- As expected, only a minimal amount of DMT cleavage occurs at pH 7.0 under ambient temperature. Elution of DMT-off nucleotide marked with red arrow.

Results 2: Optimization of the One-step On-column Purification with Concurrent Low pH DMT Cleavage

Principle:

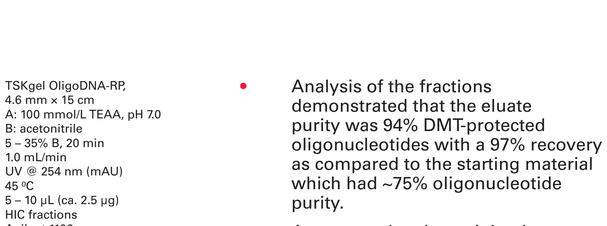
- We modified our purification process to include on-column DMT cleavage.
- DMT-protected oligonucleotides were bound to a HIC column and eluted by cleavage under acidic conditions (pH ~4).
- To optimize the DMT cleavage, an on-column low pH hold time was evaluated with a 0.2 mg-oligo/mL-resin load ratio.

On-column Purification with Low pH DMT Cleavage (0.2 mg-oligo/mL-resin load):

Media: TSKgel Phenyl-3PW (20)
Column: 5 mm × 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)

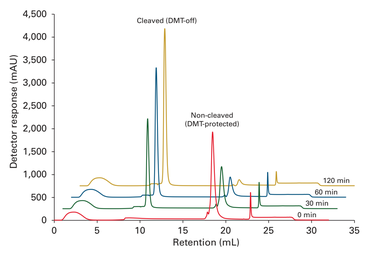
Step	Mobile phase	CV
Equilibration	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	10
Load	DMT-protected 20mer, 2 mL @ 0.1 mg/mL (0.2 mg-oligo/mL-resin)	2
Wash	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	5
Cleavage	200 mmol/L acetic acid, 1.0 mol/L $(NH_4)_2SO_4$	10*
CIP1	water	5
CIP2	0.5 mol/L arginine	5

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



- These chromatograms show a 30-min and 120-min hold duration with no flow. Note that after a 30-min hold duration, cleavage is incomplete, and DMT-protected material is eluted during the CIP1 step.

On-column Low pH DMT Cleavage Time Course:

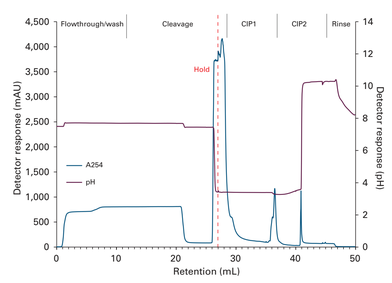


Hold duration (min)	% Conversion to DMT-off
0	7
30	57
60	78
120	93

- We evaluated hold times of 0, 30, 60, and 120 minutes to identify an optimal hold duration.
- Peaks were collected and analyzed by RP-HPLC using TSKgel OligoDNA-RP column as described above.
- Integration of FPLC peak areas was done to estimate the % conversion of DMT-protected to DMT-off oligonucleotides.
- **A 2-hour hold duration was sufficient to obtain almost complete conversion to DMT-off oligonucleotides, indicating successful on-column DMT cleavage.**

Results 3: Scaling Up the Process

On-column Purification and DMT Cleavage (10 mg-oligo/mL-resin load)



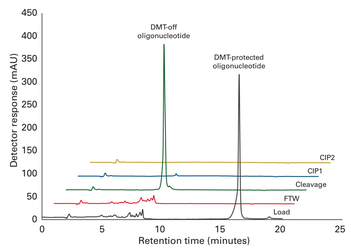
Media: TSKgel Phenyl-3PW (20)
Column: 5 mm × 5 cm (1 mL)
Flow rate: 1.0 mL/min (300 cm/hr)
Detection: UV @ 254 nm (mAU), pH
Temperature: ambient
Instrument: ÄKTA avant 25 (Unicorn 7.3)

Step	Mobile phase	CV
Equilibration	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$	10
Load	DMT-protected 20mer, 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.

- Peaks were collected and analyzed by RP-HPLC using TSKgel OligoDNA-RP column.
- Additionally, the cleavage peak was analyzed by AEX-HPLC and compared to a DMT-off crude sample.

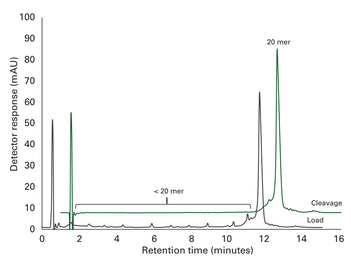
RP-HPLC:



Column: TSKgel OligoDNA-RP, 4.6 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 ºC
Injection vol.: 5 – 10 µL (ca. 2.5 µg)
Sample: HIC fractions
Instrument: Agilent 1100
(Chromatograms are baseline corrected based on a water blank)

- RP-HPLC analysis of the fractions demonstrated that the eluate was comprised of > 99% DMT-off oligonucleotides with a 97% recovery.
- The original DMT-off impurities present in the crude (load) material were also removed during HIC process.

