Fig. 1

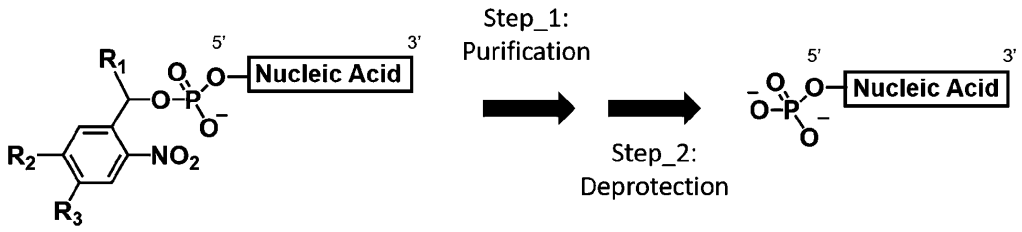
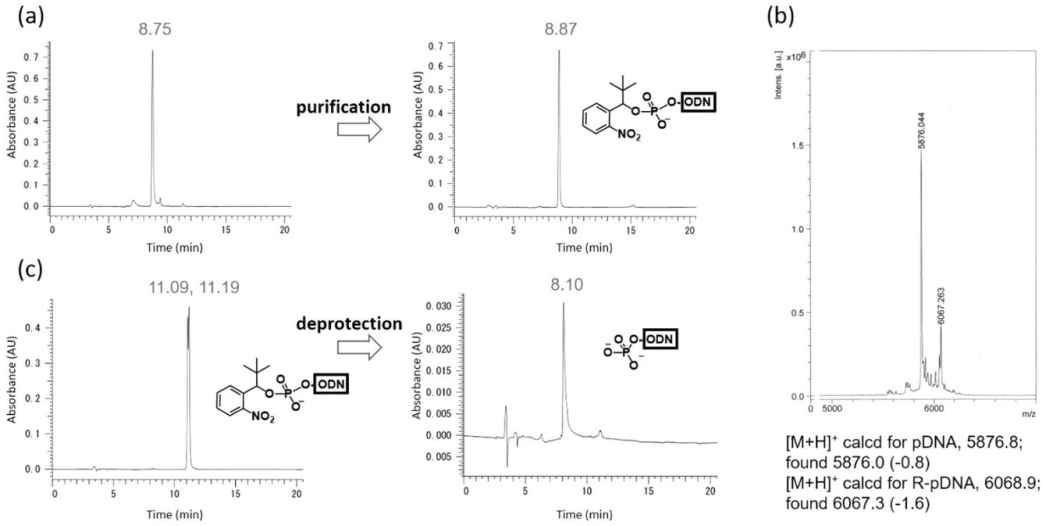
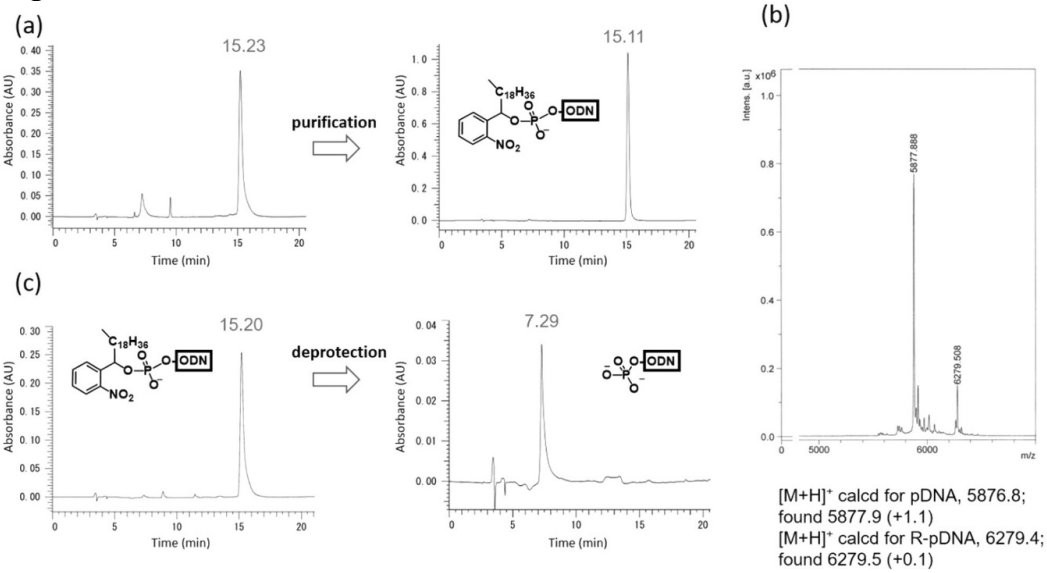


Fig. 2



[M+H]⁺ calcd for pDNA, 5876.8;
found 5876.0 (-0.8)
[M+H]⁺ calcd for R-pDNA, 6068.9;
found 6067.3 (-1.6)

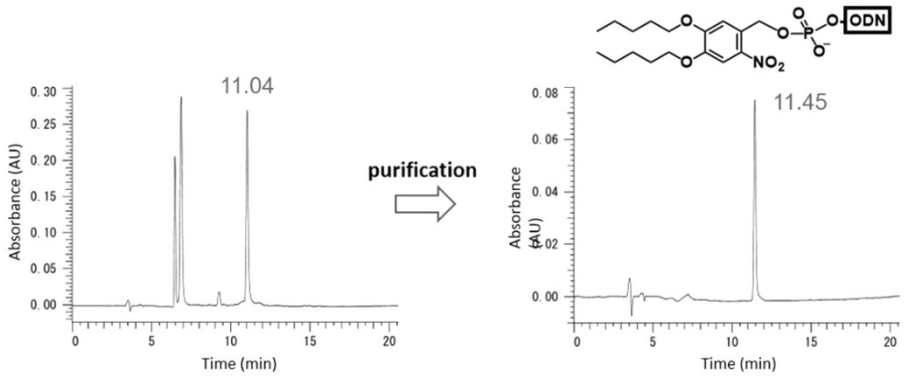
Fig. 3



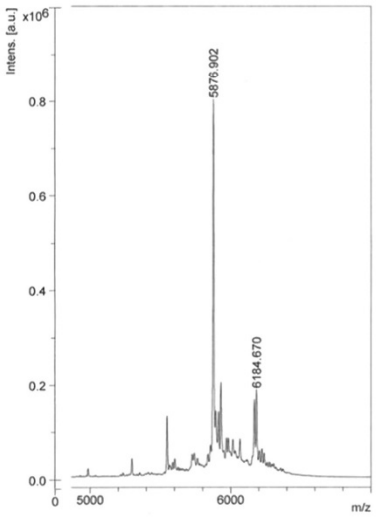
[M+H]⁺ calcd for pDNA, 5876.8;
found 5877.9 (+1.1)
[M+H]⁺ calcd for R-pDNA, 6279.4;
found 6279.5 (+0.1)

Fig. 4

(a)



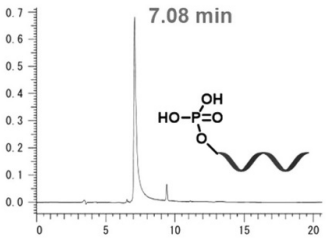
(b)



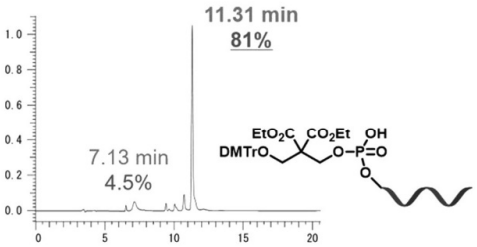
$[M+H]^+$ calcd for pDNA, 5876.8;
found 5876.9 (+0.1)
$[M+H]^+$ calcd for R-pDNA, 6185.0;
found 6184.7 (-0.3)

Fig. 5

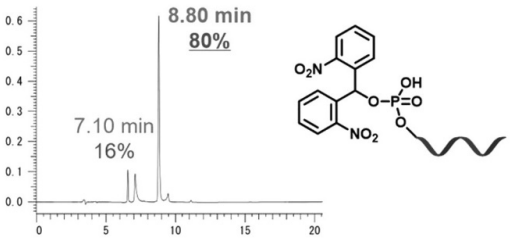
a) **CPR I** (commercially available)



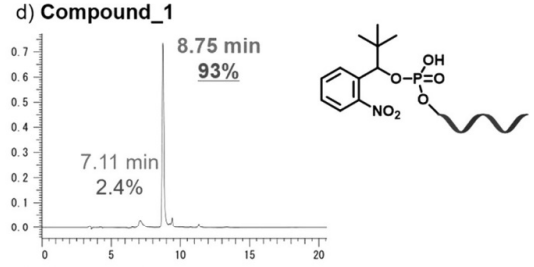
b) **CPR II** (commercially available)



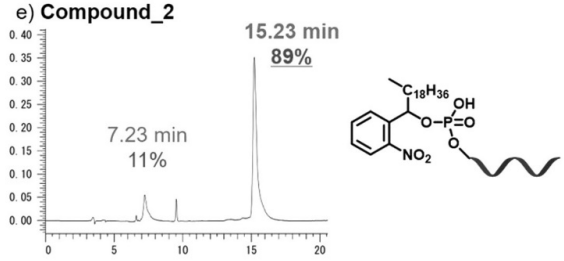
c) A reagent in *Chem Eur J* **23,** 5210 (2017)



d) **Compound_1**



e) **Compound_2**



f) Summary of this study

Name of reagent	Retention time in RP-HPLC (min)	Yield (%)
CPR II	11.31	81
A reagent in the literature*	8.80	80
Compound_1	8.75	93
Compound_2	15.23	89

Fig. 6

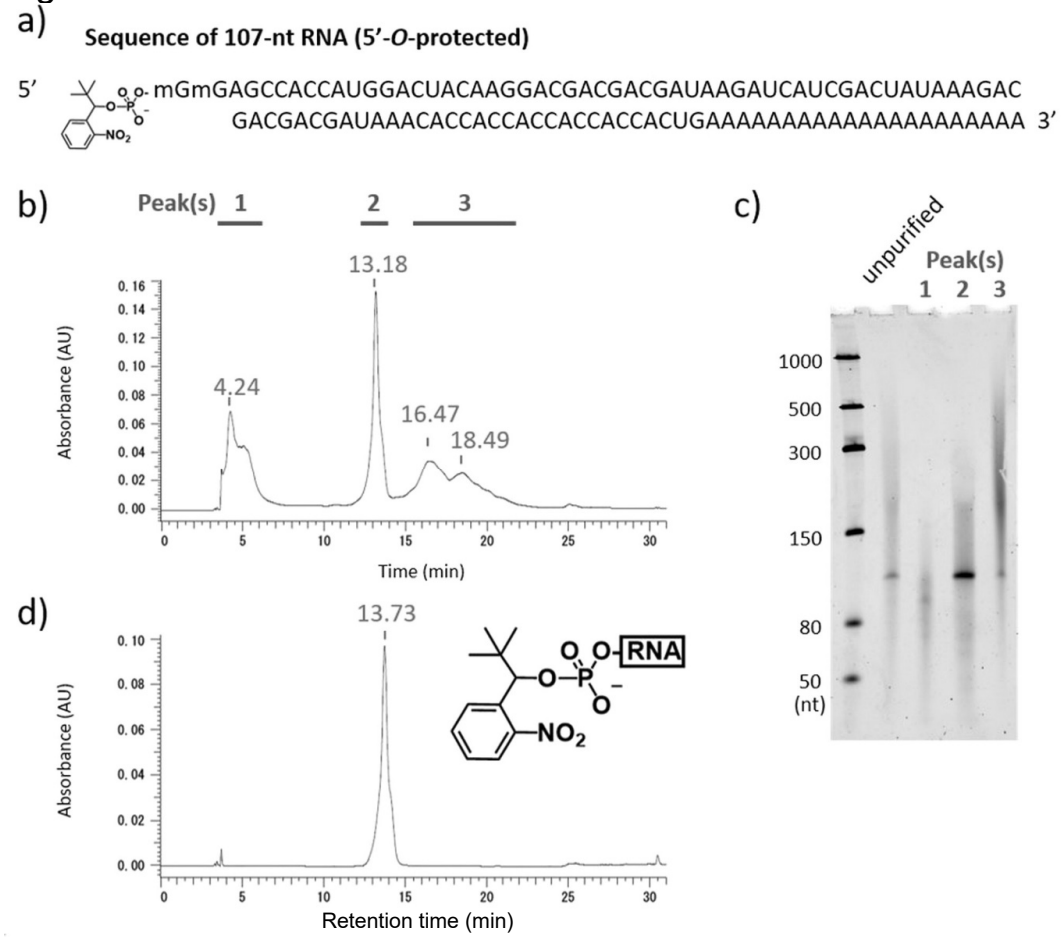

a)
Sequence of 107-nt RNA (5'-O-protected)
5'
mGmGAGCCACCAUGGACUACAAGGACGACGACGAUAAGAUCAUCGACUAUAAAGAC
GACGACGAUAAACACCACCACCACCACCACUGAAAAAAAAAAAAAAAAAAAAA 3'
b)
Peak(s) 1 2 3
13.18
4.24
16.47
18.49
Absorbance (AU)
Time (min)
c)
unpurified
Peak(s)
1 2 3
1000
500
300
150
80
50
(nt)
d)
13.73
RNA
Absorbance (AU)
Retention time (min)

Fig. 7

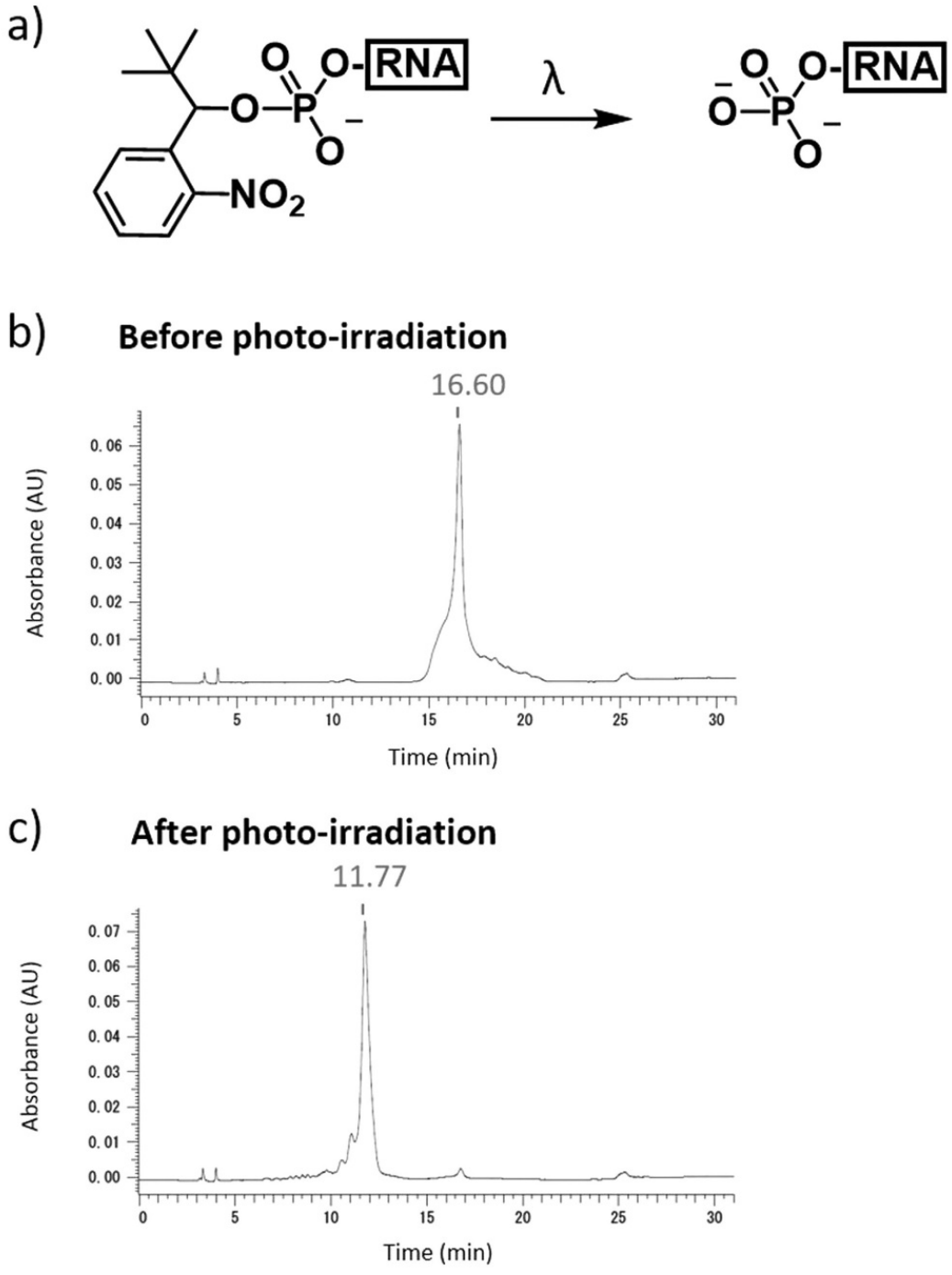

a)
RNA
λ
RNA
b)
Before photo-irradiation
16.60
Absorbance (AU)
Time (min)
c)
After photo-irradiation
11.77
Absorbance (AU)
Time (min)

Fig. 8

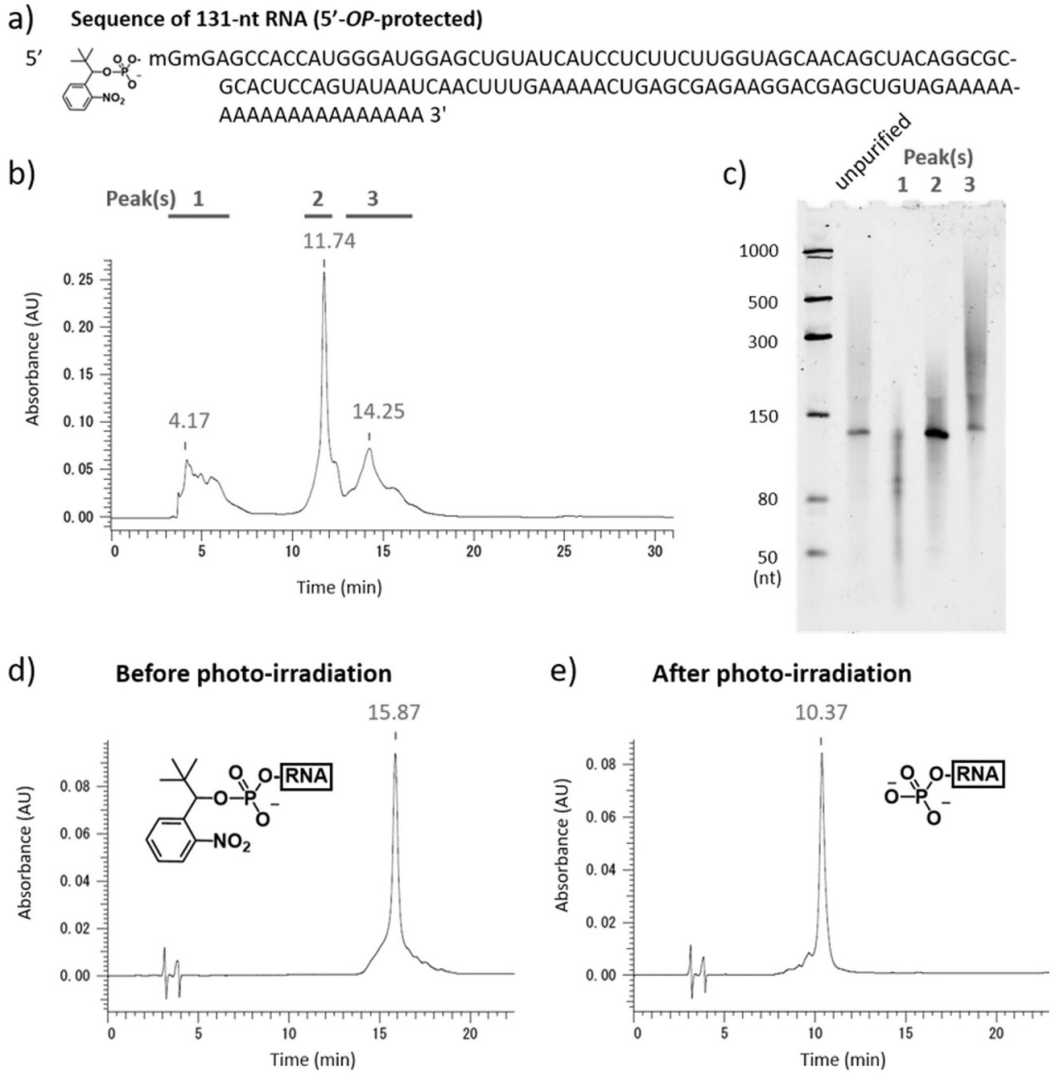

a)
Sequence of 131-nt RNA (5′-OP-protected)
5′
mGmGAGCCACCAUGGGAUGGAGCUGUAUCAUCCUCUUCUUGGUAGCAACAGCUACAGGCGC-
GCACUCCAGUAUAAUCAACUUUGAAAAACUGAGCGAGAAGGACGAGCUGUAGAAAAA-
AAAAAAAAAAAAAAA 3'
b)
Peak(s) 1
2
3
11.74
4.17
14.25
Absorbance (AU)
Time (min)
c)
unpurified
Peak(s)
1 2 3
1000
500
300
150
80
50
(nt)
d)
Before photo-irradiation
15.87
RNA
Absorbance (AU)
Time (min)
e)
After photo-irradiation
10.37
RNA
Absorbance (AU)
Time (min)

