Supplementary Information

The Scope and Limitation of the Nicking Enzyme Amplification Reaction for the Synthesis of Base-Modified Oligonucleotides and Primers for PCR

Petra Ménová, Veronika Raindlová and Michal Hocek

Contents

Single-stranded DNA Ladder

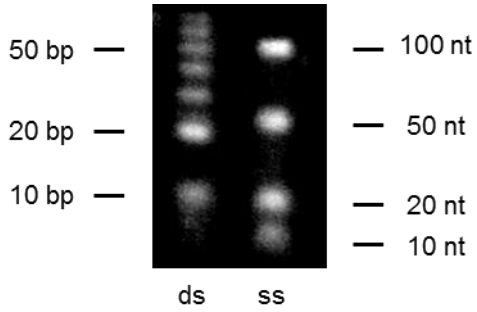


Figure S1. Comparison of different mobilities of ss and ds ONs. Run on 4% agarose gel, 120 V, 70 min.

Table S1. Sequences of commercial oligonucleotides used for ss DNA ladder.

Oligo	Length	Sequence
L10	10 nt	5´-CAGTGACTAG-3´
L20	20 nt	5´-CAGTGACTAGCTTACGGACT-3´
L50	50 nt	5´-CAGTGCATGACTATCGGACCGTATGACTAGCTCAGGTATCCAGTG ACTAG-3´
L100	100 nt	5´-GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCT GTAAGAGCAGATCCCTGGACAGGCAAGGAATACAGGTATTTTGTCCTTG-3´

Supplementary Tables

Table S2. Comparison of our original[a] and optimized conditions for NEAR.

	Original conditions	Optimized conditions	
	Modified dNTPs	Modified dNTPs	Natural dNTPs
Template	0.125 µM	0.125 µM	0.125 µM
Primer	0.125 µM	0.125 µM	0.125 µM
dNTPs[b]	125/188 µM	125/156 µM	125 µM
Nt.BstNBI	0.6 U/µL	0.6 U/µL	0.9 U/µL
Vent(exo-)	0.15 U/µL	0.10 U/µL	0.075 U/µL
ThermoPol Buffer	1x	1x	1x
NEBuffer 3	0.5x	0.5x	0.5x

[a] see lit [S1].

[b] natural/modified.

Supplementary Figures

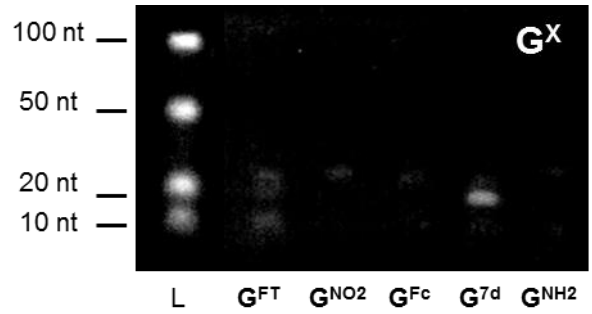


Figure S2. Incorporation of modified 7-deazaguanosine triphosphates in NEAR. In all the experiments, natural dATP, dCTP and dTTP were used together with the modified **dG^{X}TP** as indicated in the title of the line. **L** = DNA ladder. Template **Nick2**. The standard reaction conditions were used.

