Purification and quality control of an RNA 21-mer oligonucleotide using Capto™ Q ImpRes and LC-MS

Justin Townsend[1], Linda Hagman[2], Filip Bergqvist, Simon Åberg, Ganesh Nawale, Per Denker, Peter Guterstam
Cytiva, Björkgatan 30, 751 84 Uppsala, Sweden

Introduction

Therapeutic oligonucleotides are a chemical modality with diverse structures and great potential to treat diseases by modifying protein expression. These oligonucleotides, which can include siRNA or antisense oligonucleotides (ASO) that are single-stranded DNA or RNA consisting of ~ 20 nucleotides. These therapeutics are synthesized chemically base-to-base, and the resulting crude synthesis requires sophisticated purification to isolate the target oligonucleotide. Modern Capto™ resins have improved pressure/flow properties for high-resolution purifications that are fully scalable from milliliters to liters with single-use possibilities. We purified a 21-mer RNA oligonucleotide in a salt gradient using strong anion exchange Capto Q ImpRes resin with concentration and purity determinations by analytical anion-exchange chromatography. In addition, we characterized the oligonucleotide and product-related impurities with ion-pair, reversed-phase chromatography (IP-RPC) coupled to mass spectrometry (MS)—a method that is accurate, precise, and robust with good limit-of-detection and high resolution in liquid chromatography (LC) and high resolving power in MS.

Synthesis

The RNA oligonucleotide (21-mer) was synthesized on ÄKTA oligosynt™ system at 1.4 mmol scale using Primer Support™ 5G T300 solid support at ~ 308 µmol/g packed in a fixed bed stainless steel column (3.1 cm bed height, 48 mL column volume). Cleavage from solid support and deprotection of the base protecting group was performed with 1:1 mixture of aqueous ammonia and methylamine at room temperature followed by 2′ silyl group deprotection in one-pot approach with TEA.3HF. The crude synthesis yield was 84% with a concentration of 260 OD/mL.

Capto Q ImpRes buffer screening

Screening of buffers at pH 7.5 and 9.0 was performed for a 21-mer using Capto Q ImpRes resin. Before loading on the anion exchanger, crude oligo was desalted to Tris at pH 7.5 or 9.0. Urea or glycerol conditions were conditioned just before load. The column load was 2.6 mg/mL of desalted crude with 84% full length product (FLP) purity.

The main peak was fractionated into two fractions and analyzed by HPLC resulting in > 95% purity observed for all investigated elution buffers conditions.

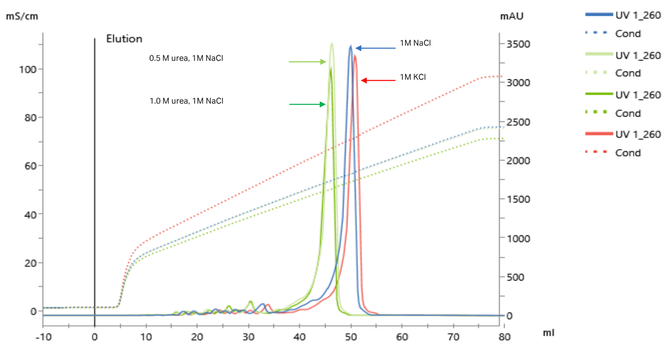

Fig 1. Capto Q ImpRes salt screening using Tris buffer pH 7.5 (with and without urea) supplemented with different salts in the elution buffer.

Urea containing samples eluted earlier at pH 7.5 compared to non-urea conditions. Urea did not seem to impact the purity in the evaluated conditions (Fig 1).

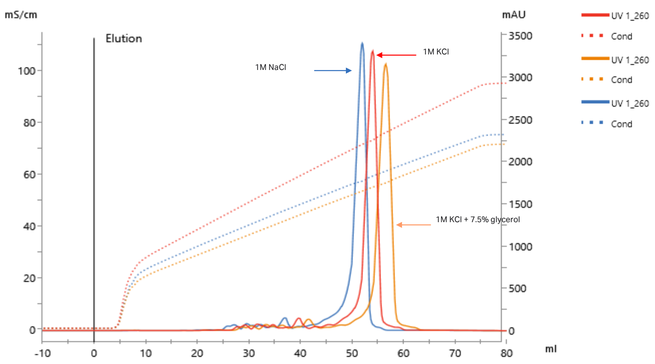

Fig 2. HiScreen™ Capto Q ImpRes salt screening using Tris buffer pH 9.0 (with and without glycerol) supplemented with different salts in the elution buffer.