AEX-HPLC:



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 ºC
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)

- We analyzed the HIC purification product also using TSKgel DNA-NPR anion exchange column.
- On-column HIC cleavage results in a product with > 90% purity (20-mer oligonucleotides).
- Most of the smaller (< 20-mer) contaminants did not bind to the HIC column and thus, as compared to a load sample, were absent from the final product.

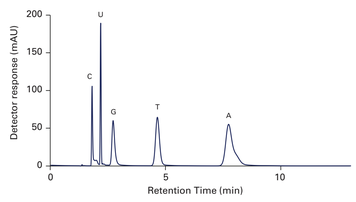
Summary of On-column Purification with Concurrent Low pH DMT Cleavage (10 mg-oligo/mL-resin load):

Fraction	Volume (mL)	Purity (%)	Purity (% 20mer)	Yield (% DMT-off)
Load	20.0	75.3 (DMT-protected)	70.9	--
Eluate after low pH hold	8.8	> 99 (DMT-off)	90.3	96.9

Results 4: Detection of Possible Depurination

- 5′-DMT is a protecting group that prevents unwanted side reactions at the 5′ hydroxy site but needs to be removed (“detritylation”) from the final oligonucleotide product.
- The process of detritylation may also cause depurination, a type of DNA damage in which the N-glycosidic bond is cleaved, releasing the free purines (adenine and guanine).
- Depurination results in an apurinic site (i.e. non-readable DNA encoding), which can lead to inefficiency with cellular function and genome stability.
- Depurination can be followed using RP chromatography (PLoS ONE, 9(12): e115950, 2014).

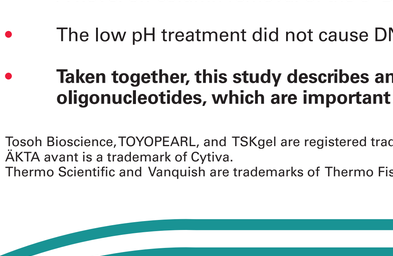
Nucleobase Standards Mix:



Column: TSKgel ODS-100V, 2 mm × 15 cm, 5 µm
Mobile phase: A: 50 mmol/L ammonium formate, pH 7.0
B: 100% acetonitrile
Gradient: 0-4% B (13 min), 80% B (3 min), 0% B (5 min)
Flow rate: 0.25 mL/min
Detection: UV absorbance @ 254 nm (mAU)
Temperature: 40 ºC
Instrument: Thermo Scientific™ Vanquish™ (Chromeleon 7.2.10)

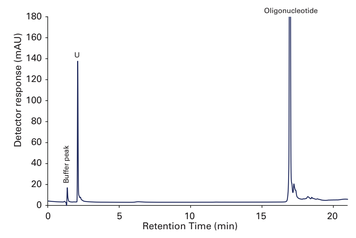
- We prepared a nucleobase standards mix by combining the 5 nucleobases, cytosine (C), uracil (U), guanine (G), thymine (T), and adenine (A), and separated them using RP column.
- In the depurination RP assay with oligonucleotide samples, the appearance of adenine and guanine nucleobases indicates depurination damage to the oligonucleotide.
- Uracil was added to all samples as an internal standard prior to RP analysis.

Induced Complete Depurination of DMT-off Oligonucleotide



- Here, total depurination was induced to prepare a positive control for the assay.
- DMT-off oligonucleotide product was kept at pH 1.6 for 1 hour with heating (90 °C).
- Uracil was added as an internal standard, and elution of oligonucleotide occurred at 17 min retention time.
- The appearance of guanine (G) and adenine (A) peaks indicates depurination process.
- **The result indicates that the depurination assay works as expected.**

Depurination Analysis of Final Product after On-column Removal of DMT-cap at pH 4



- **No sign of depurination (appearance of adenine (A) or guanine (G)) was observed in the sample.**
- Uracil was added as an internal standard.

Conclusions

- Here, we present an efficient one-step DMT-protected oligonucleotide purification process using TSKgel Phenyl-3PW (20) HIC resin.
- A novel on-column removal of the 5′-DMT-protective cap from the oligonucleotide at pH 4.0 is described.
- The low pH treatment did not cause DNA damage, and the process demonstrates good scalability.
- **Taken together, this study describes an effective purification and analytical methodology for antisense oligonucleotides, which are important modalities for targeted and selective cell & gene therapy medicines.**

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