Fig. 9

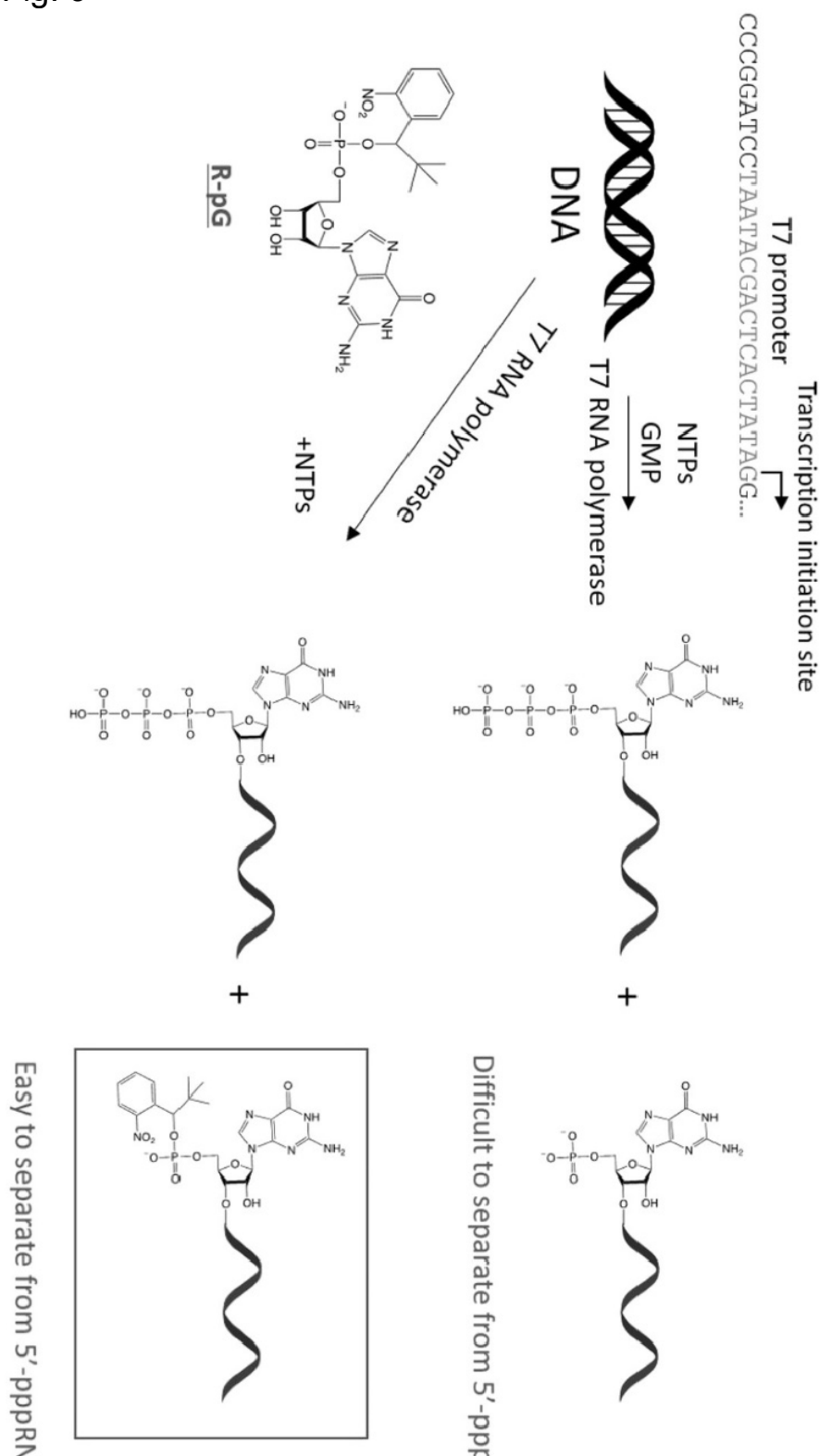
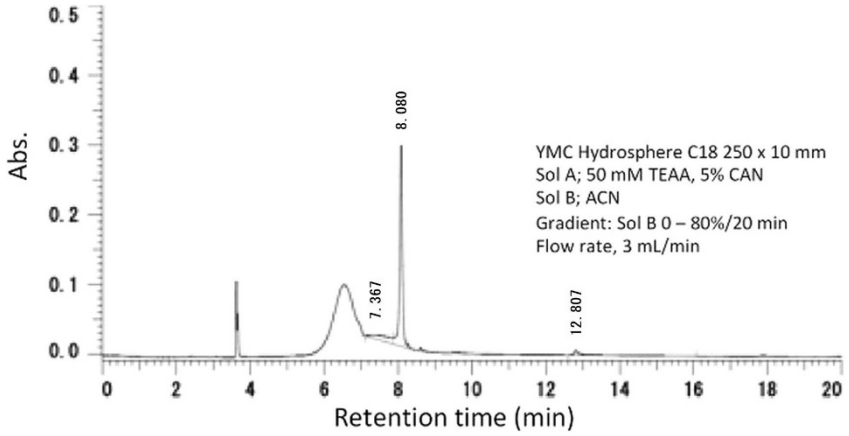

T7 promoter
Transcription initiation site
CCCGGATCCTAATACGACTCACTATAGG...
DNA
NTPs
GMP
T7 RNA polymerase
T7 RNA polymerase
+NTPs
R-pG
Difficult to separate from 5'-pppRNA
Easy to separate from 5'-pppRNA

Fig. 10

(a) pG (GMP)



(b) R-pG

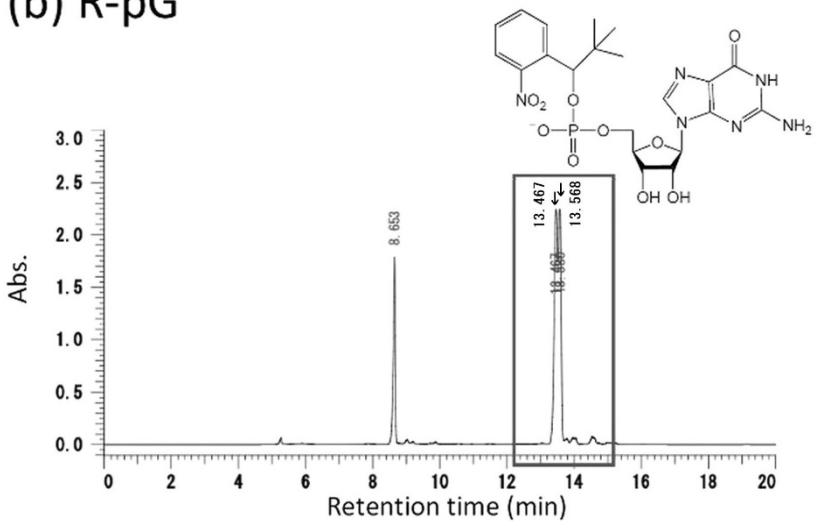
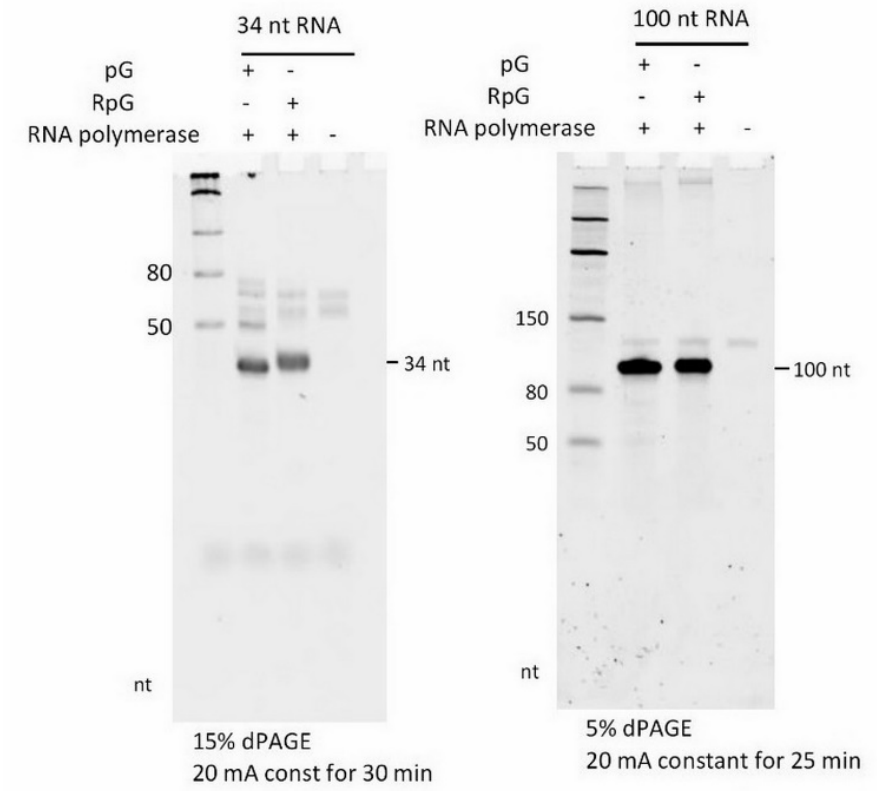


Fig. 11


34 nt RNA
pG + -
RpG - +
RNA polymerase + + -
80
50
34 nt
nt
15% dPAGE
20 mA const for 30 min
100 nt RNA
pG + -
RpG - +
RNA polymerase + + -
150
80
50
100 nt
nt
5% dPAGE
20 mA constant for 25 min

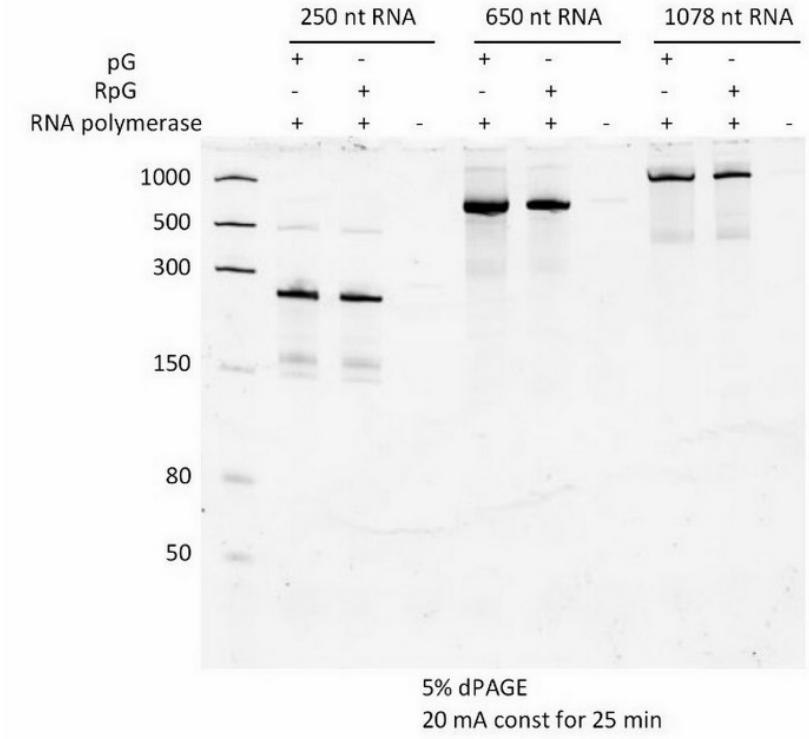

250 nt RNA
650 nt RNA
1078 nt RNA
pG + - + - + -
RpG - + - + - +
RNA polymerase + + - + + - + + -
1000
500
300
150
80
50
5% dPAGE
20 mA const for 25 min

Fig. 12

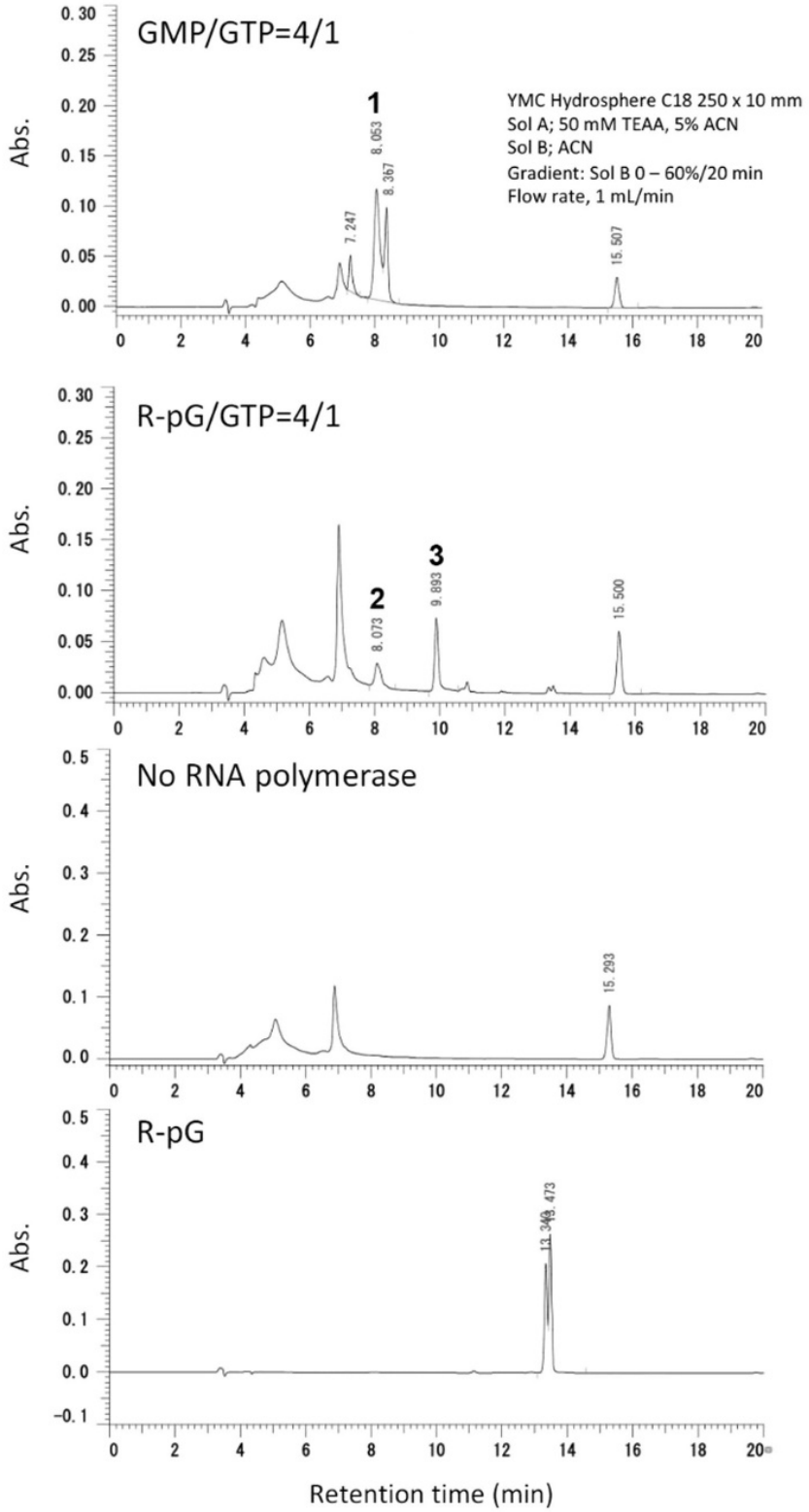

GMP/GTP=4/1
YMC Hydrosphere C18 250 x 10 mm
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 60%/20 min
Flow rate, 1 mL/min
1
8.063
8.367
7.247
15.507
R-pG/GTP=4/1
2
8.073
3
9.893
15.500
No RNA polymerase
15.293
R-pG
13.473
Abs.
Retention time (min)

