The challenge of phosphorothioate oligonucleotide purifications – optimization of AIEX parameters

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Introduction

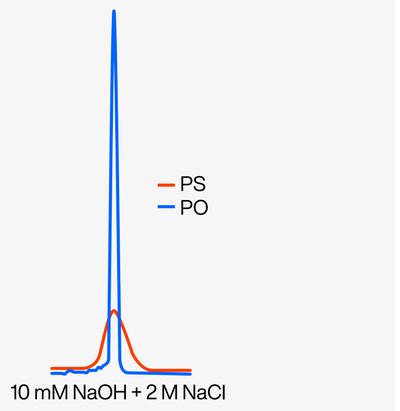
Since oligonucleotides (ONs) are mainly synthetically produced by solid-phase synthesis it is easy to modify them during the synthesis process. The most commonly used modification, phosphorothioate (PS) linkages in which the oxygen atom is replaced by a sulfur atom in the backbone, increases the in vivo stability by reducing the risk of decay by nucleases. But it also makes the downstream purification of oligonucleotides more challenging.

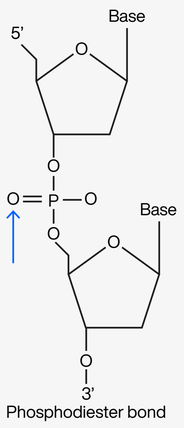
PO oligonucleotides

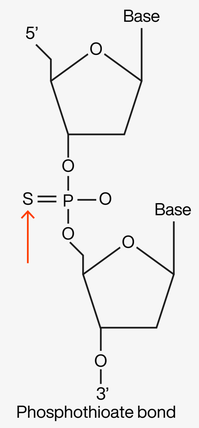
Native ONs, i.e. non-modified phosphorodiester ON (PO), can be purified using normal buffers without the requirements of any additives (e.g. 20 mM Tris, pH 8 + 1.5 M NaCl).

Narrow peaks → good separation → easy pool collection → purity ≥95% with high yields at most scales

PS oligonucleotides = challenge due to increased hydrophobicity







Considerations for PS oligonucleotides when purifying with IEX

- Broad peaks due to presence of stereoisomers and increased hydrophobic properties
- Number of stereoisomers for a sequence containing n PS linkages = n_{PS}^2

Native 20-mer ONs are relatively easy to purify with standard buffers compared to same PS modified sequence → broad peaks obtained for PS ONs.

Chaotropic factors

Chaotropic agents (complex-breaking agents) are needed to prevent and reduce the hydrophobic complex formations within or between the PS ONs in ion exchange chromatography (IEX) purification setups.

- Higher pH
- Organic modifiers (acetonitrile)
- Elution salts (NaBr and $NaClO_4$)
- Elevated temperature

} chaotropic agents/conditions

Screening for optimal IEX running conditions

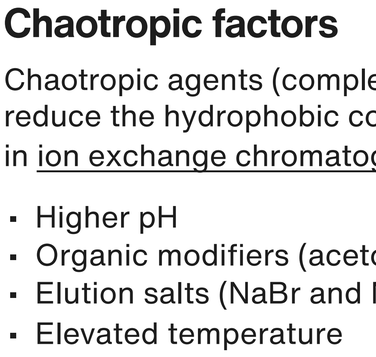
Sample: 20 nt PS ON
Buffer: 10 mM NaOH (pH 12)
Resin: WorkBeads™ 40Q (45 µm beads)

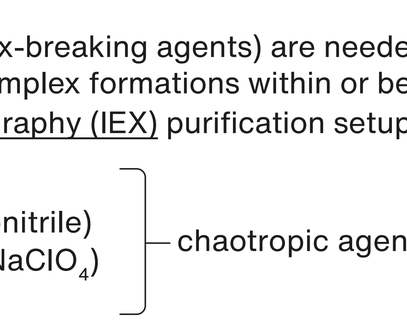
Parameters tested/included in buffers:

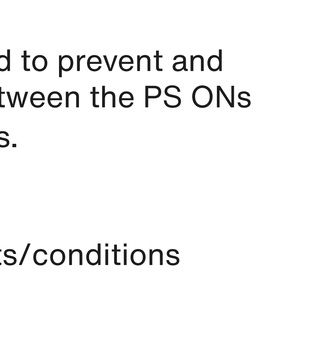
- Organic modifiers: 0-20% acetonitrile (ACN)
- Elution salts of increasing chaotrophy: NaCl - NaBr - $NaClO_4$
- Elevated temperature

