Optimization of oligonucleotide separations on ion-exchange chromatography

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Introduction

Nucleic acids such as antisense, siRNA and aptamer are expected as next-generation pharmaceuticals following antibody drugs. For providing these drugs, purification and separation analysis that can recognize slight structural differences after synthesis are important issues.

Non-porous anion exchange column is generally suitable for analysis of oligonucleotides. Thus we tried to optimize an analysis method of single-stranded DNA and RNA of about 20 mer, using BioPro IEX QF which is a nonporous high performance anion exchange column. For optimization, we changed some conditions such as type of mobile phase and column temperature. As the result, good separations could be obtained for the oligonucleotides with single-base difference in length.

In this poster, we will introduce further details about the optimization of separation conditions.

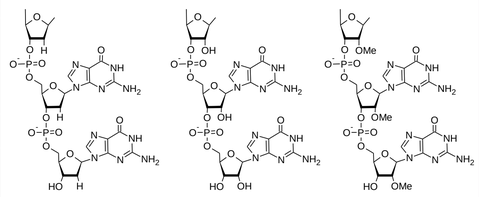
Specifications of BioPro IEX columns

	BioPro IEX QF	BioPro IEX SF	BioPro IEX QA	BioPro IEX SP
Matrix	non-porous hydrophilic polymer beads		porous hydrophilic polymer beads	
Particle size (μm)	3, 5		5	
Charged group	$-CH_2N^+(CH_3)_3$	$-CH_2CH_2CH_2SO_3^-$	$-CH_2N^+(CH_3)_3$	$-CH_2CH_2CH_2SO_3^-$
Counter ion	Cl^-	Na^+	Cl^-	Na^+
Ion-exchange capacity (meq/ml-resin)	0.075-0.110	0.230-0.290	0.075-0.100	0.070-0.095
Dynamic binding capacity (mg/ml-resin)	>12 (BSA)	>10 (human-IgG)	>110 (BSA)	>70 (human-IgG)
Usable pH range	2-12			
Column size (length X i.d.(mm))	30 X 4.6, 50 X 4.6, 100 X 4.6			

Sample Group 1 (Phosphodiester oligonucleotides ; PO)

1	Single-stranded DNA (ssDNA)	5′-TCATCACACTGAATACCAAT-3′ (DNA 20 mer)
2		5′-GTCATCACACTGAATACCAAT-3′ (DNA 21 mer)
3	Single-stranded RNA (ssRNA)	5′-UCAUCACACUGAAUACCAAU-3′ (RNA 20 mer)
4		5′-GUCAUCACACUGAAUACCAAU-3′ (RNA 21 mer)
5		5′-U(M)C(M)A(M)U(M)C(M)A(M)C(M)A(M)C(M)U(M)G(M)A(M)A(M)U(M)A(M)C(M)C(M)A(M)A(M)A(M)U(M)-3′ (2′-OMe RNA 20 mer)
6		5′-G(M)U(M)C(M)A(M)U(M)C(M)A(M)C(M)A(M)C(M)U(M)G(M)A(M)A(M)U(M)A(M)C(M)C(M)A(M)A(M)A(M)U(M)-3′ (2′-OMe RNA 21 mer)

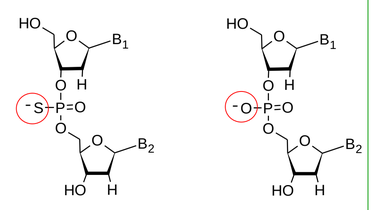
N(M)=2′-OMe RNA



Sample Group 2 (Phosphorothioate oligonucleotides ; PS)

10	Single-stranded DNA	5′-T^T^T^T^T^T^T^T^T^T^T^T^TTT-3′ (DNA 15 mer 12PS, 2PO)
11		5′-T^T^T^T^T^T^T^T^T^T^T^T^TT-3′ (DNA 15 mer 13PS, 1PO)
12		5′-T^T^T^T^T^T^T^T^T^T^T^T^T-3′ (DNA 15 mer All PS)
13	Single-stranded RNA	5′-U^C^A^U^C^A^C^A^C^U^G^A^A^U^A^C^C^A^A^U-3′ (RNA 20 mer All PS)
14		5′-G^U^C^A^U^C^A^C^A^C^U^G^A^A^U^A^C^C^A^A^U-3′ (RNA 21 mer All PS)

^=Phosphorothioated

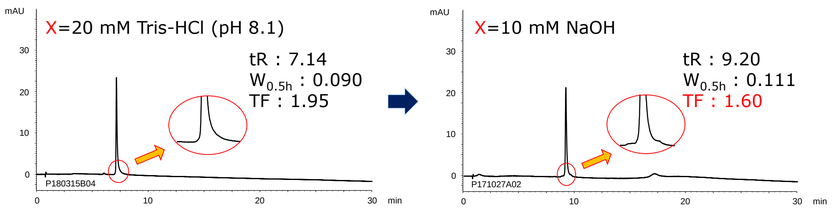


(i) Improvement of carryover peak

If initial gradient concentration of NaCl was low(ex. 50 mM), carryover occurred.
But increased initial gradient concentration of NaCl up to 400 mM enabled to avoid carryover with good reproducibility.

