Points to Note in Bioanalysis of Therapeutic Oligonucleotides by Ion-pair LC-MS/MS

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Introduction

Therapeutic Oligonucleotides in Japan, the US, and Europe (as of October 2022) *

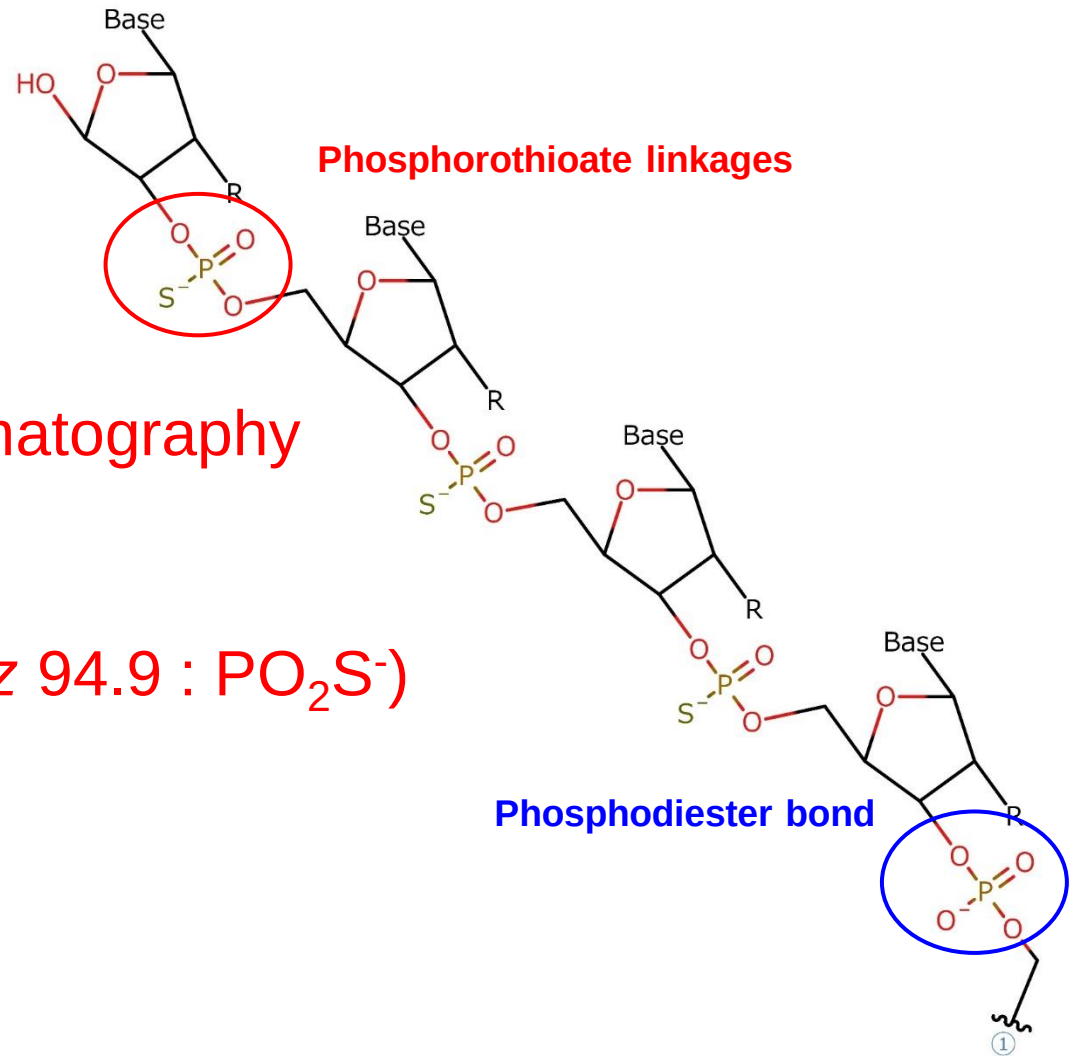
Product name	International nonproprietary name	Classification	Modification, etc	DDS, ect	Country/Year	Target	Indication	Route of Administration
Vitravene	fomivirsen	Antisense	PS (full)	Naked	US 1998 EU 1999	CMV IE2 mRNA	CMV retinitis (AIDS patient)	Intravitreal
Macugen	pegaptanib	Aptamer	2′-OMe, 2′-F	Naked (PEG-conjugate)	US 2004 EU 2006 JP 2008	VEGF165 (Protein)	Exudative age-related macular degeneration	Intravitreal
Kynamro	mipomersen	Antisense	PS (full) 2′-MOE	Naked	US 2013	ApoB-100 mRNA	Homozygous familial hypercholesterolemia	Intravenously
Exondys 51	eteplirsen	Antisense	PS (full)	Naked	US 2016	Dystrophin pre-mRNA	Duchenne type muscular dystrophy	Intrathecal
Spinraza	nusinersen	Antisense	PS (full) 2′-MOE	Naked	US 2016 EU 2017 JP 2017	SMN2 pre-mRNA	Spinal muscular atrophy	Intramuscular
HEPLISAV-B	CpG1018	CpG oligo	PS (full)	Naked	US 2017 EU 2021	TLR9 (Protein)	Hepatitis B (prevention)	Subcutaneous
Tegsedi	inotersen	Antisense	PS (full) 2′-MOE	Naked	US 2018 EU 2018	TTR mRNA	Hereditary ATTR amyloidosis	Intravenously
Onpattro	patisiran	siRNA	2′-OMe	Lipid nanoparticle	US 2018 EU 2018 JP 2019	TTR mRNA	Hereditary ATTR amyloidosis	Subcutaneous
Waylivra	volanesorsen	Antisense	PS (full) 2′-MOE	Naked	EU 2019	ApoCIII mRNA	Familial hyperchylomicronemia	Subcutaneous
Givlaari	givosiran	siRNA	PS (partial) 2′-OMe, 2′-F	Naked (GalNAc-conjugate)	US 2019 EU 2020 JP 2021	ALAS1 mRNA	Acute hepatic porphyria	Subcutaneous
Vyondys 53	golodirsen	Antisense	Morpholino	Naked	US 2019	Dystrophin pre-mRNA	Duchenne type muscular dystrophy	Intravenously
Viltepso	viltolarsen	Antisense	Morpholino	Naked	JP 2020 US 2020	Dystrophin pre-mRNA	Duchenne type muscular dystrophy	Intravenously
Oxlumo	lumasiran	siRNA	PS (partial) 2′-OMe, 2′-F	Naked (GalNAc-conjugate)	US 2020 EU 2020	HAO mRNA	Primary Hyperoxaluria type I	Subcutaneous
Leqvio	inclisiran	siRNA	PS (partial) 2′-OMe, 2′-F	Naked (GalNAc-conjugate)	EU 2020 US 2021	PCSK9 mRNA	Hypercholesterolemia mixed dyslipidemia	Subcutaneous
Amondys 45	casimersen	Antisense	Morpholino	Naked	US 2021	Dystrophin pre-mRNA	Duchenne type muscular dystrophy	Intravenously
Amvuttra	vutrisiran	siRNA	PS (partial) 2′-OMe, 2′-F	Naked (GalNAc-conjugate)	US 2022 EU 2022 JP 2022	TTR mRNA	Hereditary ATTR amyloidosis	Subcutaneous

*** : Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences HP**
URL : https://www.nihs.go.jp/mtgt/section2.html

Introduction

Therapeutic Oligonucleotides Chemical Structure Affecting Analysis

- Highly charged poly-anionic backbone
  ⇒ Sensitivity loss due to adduct ion formation
- High polarity
  ⇒ Difficult separation in reversed-phase chromatography
- Phosphorothioate linkages
  ⇒ Mutual interference of precursor ions (of *m/z* 94.9 : PO_2S^-) with each other
- Phosphodiester bond
  ⇒ Carry over caused by metal adsorption



Introduction

Critical Point

- **ESI-MS (Selection of precursor ion)**
  Multiple negative charge states (H^+, Na^+ and K^+ adduct ion)
- **MS/MS (Selection of product ion)**
  Fragmentation of multiple charged oligonucleotides
- **Ion-pair LC-MS/MS Efficiency**
  Retention vs ionization
- **Quantitation**
  Internal standard selection
- **Sample Preparation and Separation Conditions**
  Introduction and degradation of ion-pair reagent

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ESI-MS (Selection of precursor ion) : Method

Mipomersen sodium

5′-G^{Me}C^{Me}C^{Me}U^{Me}C AGT^{Me}CTG^{Me}CTT^{Me}C G^{Me}CA^{Me}C^{Me}C- 3′

Molecular formula : $C_{230}H_{305}N_{67}O_{122}P_{19}S_{19}Na_{19}$

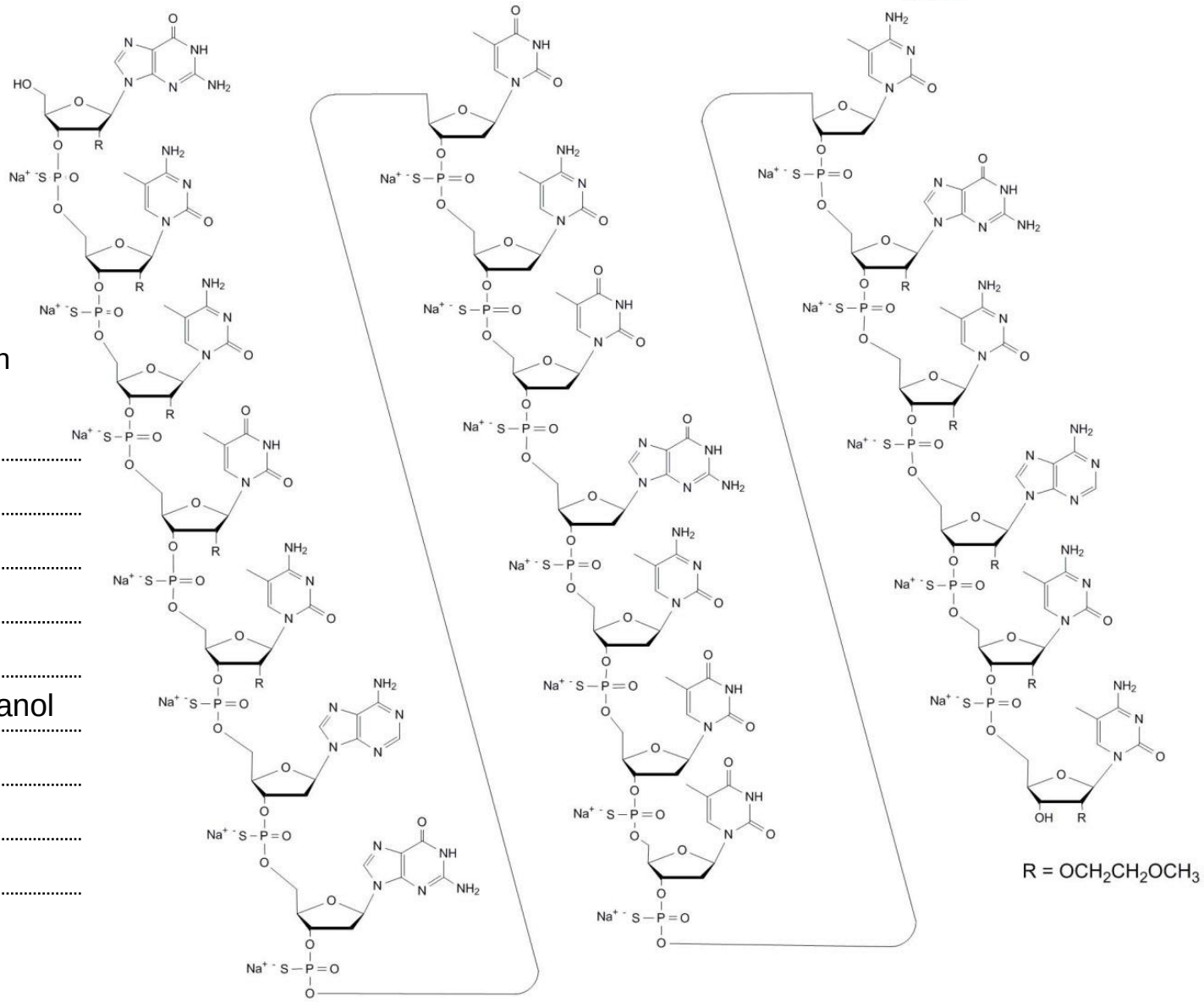
Monoisotopic mass: 7589.7486

Average mass: 7594.8005

The underlined residues are 2′-*O*-(2-methoxyethyl) nucleoside (Me).

All other residues are 2′-deoxynucleosides.

Substitution at the 5-position of the cytosine (C) and uracil (U) bases with a methyl group is indicated by ^{Me}.