Fig. 13

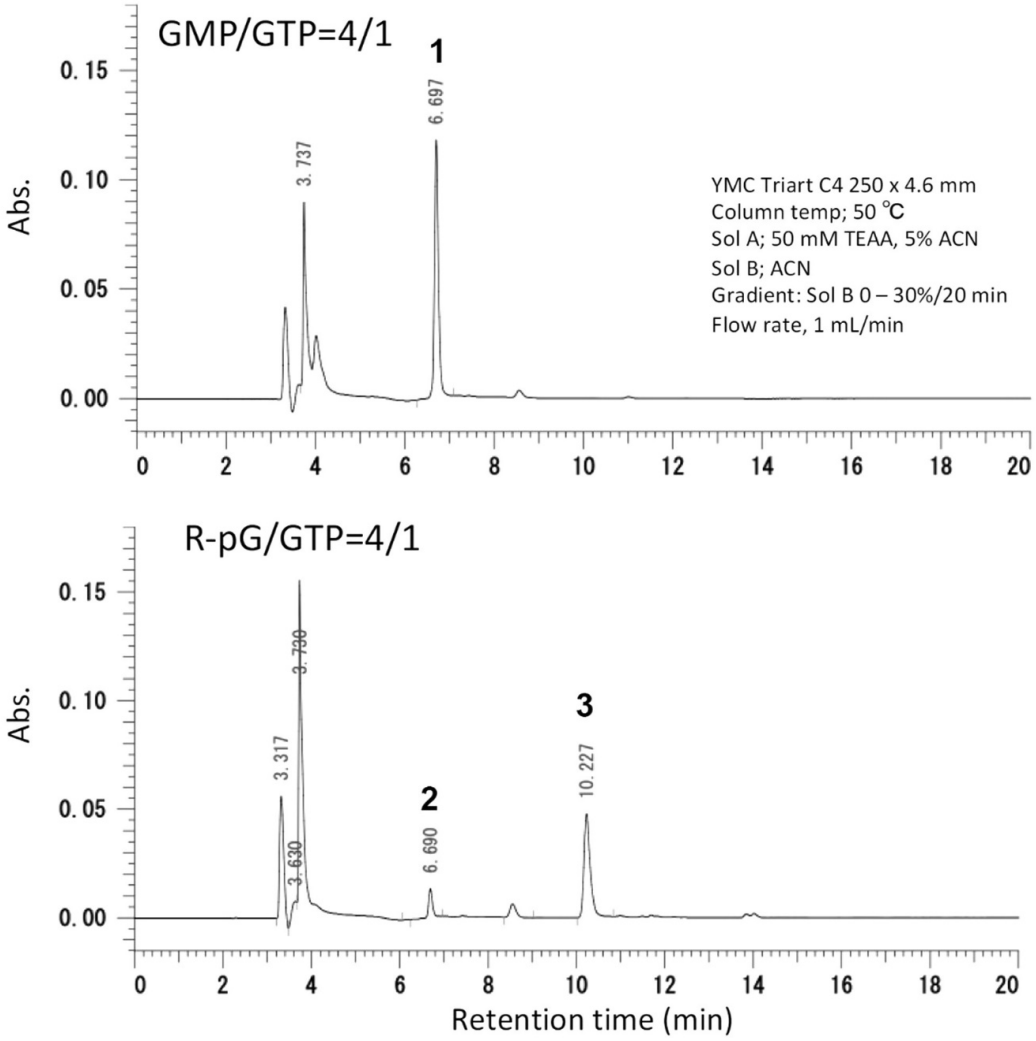

GMP/GTP=4/1
1
6.697
3.737
YMC Triart C4 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
R-pG/GTP=4/1
3.730
3.317
3.630
2
6.690
3
10.227
Abs.
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
16
18
20
Retention time (min)

Fig. 14

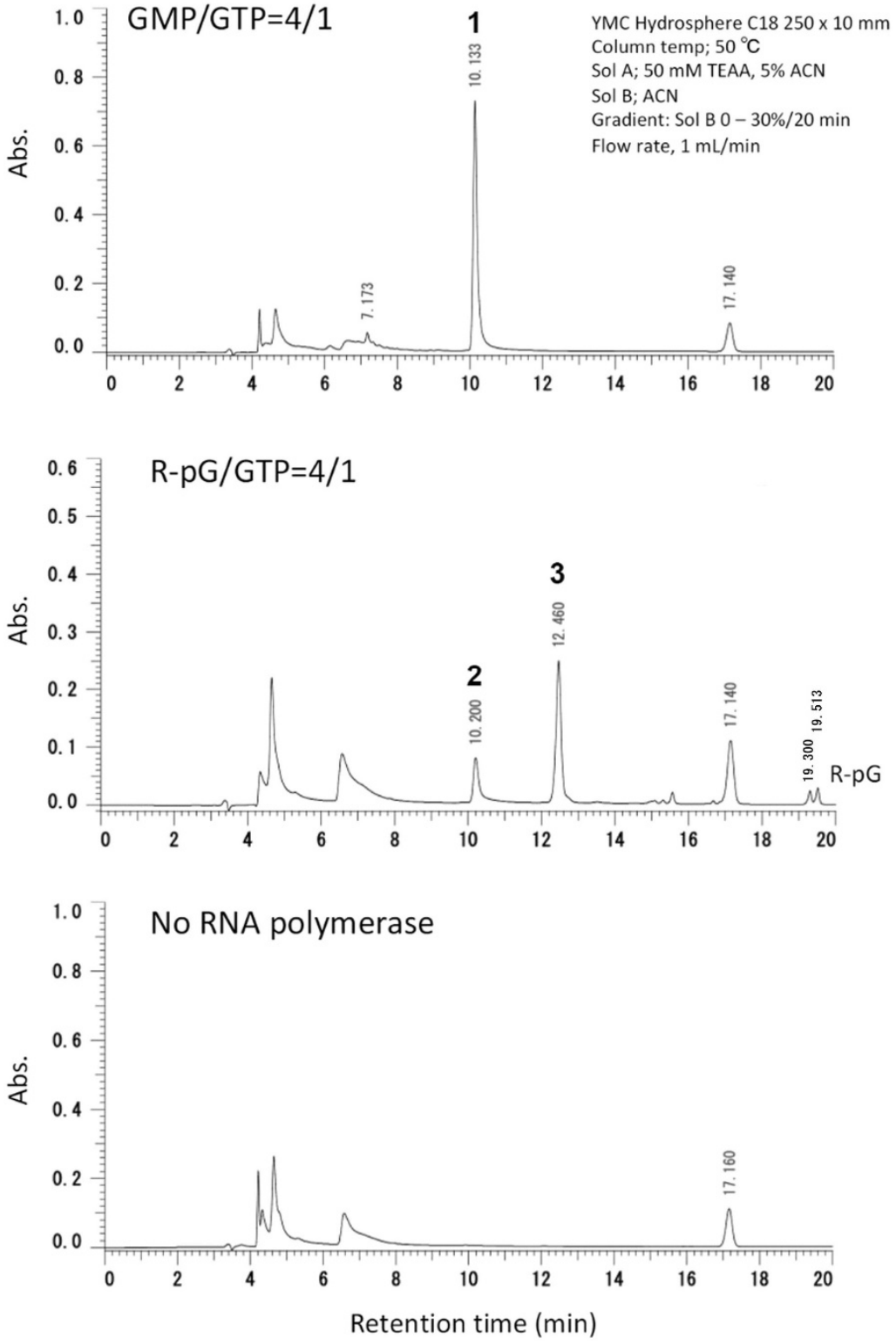

GMP/GTP=4/1
1
10.133
7.173
17.140
YMC Hydrosphere C18 250 x 10 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
R-pG/GTP=4/1
2
10.200
3
12.460
17.140
19.300
19.513
R-pG
No RNA polymerase
17.160
Abs.
Retention time (min)

Fig. 15

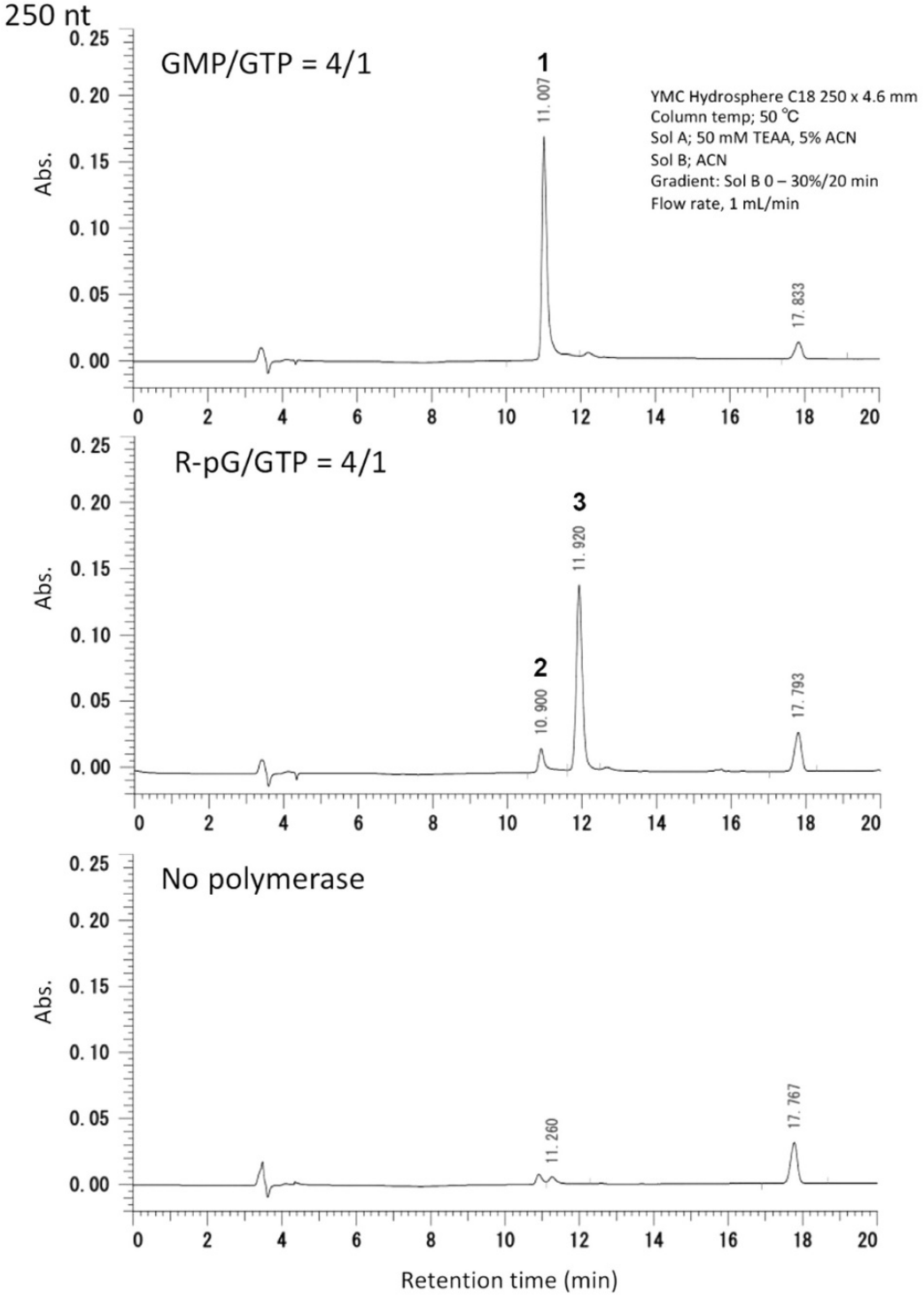

250 nt
GMP/GTP = 4/1
1
11.007
17.833
YMC Hydrosphere C18 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
R-pG/GTP = 4/1
2
10.900
3
11.920
17.793
No polymerase
11.260
17.767
Abs.
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
16
18
20
Retention time (min)

Fig. 16

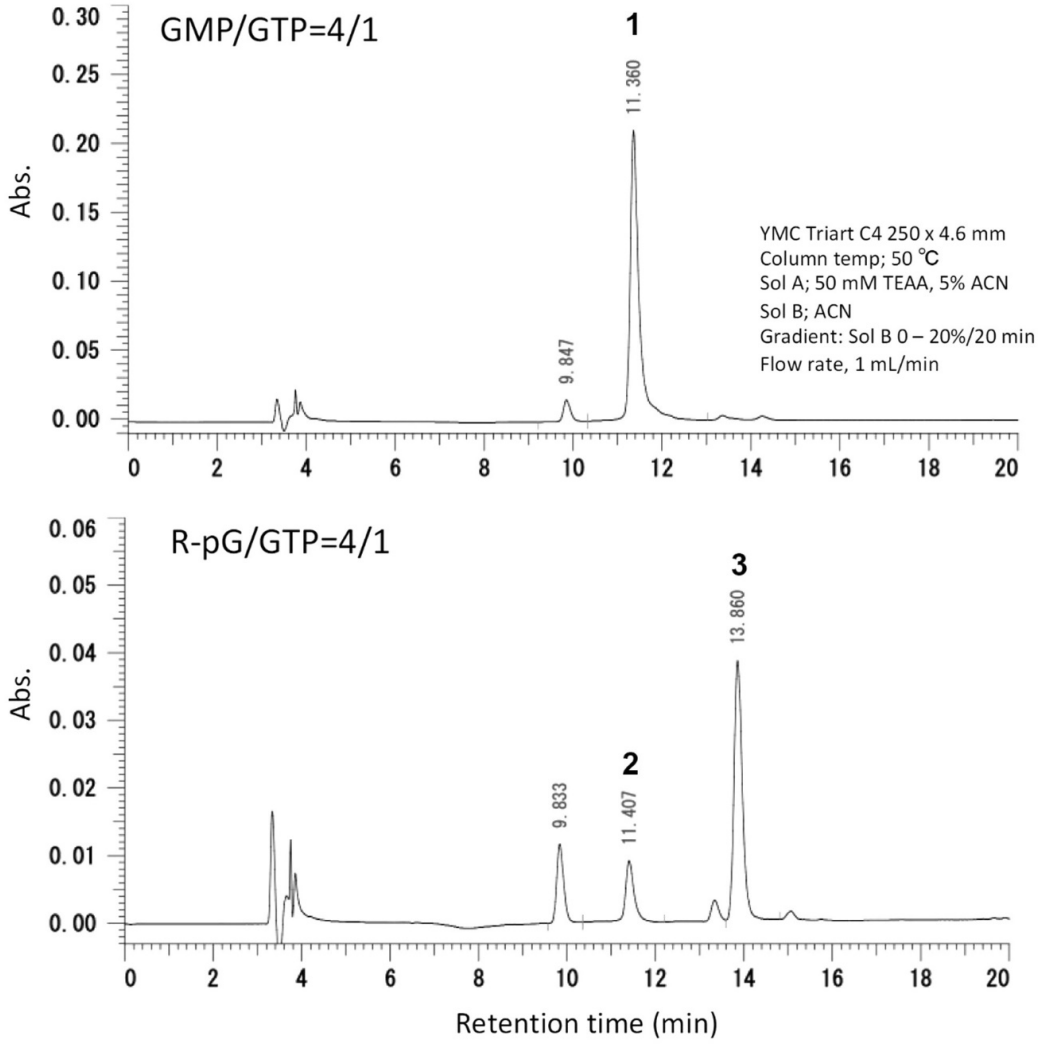

GMP/GTP=4/1
1
11.360
9.847
YMC Triart C4 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 20%/20 min
Flow rate, 1 mL/min
R-pG/GTP=4/1
3
13.860
2
11.407
9.833
Abs.
Retention time (min)

Fig. 17

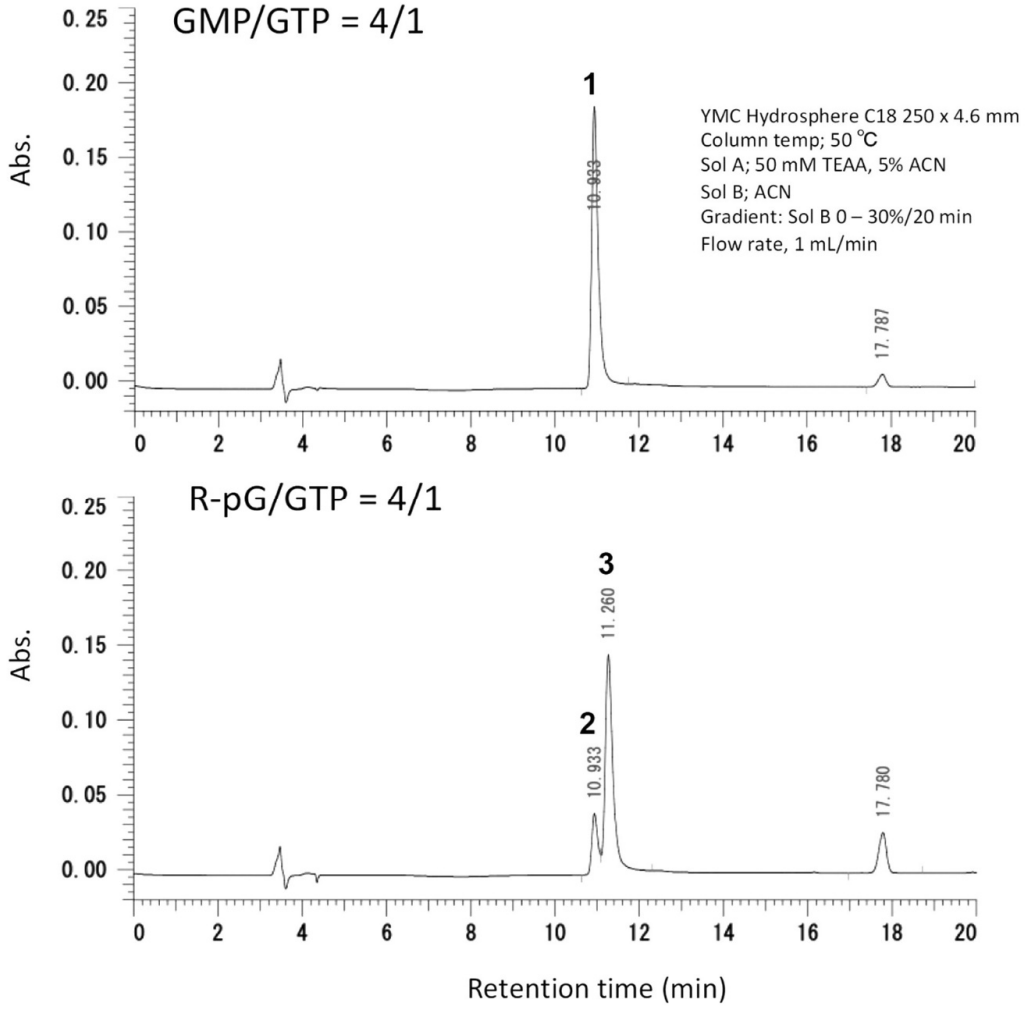

GMP/GTP = 4/1
1
10. 933
17. 787
YMC Hydrosphere C18 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
R-pG/GTP = 4/1
3
11. 260
2
10. 933
17. 780
Abs.
0. 25
0. 20
0. 15
0. 10
0. 05
0. 00
0
2
4
6
8
10
12
14
16
18
20
Retention time (min)

Fig. 18

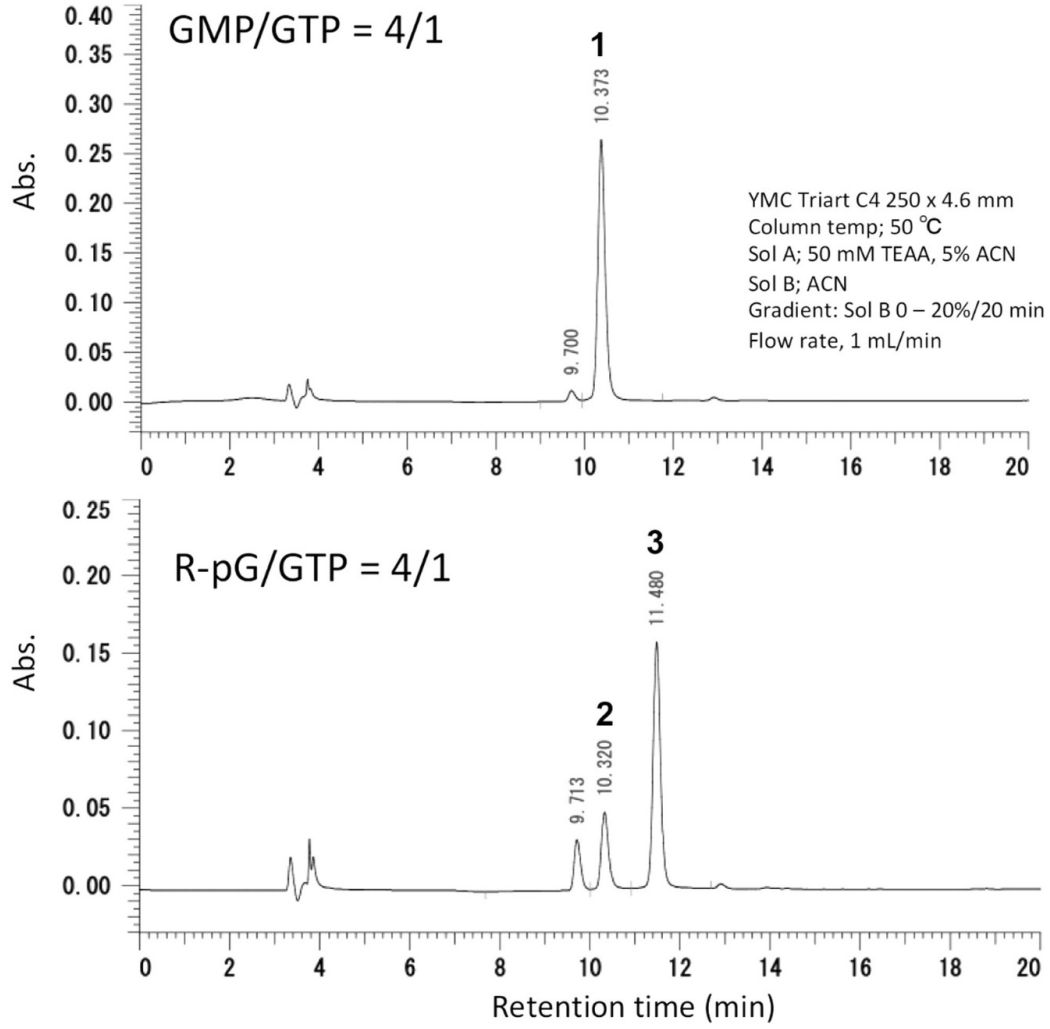

GMP/GTP = 4/1
1
10.373
9.700
YMC Triart C4 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 20%/20 min
Flow rate, 1 mL/min
Abs.
R-pG/GTP = 4/1
3
11.480
2
10.320
9.713
Abs.
Retention time (min)

