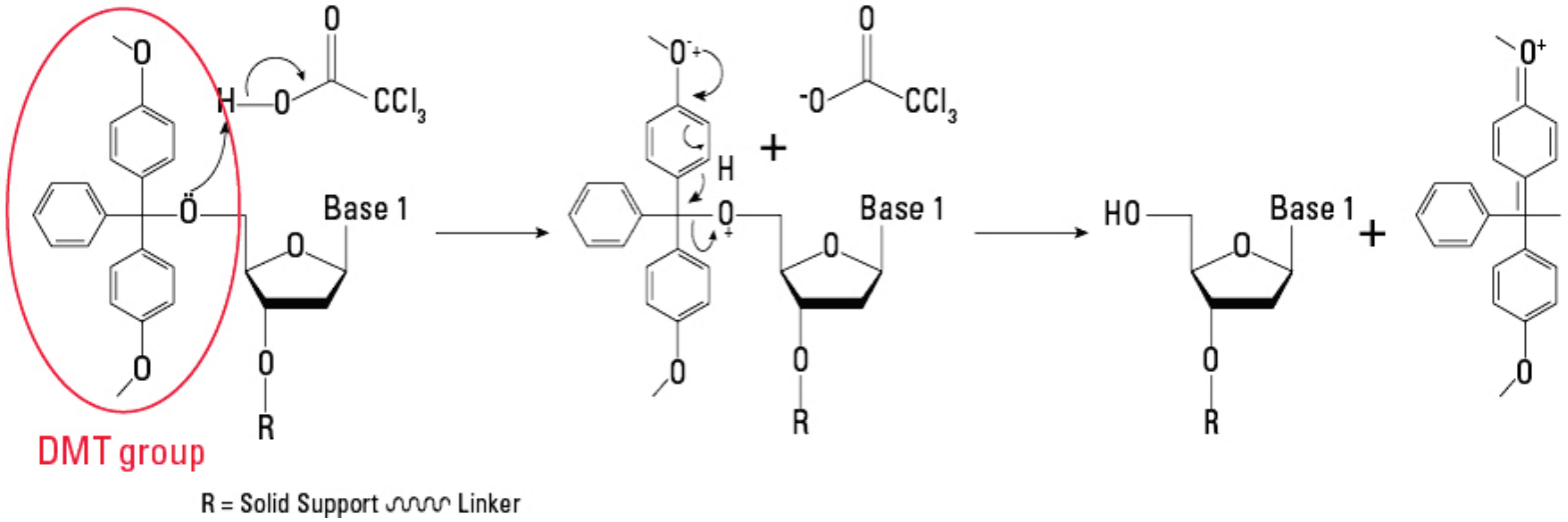
An Effective One-step Purification of 5'-DMT-On Oligonucleotides Using Hydrophobic Interaction Chromatography

William E. Evans, M.S. and Jukka Kervinen, Ph.D.
Tosoh Bioscience LLC, King of Prussia, PA 19406

Introduction

One-step purification of oligonucleotides is presented using hydrophobic interaction chromatography (HIC). This one-step purification includes the isolation of the DMT-on oligonucleotide from its impurities from the feedstock. This presentation also demonstrates the removal of the DMT-protecting group from the DMT-on oligonucleotide directly on the column to obtain a purified oligonucleotide without a DMT group.

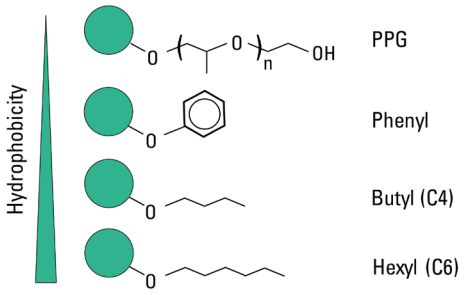


Materials and Methods

- A crude, 20-mer, DMT-on oligonucleotide was obtained from Trilink Technologies (San Diego, CA) with the following sequence:

  5'-GAA TTC ATC GGT TCA GAG AC-3’

