Optimisation of oligonucleotide separations on ion exchange chromatography

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Introduction

Nucleic acids such as antisense, siRNA and aptamer are expected to be the next generation pharmaceuticals following antibody drugs. In order to provide these drugs, purification and separation analysis that can recognize slight structural differences after synthesis are important issues.

Nonporous anion exchange column is generally suitable for analysis of oligonucleotides. Therefore we chose to optimise an analytical method for single-stranded DNA and RNA of about 20 mer, using BioPro IEX QF which is a nonporous high performance anion exchange column. For optimisation, 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 differences in length.

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

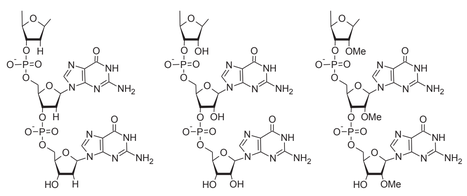
Specifications of BioPro IEX columns

	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 ID (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)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)U(M)-3′ (2′-OMe RNA 21 mer)

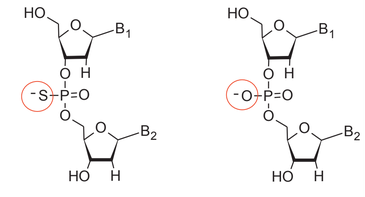
N(M)=2′-OMe RNA



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^T^TT-3′ (DNA 15 mer 13PS, 1PO)
12		5′-T^T^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

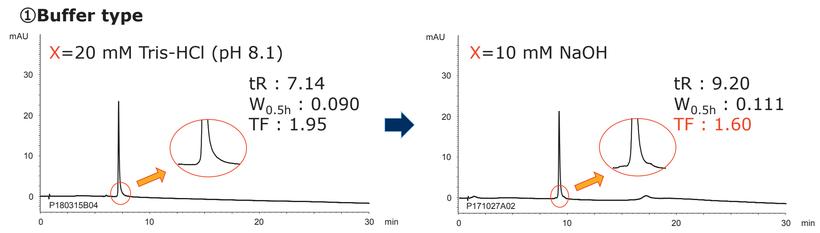


(i) Reduction of carryover peak

When the initial gradient concentration of NaCl was low (ex. 50 mM), carryover was observed.
By increasing the initial gradient concentration of NaCl up to 400 carryover could be avoided with good reproducibility.

(ii) Reduction of peak tailing

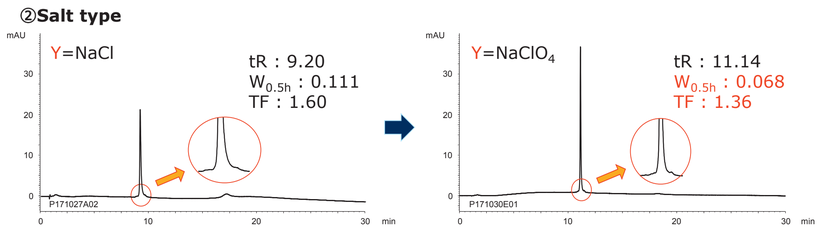
①Buffer type



Column	: BioPro IEX QF 5 μm, 100 x 4.6 mm ID
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.

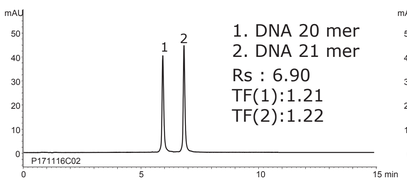
By changing the buffer from 20 mM Tris-HCl (pH 8.1) to 10 mM NaOH, the tailing factor for an oligonucleotide was reduced. Furthermore, the peak tailing was further suppressed when $NaClO_4$ was added to 10 mM NaOH instead of NaCl.

(i ~ ii) Summary

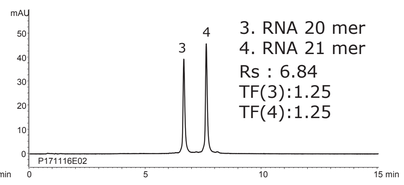
By using BioPro IEX QF, the conditions for the analysis of oligonucleotides (PO) was optimised. We concluded that 10 mM NaOH (as buffer solution) , $NaClO_4$ (as salt) and higher initial gradient concentration of salt were preferable to suppress carryover and peak tailing.