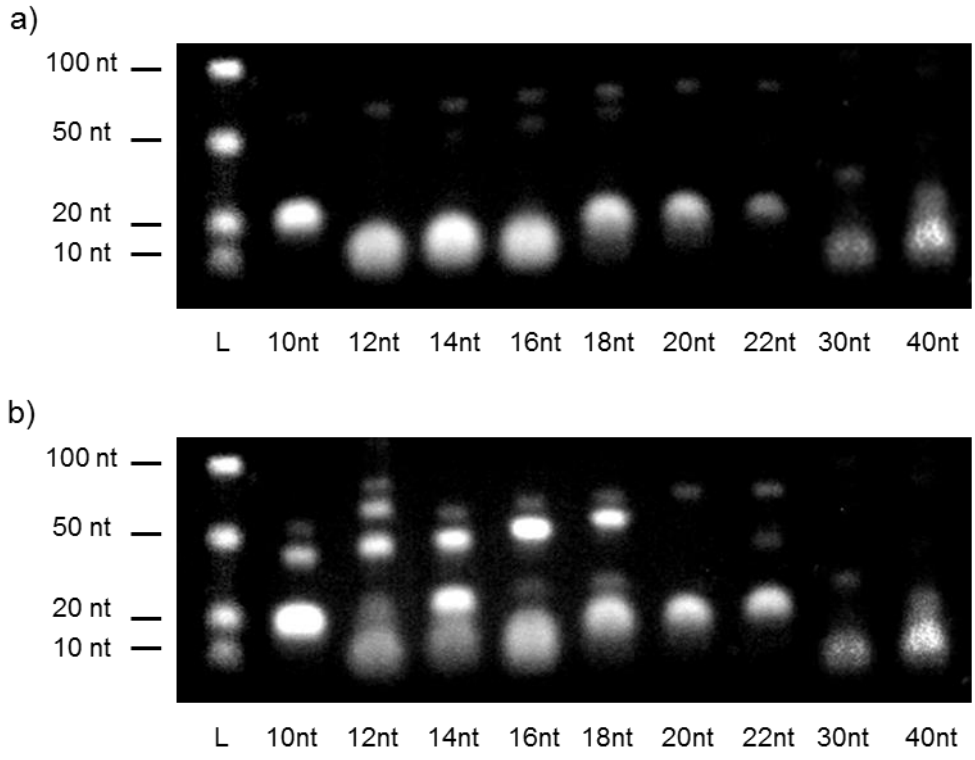
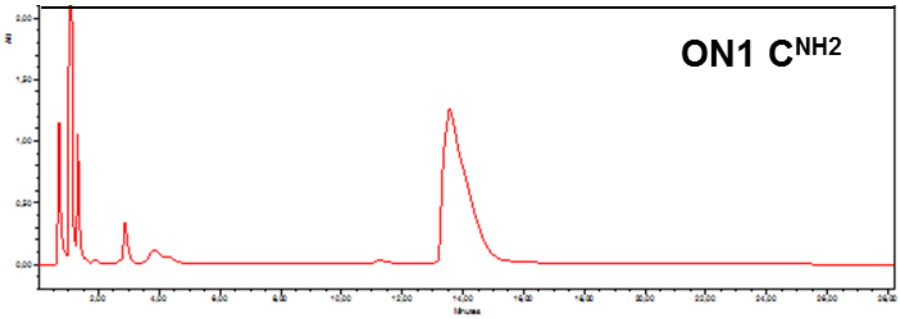
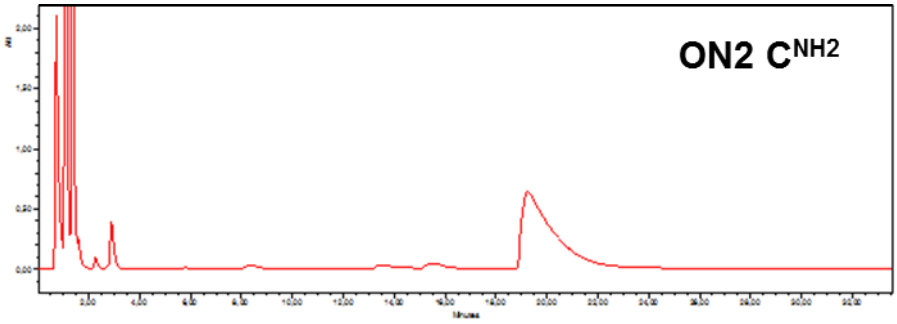
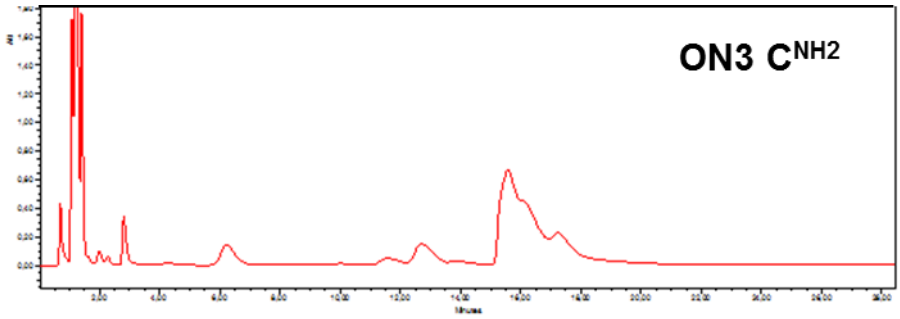
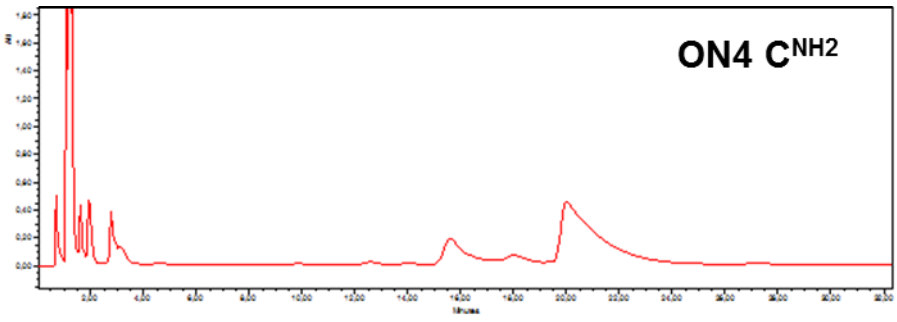