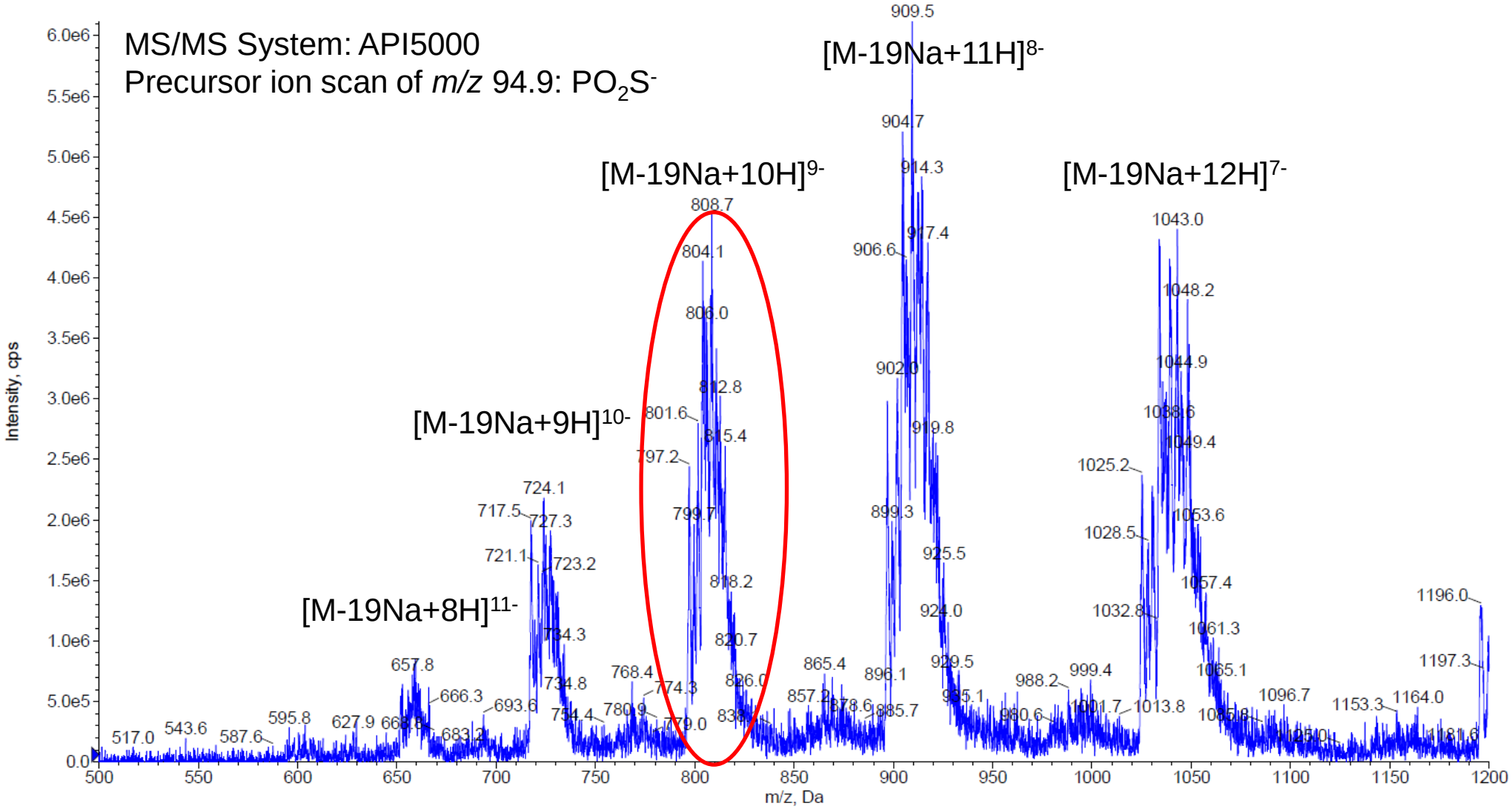


MS/MS System	API5000 or Triple TOF 5600+
Scan range	*m/z* 500-1200
Mobile phase A	Ultrapure water
Mobile phase B	Methanol : acetonitrile = 75 : 25
Mobile phase C	1 mol/L HFIP and 150 mmol/L DIPEA in methanol
Flow rate	0.3 mL/min
Composition	A : B : C = 60 : 30 : 10
T-infusion	20 µL/min (Analyte : 100 ng/mL)

HFIP: Hexafluoroisopropanol
DIPEA: *N,N*-Diisopropylethylamine

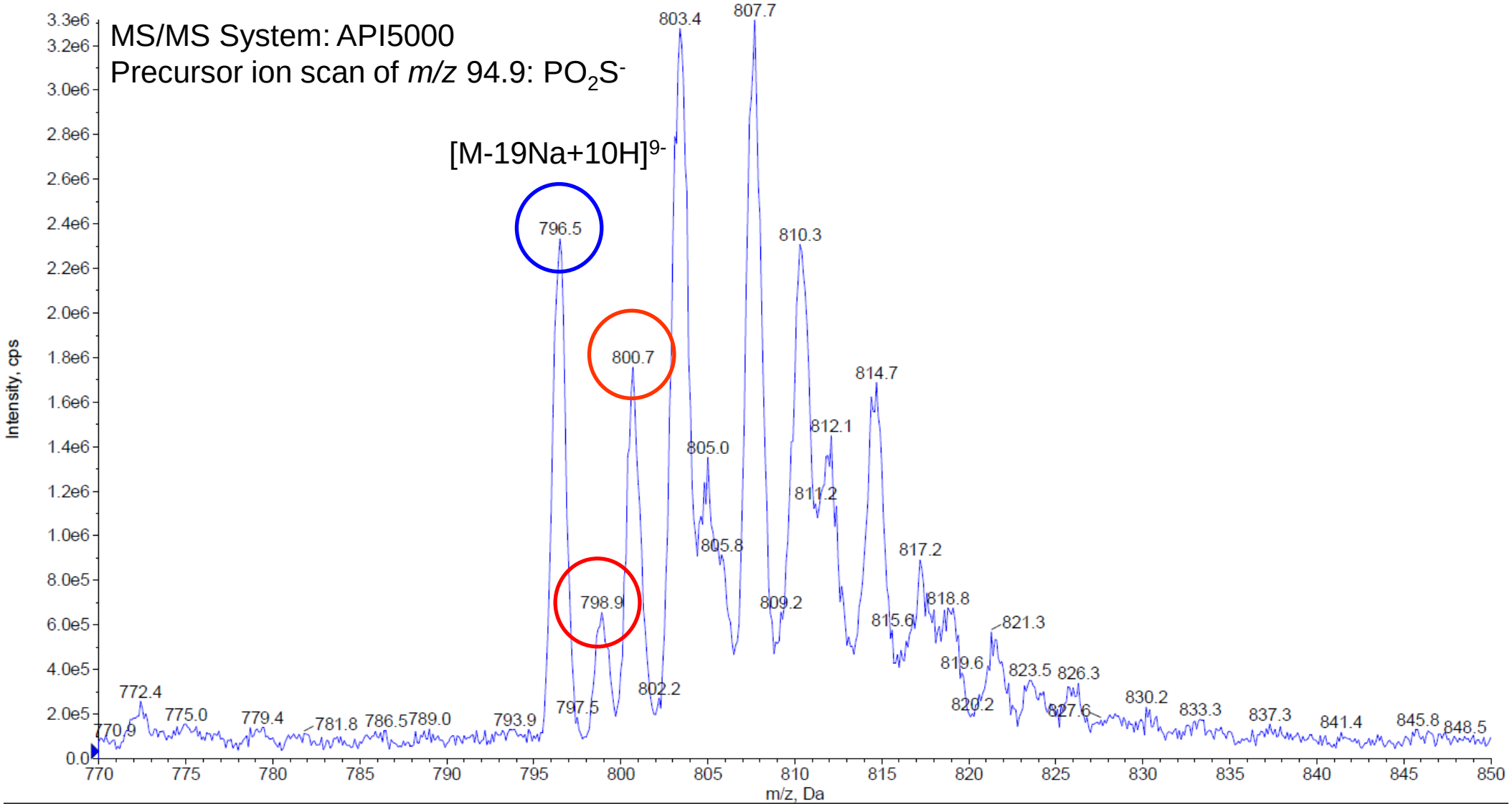
ESI-MS (Selection of precursor ion) : Results

Formation of multiple negative charge states



ESI-MS (Selection of precursor ion) : Results

Multiple negative charge states



ESI-MS (Selection of precursor ion) : Results

Multiple negative charge states (H^+, Na^+ and K^+ adduct ion)