- Separations were performed with TOYOPEARL® porous polymethacrylate media:
  - TOYOPEARL PPG-600M
  - TOYOPEARL Phenyl-650M
  - TOYOPEARL Butyl-650M
  - TOYOPEARL Hexyl-650C



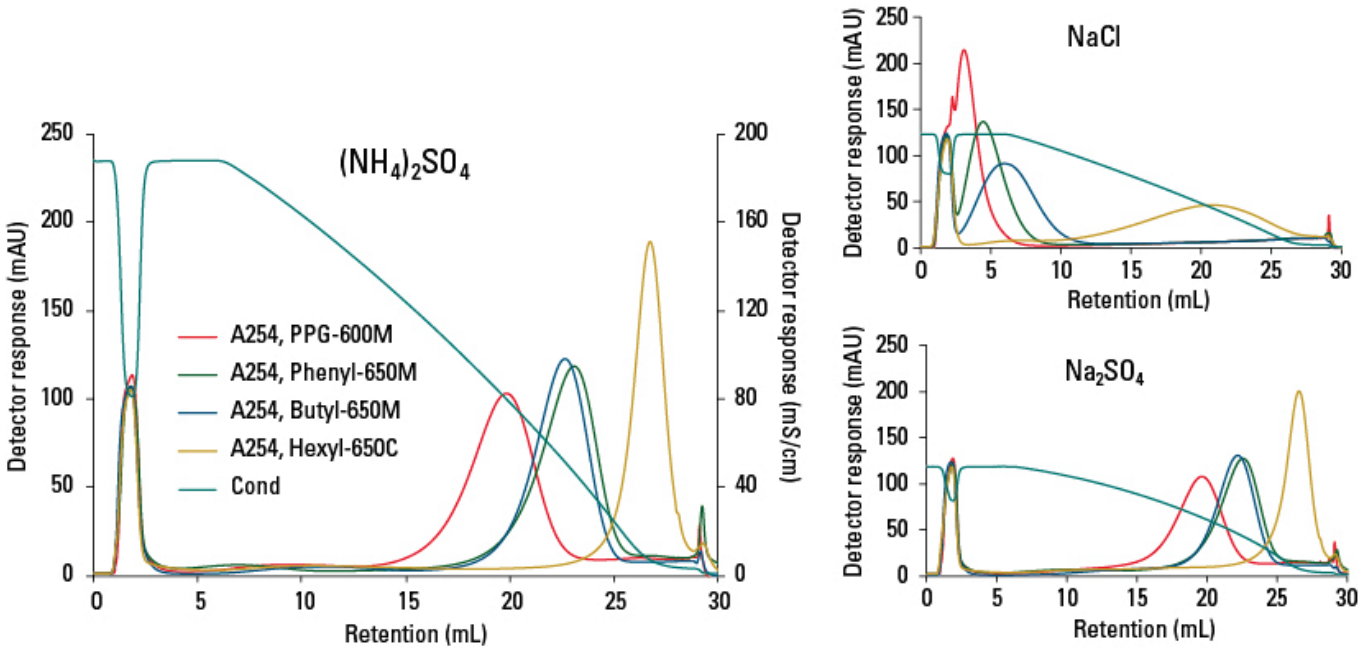
- Instrumentation ÄKTA™ avant 25 FPLC

Effects of Different Salts on Oligonucleotide Elution from HIC

Phase	Volume (mL)	Buffer
Equilibration	10	20 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 7.5 (TE) + 1.5 mol/L salt as indicated
Load	1	DMT-on 20-mer, 0.1 mg/mL in TE + 1 mol/L $(NH_4)_2SO_4$
Wash	4	TE + 1.5 mol/L salt as indicated
Gradient elution	20	TE + 1.5 – 0 mmol/L salt as indicated
Wash	4	TE
CIP	5	30% isopropyl alcohol