Fig. 19

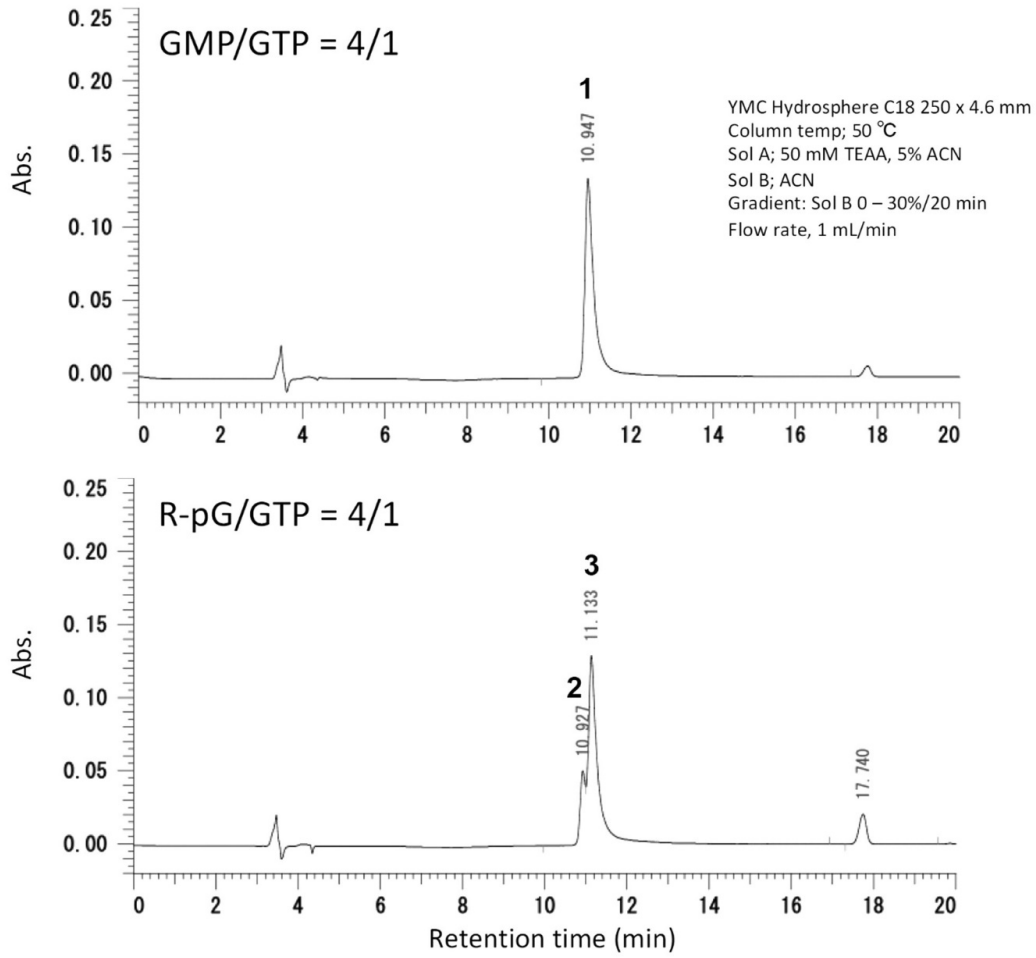

GMP/GTP = 4/1
1
10.947
YMC Hydrosphere C18 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
R-pG/GTP = 4/1
3
11.133
2
10.927
17.740
Abs.
Retention time (min)

Fig. 20

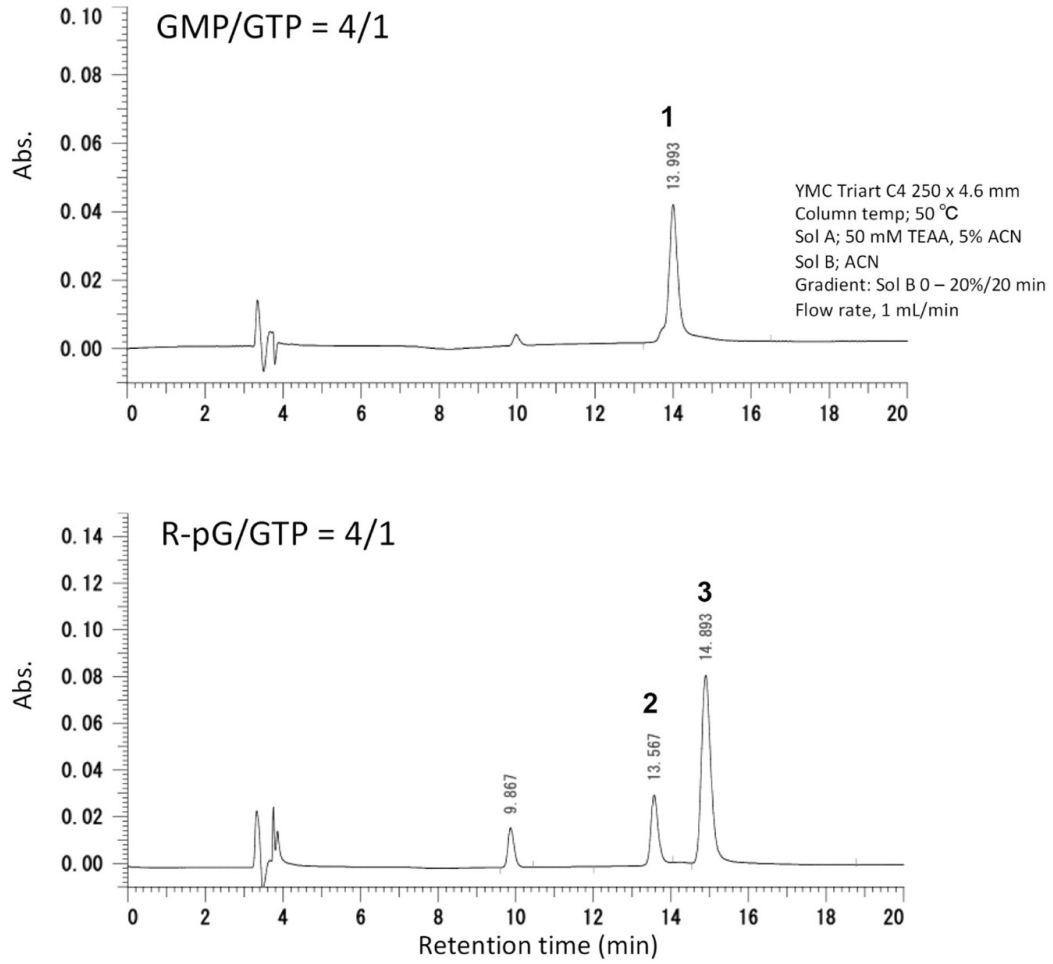

GMP/GTP = 4/1
Abs.
0.10
0.08
0.06
0.04
0.02
0.00
1
13.993
YMC Triart C4 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 20%/20 min
Flow rate, 1 mL/min
0 2 4 6 8 10 12 14 16 18 20
R-pG/GTP = 4/1
Abs.
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
3
14.893
2
13.567
9.867
0 2 4 6 8 10 12 14 16 18 20
Retention time (min)

Fig. 21

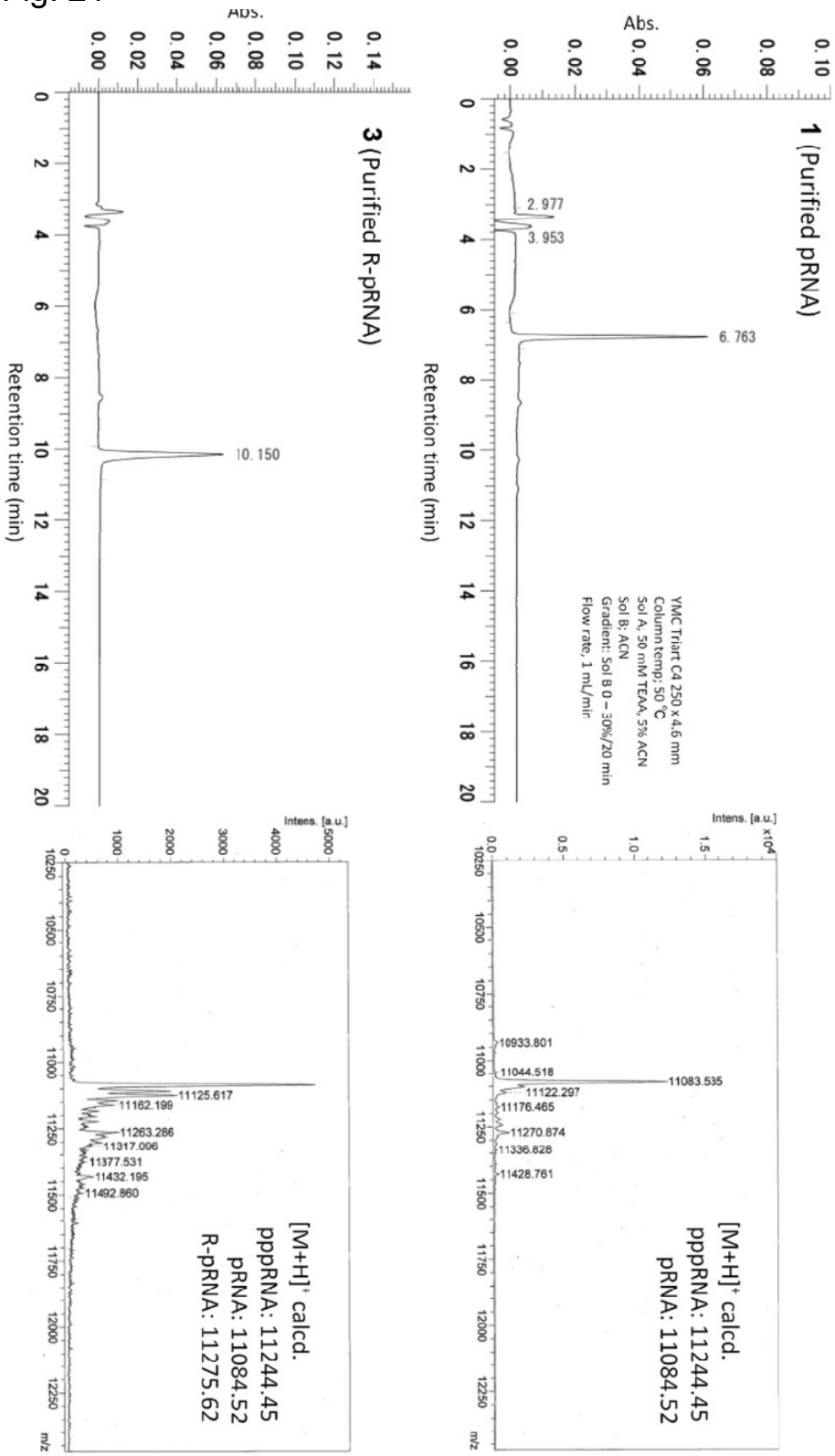

1 (Purified pRNA)
Abs.
Retention time (min)
2.977
3.953
6.763
YMC Triart C4 250 x 4.6 mm
Column temp: 50 °C
Sol A: 50 mM TEAA, 5% ACN
Sol B: ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate: 1 mL/min
Intens. [a.u.]
10933.801
11044.518
11083.535
11122.297
11176.465
11270.874
11336.828
11428.761
[M+H]+ calcd.
pppRNA: 11244.45
pRNA: 11084.52
3 (Purified R-pRNA)
Abs.
Retention time (min)
10.150
Intens. [a.u.]
11125.617
11162.199
11263.286
11317.096
11377.531
11432.195
11492.860
[M+H]+ calcd.
pppRNA: 11244.45
pRNA: 11084.52
R-pRNA: 11275.62
m/z

Fig. 22

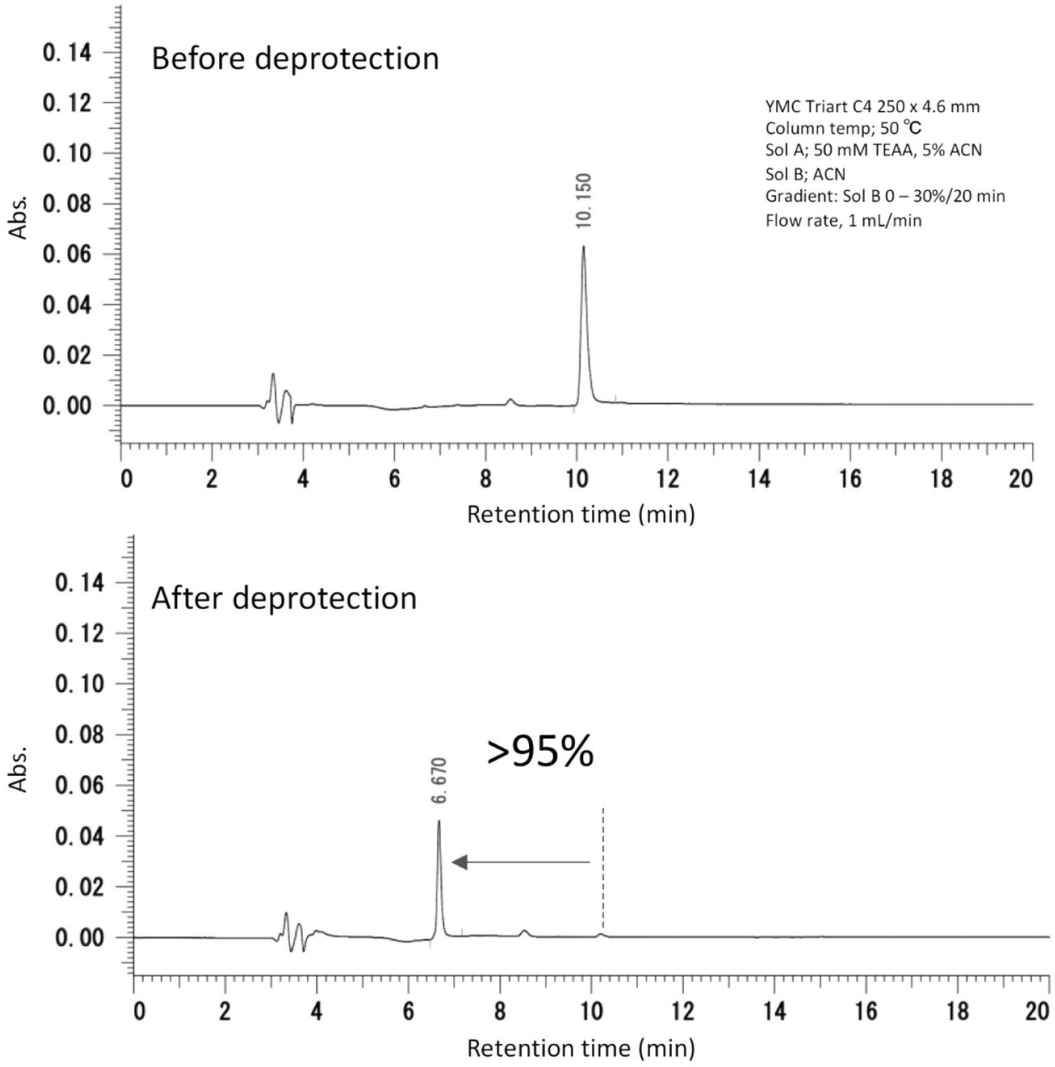

Before deprotection
YMC Triart C4 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
10.150
Abs.
Retention time (min)
After deprotection
>95%
6.670
Abs.
Retention time (min)