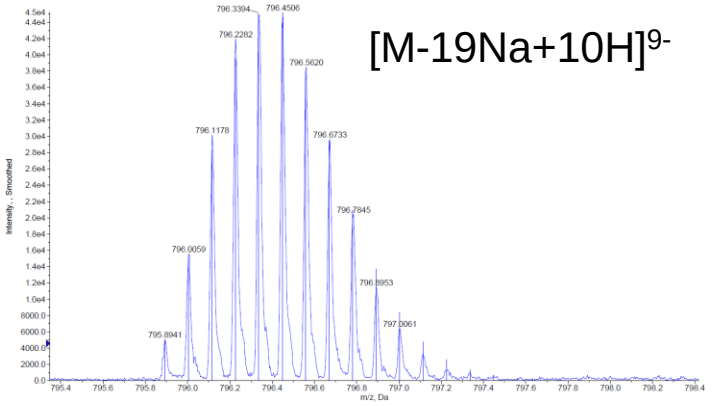
MS/MS System: Triple TOF 5600+

TOF MS scan

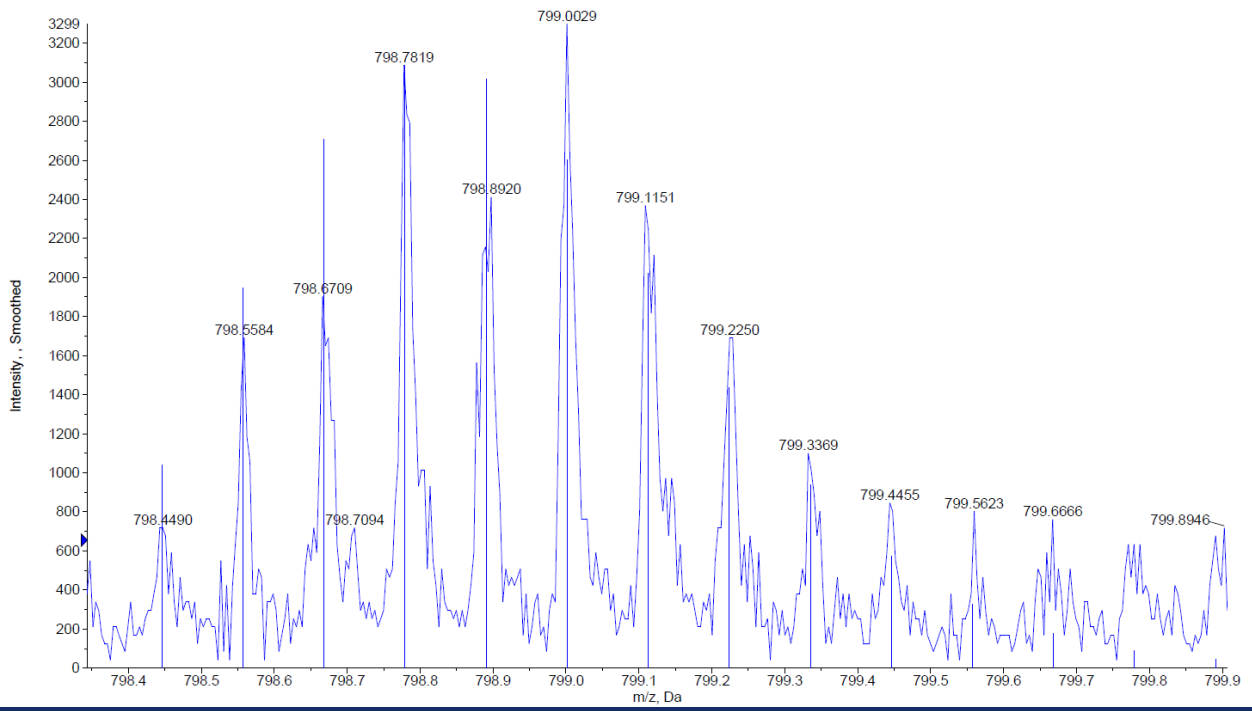
X axis: monoisotopic ion

Y axis: ion intensity

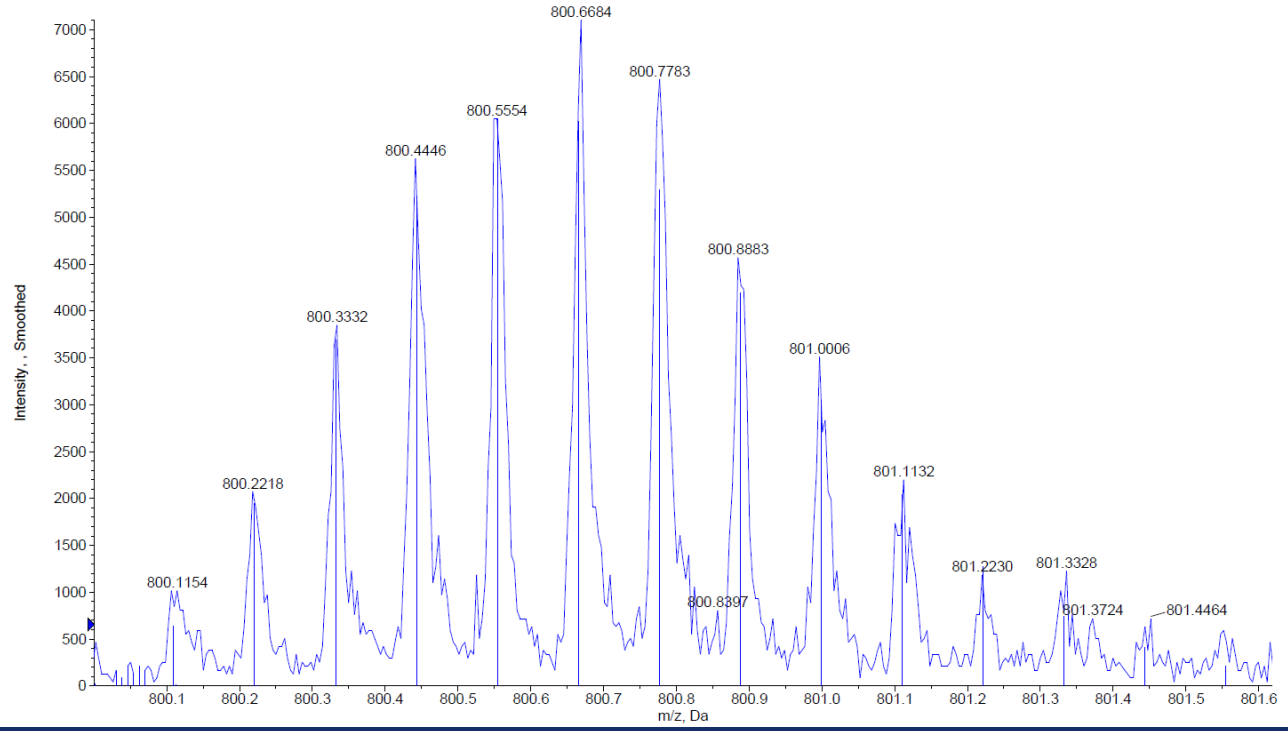
$[M-19Na+10H]^{9-}$



$[M-18Na+10H]^{9-}$

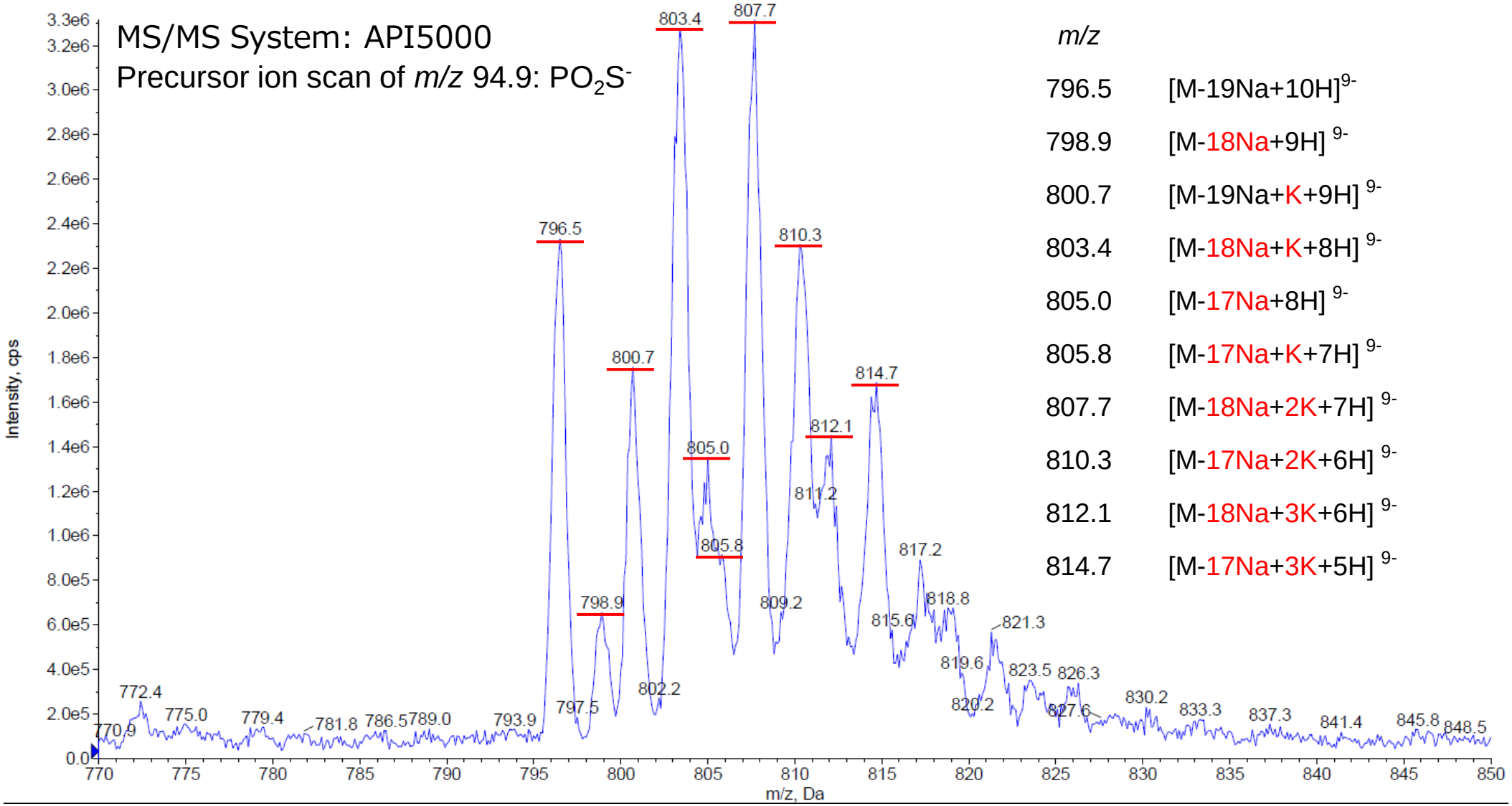


$[M-19Na+K+9H]^{9-}$



ESI-MS (Selection of precursor ion) : Results

Multiple negative charge states (H^+, Na^+ and K^+ adduct ion)



m/z	
796.5	$[M-19Na+10H]^{9-}$
798.9	$[M-18Na+9H]^{9-}$
800.7	$[M-19Na+K+9H]^{9-}$
803.4	$[M-18Na+K+8H]^{9-}$
805.0	$[M-17Na+8H]^{9-}$
805.8	$[M-17Na+K+7H]^{9-}$
807.7	$[M-18Na+2K+7H]^{9-}$
810.3	$[M-17Na+2K+6H]^{9-}$
812.1	$[M-18Na+3K+6H]^{9-}$
814.7	$[M-17Na+3K+5H]^{9-}$

ESI-MS (Selection of precursor ion) : Materials

➢**Analyte**:

ASO-1: *MALAT1* (metastasis associated in lung adenocarcinoma transcript-1) - ASO

【Sequence】A(L)^G(L) ^T(L) ^a^c^t^a^t^a^g^c^a^t^5(L) ^T(L) ^G(L)＊1 (16 mer, MW：5303.32) ＊2

➢**Internal Standard Candidate**:

① **ASO-2**: ASO with the same modification as the above ASO but with a different sequence

【Sequence】G(L)^G(L) ^5(L) ^c^a^a^t^a^c^g^c^c^g^ T(L) ^ 5(L) ^A(L) ＊1 (16 mer, MW：5288.29)

② **PS ASO-1**: The above ASO with phosphorothioate modification only

【Sequence】A^G^U^a^c^t^a^t^a^g^c^a^t^C^U^G＊1 (16 mer, MW：5189.23)

③ **2′-OMe ASO-1**: The above ASO with 2′-OMe modification instead of LNA modification

【Sequence】A(M)^G(M) ^U(M) ^a^c^t^a^t^a^g^c^a^t^C(M) ^U(M) ^G(M) ＊1 (16 mer, MW：5273.41)

④ **MOE ASO-1**: The above ASO with MOE modification instead of LNA modification

【Sequence】A(m)^G(m) ^U(m) ^a^c^t^a^t^a^g^c^a^t^C(m) ^U(m) ^G(m) ＊1 (16 mer, MW：5579.80)

⑤ **ASO-3**: The 5′ terminal G(L) was deleted from the above ASO

【Sequence】A(L)^G(L) ^T(L) ^a^c^t^a^t^a^g^c^a^t^5(L) ^T(L) ＊1 (15 mer, MW：4930.05)

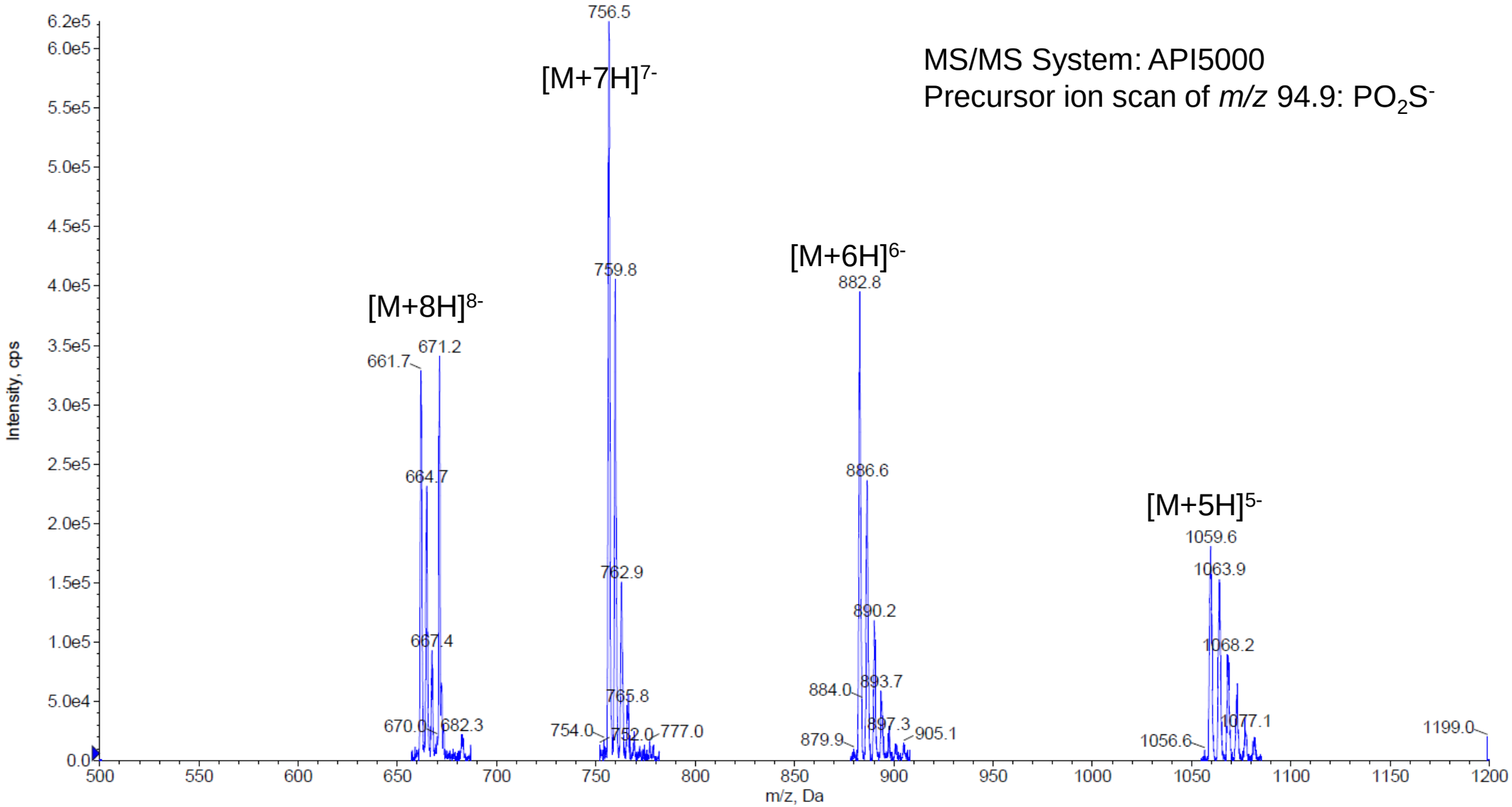
＊1: Upper Case=RNA / Lower Case=DNA / N(L)=LNA / N(M)=2′-OMe / N(m)=MOE / 5(L)=LNA_mC / 5(m)= MOE _mC / ^=Phosphorothioate

＊2: Paymaan Jafar-Nejad, et al. The atlas of RNase H antisense oligonucleotide distribution and activity in the CNS of rodents and non-human primates following central administration. Nucleic Acids Res. 2021 Jan 25;49(2):657-673.

LNA：Locked nucleic acid, **mC**：5-Methylcytosine, **2′-OMe**：2′-O-Methyl, **MOE**：2′-Methoxyethyl

ESI-MS (Selection of precursor ion) : Results

ASO1: Multiple negative charge states (Na^+ adduct ion)

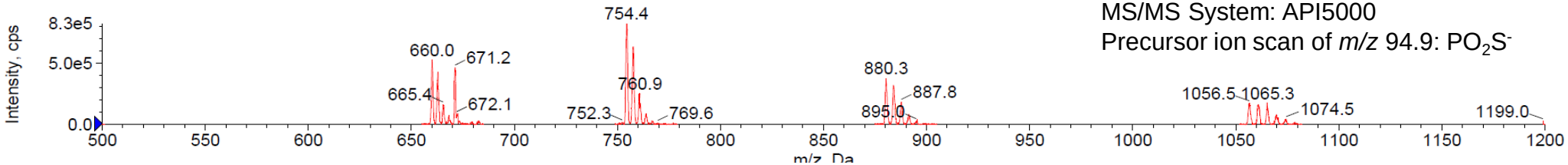


ESI-MS (Selection of precursor ion) : Results

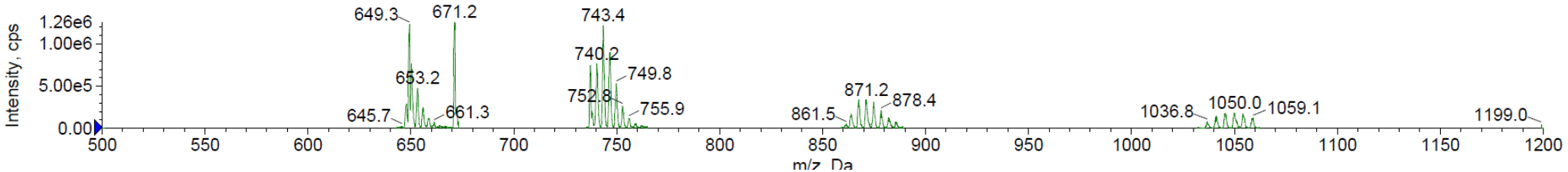
Internal standard candidate : Multiple negative charge states (Na^+ adduct ion)

MS/MS System: API5000
Precursor ion scan of *m/z* 94.9: PO_2S^-

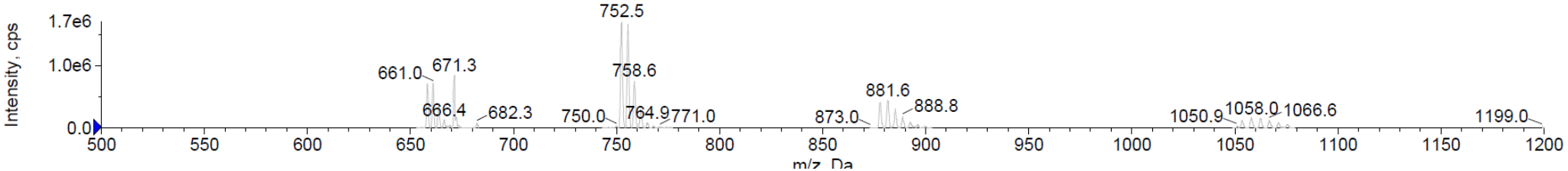
ASO-2



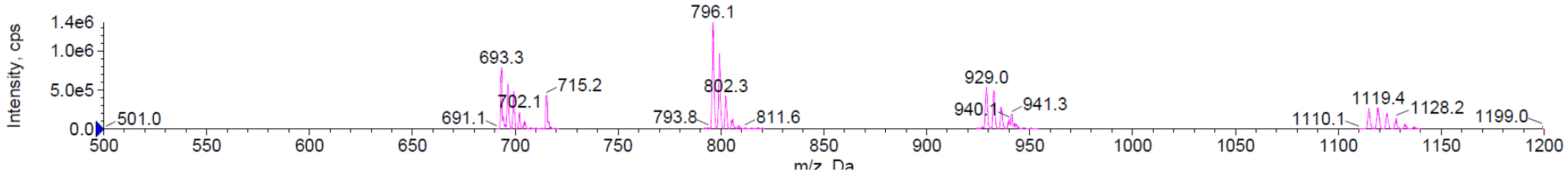
PS ASO-1



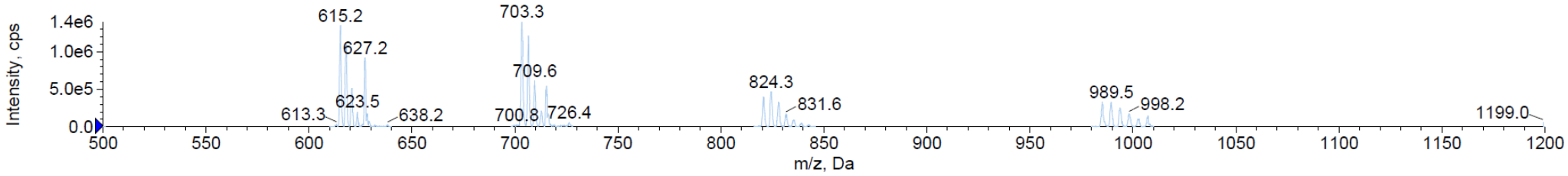
2′-OMe ASO-1



MOE ASO-1



ASO-3



ESI-MS (Selection of precursor ion) : Method

Analysis target	ASO-1
HPLC System	Shimadzu 30A system
Column	ACQUITY Premier BEH C18 Column with VanGuard FIT (1.7 µm, 2.1 x 100 mm, Waters)
Column temperature	60°C
Mobile phase A	Ultrapure water
Mobile phase B	Methanol : acetonitrile = 75 : 25
Mobile phase C	1 mol/L HFIP and 150 mmol/L DIPEA in methanol
Injection volume	3 µL
Needle wash solvent	Water / methanol / isopropanol / acetylacetone / 0.5 M EDTA (pH 8.0) (500:250:250:2:2)
Flow rate	0.3 mL/min
Gradient	See table below
MS/MS system	API5000
Scan type	Selected Reaction Monitoring
Monitored Ion	See Figure (Next page)