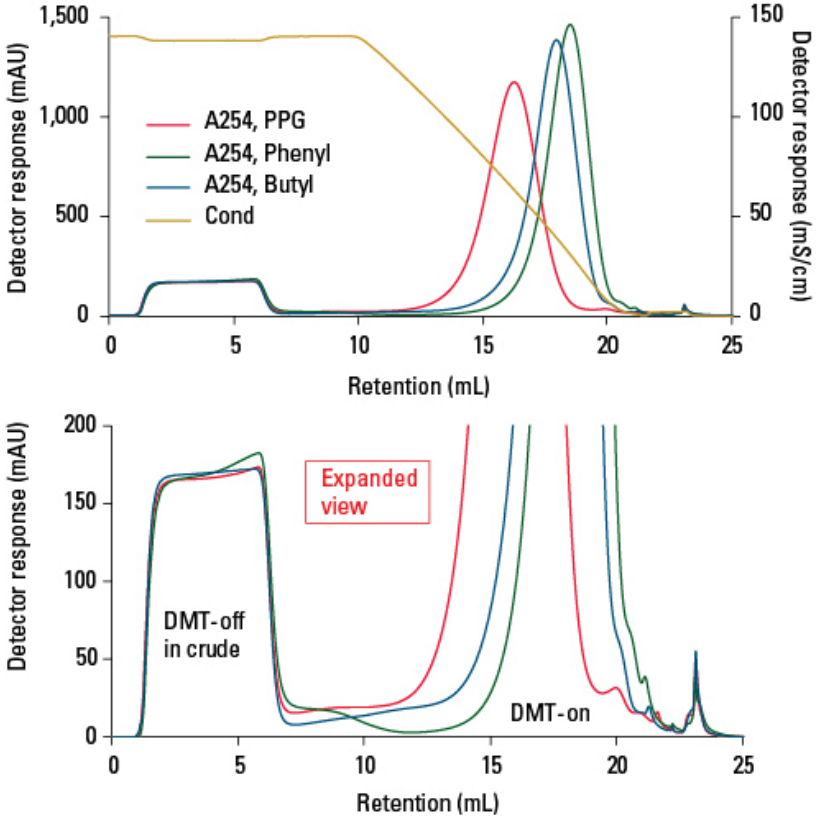
Media	TOYOPEARL PPG-600M, Phenyl-650M, Butyl-650M, or Hexyl-650C
Column	5 mm ID × 5 cm (1 mL)
Flow rate	1.0 mL/min (1 min residence time)
Detection	UV @ 254 nm (mAU), conductivity (mS/cm)
Temperature	ambient

Effects of Different Salts on Oligonucleotide Elution from HIC



- The order of hydrophobicity from least to most hydrophobic is: PPG < butyl < phenyl < hexyl
- Ammonium sulfate was the most effective salt for separating DMT-on oligonucleotides from its impurities for all HIC resins.
- Ammonium sulfate was selected as the salt for all experiments based on binding effectiveness and linear conductivity response.

The Effect of HIC Stationary Phases on DMT-on Oligonucleotide Purification



- Crude preparation contains both DMT-off (primarily < 20-mer) and DMT-on oligonucleotides
- All ligands gave similar elution profiles
- All resins effectively separated DMT-off and DMT-on oligos
- Phenyl and butyl had a stronger binding compared to PPG

Effects of Media on Purity and Recovery

Fraction	Fraction Volume (mL)	Avg. Conductivity (mS/cm)	Purity (% DMT-on)	Recovery (% DMT-on)
Load			*77.9*	
PPG elution	4.9	68.0	98.7	89.1
Butyl elution	4.5	45.9	99.0	89.0
Phenyl elution	4.2	36.8	99.0	88.9

- All media give similar performance in the purity (99%) and recovery (89%) of DMT-protected oligonucleotide as determined by reversed phase HPLC (RP-HPLC).
- Due to higher recovery, TOYOPEARL Phenyl-650M was chosen for further study.

On-column DMT Cleavage to Release Oligonucleotides

To demonstrate the on-column removal of the DMT protecting group from the oligonucleotide, DMT-on oligonucleotide was bound to a phenyl stationary phase at 1 mol/L ammonium sulfate, followed by wash to remove unbound material. Cleavage of the DMT protecting group was performed by **acidification**. A two-step wash was performed to remove bound DMT between cycles.