(ii) Improvement of peak tailing

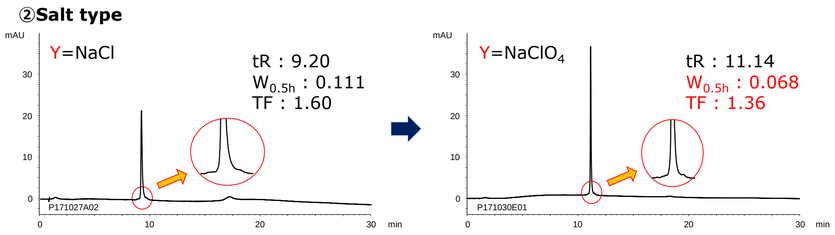
①Buffer type



Column	: BioPro IEX QF 5 μm, 100 X 4.6 mm i. d.
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 2 μl (10 nmol/ml)
Sample	: RNA 20 mer (sample 3)

Eluent	: A) X B) X containing 2.0 M NaCl 15-100%B (0-30 min)

②Salt type



Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 2.0 M Y 15-100%B (0-30 min) for NaCl 5-50%B (0-30 min) for $NaClO_4$

When $NaClO_4$ is used for ion exchange mode chromatography, it is said the elution force becomes 2-3 times larger than NaCl. Thus the gradient condition is properly adjusted.

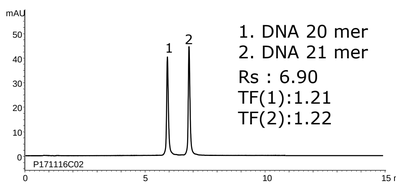
By changing the buffer from 20 mM Tris-HCl (pH 8.1) to 10 mM NaOH, the tailing factor of oligonucleotide was improved. Furthermore, the peak tailing was suppressed when $NaClO_4$ was added to 10 mM NaOH instead of NaCl.

(i ~ ii) Summary

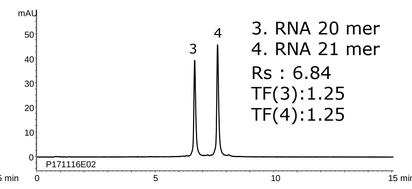
By using BioPro IEX QF, the condition for the analysis of oligonucleotides (PO) was optimized. We concluded 10 mM NaOH(as buffer solution) , $NaClO_4$(as salt) and higher initial gradient concentration of salt were preferable to suppress carryover and peak tailing.