Fig. 23

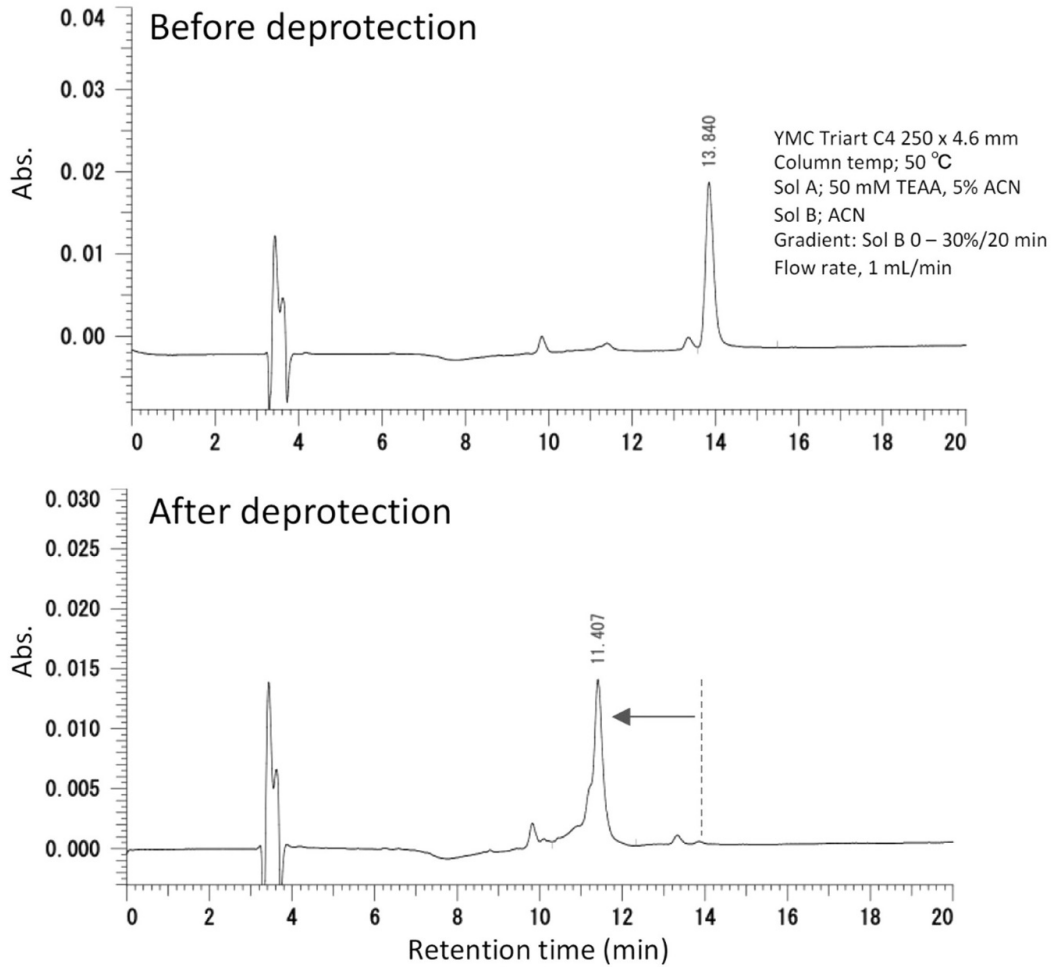
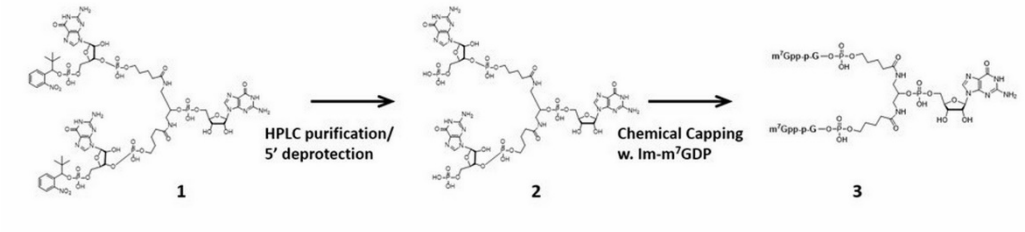
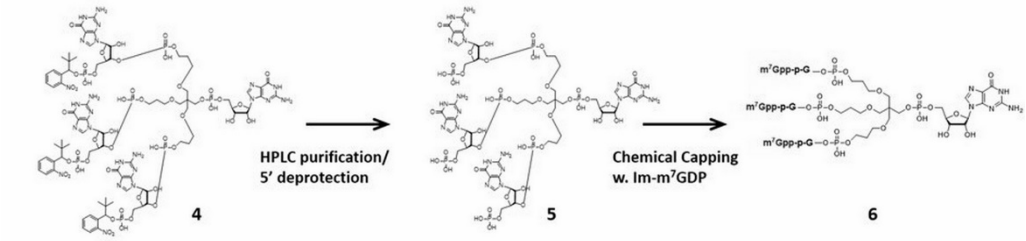

Before deprotection
Abs.
0.04
0.03
0.02
0.01
0.00
13.840
YMC Triart C4 250 x 4.6 mm
Column temp; 50 ℃
Sol A; 50 mM TEAA, 5% ACN
Sol B; ACN
Gradient: Sol B 0 – 30%/20 min
Flow rate, 1 mL/min
0 2 4 6 8 10 12 14 16 18 20
After deprotection
Abs.
0.030
0.025
0.020
0.015
0.010
0.005
0.000
11.407
0 2 4 6 8 10 12 14 16 18 20
Retention time (min)

Fig. 24

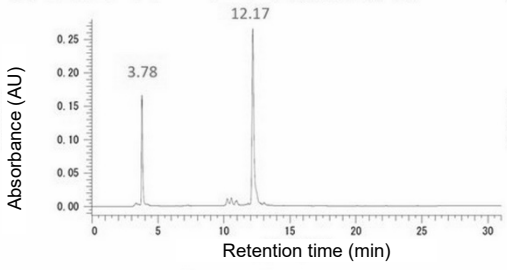
(a) Bi-branched cap analog compound



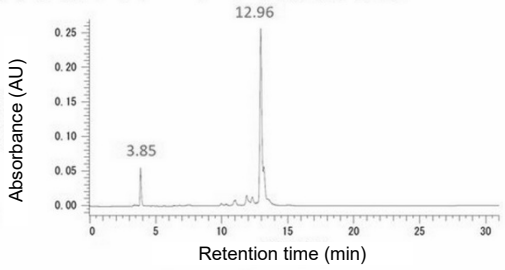
(b) Tri-branched cap analog compound



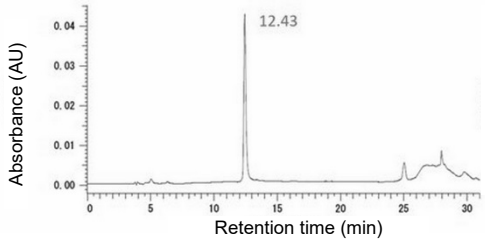
(c) Bi-branched cap analog compound precursor (1)



(d) Tri-branched cap analog compound precursor (4)



(e) Bi-branched cap analog compound precursor (2)



(f) Tri-branched cap analog compound precursor (5)

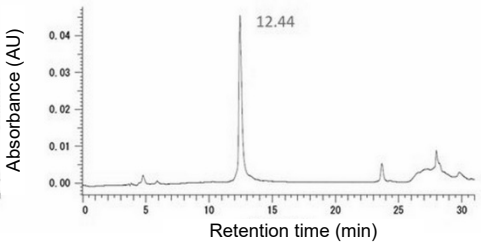


Fig. 25

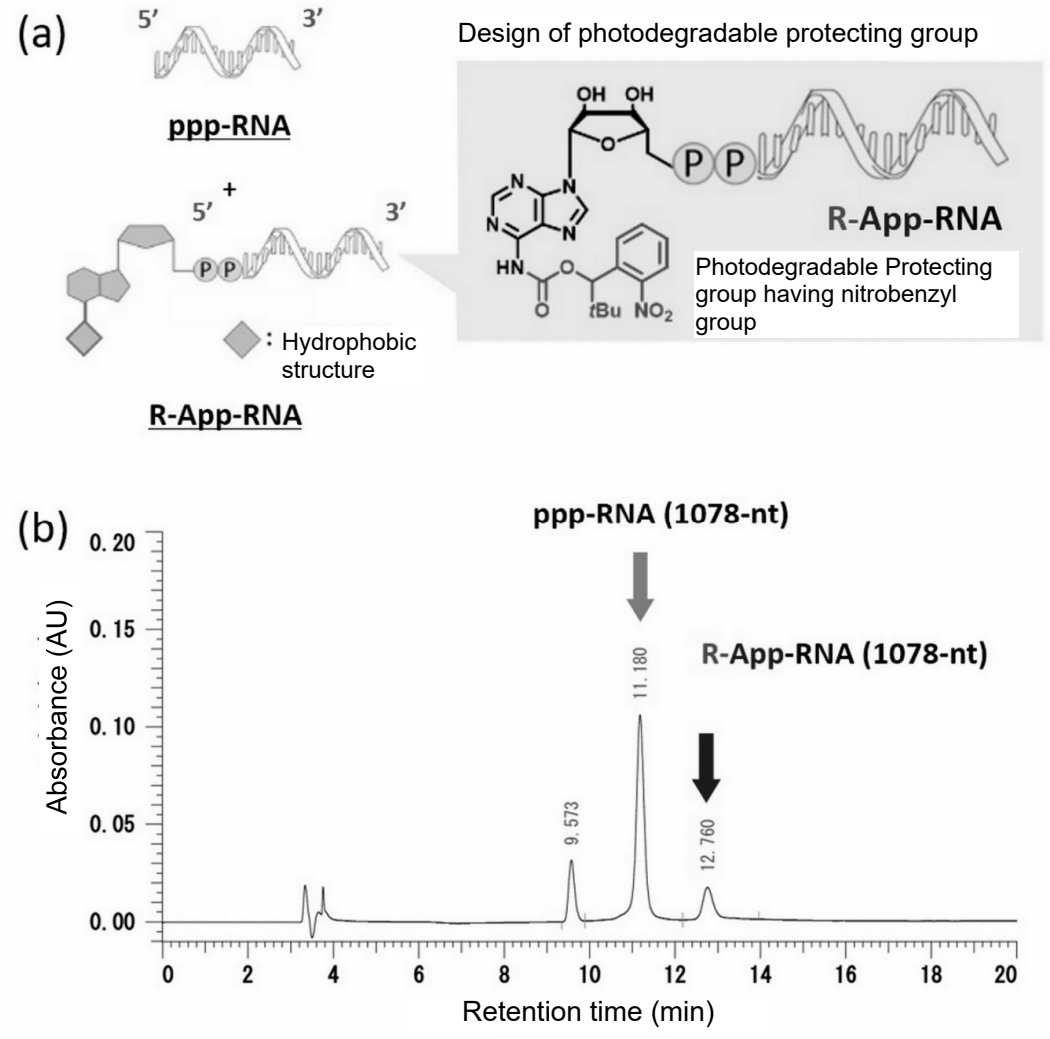

(a)
5′
3′
ppp-RNA
+
5′
3′
P P
: Hydrophobic structure
R-App-RNA
Design of photodegradable protecting group
OH OH
P P
R-App-RNA
Photodegradable Protecting group having nitrobenzyl group
tBu NO2
(b)
ppp-RNA (1078-nt)
R-App-RNA (1078-nt)
11.180
9.573
12.760
Absorbance (AU)
0.20
0.15
0.10
0.05
0.00
0 2 4 6 8 10 12 14 16 18 20
Retention time (min)

Fig. 26

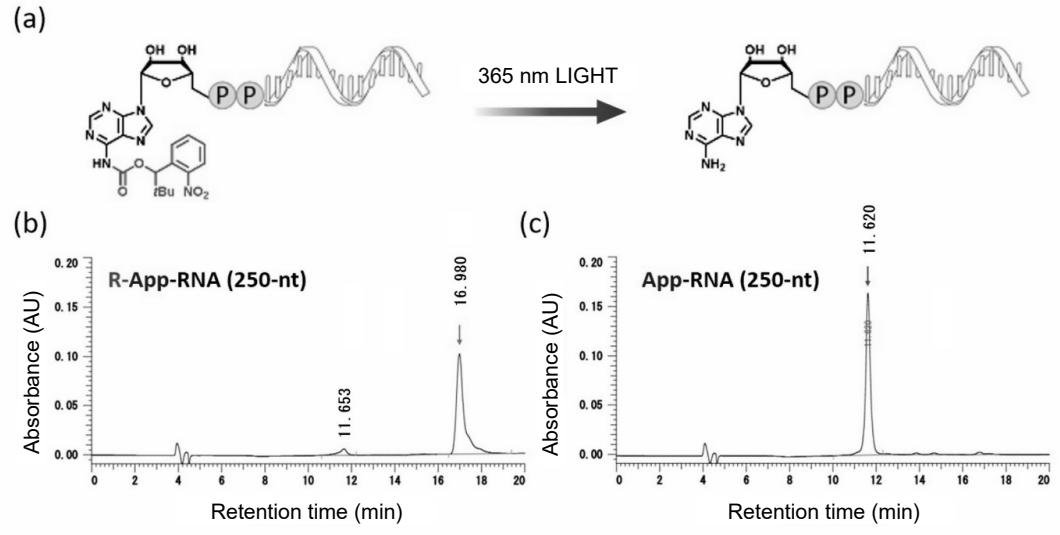

(a)
365 nm LIGHT
(b)
R-App-RNA (250-nt)
11.653
16.980
(c)
App-RNA (250-nt)
11.620
Absorbance (AU)
Retention time (min)

Fig. 27

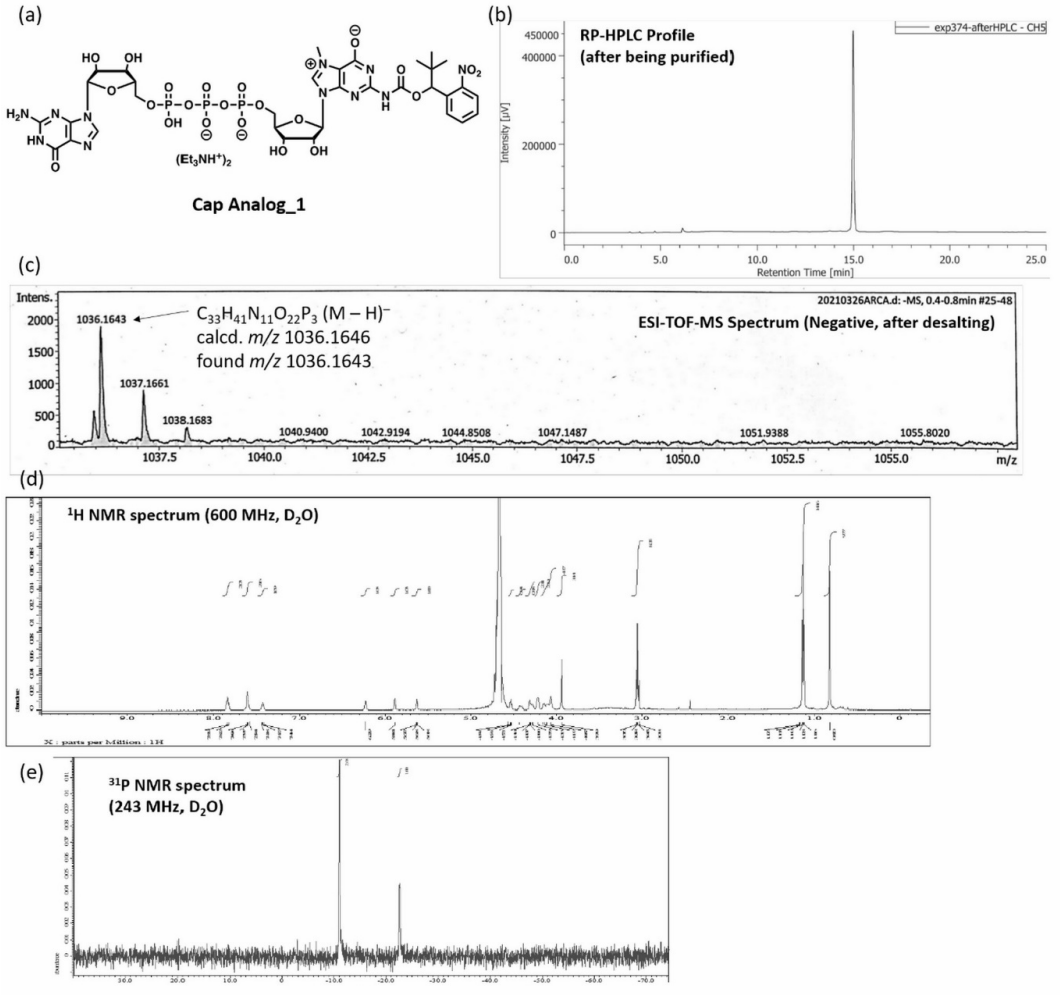

(a)
Cap Analog_1
(b)
RP-HPLC Profile
(after being purified)
exp374-afterHPLC - CH5
Intensity [μV]
Retention Time [min]
(c)
Intens.
1036.1643
C33H41N11O22P3 (M – H)−
calcd. m/z 1036.1646
found m/z 1036.1643
1037.1661
1038.1683
1040.9400
1042.9194
1044.8508
1047.1487
1051.9388
1055.8020
20210326ARCA.d: -MS, 0.4-0.8min #25-48
ESI-TOF-MS Spectrum (Negative, after desalting)
m/z
(d)
1H NMR spectrum (600 MHz, D2O)
(e)
31P NMR spectrum
(243 MHz, D2O)

Fig. 28

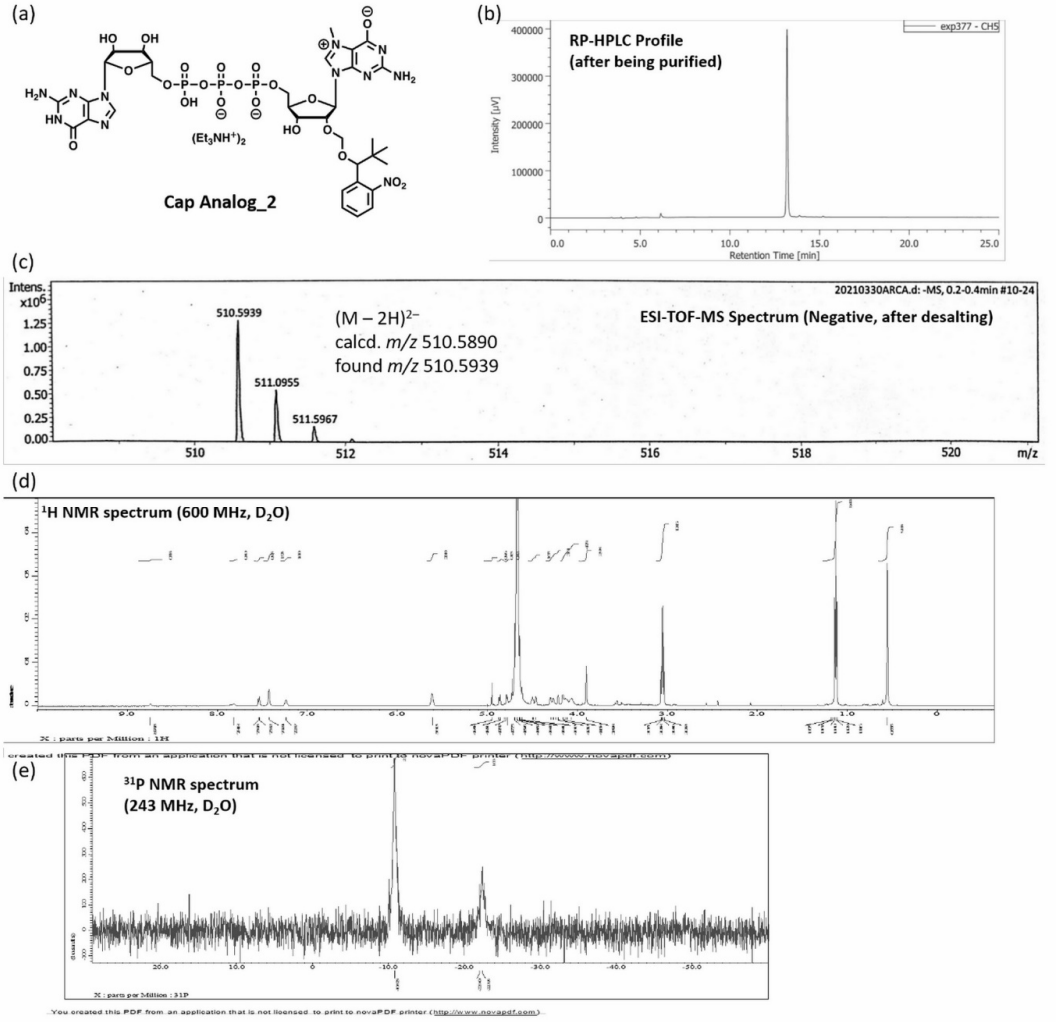

(a)
Cap Analog_2
(b)
RP-HPLC Profile
(after being purified)
exp377 - CH5
Intensity [uV]
Retention Time [min]
(c)
Intens.
x10^6
510.5939
511.0955
511.5967
(M – 2H)2–
calcd. m/z 510.5890
found m/z 510.5939
ESI-TOF-MS Spectrum (Negative, after desalting)
20210330ARCA.d: -MS, 0.2-0.4min #10-24
m/z
(d)
1H NMR spectrum (600 MHz, D2O)
(e)
31P NMR spectrum
(243 MHz, D2O)