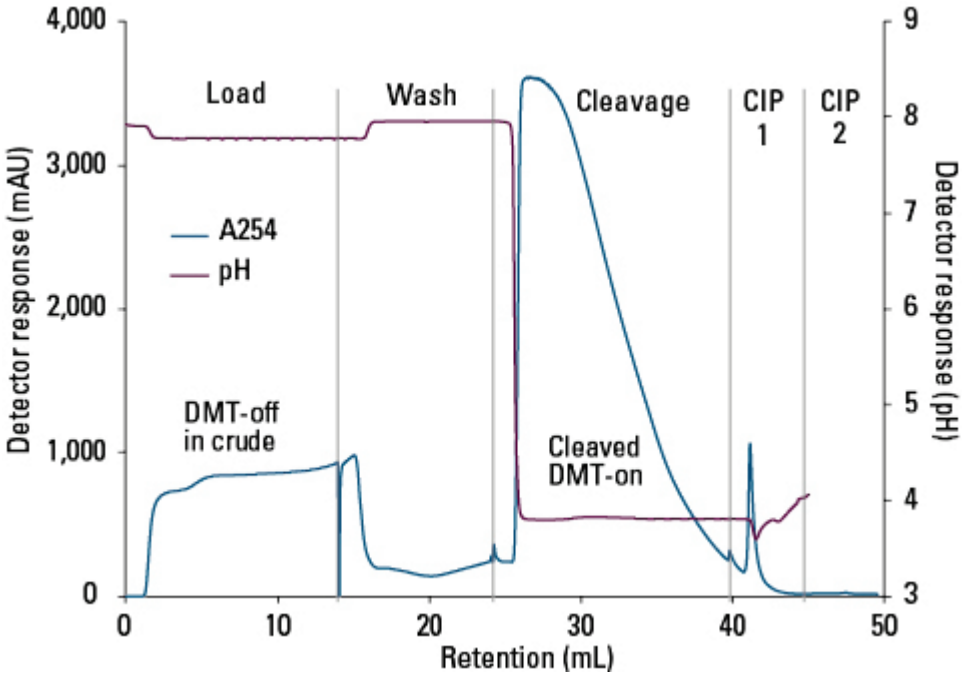
Material from the cleavage fraction was analyzed by reversed phase HPLC to determine recovery and purity of DMT-on oligonucleotides.

On-column DMT Group Cleavage Condition

Media	TOYOPEARL Phenyl-650M
Column	6.6 mm ID × 3.0 cm (1 mL)
Flow rate	0.25 mL/min (4 min residence time)
Detection	UV @ 254 nm (mAU), pH
Temperature	ambient

Phase	Volume (mL)	Buffer
Equilibration	10	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$
Load	14	DMT-oligo, 0.5 mg/mL (7 mg)
Wash	10	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$
Cleavage	15	50 mmol/L acetic acid, 1.0 mol/L $(NH_4)_2SO_4$
CIP 1	5	water
CIP 2	5	30% (v/v) 2-propanol