(iii) Examples of analysis under the optimized condition

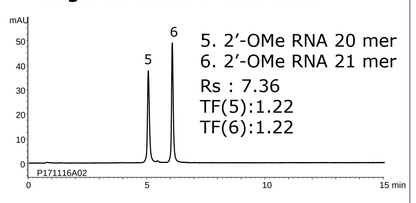
Single-stranded DNA



Single-stranded RNA



Single-stranded 2′-OMe RNA

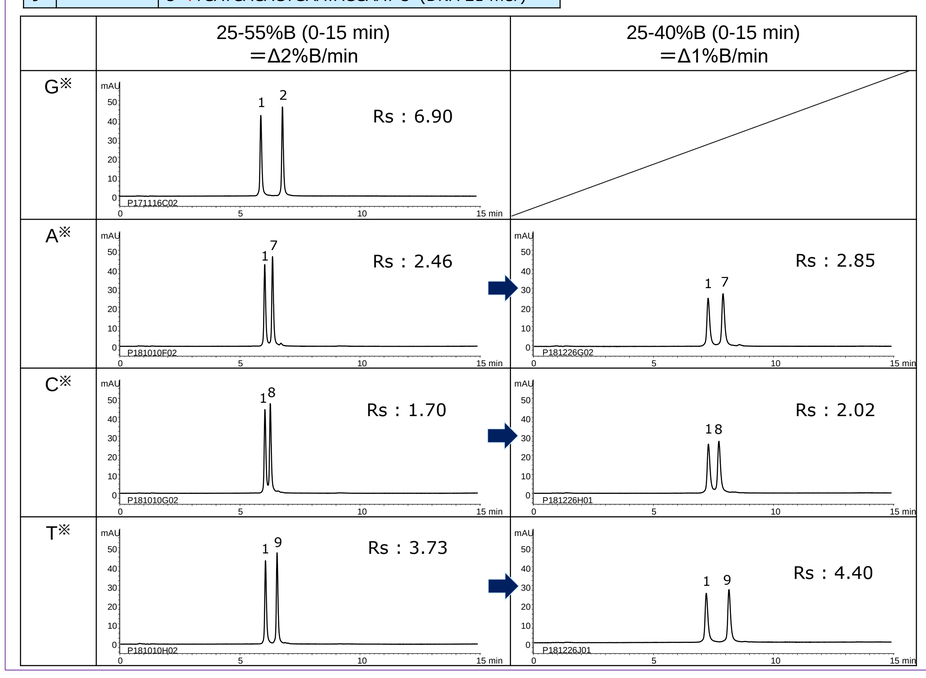


Each ssDNA, ssRNA and 2'-OMe ssRNA with single-base difference in length could be separated under the optimized condition.

Column	: BioPro IEX QF, 5 μm, 100 X 4.6 mm i. d.
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$ 25-55%B (0-15 min), 100%B (15-20 min)
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 4 μl (each 5 nmol/ml)

(iv) Separation of ssDNAs with single-base difference in length (differ in type of base of 5′end of DNA 21mer)

1	Single-stranded DNA	5′-TCATCACACTGAATACCAAT-3′ (DNA 20 mer)
2		5′-GTCATCACACTGAATACCAAT-3′ (DNA 21 mer)
7		5′-ATCATCACACTGAATACCAAT-3′ (DNA 21 mer)
8		5′-CTCATCACACTGAATACCAAT-3′ (DNA 21 mer)
9		5′-TTCATCACACTGAATACCAAT-3′ (DNA 21 mer)



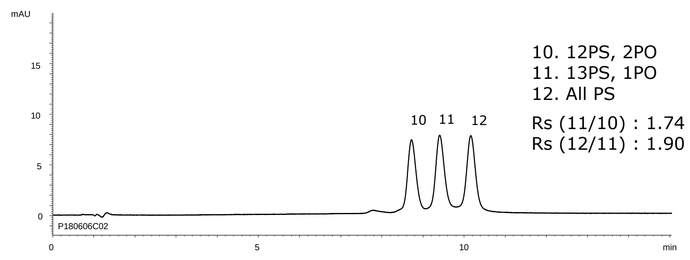
Column	: BioPro IEX QF 5 μm, 100 X 4.6 mm i. d.
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 4 μl (each 5 nmol/ml)

※base of 5′end of DNA 21 mer

When ssDNAs with single-base difference in length (differ in type of base of 5′end of DNA 21 mer) were analyzed under the described condition in (iii), all of peak separations got worse.
By using shallow gradient, better separations could be achieved.

(v) Resolution of phosphorothioate oligonucleotides with different degrees of thiolation

DNA 15 mer group (sample 10-12)

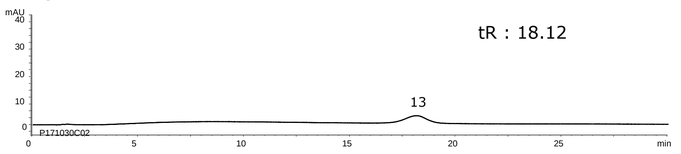


Column	: BioPro IEX QF 5 μm, 100 X 4.6 mm i. d.
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$ 40-70%B (0-15 min), 100%B (15-20 min)
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 6 μl (each 3.3 nmol/ml)

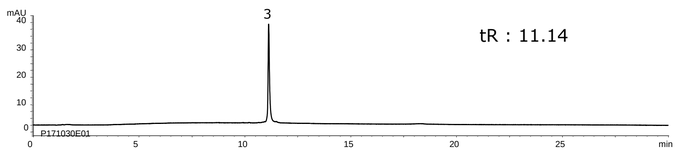
Under the optimized condition described in the left side, [All PS], [13PS, 1PO] and [12PS, 2PO] of DNA 15 mer (sample 10-12) were clearly separated by ion exchange chromatography.