Fig. 29

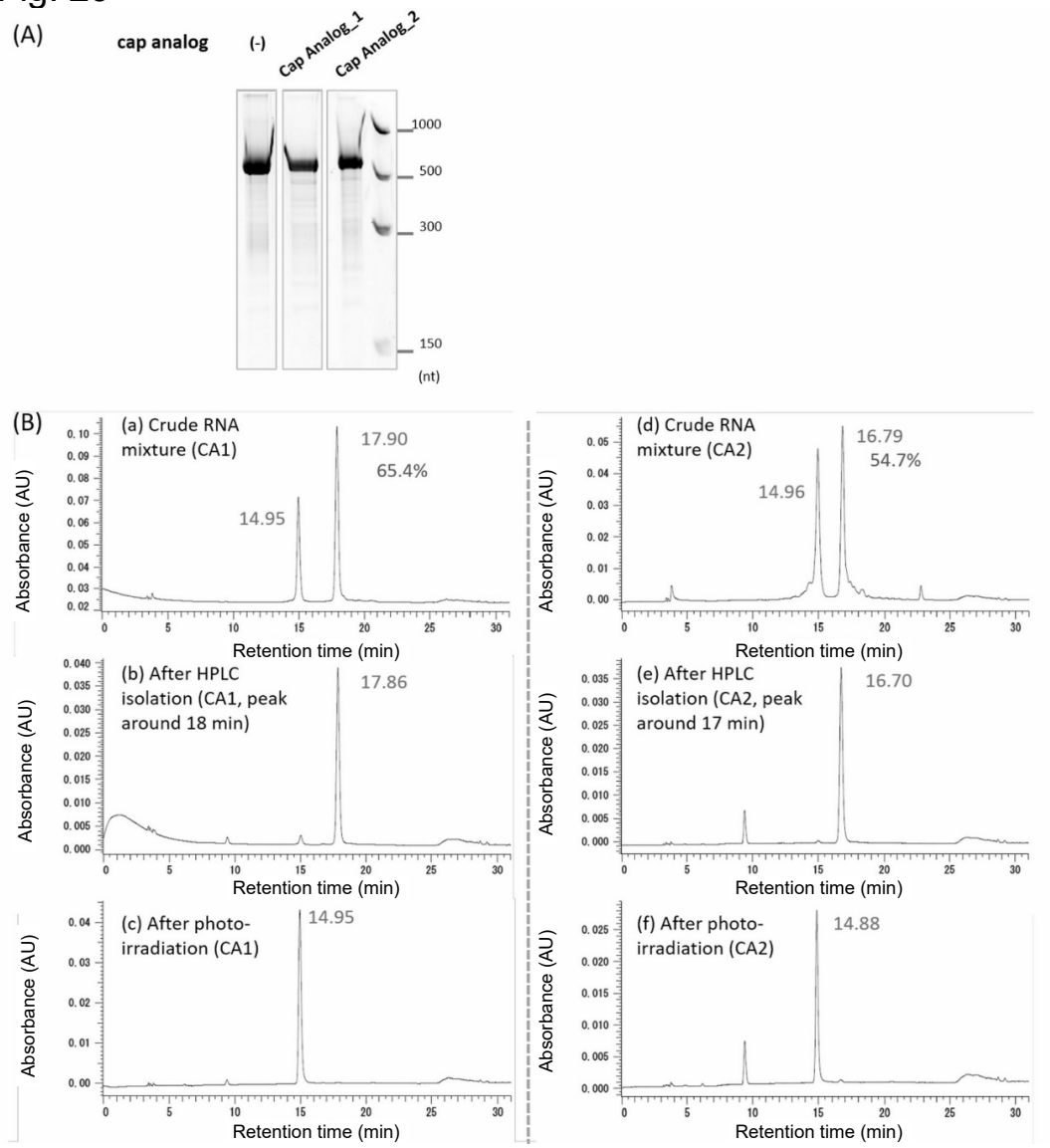

(A)
cap analog
(-)
Cap Analog_1
Cap Analog_2
1000
500
300
150
(nt)
(B)
(a) Crude RNA mixture (CA1)
17.90
65.4%
14.95
(b) After HPLC isolation (CA1, peak around 18 min)
17.86
(c) After photo-irradiation (CA1)
14.95
(d) Crude RNA mixture (CA2)
16.79
54.7%
14.96
(e) After HPLC isolation (CA2, peak around 17 min)
16.70
(f) After photo-irradiation (CA2)
14.88
Absorbance (AU)
Retention time (min)

Fig. 30

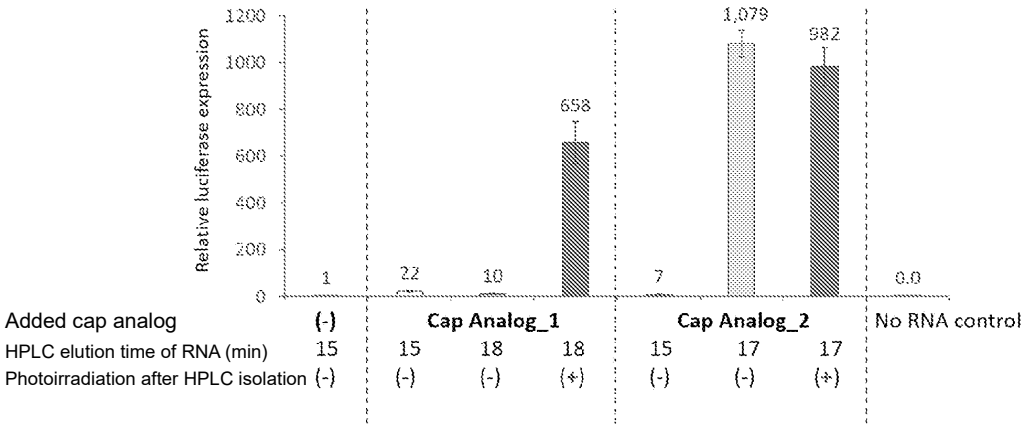

Relative luciferase expression
1200
1000
800
600
400
200
0
1
22
10
658
7
1,079
982
0.0
Added cap analog
(-)
Cap Analog_1
Cap Analog_2
No RNA control
HPLC elution time of RNA (min)
15
15
18
18
15
17
17
Photoirradiation after HPLC isolation
(-)
(-)
(-)
(+)
(-)
(-)
(+)

Fig. 31

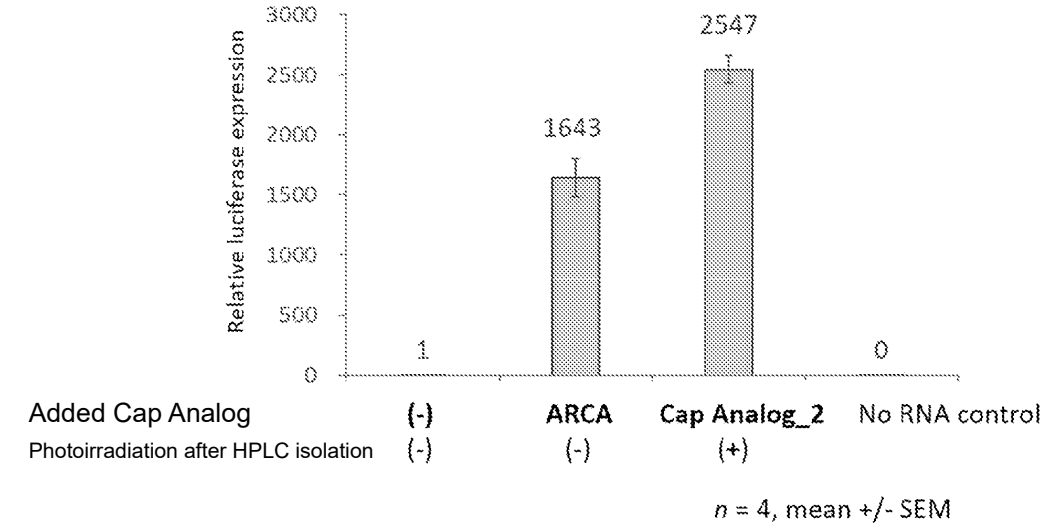

Relative luciferase expression
3000
2500
2000
1500
1000
500
0
1
1643
2547
0
Added Cap Analog
(-)
ARCA
Cap Analog_2
No RNA control
Photoirradiation after HPLC isolation
(-)
(-)
(+)
n = 4, mean +/- SEM

Fig. 32

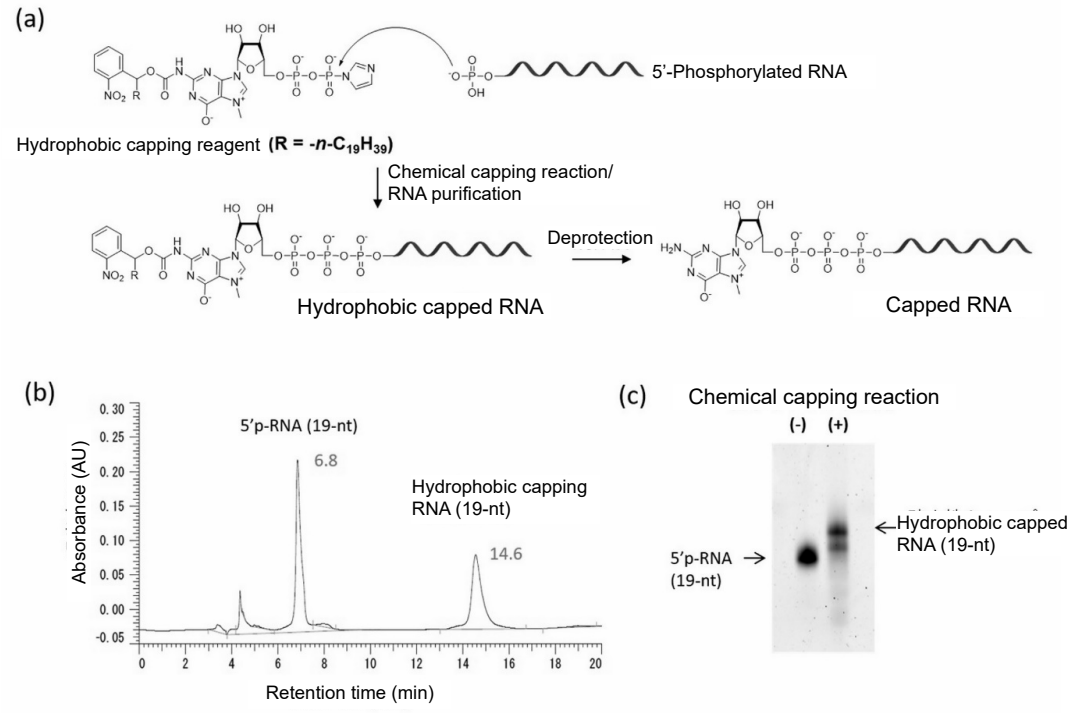

(a)
5'-Phosphorylated RNA
Hydrophobic capping reagent (R = -n-C19H39)
Chemical capping reaction/
RNA purification
Deprotection
Hydrophobic capped RNA
Capped RNA
(b)
5'p-RNA (19-nt)
6.8
Hydrophobic capping
RNA (19-nt)
14.6
Absorbance (AU)
Retention time (min)
(c)
Chemical capping reaction
(-) (+)
5'p-RNA
(19-nt)
Hydrophobic capped
RNA (19-nt)

Fig. 33

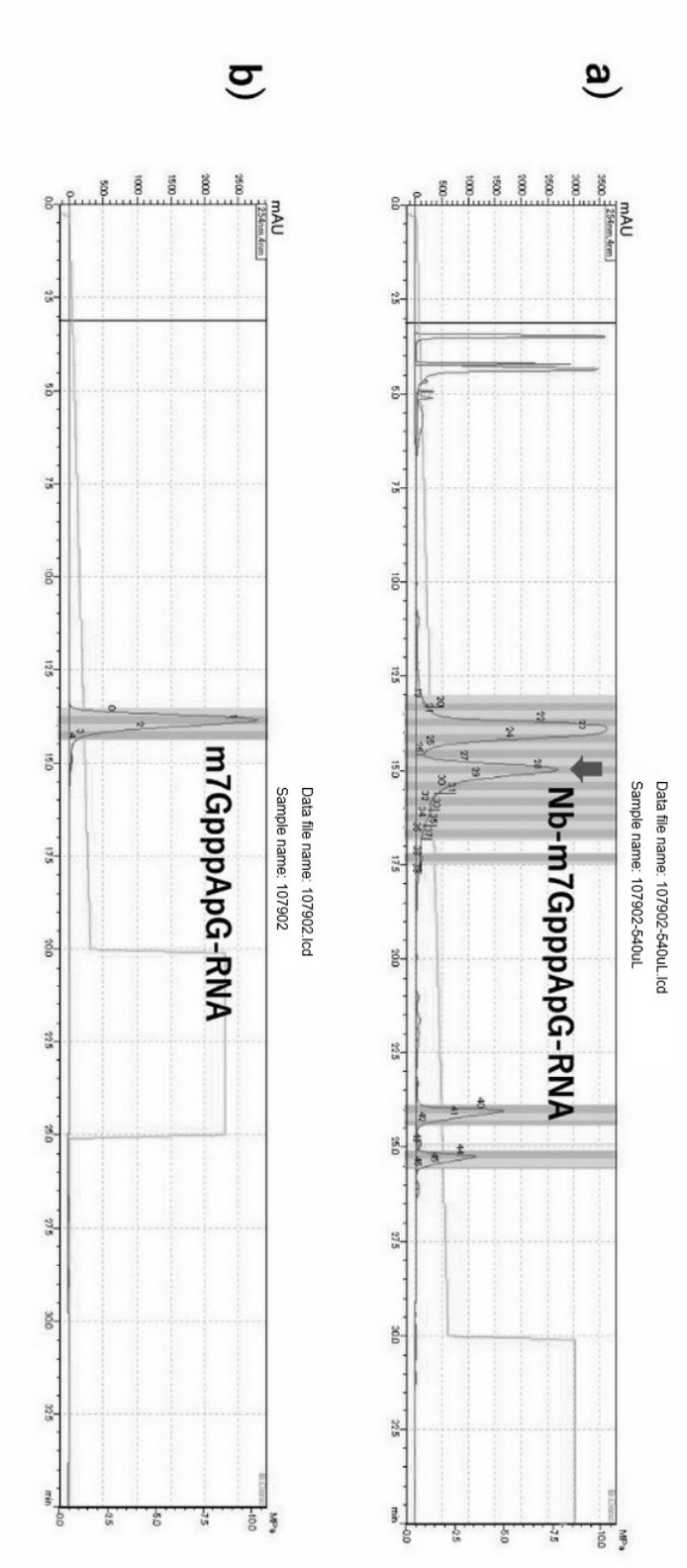

a)
mAU
Data file name: 107902-540uL.lcd
Sample name: 107902-540uL
Nb-m7GpppApG-RNA
b)
mAU
Data file name: 107902.lcd
Sample name: 107902
m7GpppApG-RNA

Fig. 34

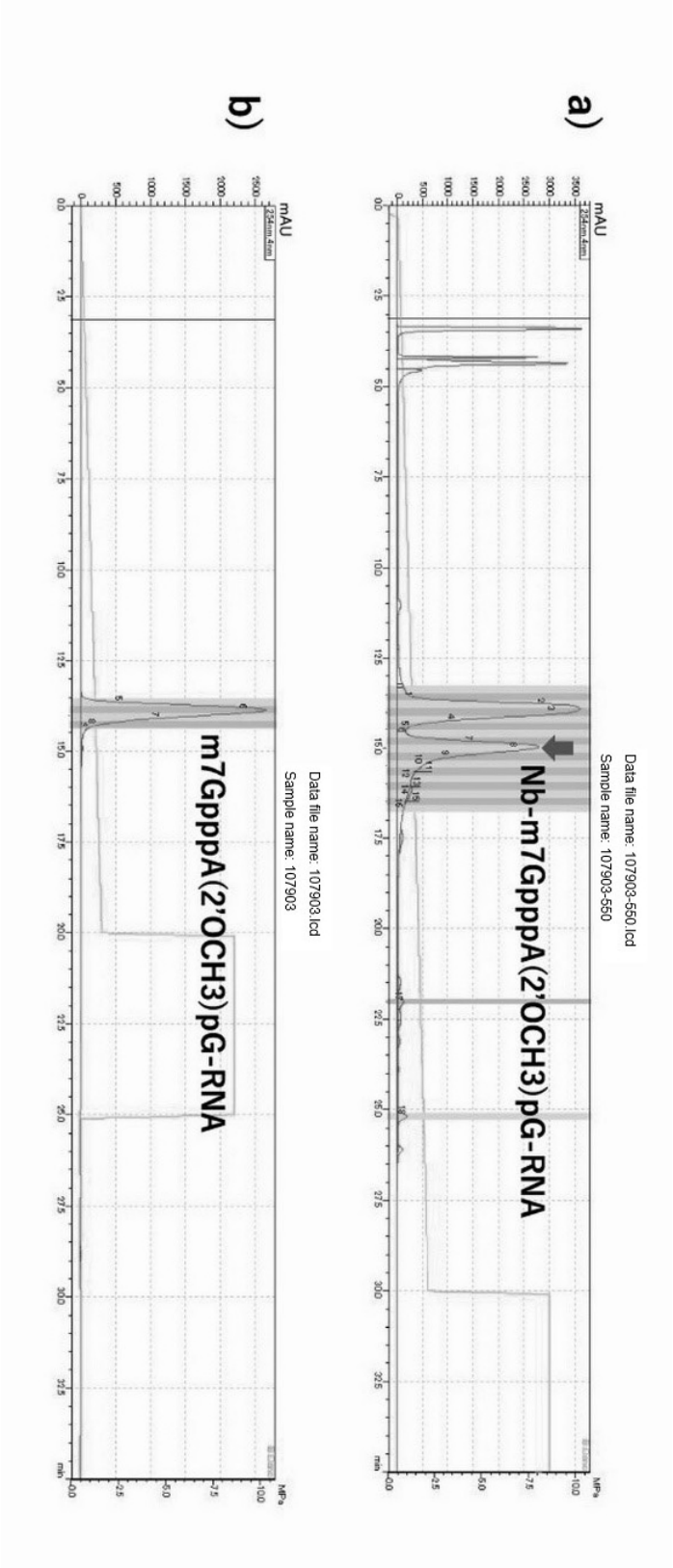

a)
mAU
Data file name: 107903-550.lcd
Sample name: 107903-550
Nb-m7GpppA(2'OCH3)pG-RNA
b)
mAU
Data file name: 107903.lcd
Sample name: 107903
m7GpppA(2'OCH3)pG-RNA