Result of On-column Cleavage of DMT-group



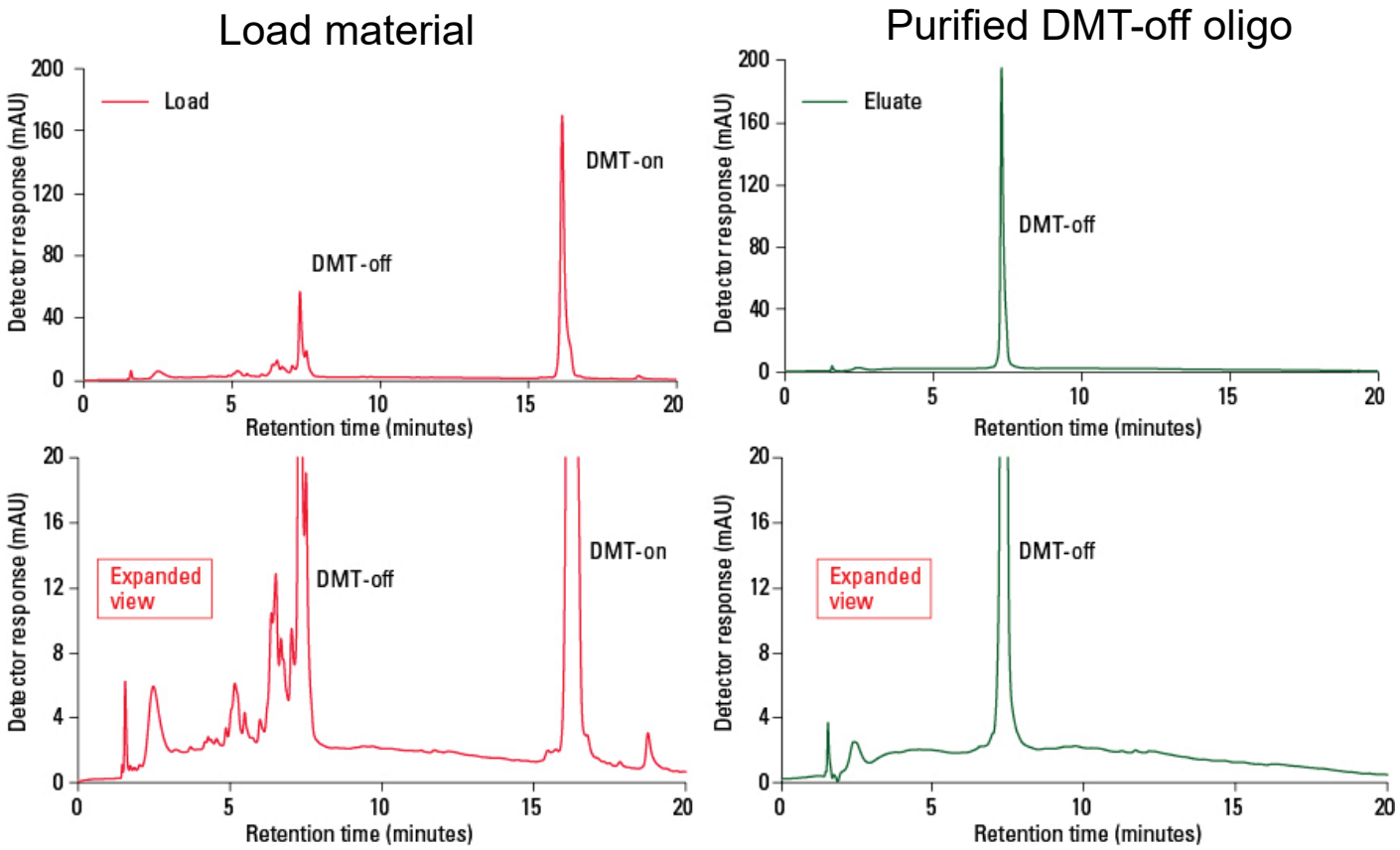
- DMT-off impurities present in the crude flow-through - primarily <20-mer oligos
- DMT-on oligos are bound
- Lowering pH to ~4 initiated DMT cleavage and DMT-off oligonucleotides were eluted from the column.

Oligonucleotide Analysis by Reversed Phase HPLC Column

To evaluate the removal of the DMT-protecting group, samples were analyzed by reversed phase HPLC. The purity and recovery of DMT-off oligonucleotide was calculated from the relative peak areas in the crude (load) and cleavage (eluate) fractions.

Column	TSKgel® OligoDNA-RP, 4.6 mm ID × 15 cm
Mobile phase A	100 mmol/L TEAA, pH 7.0
Mobile phase B	acetonitrile
Gradient	5 - 35% B, 20 min
Temperature	45 °C
Flow Rate	1.25 mL/min
Detection	UV @ 254 nm (mAU)
Injection Vol.	10 µL

Analysis of Crude vs Purified Sample Using Reversed Phase Chromatography



- Analysis by RPC confirms that DMT group is removed (not present in eluted oligonucleotide)
- On-column DMT cleavage resulted in > 99% pure DMT-off oligo at 99% recovery

Conclusions

- Hydrophobic interaction chromatography with ammonium sulfate is effective in separating DMT-on from DMT-off oligonucleotides in a crude preparation.
- On-column cleavage at low pH may be used to remove DMT group and elute DMT-off oligonucleotides.
- High purity and recovery was achieved for on-column cleavage procedure at pH ~4.