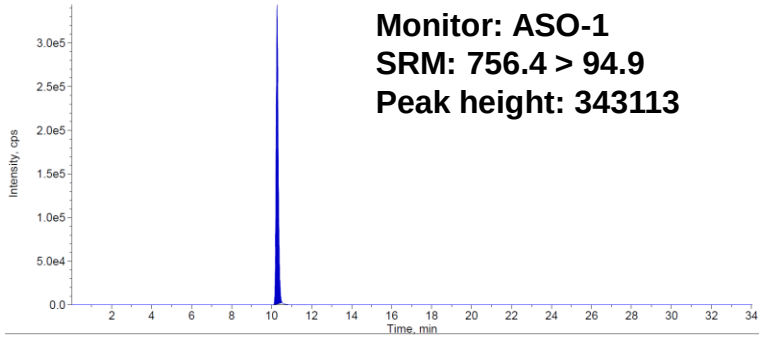
Gradient

Time (min)	A Conc. (%)	B Conc. (%)	C Conc. (%)
0.00	90.0	0.0	10.0
0.50	90.0	0.0	10.0
27.00	0.0	90.0	10.0
29.00	0.0	90.0	10.0
29.01	90.0	0.0	10.0
34.00	90.0	2.5	10.0

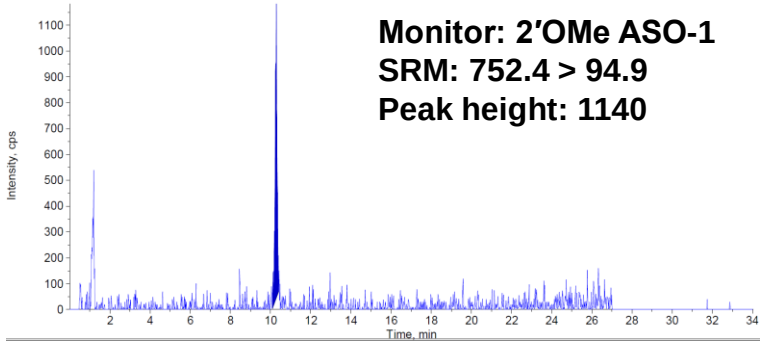
HFIP: Hexafluoroisopropanol
DIPEA: *N,N*-Diisopropylethylamine

ESI-MS (Selection of precursor ion) : Results

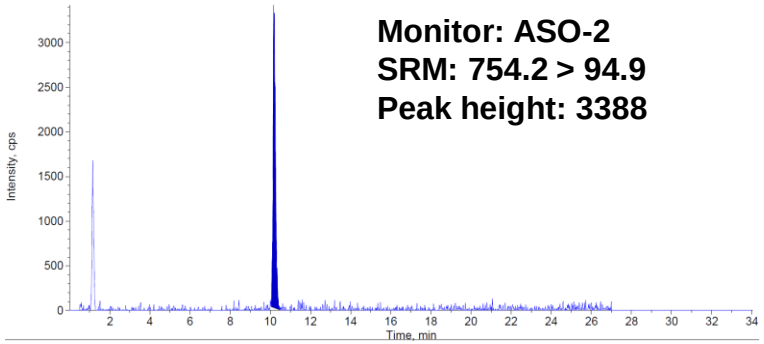
The peak (ASO-1) was confirmed at the same retention time.
ASO-1 interfered with monitoring of other oligonucleotides.



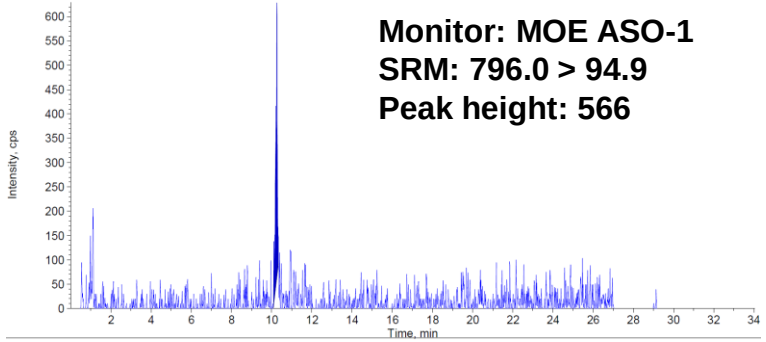
Monitor: ASO-1
SRM: 756.4 > 94.9
Peak height: 343113



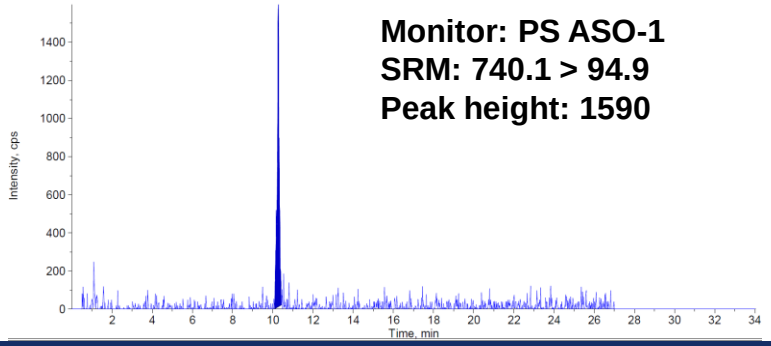
Monitor: 2′OMe ASO-1
SRM: 752.4 > 94.9
Peak height: 1140



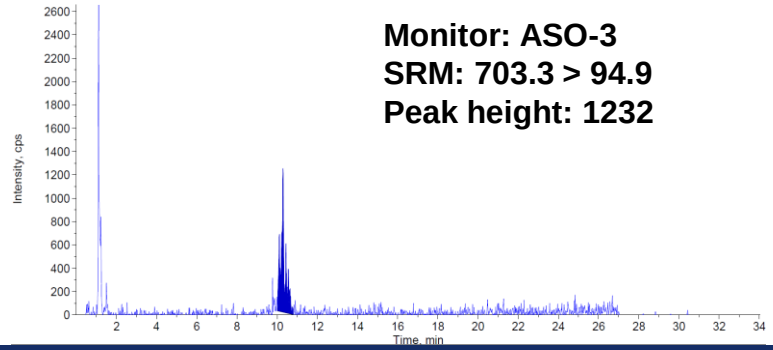
Monitor: ASO-2
SRM: 754.2 > 94.9
Peak height: 3388



Monitor: MOE ASO-1
SRM: 796.0 > 94.9
Peak height: 566



Monitor: PS ASO-1
SRM: 740.1 > 94.9
Peak height: 1590



Monitor: ASO-3
SRM: 703.3 > 94.9
Peak height: 1232

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- **MS/MS (Selection of product ion)**
  Fragmentation of multiple charged oligonucleotides
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ESI-MS (Selection of product ion) : Method

Question: Is it possible to give selectivity to product ions?

Answer: It is possible, but at the expense of sensitivity.

➢Analyte:

ASO-1: *MALAT1* (metastasis associated in lung adenocarcinoma transcript-1) – ASO

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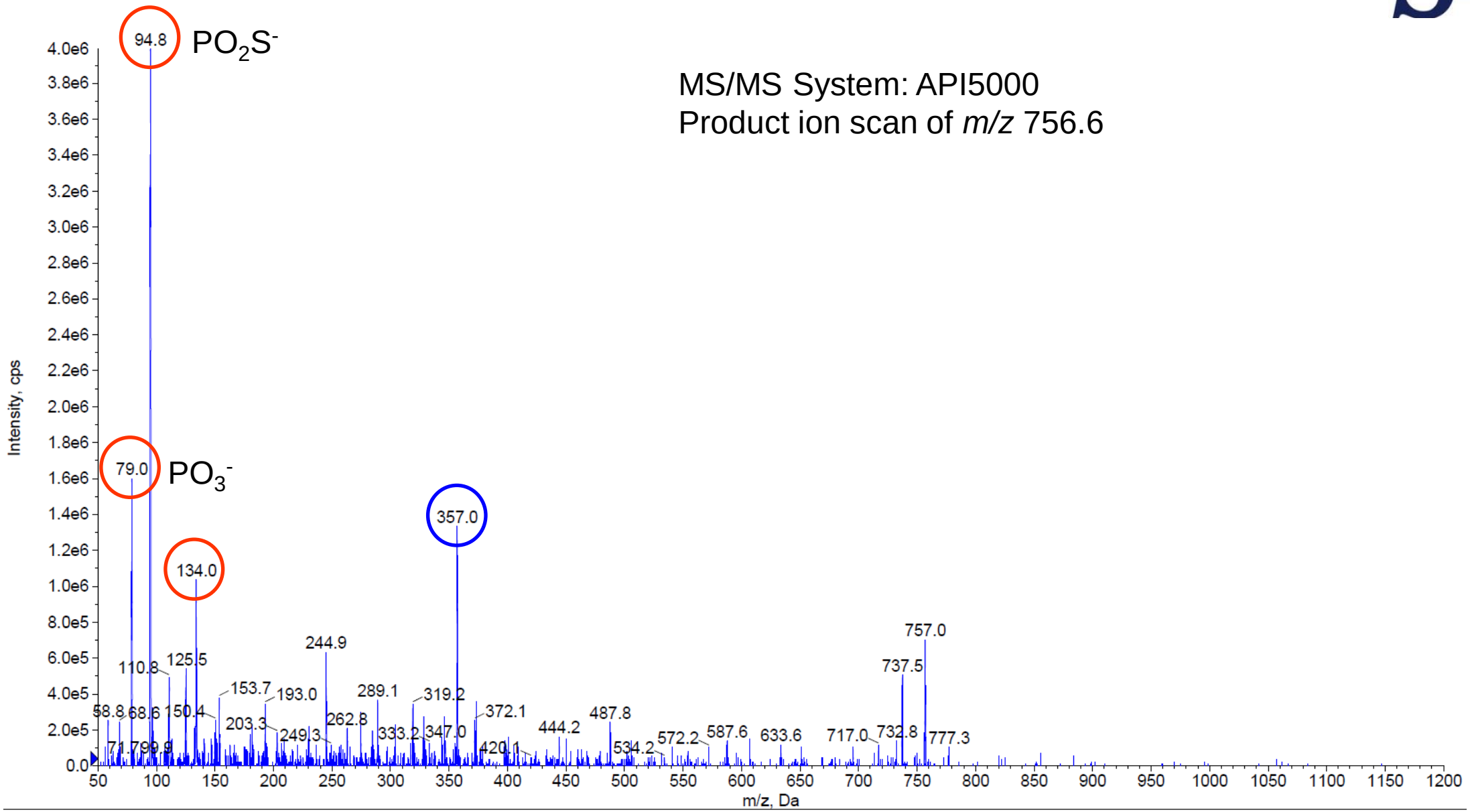
＊1: Upper Case=RNA / Lower Case=DNA / N(L)=LNA / 5(L)=LNA_mC / ^=Phosphorothioate

LNA：Locked nucleic acid
mC： 5-Methylcytosine

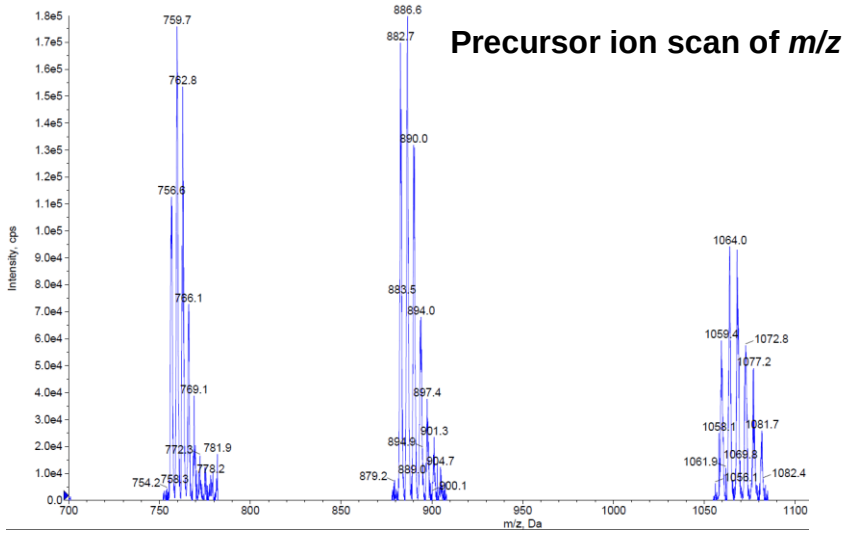
MS/MS System	API5000 or Triple TOF 5600+
Scan range	*m/z* 50-1200 (Product ion scan)
Mobile phase A	Ultrapure water
Mobile phase B	Methanol : acetonitrile = 75 : 25
Mobile phase C	1 mol/L HFIP and 150 mmol/L DIPEA in methanol
Flow rate	0.3 mL/min
Composition	A : B : C = 60 : 30 : 10
T-infusion	40 µL/min (Analyte : 100 ng/mL)