Fig. 35

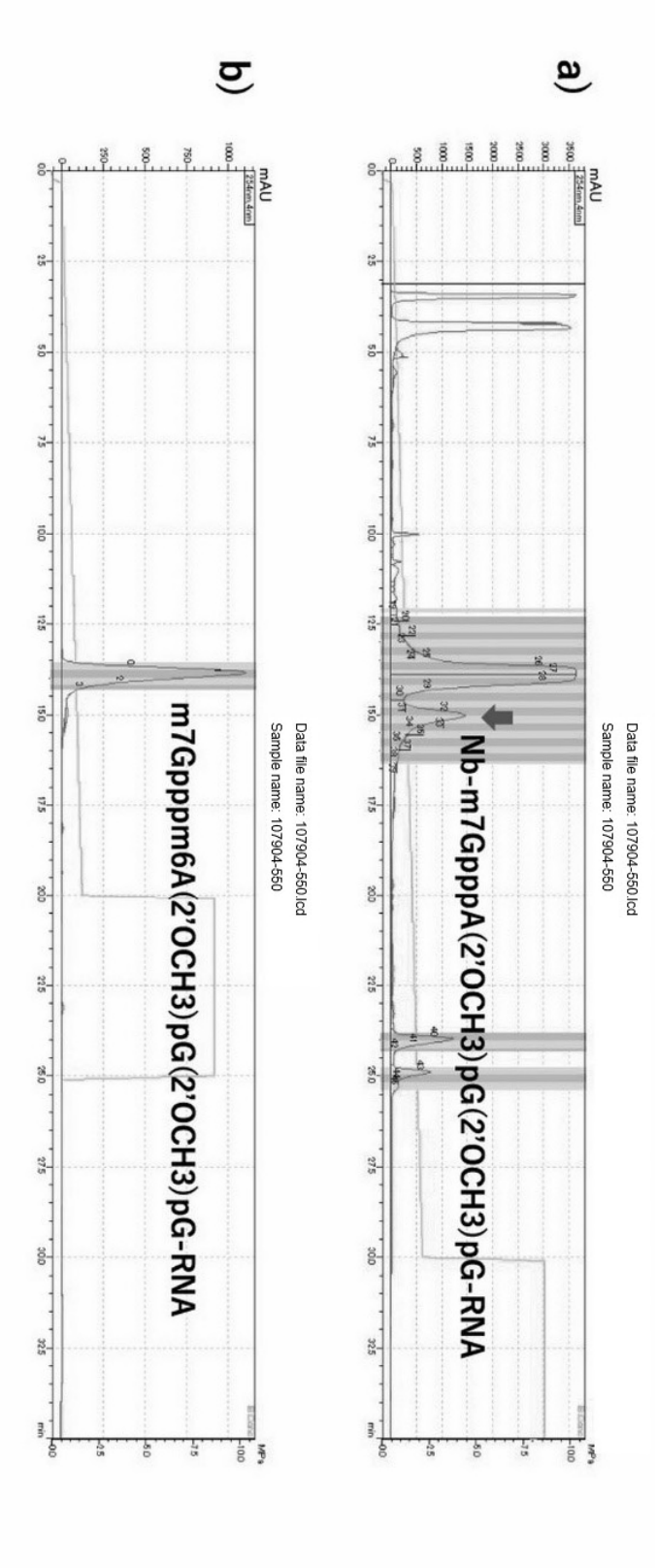

a)
mAU
254nm,4nm
Data file name: 107904-550.lcd
Sample name: 107904-550
Nb-m7GpppA(2'OCH3)pG(2'OCH3)pG-RNA
min
b)
mAU
254nm,4nm
Data file name: 107904-550.lcd
Sample name: 107904-550
m7Gpppm6A(2'OCH3)pG(2'OCH3)pG-RNA
min

Fig. 36

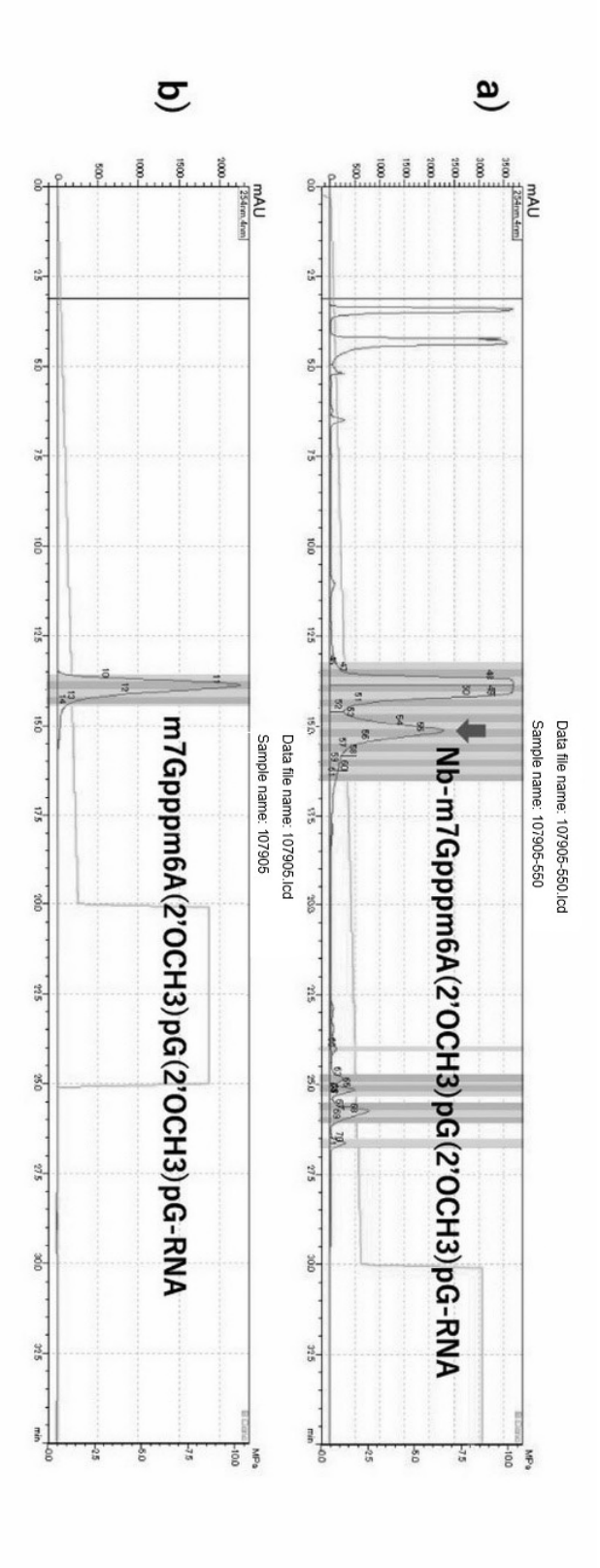

a)
mAU
254nm,4nm
Data file name: 107905-550.lcd
Sample name: 107905-550
Nb-m7Gpppm6A(2'OCH3)pG(2'OCH3)pG-RNA
min
b)
mAU
254nm,4nm
Data file name: 107905.lcd
Sample name: 107905
m7Gpppm6A(2'OCH3)pG(2'OCH3)pG-RNA
min

Fig. 37

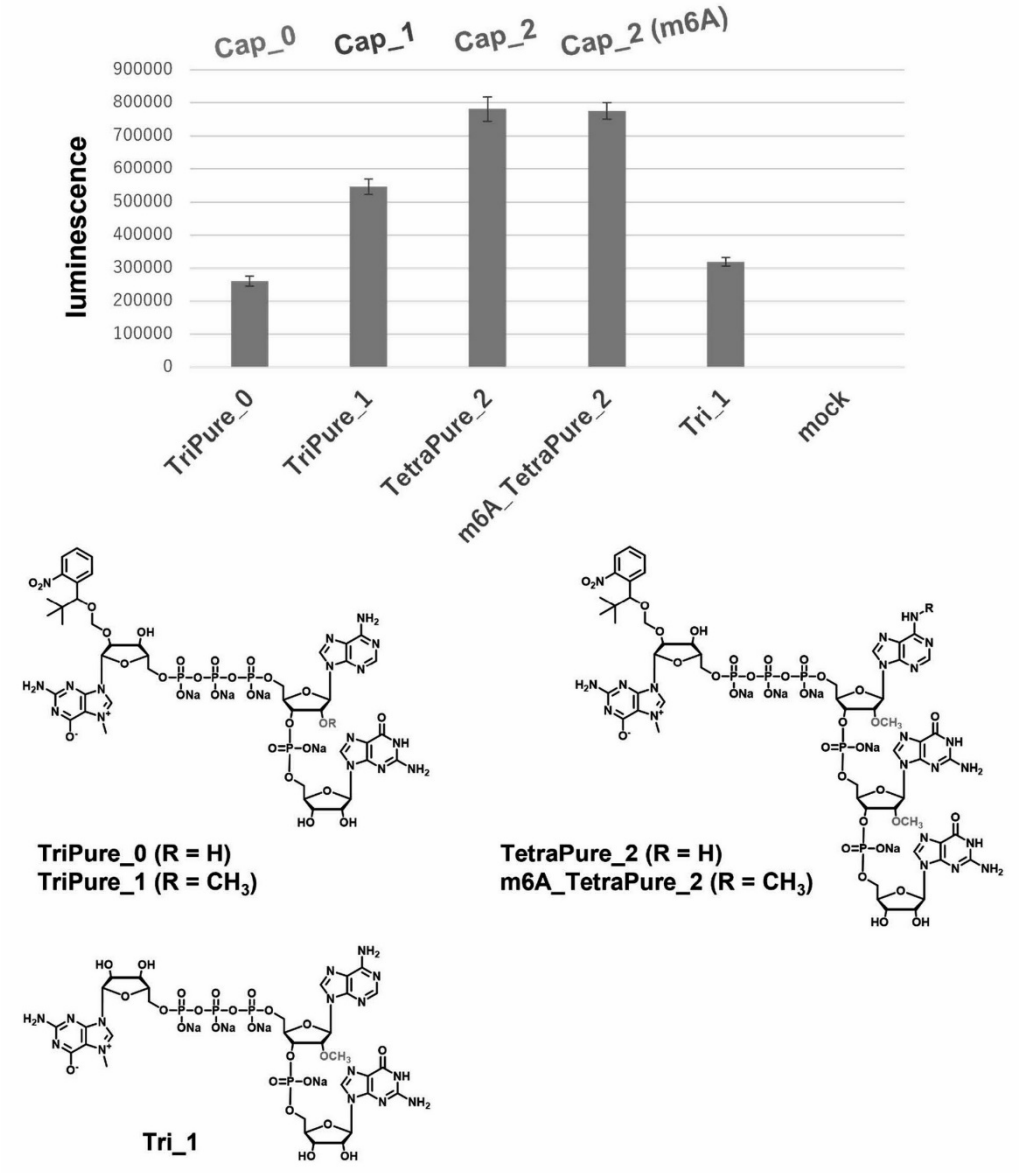

Cap_0
Cap_1
Cap_2
Cap_2 (m6A)
luminescence
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
TriPure_0
TriPure_1
TetraPure_2
m6A_TetraPure_2
Tri_1
mock
TriPure_0 (R = H)
TriPure_1 (R = CH3)
TetraPure_2 (R = H)
m6A_TetraPure_2 (R = CH3)
Tri_1

Fig. 38

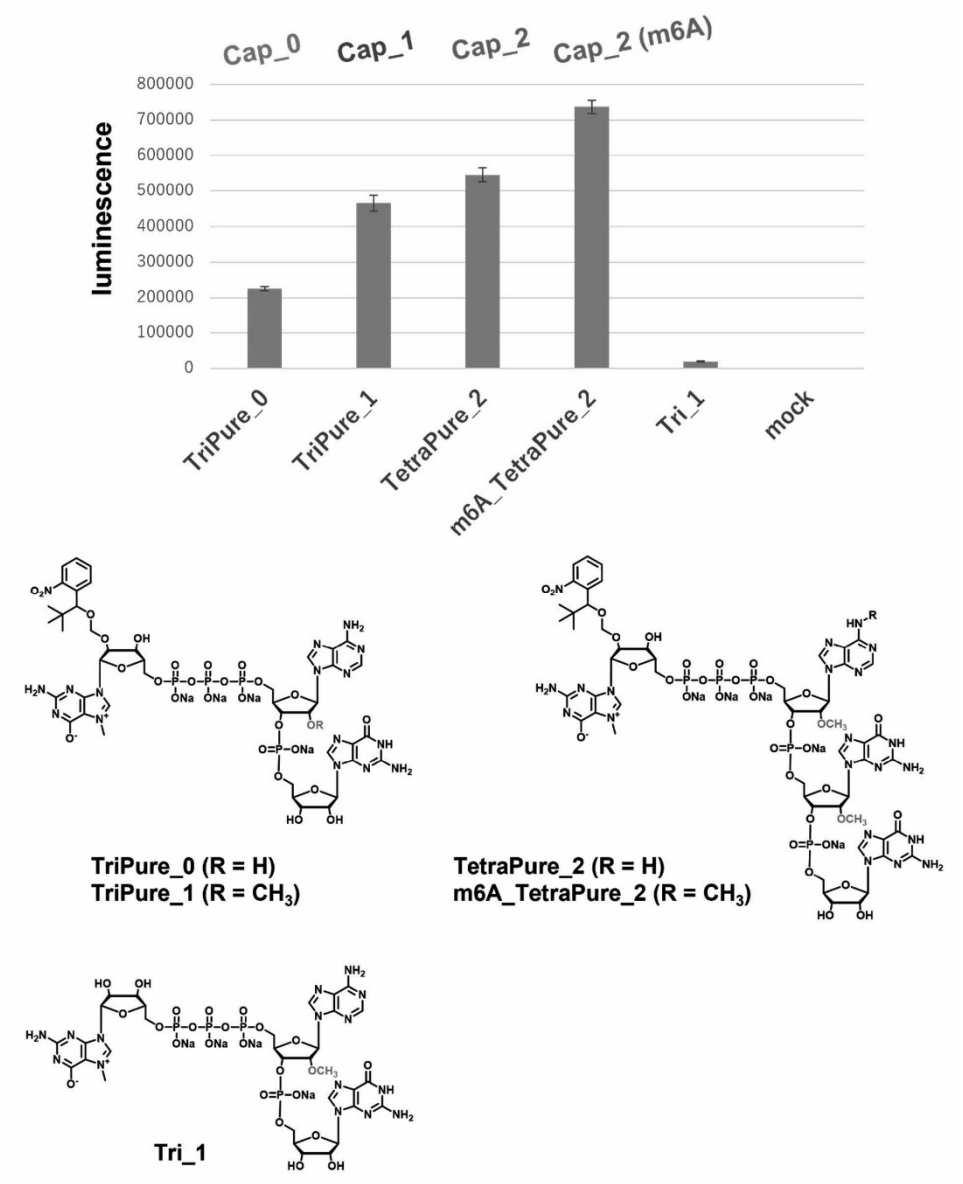

Cap_0
Cap_1
Cap_2
Cap_2 (m6A)
luminescence
800000
700000
600000
500000
400000
300000
200000
100000
0
TriPure_0
TriPure_1
TetraPure_2
m6A_TetraPure_2
Tri_1
mock
TriPure_0 (R = H)
TriPure_1 (R = CH3)
TetraPure_2 (R = H)
m6A_TetraPure_2 (R = CH3)
Tri_1

Fig. 39

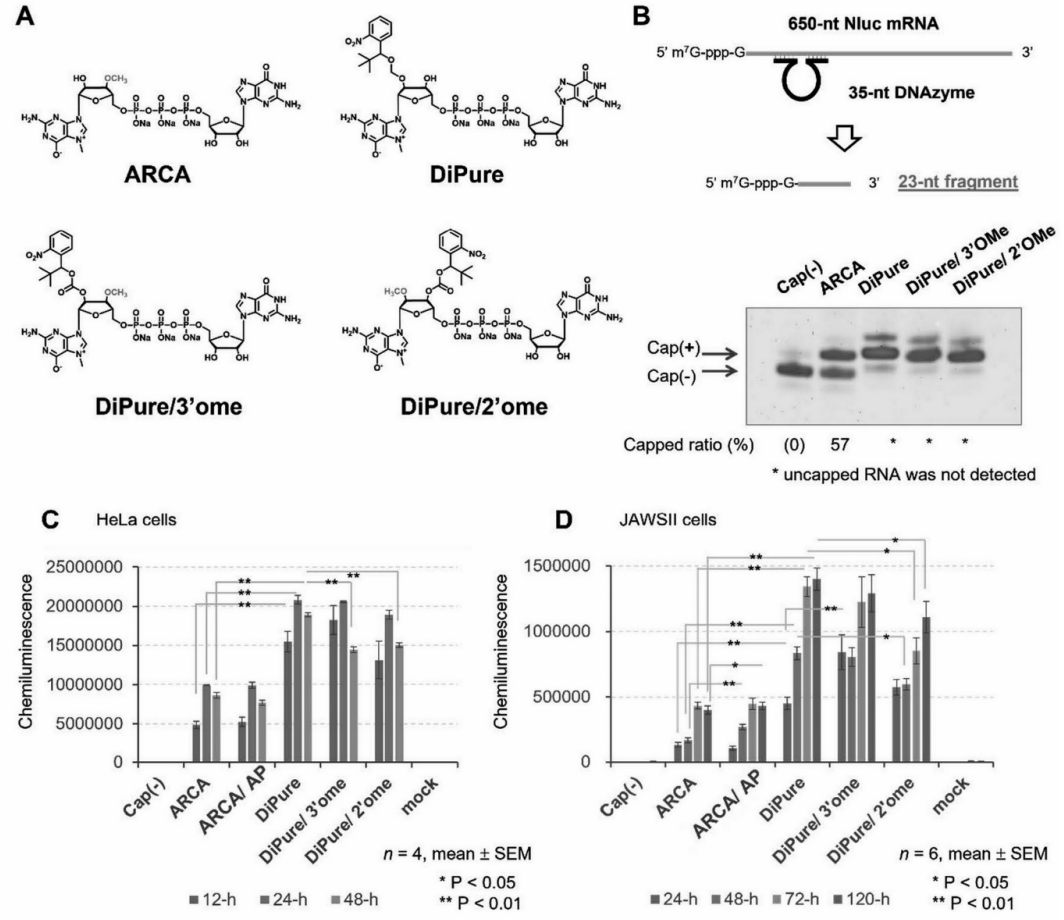

A
ARCA
DiPure
DiPure/3'ome
DiPure/2'ome
B
650-nt Nluc mRNA
5' m7G-ppp-G
3'
35-nt DNAzyme
5' m7G-ppp-G
3'
23-nt fragment
Cap(-)
ARCA
DiPure
DiPure/ 3'OMe
DiPure/ 2'OMe
Cap(+)
Cap(-)
Capped ratio (%)
(0)
57
* uncapped RNA was not detected
C
HeLa cells
Chemiluminescence
25000000
20000000
15000000
10000000
5000000
0
Cap(-)
ARCA
ARCA/ AP
DiPure
DiPure/ 3'ome
DiPure/ 2'ome
mock
n = 4, mean ± SEM
* P < 0.05
** P < 0.01
12-h
24-h
48-h
D
JAWSII cells
Chemiluminescence
1500000
1000000
500000
0
Cap(-)
ARCA
ARCA/ AP
DiPure
DiPure/ 3'ome
DiPure/ 2'ome
mock
n = 6, mean ± SEM
* P < 0.05
** P < 0.01
24-h
48-h
72-h
120-h