Output values in the screening: yield, peak width and conductivity

WorkBeads 40Q - resin with small pore sizes optimal for ON purifications

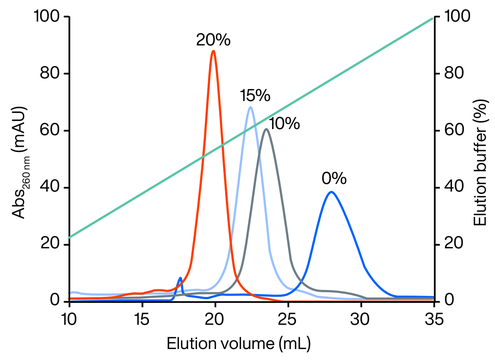




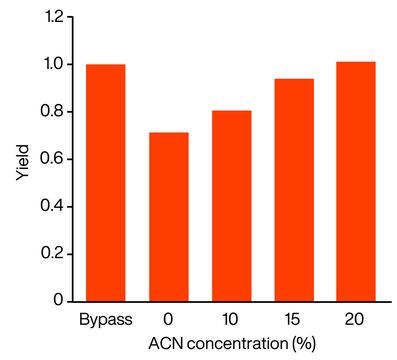


- Smaller pore sizes → higher available interaction area → higher binding capacities
- More narrow pore size distribution (PSD) → better mass transport → increased purity

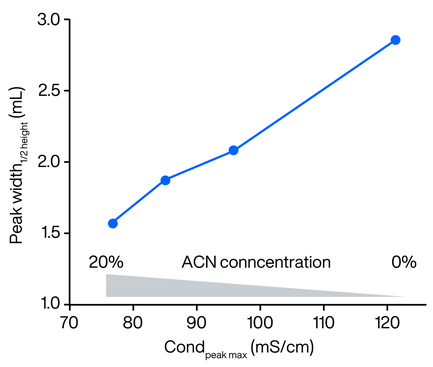
ACN additions



ACN additions



Elution salt effects



- 20% ACN needed for full recovery and efficiency
- $NaClO_4$, the most chaotropic salt, promoted highest efficiency
- Elevated temperature not required
- Both ACN and $NaClO_4$ minimized non-specific interactions → elution at lower conductivity

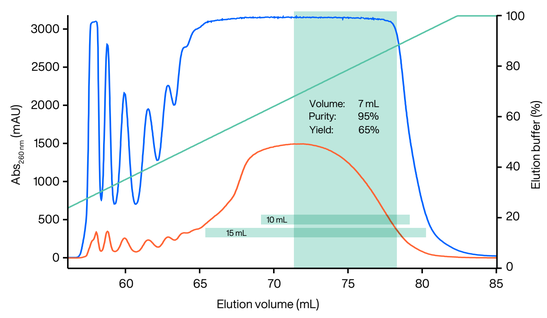
→ Optimal conditions: 10 mM NaOH, 20% ACN, + 0.4 M $NaClO_4$ (22°C)
→ Up to 40 mg PS ON can be loaded/mL resin without breakthrough

PS ON purifications

Preparative sample loading, of 20 mg PS ON feed (78% purity)/mL resin.
Aim: Target purity of 95%

Column: WorkBeads 40Q, 6.6 x 50 mm
Binding buffer: 10 mM NaOH, 20% ACN
Elution buffer: 10 mM NaOH, 20% ACN, 0.4 M $NaClO_4$
Gradient: 0-100% elution buffer in 20 CV
Flow rate: 0.45 mL/min (4 min residence time (RT))

(A)



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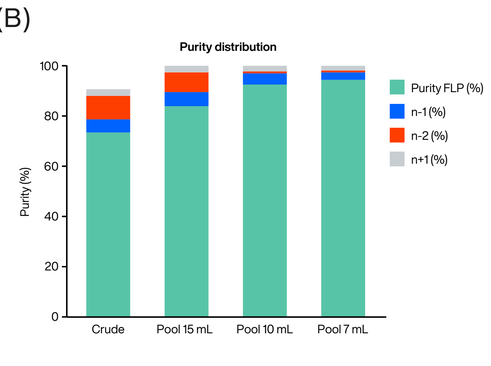

(A) PS ON elution pattern on AIEX. 20 mg PS ON crude was purified per mL WorkBeads 40Q resin. Elution buffer gradient (green), UV traces 260 nm (blue), and 295 nm (orange). Green boxes represent collected pools. (B) PS ON purity distribution within the crude, and each pool, for the resolved species: FLP (green), n-1 (blue), n-2 (red) and n+1 (grey) using RPC analysis.

Volume (mL)	Yield (%)	Purity (%)
7	**65**	**95**
10	85	92
15	93	85

- 95% purity at 65% yield (7 mL)
- Larger pools: reduced purity, increased yield
- A_{295} facilitates pool collection

Conclusions

- PS ON preparation (20-mer DNA) from solid phase was purified from 78% to 95% at preparative loading scale (yield 65%) using IEX as stand-alone step
- Optimizations of running conditions are essential for PS ON crudes
- Chaotropic additives required, such as ACN and $NaClO_4$
- When chaotropic additives were present, elevated temperature had no effect

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