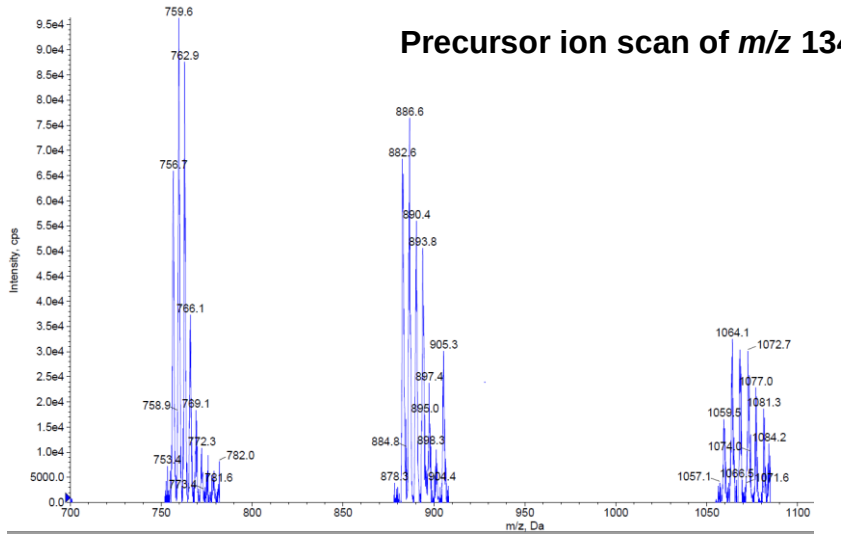
ESI-MS (Selection of product ion) : Results

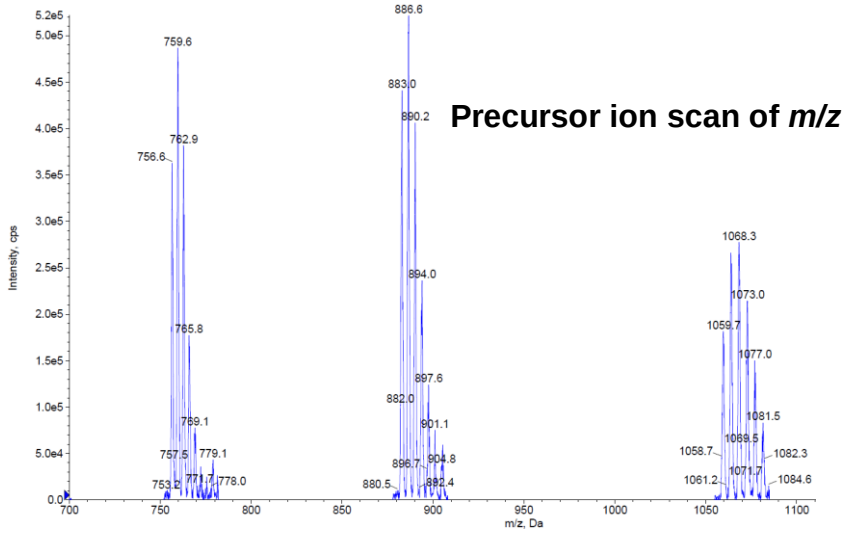


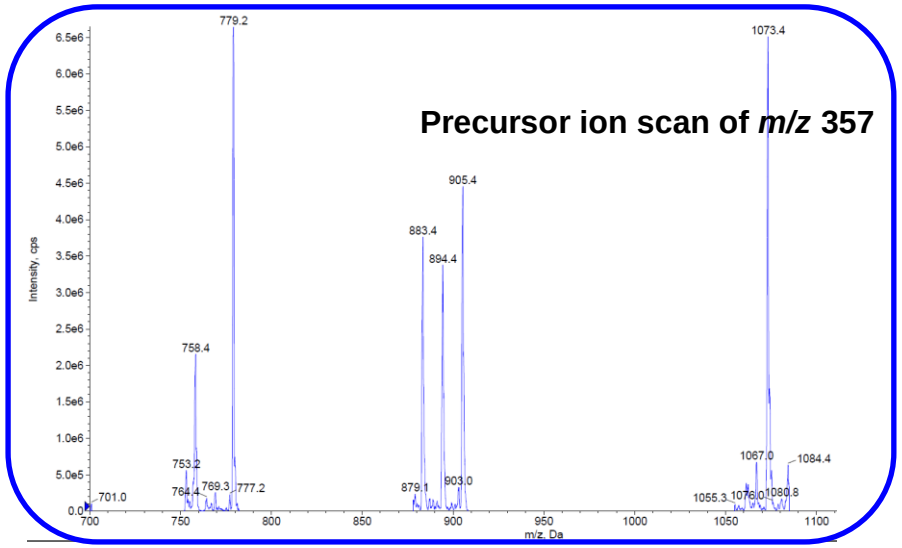
MS/MS System: API5000
Product ion scan of *m/z* 756.6

ESI-MS (Selection of product ion) : Results









Unlike other product ions, adduct ions are not observed in the breaker ions.

Meaning it is not a spectrum derived from oligonucleotides.

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Ion-pair LC-MS/MS Efficiency

Point at issue

① Sensitivity is dispersed due to the generation of multiply charged ions and adduct ions.

② Oligonucleotides interfere with each other’s monitoring.

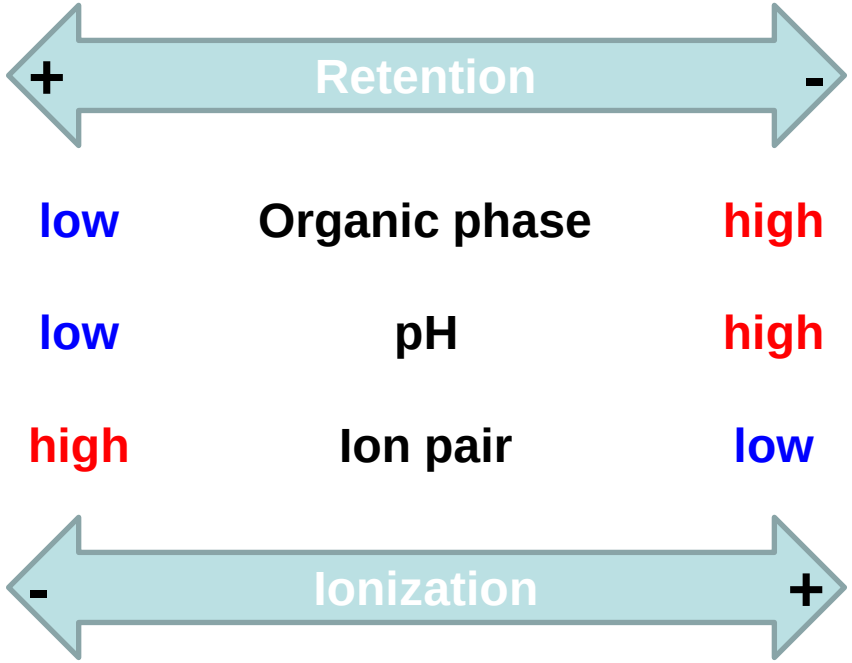
Measures

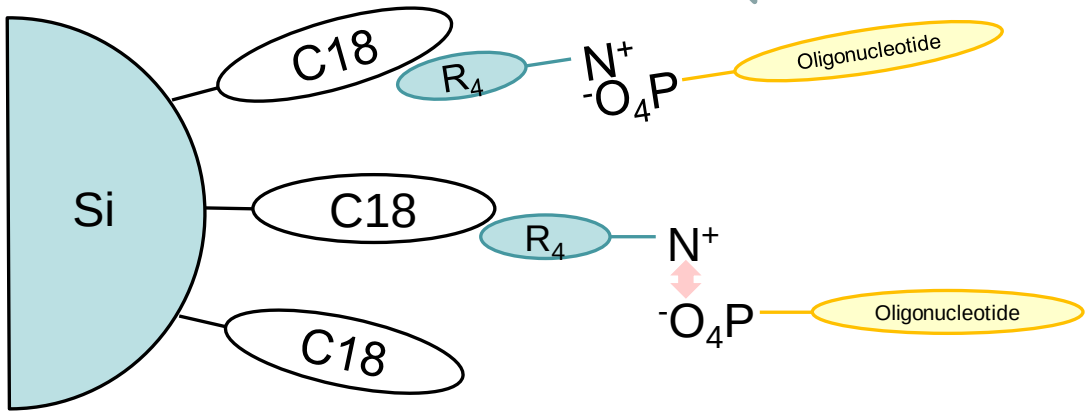
① Change fluorinated alcohol and alkylamine (ion-pair reagent).

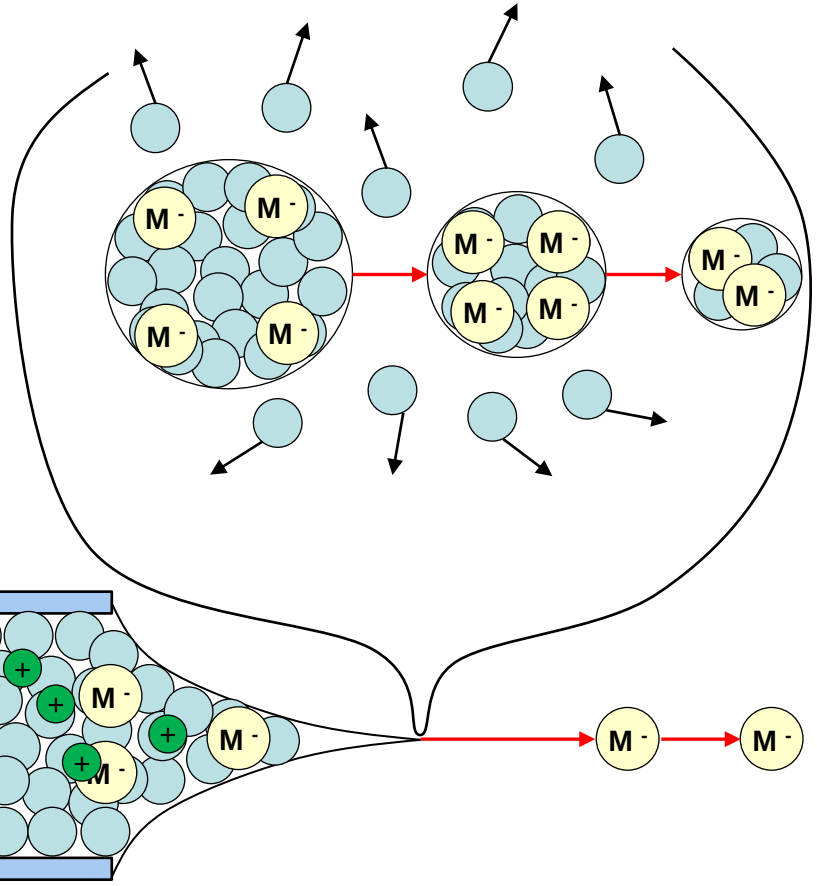
② Separate the target of analysis and the internal standard.

Ion-pair LC-MS/MS Efficiency

Chromatographic retention vs ionization efficiency in oligonucleotides analysis (Trade Off)







Ion-pair LC-MS/MS Efficiency : Method

HPLC System	Shimadzu 30A system
Column	ACQUITY Premier BEH C18 Column with VanGuard FIT (1.7 µm, 2.1 x 100 mm, Waters)
Column temperature	60°C
Mobile phase A	Ultrapure water
Mobile phase B	Methanol : acetonitrile = 75 : 25
Mobile phase C	1 mol/L Fluorinated alcohol and 150 mmol/L Alkylamine in methanol
Injection volume	10 µL
Needle wash solvent	Water / methanol / isopropanol/ acetylacetone / 0.5 M EDTA (pH 8.0) (500:250:250:2:2)
Flow rate	Sum of A and B : 0.27 mL/min, C : 0.03 mL/min
Gradient	

Time (min)	A Conc. (%)	B Conc. (%)
0.00	97.5	2.5
0.20	97.5	2.5
8.00	70	30
8.01	10	90
11.00	10	90
11.01	97.5	2.5
15.00	97.5	2.5

Fluorinated alcohol
- Hexafluoroisopropanol (HFIP)
- Trifluoroethanol (TFE)
- Pentafluoropropanol (PFP)

Alkylamine
- *N,N*-Diisopropylethylamine (DIPEA)
- Octylamine (OA)
- Diisopropylamine (DIPA)
- Hexylamine (HA)
- Tributylamine (TBA)
- *N,N*-Dimethylcyclohexylamine (DMCHA)

Ion-pair LC-MS/MS Efficiency : Materials and Evaluation

Materials

- Ion-pairs (6 reagents) and fluorinated alcohols (3 reagents) : 18 ways in total
- Analyte:

  ASO-1: *MALAT1* (metastasis associated in lung adenocarcinoma transcript-1) - ASO

  【Sequence】A(L)^G(L) ^T(L) ^a^c^t^a^t^a^g^c^a^t^5(L) ^T(L) ^G(L)＊1 (16 mer, MW：5303.32)
- Internal Standard Candidate:

  PS ASO-1: The above ASO with phosphorothioate modification only

  【Sequence】A^G^U^a^c^t^a^t^a^g^c^a^t^C^U^G＊1 (16 mer, MW：5189.23)
- Sample: Reference