Fig. 40

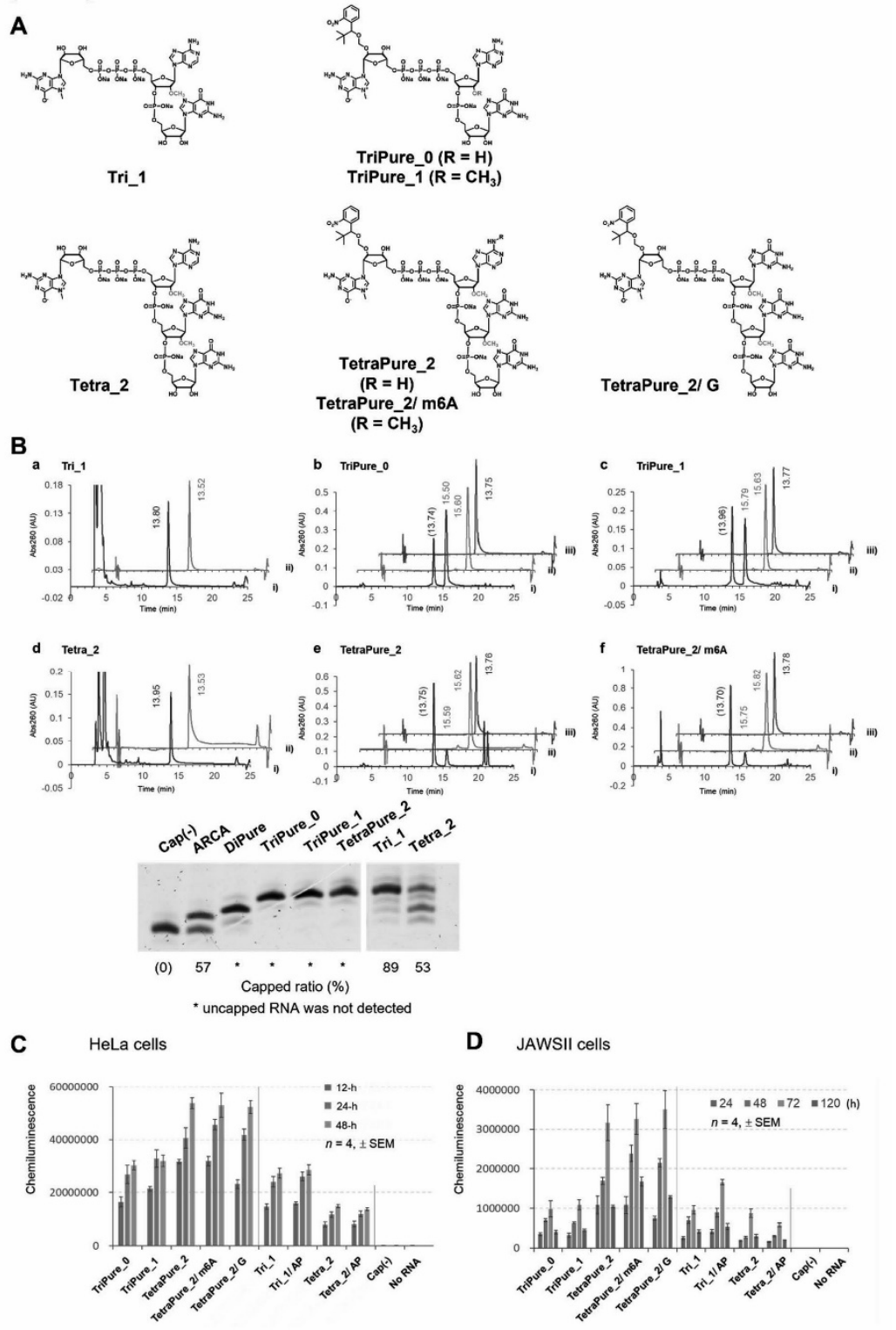
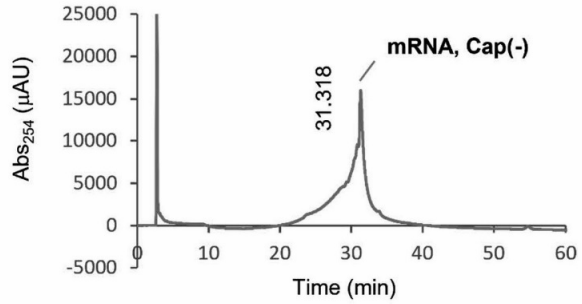
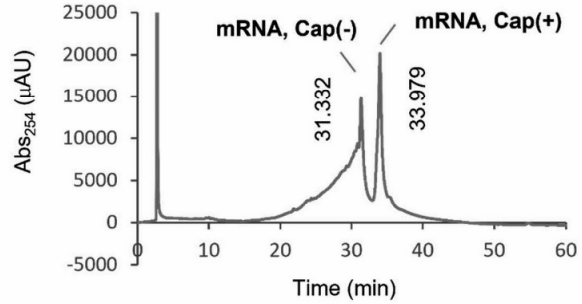

A
Tri_1
TriPure_0 (R = H)
TriPure_1 (R = CH3)
Tetra_2
TetraPure_2
(R = H)
TetraPure_2/ m6A
(R = CH3)
TetraPure_2/ G
B
a Tri_1
b TriPure_0
c TriPure_1
d Tetra_2
e TetraPure_2
f TetraPure_2/ m6A
Abs260 (AU)
Time (min)
Cap(-)
ARCA
DiPure
TriPure_0
TriPure_1
TetraPure_2
Tri_1
Tetra_2
(0) 57 * * * * 89 53
Capped ratio (%)
* uncapped RNA was not detected
C HeLa cells
D JAWSII cells
Chemiluminescence
12-h
24-h
48-h
n = 4, ± SEM
24 48 72 120 (h)
TriPure_0
TriPure_1
TetraPure_2
TetraPure_2/ m6A
TetraPure_2/ G
Tri_1
Tri_1/ AP
Tetra_2
Tetra_2/ AP
Cap(-)
No RNA

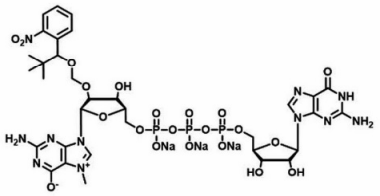
Fig. 41

(a) Cap(-)



(b) DiPure(+)





DiPure

(c)

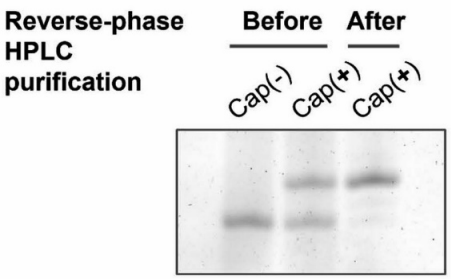
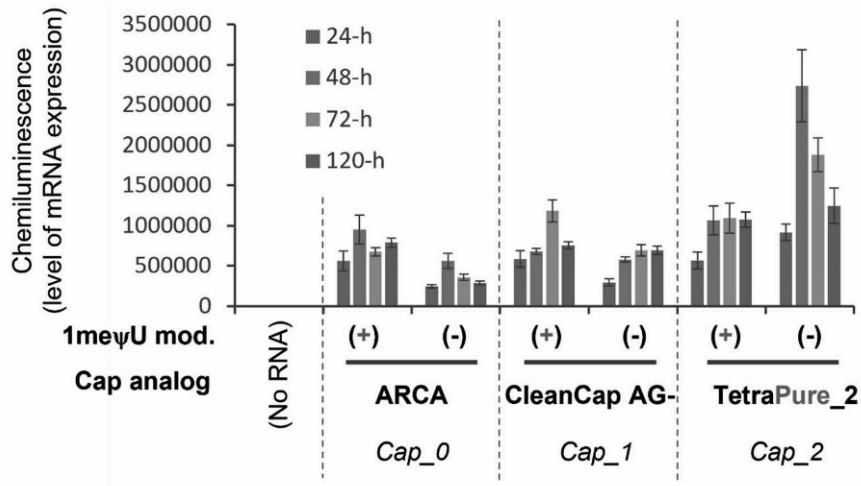
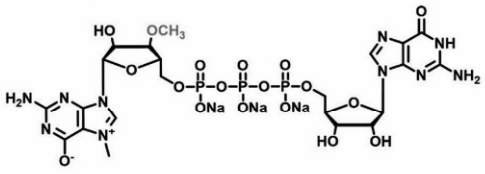
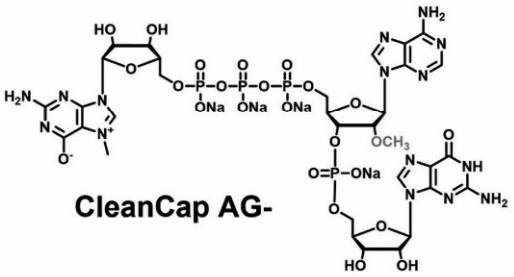


Fig. 42

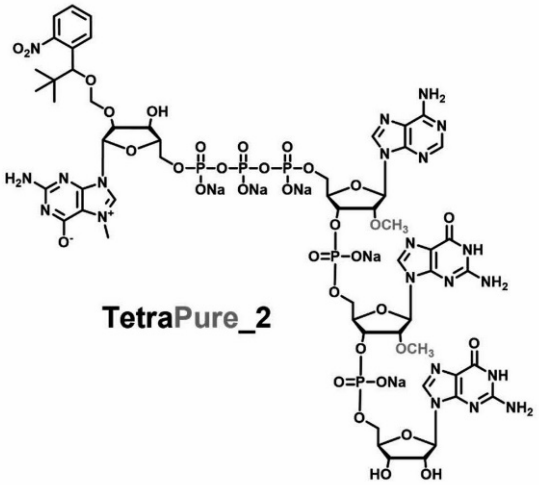




ARCA

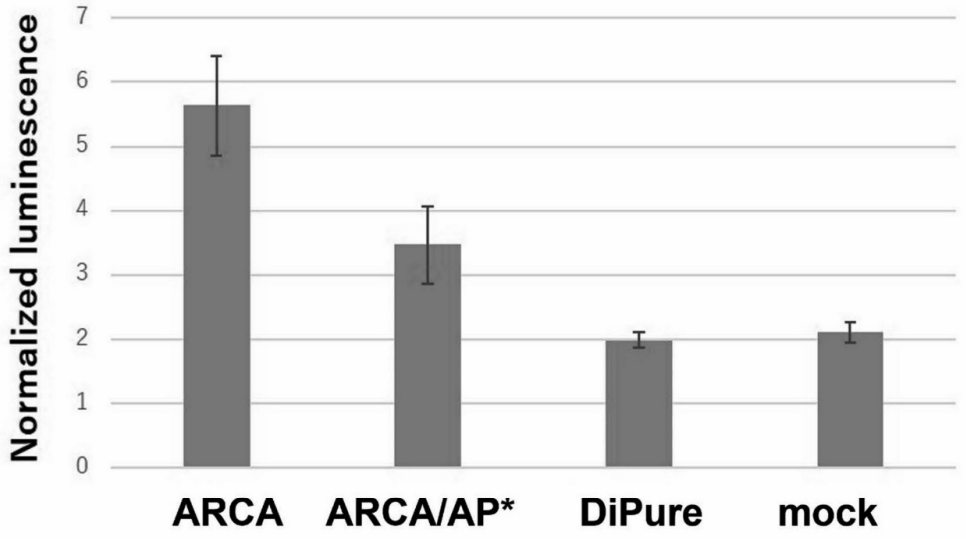


CleanCap AG-

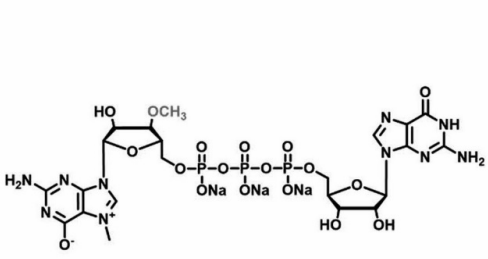


TetraPure_2

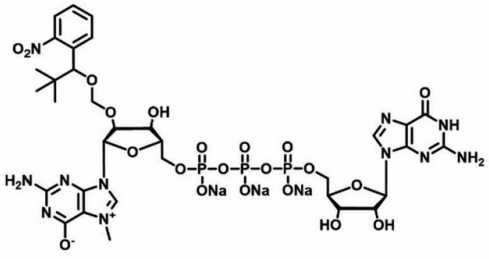
Fig. 43



*AP, mRNA was dephosphorylated using Antarctic phosphatase



ARCA



DiPure

Fig. 44

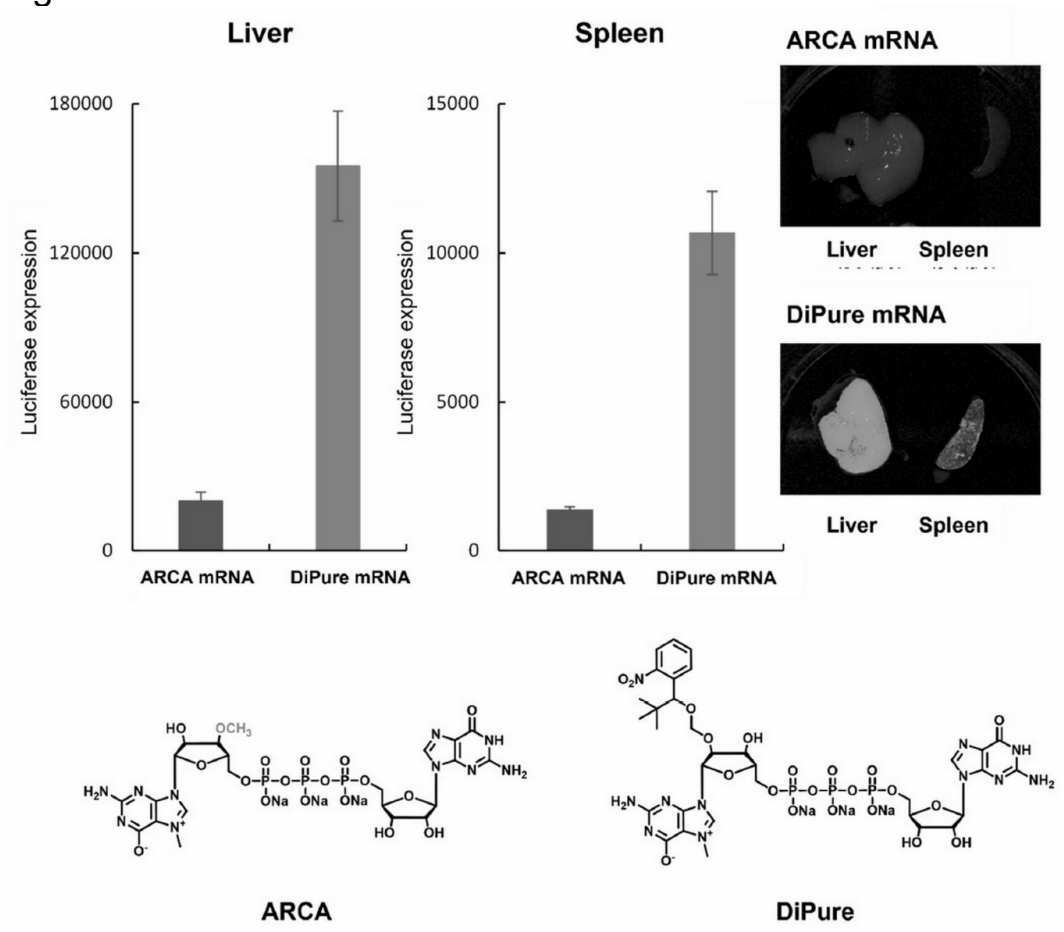
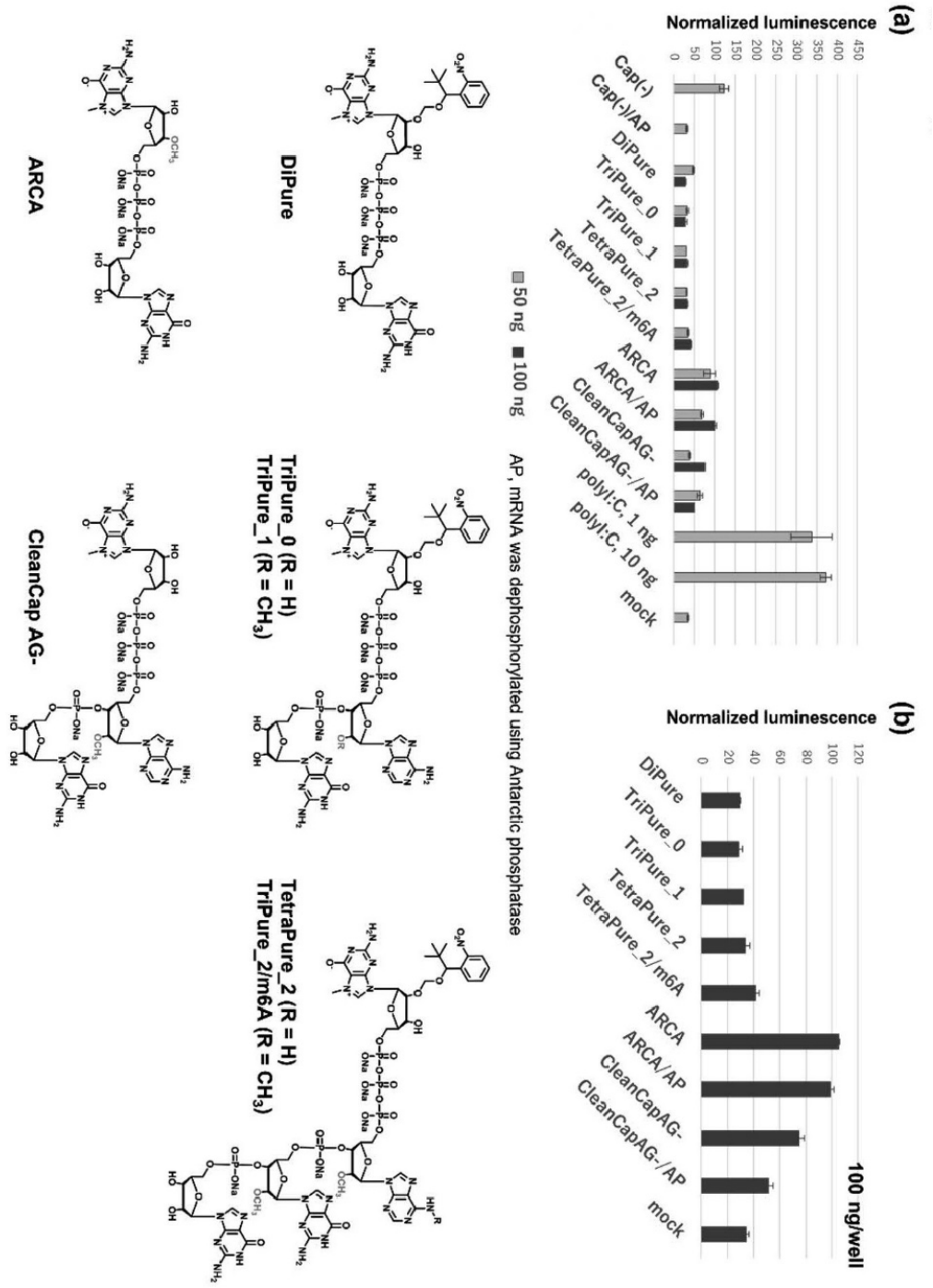

Liver
Spleen
ARCA mRNA
180000
120000
60000
0
Luciferase expression
ARCA mRNA
DiPure mRNA
15000
10000
5000
0
Luciferase expression
ARCA mRNA
DiPure mRNA
Liver
Spleen
DiPure mRNA
Liver
Spleen
ARCA
DiPure

Fig. 45


(a)
Normalized luminescence
0
50
100
150
200
250
300
350
400
450
Cap(-)
Cap(-)/AP
DiPure
TriPure_0
TriPure_1
TetraPure_2
TetraPure_2/m6A
ARCA
ARCA/AP
CleanCapAG-
CleanCapAG-/AP
polyI:C, 1 ng
polyI:C, 10 ng
mock
50 ng
100 ng
AP, mRNA was dephosphorylated using Antarctic phosphatase
(b)
Normalized luminescence
0
20
40
60
80
100
120
100 ng/well
DiPure
TriPure_0
TriPure_1
TetraPure_2
TetraPure_2/m6A
ARCA
ARCA/AP
CleanCapAG-
CleanCapAG-/AP
mock
ARCA
DiPure
CleanCap AG-
TriPure_0 (R = H)
TriPure_1 (R = CH3)
TetraPure_2 (R = H)
TriPure_2/m6A (R = CH3)