At pH 9.0 KCl, the oligo elutes later in the gradient and at higher conductivity compared to NaCl. Comparing FLP elution peak at peak max, the conductivity is ~ 56 mS/cm in the glycerol and KCl combination and ~ 73 mS/cm in the KCl buffer (Fig 2).

All evaluated conditions shows high purity and have potential to work at higher column loads.

Urea or glycerol can be used as a supplement to limit undesired intra- or inter-oligo interactions (1). But using them does also add complexity and possible wastewater treatment in case of urea.

1. Kanwal F, Lu C. A review on Native and denaturing purification methods for non-coding RNA (nc RNA). *J Chromatogr B.* 2019;1120:71-79. doi:10.1016/j.jchromb.2019.04.034

Dynamic binding capacity (DBC)

Table 1. DBC at 20 mM Tris pH 7.5 and 25 mM Tris pH 9.0

Buffer	Qb_{10} DCB
20 mM Tris pH 7.5	44 mg/mL
25 mM Tris pH 9.0	46 mg/mL

High purity on Capto Q ImpRes at high load

Crude oligo from the synthesis was desalted using a HiPrep™ desalting column to 25 mM Tris pH 9.0. The desalted crude was loaded on a HiScreen Capto Q ImpRes column at 80% of DBC (total 174 mg) and the full-length product (FLP) was eluted in a 15 CV linear gradient (10% to 80% B) of 25 mM Tris, 1 M NaCl pH 9.0. The eluate was fractionated and analyzed with IEX-HPLC for FLP purity (Fig 3).

Pooling fractions with > 90% purity resulted in 86% recovery with 94% purity (Fig 4). Similar results were obtained for KCl gradient at pH 9.0.

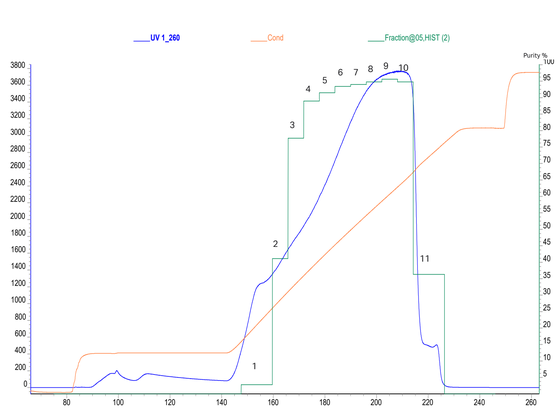

Fig 3. Purification of desalted R21-mer on HiScreen Capto Q ImpRes using an ÄKTA pure™ 25

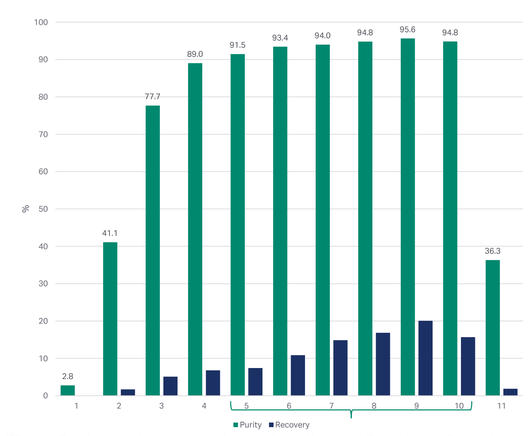

Fig 4. Purity and recovery in each collected fraction of total amount crude oligo according to HPLC

Quality confirmation by intact mass analysis using LC-MS

The desalted crude (load) from oligo synthesis and the fractions from the purification with HiScreen Capto Q ImpRes were analyzed with ion-pair reversed-phase chromatography (IP-RPC) coupled to liquid chromatography–mass spectrometry (LC-MS). The high purity observed for fractions 5–10 with IEX-HPLC was confirmed with IP-RPC LC-MS by integrating the UV response (Table 2). In addition, the MS response for FLP and the top-five product-related impurities are presented in Figure 5 with corresponding sequences presented in Table 3.