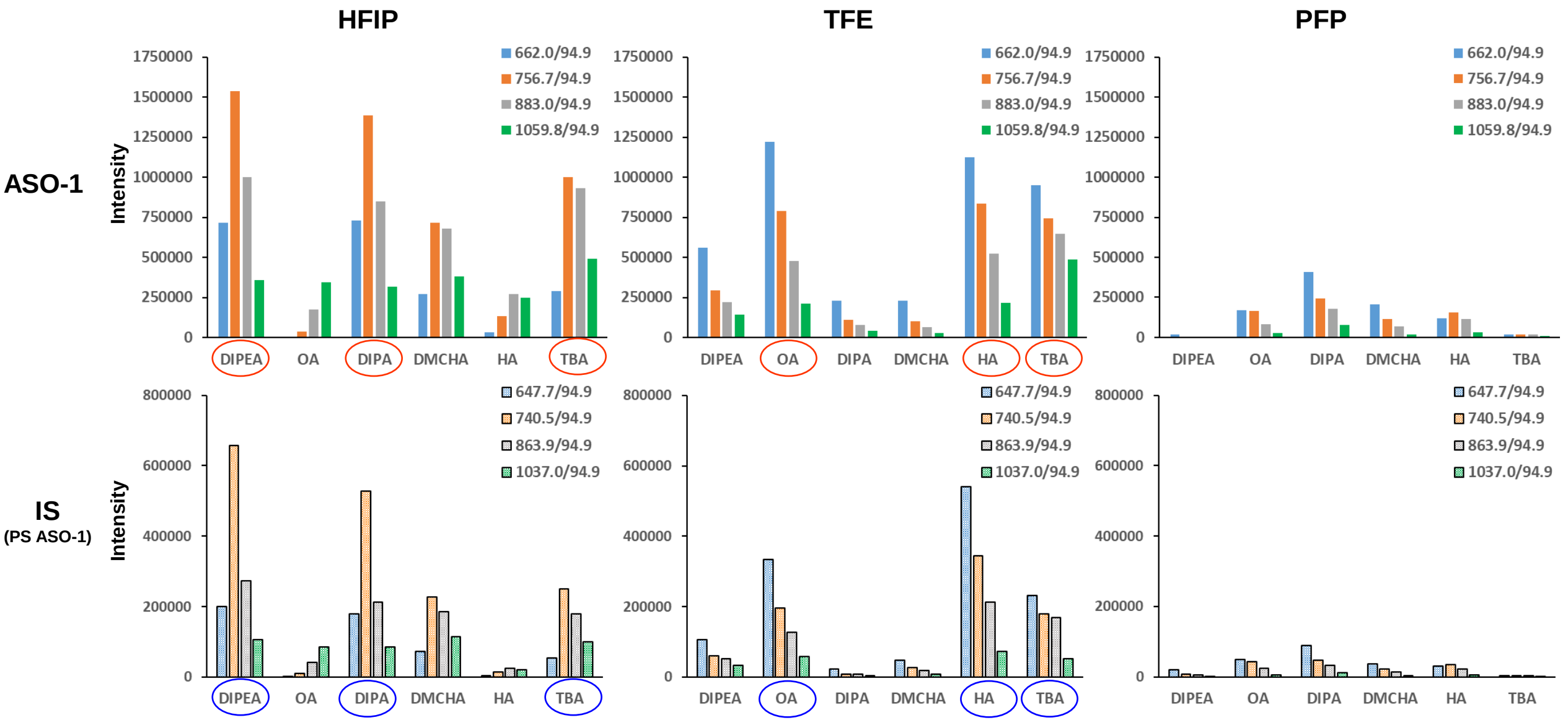
＊1: Upper Case=RNA / Lower Case=DNA / N(L)=LNA / N(M)=2′-OMe / N(m)=MOE / 5(L)=LNA_mC / 5(m)= MOE _mC / ^=Phosphorothioate

LNA：Locked nucleic acid
mC： 5-Methylcytosine

Evaluation

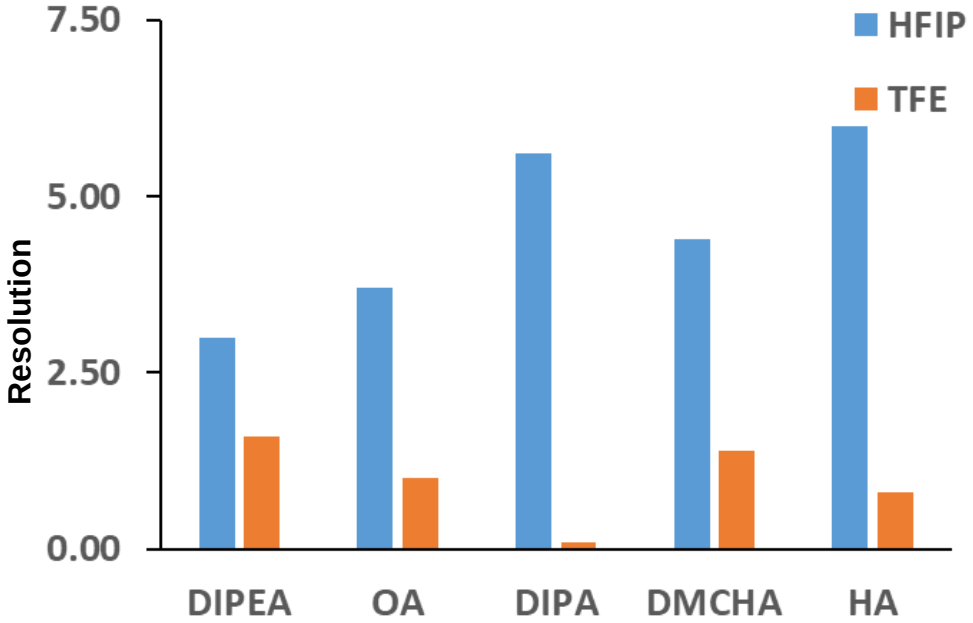
- Sensitivity comparison of each mobile phase
- Separation and chromatogram comparison of each mobile phase

Intensity (Ion-pair LC-MS/MS Efficiency) : Results



Separation (Ion-pair LC-MS/MS Efficiency) : Results

Resolution of oligonucleotides in each mobile phase



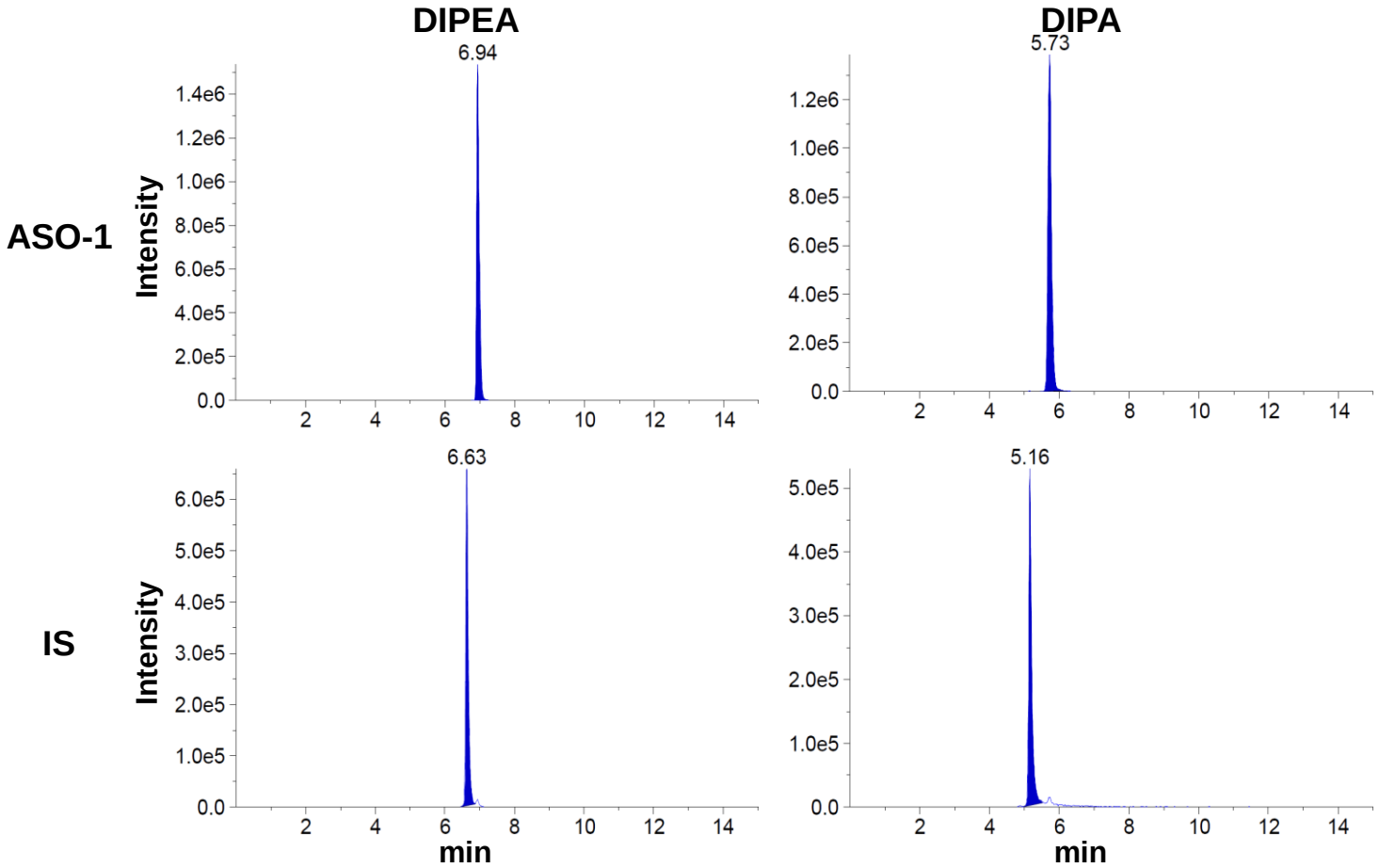
Resolution was calculated by the half-width method (below).

$$\frac{1.18 \times |tR_2 - tR_1|}{W_{0.5h1} + W_{0.5h2}}$$

t_{R1}: ASO-1 retention time, t_{R2}: IS retention time

$W_{0.5h1}$: ASO-1 half-width, $W_{0.5h2}$: IS half-width

Chromatograms of oligonucleotides in mobile phase containing HFIP



- The mobile phase was determined from the sensitivity and resolution.
  Ion pair reagent: DIPEA and DIPA
  Fluorinated alcohol: HFIP

Introduction

Critical Point

- **ESI-MS (Selection of precursor ion)**
  Multiple negative charge states (H^+, Na^+ and K^+ adduct ion)
- **MS/MS (Selection of product ion)**
  Fragmentation of multiple charged oligonucleotides
- **Ion-pair LC-MS/MS Efficiency**
  Retention vs ionization
- **Quantitation**
  Internal standard selection
- **Sample Preparation and Separation Conditions**
  Introduction and degradation of ion-pair reagent

Quantitation (Internal standard selection) : Materials

➢**Analyte**:

ASO-1: *MALAT1* (metastasis associated in lung adenocarcinoma transcript-1) - ASO

【Sequence】A(L)^G(L) ^T(L) ^a^c^t^a^t^a^g^c^a^t^5(L) ^T(L) ^G(L)＊1 (16 mer, MW：5303.32) ＊2

➢**Internal Standard Candidate**:

① **ASO-2**: ASO with the same modification as the above ASO but with a different sequence

【Sequence】G(L)^G(L) ^5(L) ^c^a^a^t^a^c^g^c^c^g^ T(L) ^ 5(L) ^A(L) ＊1 (16 mer, MW：5288.29)

② **PS ASO-1**: The above ASO with phosphorothioate modification only

【Sequence】A^G^U^a^c^t^a^t^a^g^c^a^t^C^U^G＊1 (16 mer, MW：5189.23)

③ **2′-OMe ASO-1**: The above ASO with 2′-OMe modification instead of LNA modification

【Sequence】A(M)^G(M) ^U(M) ^a^c^t^a^t^a^g^c^a^t^C(M) ^U(M) ^G(M) ＊1 (16 mer, MW：5273.41)

④ **MOE ASO-1**: The above ASO with MOE modification instead of LNA modification

【Sequence】A(m)^G(m) ^U(m) ^a^c^t^a^t^a^g^c^a^t^C(m) ^U(m) ^G(m) ＊1 (16 mer, MW：5579.80)

⑤ **ASO-3**: The 5′ terminal G(L) was deleted from the above ASO

【Sequence】A(L)^G(L) ^T(L) ^a^c^t^a^t^a^g^c^a^t^5(L) ^T(L) ＊1 (15 mer, MW：4930.05)

＊1: Upper Case=RNA / Lower Case=DNA / N(L)=LNA / N(M)=2′-OMe / N(m)=MOE / 5(L)=LNA_mC / 5(m)= MOE _mC / ^=Phosphorothioate

＊2: Paymaan Jafar-Nejad, et al. The atlas of RNase H antisense oligonucleotide distribution and activity in the CNS of rodents and non-human primates following central administration. Nucleic Acids Res. 2021 Jan 25;49(2):657-673.

LNA：Locked nucleic acid, **mC**：5-Methylcytosine, **2′-OMe**：2′-O-Methyl, **MOE**：2′-Methoxyethyl

Quantitation (Internal standard selection) : Method

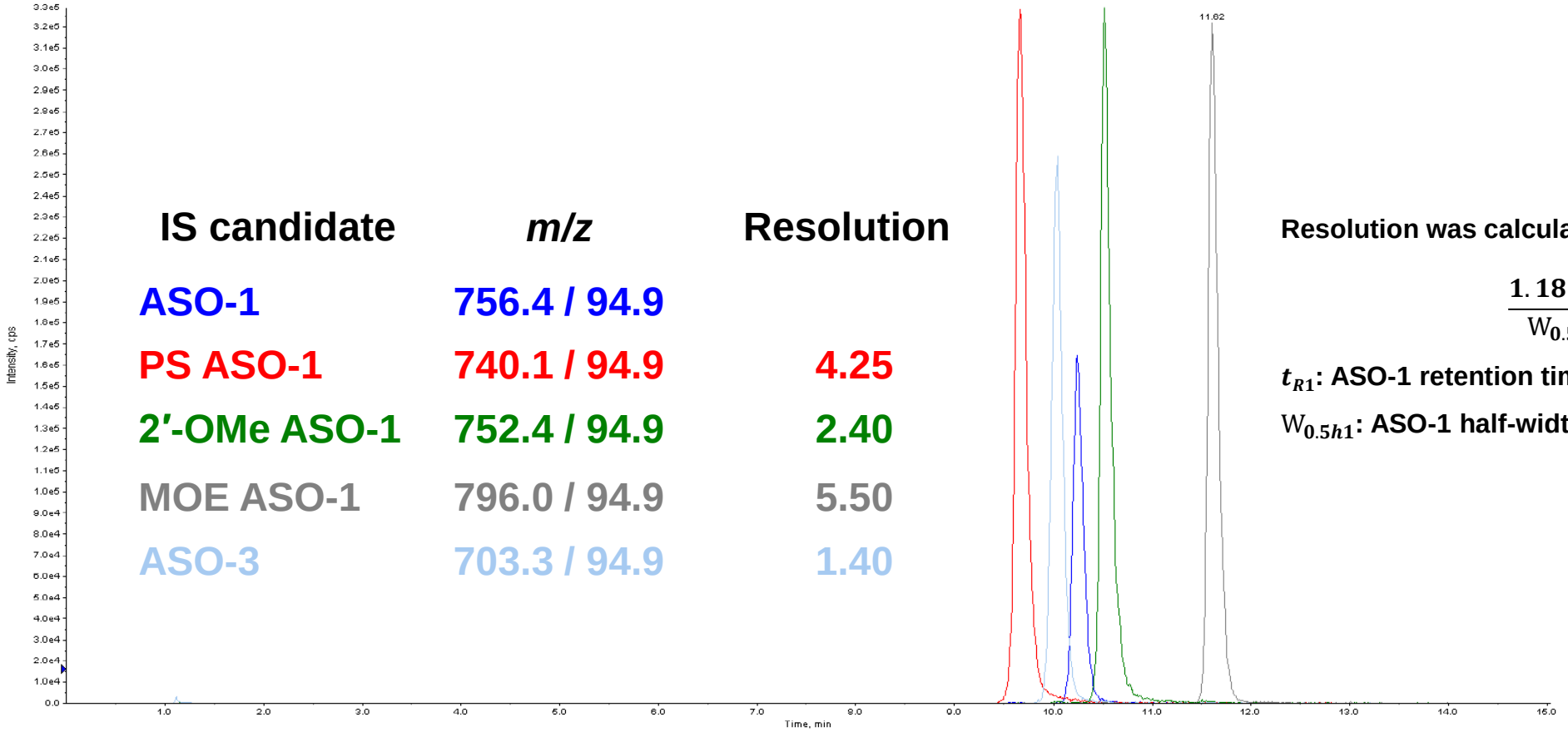
Analysis target	ASO-1, 4 candidate internal standard
HPLC System	Shimadzu 30A system
Column	ACQUITY Premier BEH C18 Column with VanGuard FIT (1.7 µm, 2.1 x 100 mm, Waters)
Column temperature	60°C
Mobile phase A	Ultrapure water
Mobile phase B	Methanol : acetonitrile = 75 : 25
Mobile phase C	1 mol/L HFIP and 150 mmol/L DIPEA in methanol
Injection volume	3 µL
Needle wash solvent	Water / methanol / isopropanol/ acetylacetone / 0.5 M EDTA (pH 8.0) (500:250:250:2:2)
Flow rate	0.3 mL/min
Gradient	See table below
MS/MS system	API5000
Scan type	Selected Reaction Monitoring
Monitored Ion	See Figure (Next page)