(iii) Examples of analysis under the optimised conditions

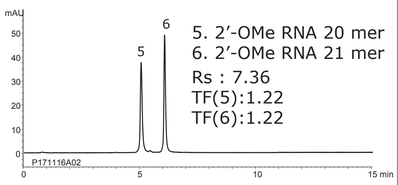
Single-stranded DNA



Single-stranded RNA



Single-stranded 2′-OMe RNA

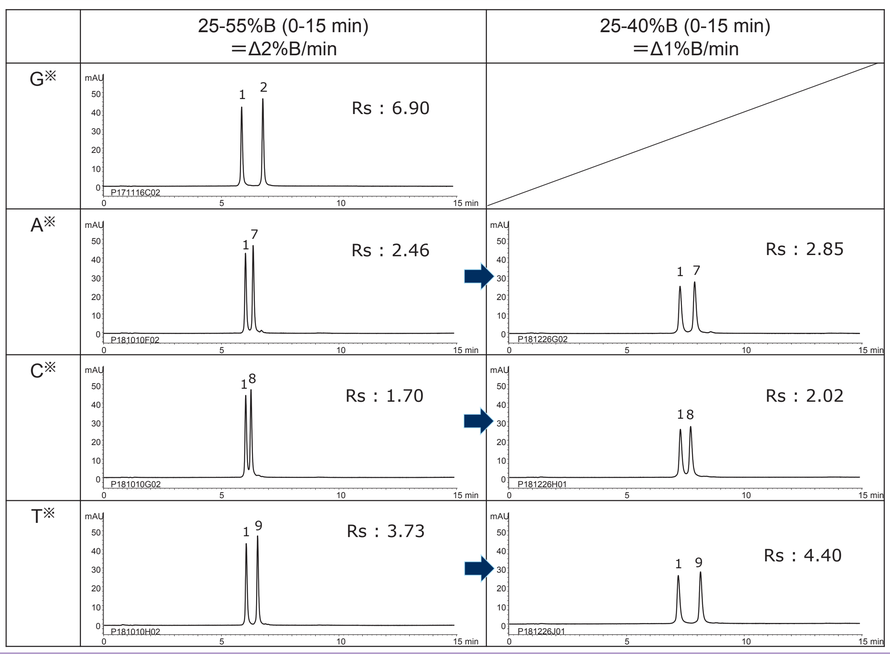


Each ssDNA, ssRNA and 2'-OMe ssRNA with single-base difference in length could be separated under the optimised condition.

Column	: BioPro IEX QF, 5 μm, 100 x 4.6 mm ID
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 differences (differing in the 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)



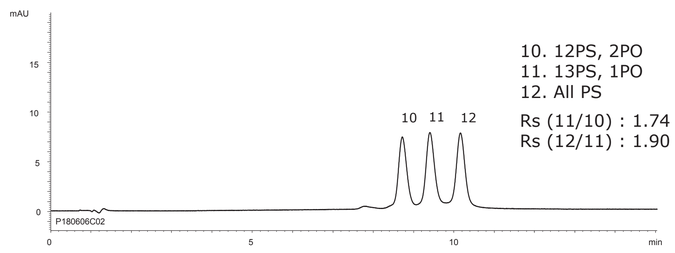
Column	: BioPro IEX QF 5 μm, 100 x 4.6 mm ID
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 4 μl (each 5 nmol/ml)

※base of 5′end of DNA 21 mer

When ssDNAs with single-base differences (differing in the type of base of 5′end of DNA 21 mer) were analysed under the described condition in (iii), all of peak separations got worse.
By using a shallower gradient, better separations could be achieved.