Sample	Purity (IEX-HPLC)	Purity (IP-RPC LC-MS)
Crude (load)	85%	90%
Fraction 1	3%	< 10%
Fraction 2	41%	46%
Fraction 3	78%	81%
Fraction 4	89%	91%
Fraction 5	92%	93%
Fraction 6	93%	94%
Fraction 7	94%	95%
Fraction 8	95%	95%
Fraction9	96%	96%
Fraction 10	95%	94%
Fraction 11	36%	52%

Table 2. Purity obtained with IEX-HPLC and IP-RPC LC-MS shows that both methods give comparable results when the oligo concentration is reasonable (see Figs 3 and 4).

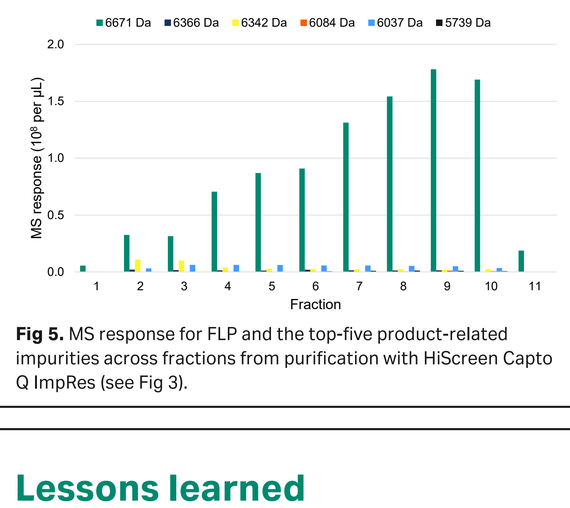

Fig 5. MS response for FLP and the top-five product-related impurities across fractions from purification with HiScreen Capto Q ImpRes (see Fig 3).

Mass (Da)	Molecule	Sequence
6671 Da	Full-length product (FLP)	ACG UUG CAG ACU CCU AAU GGT
6366 Da	FLP with loss of 3′-T	ACG UUG CAG ACU CCU AAU GG
6342 Da	FLP with loss of 5′-A	CG UUG CAG ACU CCU AAU GGT
6084 Da	FLP with loss of 587 Da	Unknown
6037 Da	FLP with loss of 5′-AC	G UUG CAG ACU CCU AAU GGT
5739 Da	FLP with loss of 932 Da	Unknown

Table 3. Measured mass for FLP and the top-five product-related impurities and their corresponding sequences.

Lessons learned

Pre-treatment improves performance of Capto Q ImpRes purification. Desalted load compared to diluted load resulted in higher recoveries with retained purity. However, cost- and time-efficiency must be considered in a manufacturing process. Higher column loads result in earlier elution due to displacement effects and must be considered in process development. Finally, residence time correlates with improved resolution using Capto Q ImpRes.

When working with native RNA, make sure to work RNase-free in downstream. Native RNA oligos rapidly degrade in high salt and high pH as compared to buffer only. Inclusion of EDTA in buffers or samples may cause undesired pH shift due to its ability to interact with anion exchange chromatography columns (2).

2. Chumanov RS, Burgess RR. Artifact-inducing enrichment of ethylenediaminetetraacetic acid and ethyleneglycoltetraacetic acid on anion exchange resins. *Anal Biochem.* 2011;412(1):34-39. doi:10.1016/j.ab.2011.01.027

Conclusions

- We purified a native 21-mer RNA oligonucleotide with 2'-OH using Capto Q ImpRes resin
- A 1 M NaCl salt gradient, without addition of solvents or chaotropic salts, was used for this native 21-mer RNA oligonucleotide
- Pooling of fractions resulted in 86% recovery with 94% purity
- Purity was confirmed with LC-MS
- This low-pressure (< 3 bar) chromatography step is suitable for scaling-up
- Capto Q ImpRes is available in prepacked ReadyToProcess™ columns or can be packed in AxiChrom™ columns

1. Presenting author
2. Corresponding author

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