Gradient

Time (min)	A Conc. (%)	B Conc. (%)	C Conc. (%)
0.00	90.0	0.0	10.0
0.50	90.0	0.0	10.0
27.00	0.0	90.0	10.0
29.00	0.0	90.0	10.0
29.01	90.0	0.0	10.0
34.00	90.0	2.5	10.0

HFIP: Hexafluoroisopropanol
DIPEA: *N,N*-Diisopropylethylamine

Quantitation (Internal standard selection) : Results



IS candidate	*m/z*	Resolution
ASO-1	756.4 / 94.9	
PS ASO-1	740.1 / 94.9	4.25
2′-OMe ASO-1	752.4 / 94.9	2.40
MOE ASO-1	796.0 / 94.9	5.50
ASO-3	703.3 / 94.9	1.40

Resolution was calculated by the half-width method (below).

$$\frac{1.18 \times |tR_2 - tR_1|}{W_{0.5h1} + W_{0.5h2}}$$

t_{R1}: ASO-1 retention time, t_{R2}: IS candidate retention time

$W_{0.5h1}$: ASO-1 half-width, $W_{0.5h2}$: IS candidate half-width

- The internal standard was determined from the resolution.
  Internal standard: **PS ASO-1,** **MOE ASO-1**

Introduction

Critical Point

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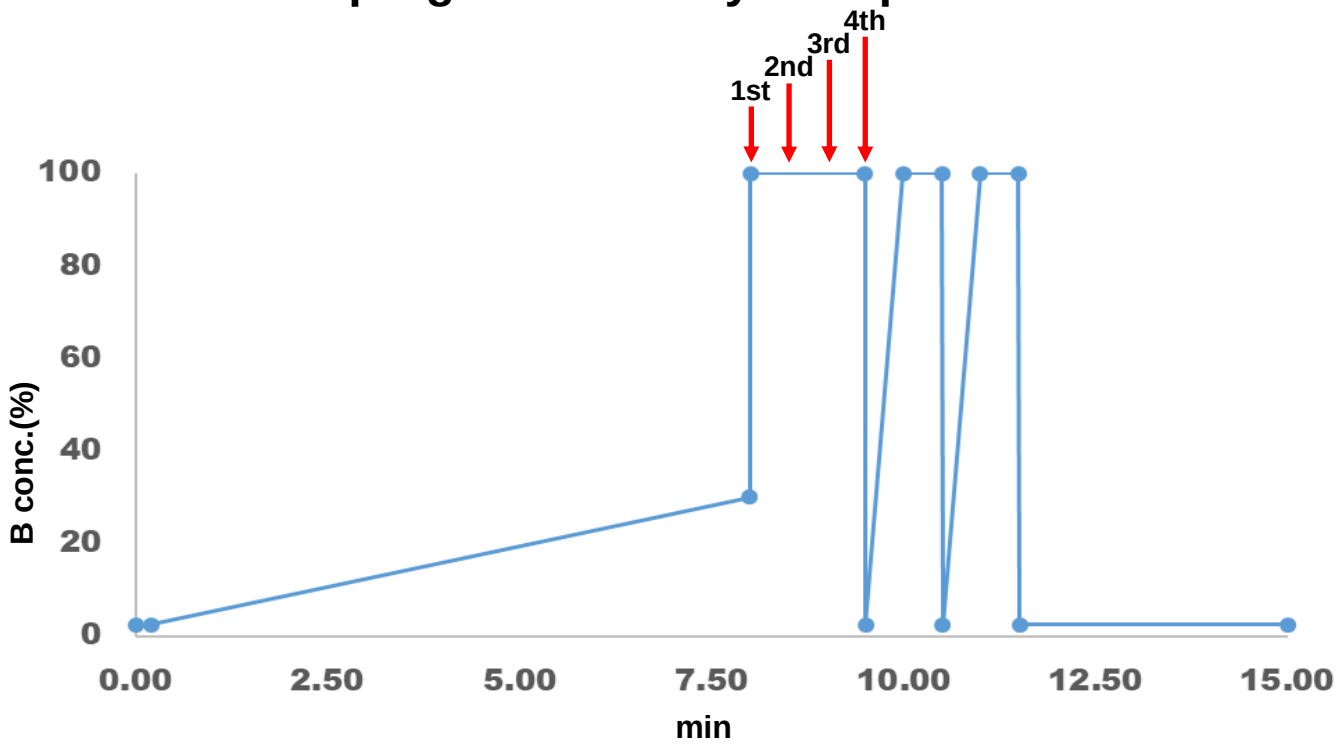
  Multiple negative charge states (H^+, Na^+ and K^+ adduct ion)
- **MS/MS (Selection of product ion)**
  Fragmentation of multiple charged oligonucleotides
- **Ion-pair LC-MS/MS Efficiency**
  Retention vs ionization
- **Quantitation**
  Internal standard selection
- **Sample Preparation and Separation Conditions**
  Introduction and degradation of ion-pair reagent

Carry over (Separation conditions) : Method

Gradient program for carry over prevention



Arrows (↓) indicate the timing of injection when multiple injections of injection reagent for carry over prevention is conducted.

Injection reagent for carry over prevention

① No injection

② 5 mM EDTA × 3 injections

③ Blank × 3 injections

④ 5% ammonia × 3 injections

⑤ Blank including MOE × 3 injections

⑥ Blank × 4 injections

⑦ Blank including MOE × 4 injections

- 5 mM EDTA and 5% ammonia are used as stripping reagents.
- Blanks and MOE are used as stripping and blocking reagents.

Sample injection order:

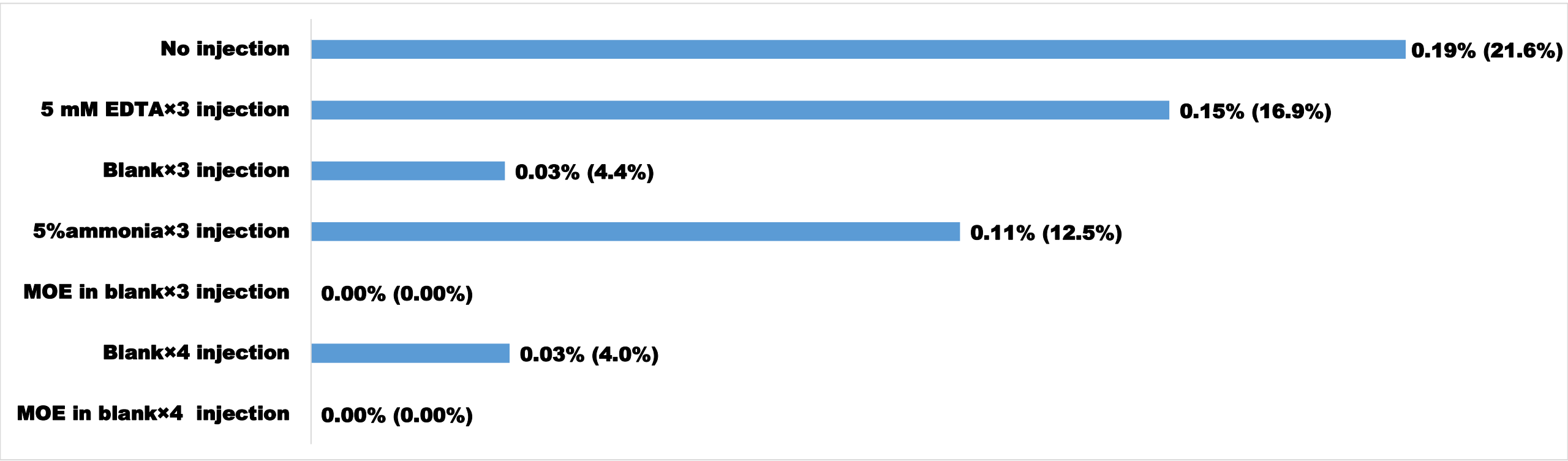
LLOQ sample → ULOQ sample → Double blank sample

LLOQ: 10 ng/mL

ULOQ: 1000 ng/mL

MOE: 2'-Methoxyethyl
LLOQ: lower limit of quantification
ULOQ: Upper limit of quantification

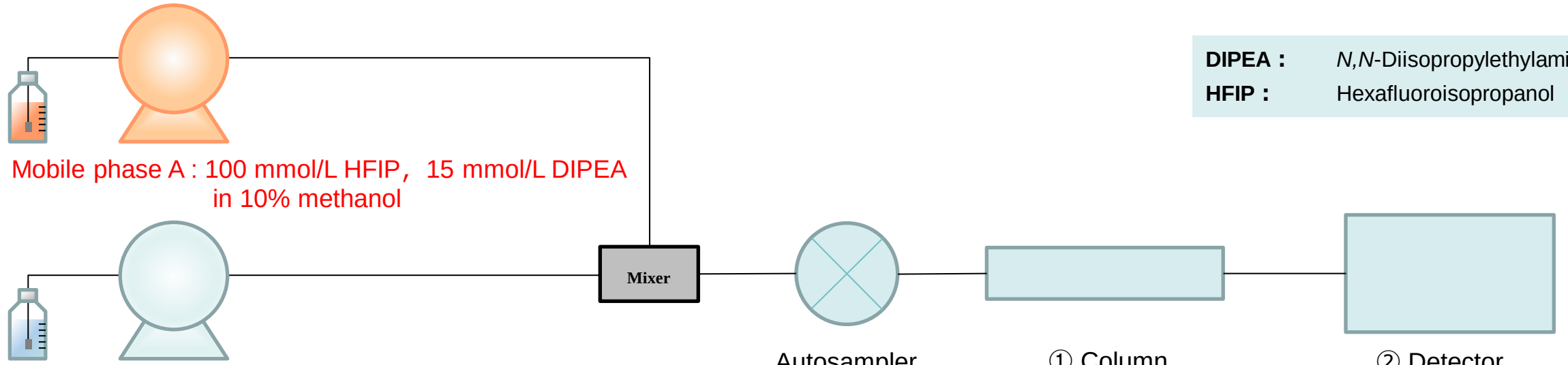
Carry over (Separation conditions) : Results



- Each stripping reagents was effective against carry over.
- Blocking reagent increase effectiveness of stripping reagents against carry over.
- The effect of the blocking reagent does not change after 3 or more injections.

Introduction and degradation of ion-pair reagent : Condition 1

Degradation of mobile phase (alkylamine/fluorinated alcohol) over time

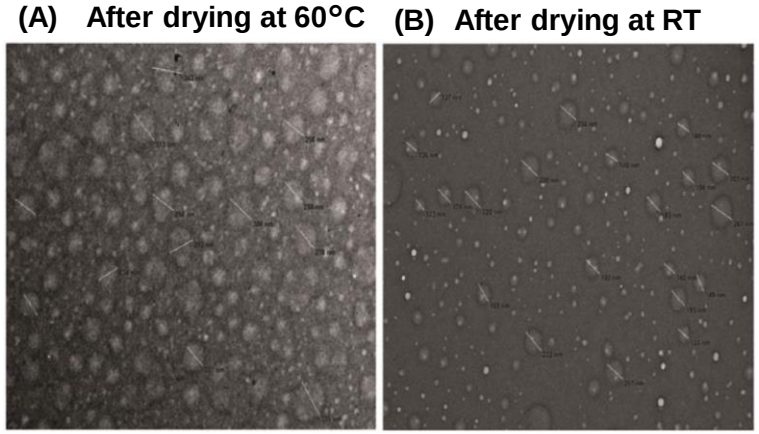


DIPEA : *N,N*-Diisopropylethylamine
HFIP : Hexafluoroisopropanol

The ion-pair reagent (alkylamine) in the aqueous mobile phase deteriorates due to pH increase and high temperature. (Expansion of micelles)

① Micellization of alkylamines shortens the retention time.

② Ionization suppression occurs due to micellization of alkylamines.