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

DNA 15 mer group (sample 10-12)

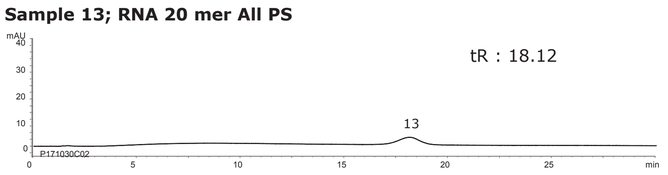


Column	: BioPro IEX QF 5 μm, 100 x 4.6 mm ID
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$, 40-70%B (0-15 min), 100%B (15-20 min)
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 6 μl (each 3.3 nmol/ml)

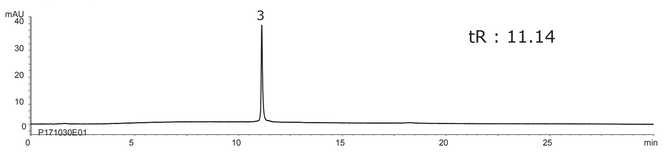
Using the optimised conditions described in section (iv), [All PS], [13PS, 1PO] and [12PS, 2PO] of DNA 15 mer (samples 10-12) were clearly separated by ion exchange chromatography.

(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

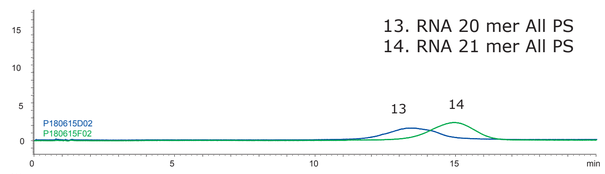


Column	: BioPro IEX QF 5 μm, 100 x 4.6 mm ID
Eluent	: A) 10 mM NaOH B) 10 mM NaOH containing 1.0 M $NaClO_4$ 10-100%B (0-30 min)
Flow rate	: 1.0 ml/min
Temperature	: 25℃
Detection	: UV at 260 nm
Injection	: 2 μl (10 nmol/ml)

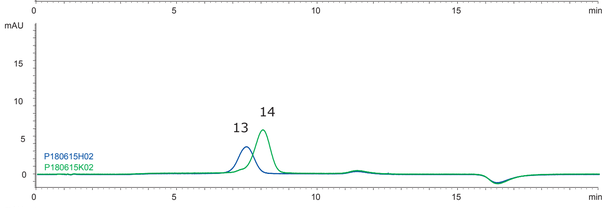
The separation of RNA 20 mer All PS was compared with RNA 20 mer All PO under the same conditions.
Since acidity of All PS is much higher than All PO, a higher salt concentration is required for elution.
The peak of All PS is much broader because it is thought that All PS contains as many as 2^{19} (524288)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

(vii) Separation of optimisation trial of phosphorothioate oligonucleotides with single-base differences in length

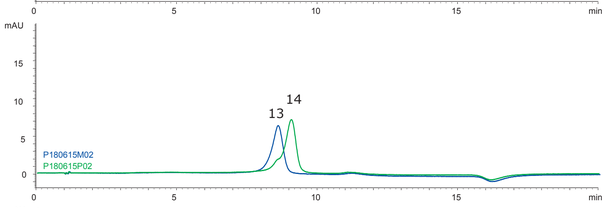
X/Y=100/0
32-80%B (0-24 min)
=Δ20 mM $NaClO_4$/min
25℃



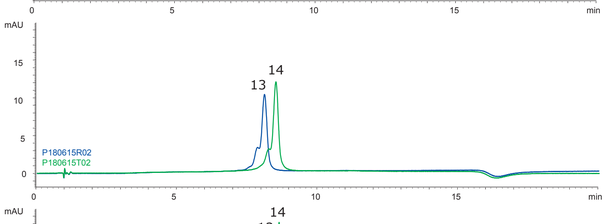
Steep gradient curve
X/Y=100/0
32-80%B (0-8 min)
=Δ60 mM $NaClO_4$/min
25℃



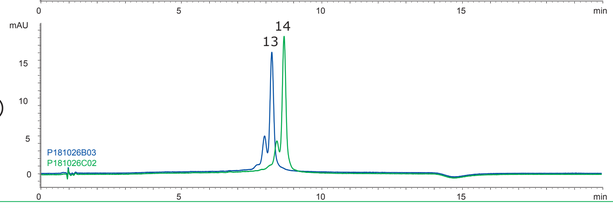
Raising column temperature
X/Y=100/0
32-80%B (0-8 min)
=Δ60 mM $NaClO_4$/min
60℃



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 ID
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 that a steeper gradient curve, increasing column temperature and adding organic solvent could improve peak separation.
However increasing of organic solvent ratio gave little improvement in 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 also be separated under the optimised conditions.
- 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 a limited extent and we continue to search for further improvements in optimised conditions.