(vi) Difference in required salt concentrations for eluting modified RNA (All PS) and normal RNA (All PO)

Sample 13; RNA 20 mer All PS



Sample 3; RNA 20 mer All PO

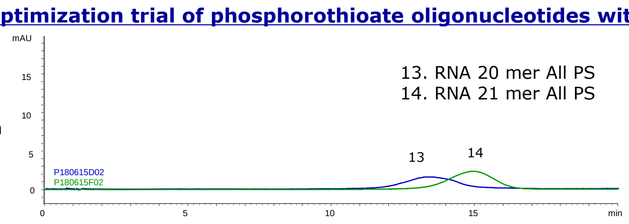


Column	: BioPro IEX QF 5 μm, 100 X 4.6 mm i. d.
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$ 10-100%B (0-30 min)
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 2 μl (10 nmol/ml)

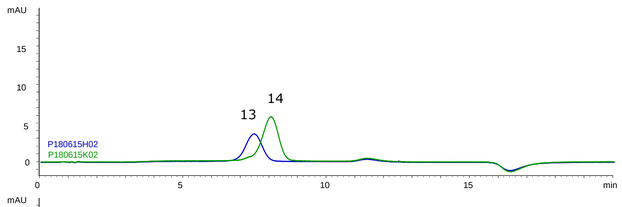
The separation of RNA 20 mer All PS was compared with RNA 20 mer All PO under the same condition.
Since acidity of All PS is much higher than All PO, higher salt concentration is required to elute it.
The peak of All PS is much broader because it is thought that All PS contains as many as 2^{19} (524288) stereoisomers.

(vii) Separation optimization trial of phosphorothioate oligonucleotides with single-base difference in length

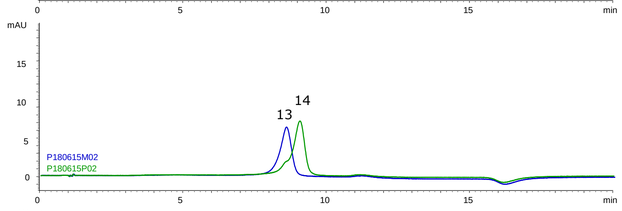
X/Y=100/0
32-80%B (0-24 min)
=Δ20 mM $NaClO_4$/min
25℃



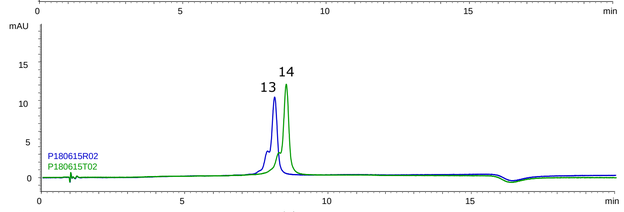
Steep gradient curve
X/Y=100/0
32-80%B (0-8 min)
=Δ60 mM $NaClO_4$/min
25℃



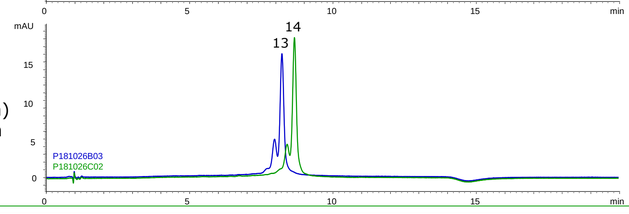
Raising column temperature
X/Y=100/0
32-80%B (0-8 min)
=Δ60 mM $NaClO_4$/min
60℃



Addition of organic solvent
X/Y=80/20
40-100%B (0-8 min)
=Δ60 mM $NaClO_4$/min
60℃



Increasing ratio of organic solvent
X/Y=70/30
46-100%B (0-6.3 min)
=Δ60 mM $NaClO_4$/min
60℃



Column	: BioPro IEX QF 5 μm, 100 X 4.6 mm i. d.
Eluent	: A) 10 mM NaOH/methanol (X/Y) B) 10 mM NaOH containing 1.0 M $NaClO_4$/methanol (X/Y)
Flow rate	: 1.0 ml/min
Detection	: UV at 260 nm
Injection	: 2 μl (10 nmol/ml)

We found out that steeper gradient curve, increasing column temperature and adding organic solvent could improve peak separation.
However increasing of organic solvent ratio hardly improved peak separation.

Conclusions

By using BioPro IEX QF:

- Each ssDNA, ssRNA and 2'-OMe ssRNA with single-base difference in length can be successfully separated.
- [All PS], [13PS, 1PO] and [12PS, 2PO], which consist of 15-mer ssDNA, can be also separated under the optimized condition.
- Higher salt concentration is required to elute All PS compared to eluting All PO.
- All PS with single-base difference in length can be separated to some extent. So we continue to establish more optimized conditions in future.