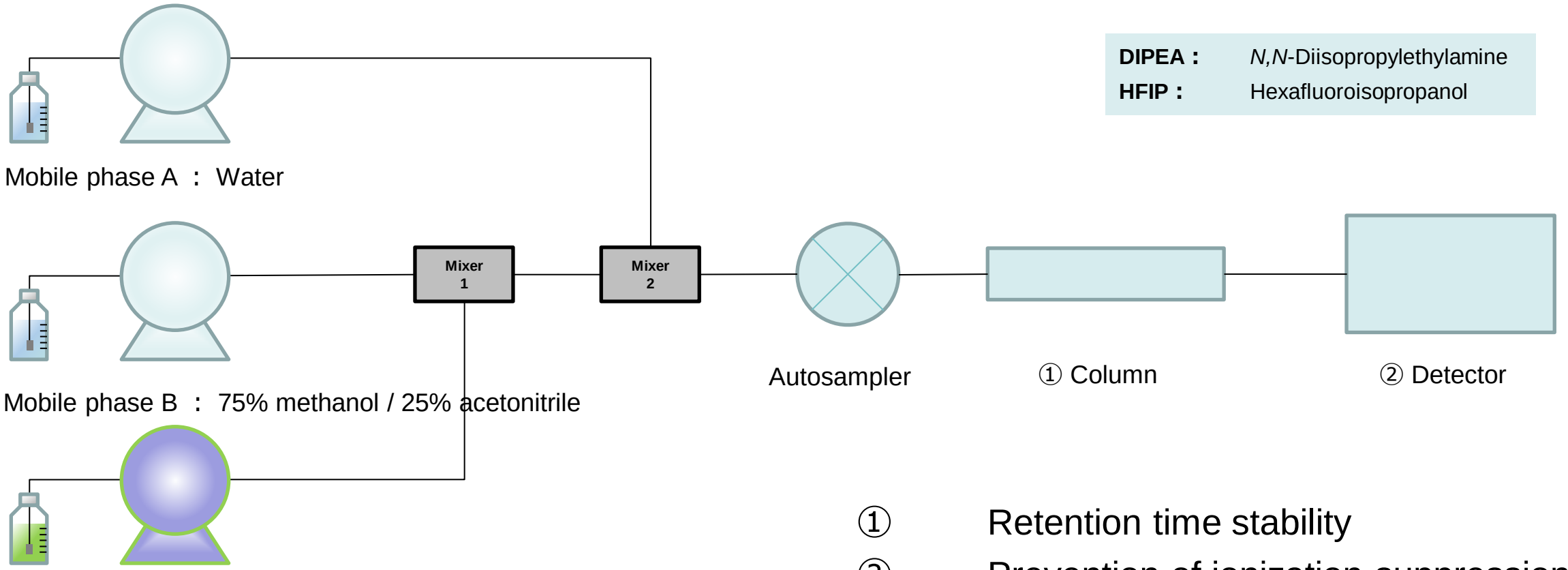


Electron micrograph of micellization of alkylamines*

* Li N, et al. Alkylamine ion-pairing reagents and the chromatographic separation of oligonucleotides. J. Chromatogr. A 1580, 110–119 (2018).

Introduction and degradation of ion-pair reagent : Condition 2

Countermeasures against deterioration of mobile phase over time



DIPEA : *N,N*-Diisopropylethylamine
HFIP : Hexafluoroisopropanol

① Retention time stability

② Prevention of ionization suppression (stable sensitivity)

Introduction and degradation of ion-pair reagent ：Method

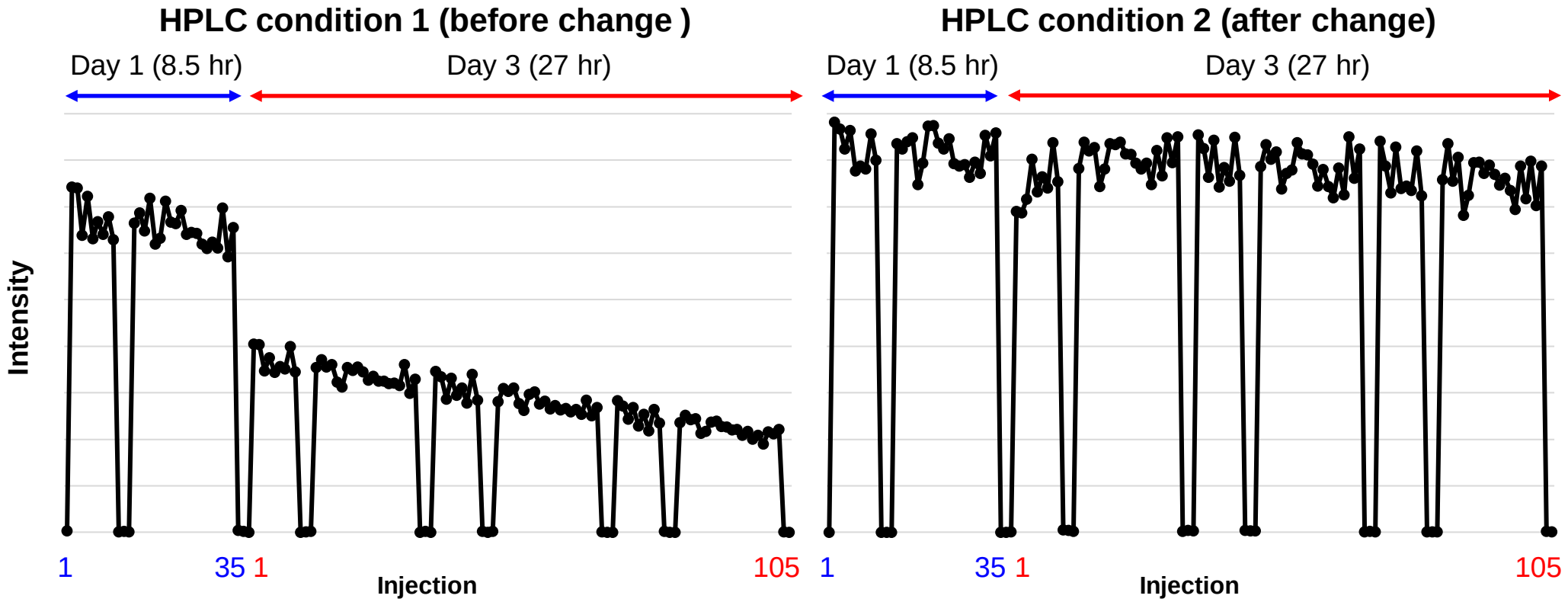
HPLC System	Shimadzu 40A system	
Column	ACQUITY Premier BEH C18 Column with VanGuard FIT (1.7 µm, 2.1 x 100 mm, Waters)	
Column temperature	60°C	
Mobile phase A	100 mmol/L HFIP, 15 mmol/L DIPEA in 10% methanol	→ Ultrapure water
Mobile phase B	100 mmol/L HFIP,15 mmol/L DIPEA in 75% methanol/ 25% acetonitrile	→ 75% methanol / 25% acetonitrile
Mobile phase C	-	→1 mol/L HFIP, 150 mmol/L DIPEA in methanol
Injection volume	5 µL	
Needle wash solvent	Water / methanol / 2-propanol/ acetylacetone/ 0.5 M EDTA (pH 8.0) (500:250:250:2:2)	
Flow rate	0.3 mL/min	→A-B：0.27 mL/min，C：0.03 mL/min

Time (min)	A Conc. (%)	B Conc. (%)
0.00	97.5	2.5
0.20	97.5	2.5
8.00	70	30
8.01	10	90
11.00	10	90
11.01	97.5	2.5
15.00	97.5	2.5

DIPEA： *N,N*-Diisopropylethylamine
HFIP： Hexafluoroisopropanol

Introduction and degradation of ion-pair reagent : Results

Sensitivity changes due to different ion-pairing reagent introduction *



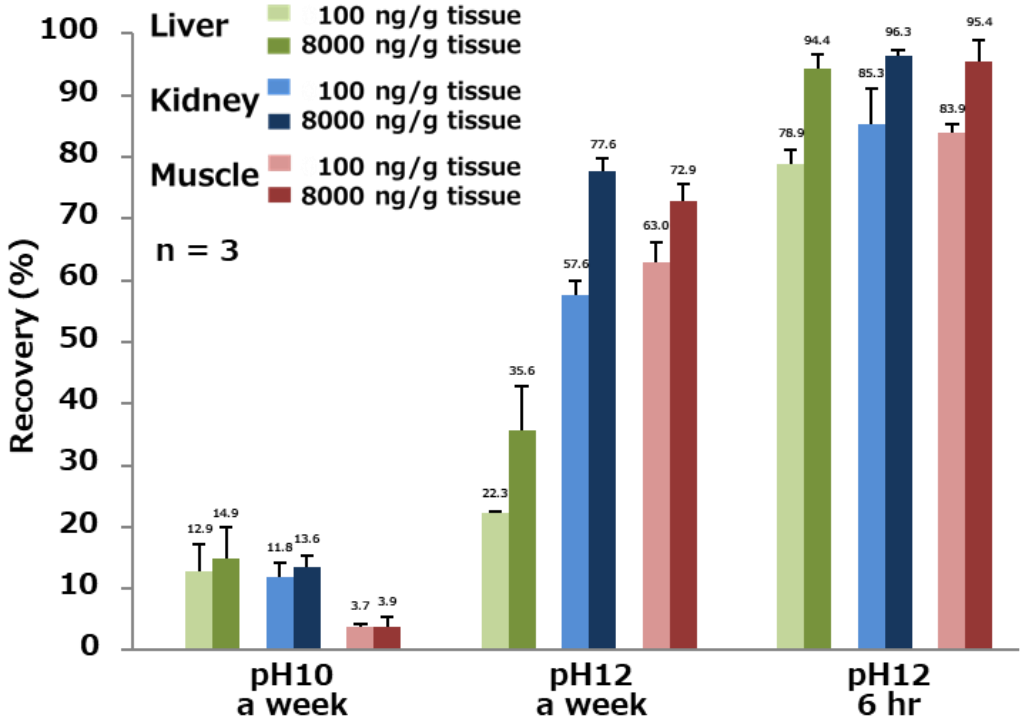
By introducing the ion-pair reagent from the water-free mobile phase C, it was possible to avoid the decrease in sensitivity of the IS during the measurement.

MS/MS system: TQ7500
Matrix: Brain homogenate
Pretreatment: LLE+SPE_HFIP+acid

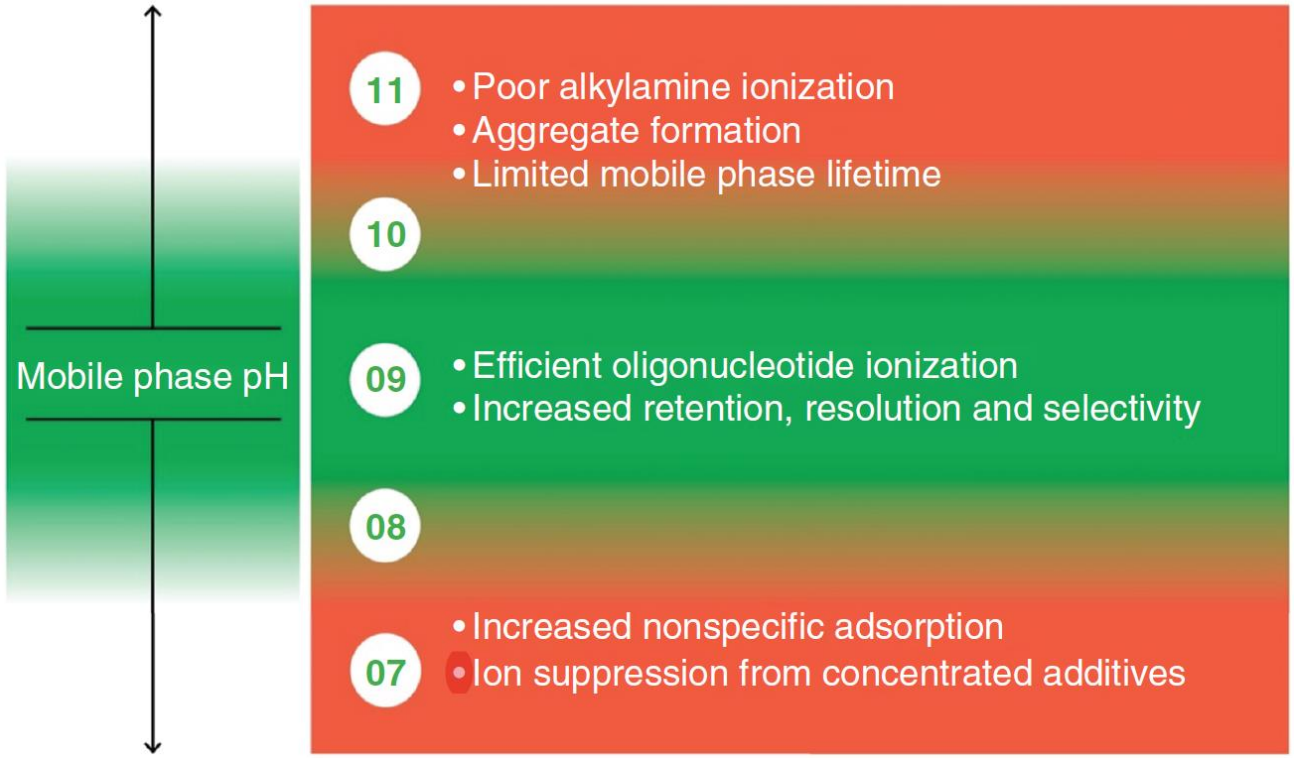
* Guilherme J Guimaraes, et al. The critical role of mobile phase pH in the performance of oligonucleotide ion-pair LC–MS methods. Future Science OA (2021) FSO753.

Degradation of ion-pair reagent (eluent) : Results

pH and freshness versus recovery of ASO-1 in SPE



General relationship between mobile phase pH and method performance*



- Degradation of alkylamine in both mobile phase and eluent adversely affects sensitivity (MS) and recovery (SPE).
- In order to prevent deterioration of the ion-pair reagent, it is necessary to pay attention to the water content and pH control of the mobile phase and elution buffer.

* Guilherme J Guimaraes, et al. The critical role of mobile phase pH in the performance of oligonucleotide ion-pair LC–MS methods. Future Science OA (2021) FSO753.

Conclusion

- **Selection of internal standard**
  In order to achieve high sensitivity analysis, it is necessary to select the product ion of *m/z* 94.9.
  In that case, the selection of the precursor ion becomes important.

- **Separation of the analyte and internal standards in HPLC**
  It is better to use HFIP (fluorinated alcohol) and DIPEA or DIPA (ion-pair reagent) in mobile phases to achieve high sensitivity and high resolution.

- **Prevention of ion-pair reagents deterioration**
  Mobile phase
  Do not mix ion-pair reagents with water, and ion-pairing reagents should be mixed at time of analysis.

  Elution buffer in SPE
  The elution buffer should be of high pH (>12) and should be freshly prepared.

- **Carry over measures**
  Carry over can be reduced by injecting a stripping reagent. Furthermore, the effect can be enhanced by using a blocking reagent together.