Figure S3. Comparison of the outcome of NEAR with natural dNTPs under the conditions optimized for natural dNTPs (a), and under the conditions used for modified dNTPs (b). The results suggest that for shorter ONs (10-18 nt), conditions (a) are more favourable, while for longer ONs (above 20 nt) the reaction should be done according to the same protocol as with modified dNTPs.


ON1 C^NH2


ON2 C^NH2


ON3 C^NH2


ON4 C^NH2

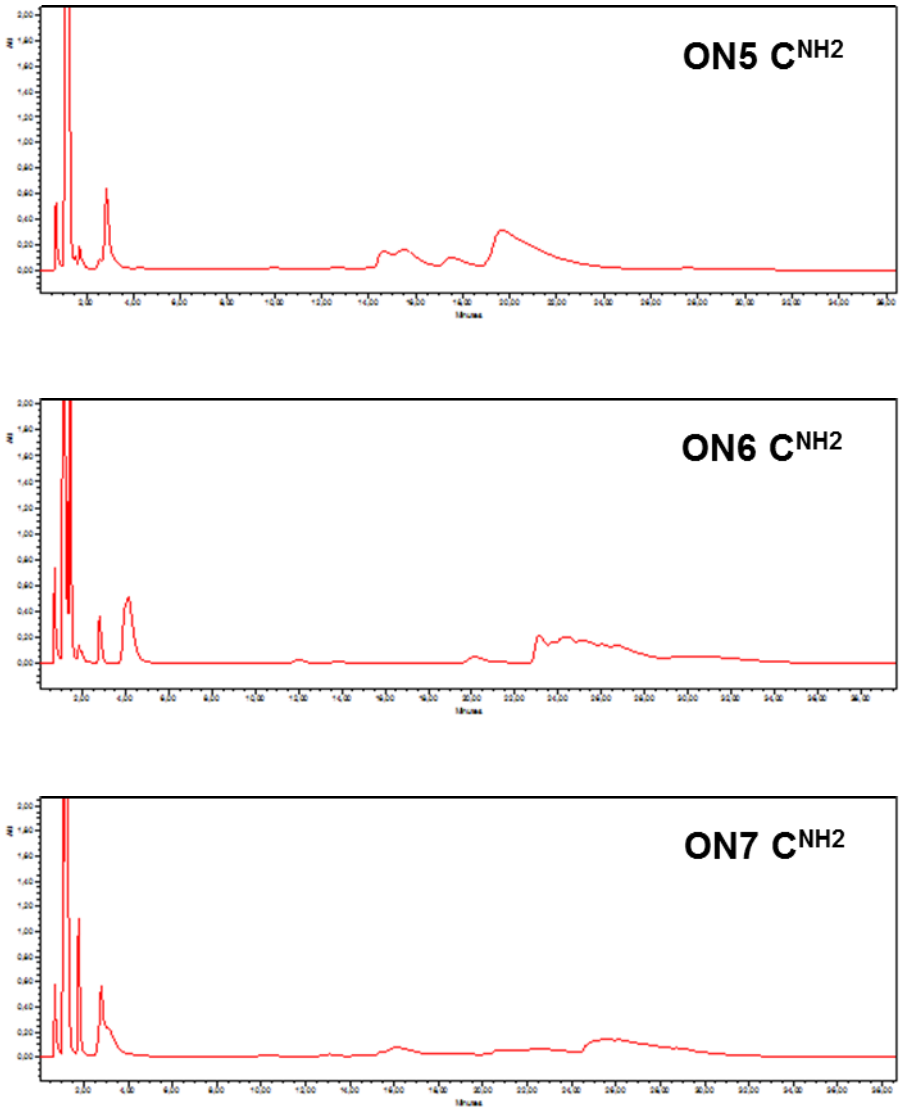


Figure S4. HPLC chromatograms of crude NEAR mixtures (semi-preparative reactions). Column XBridge OST C18 2.5 µM (4.6 × 50 mm). Mobile phase A: 0.1 M TEAA, B: acetonitrile/0.1M TEAA, 20/80 (v/v). Flow rate: 1 mL/min. Gradient 40 to 80 % B in 53 min.

Synthesis of Modified dNTPs

General remarks

Ethynylferrocene was purchased from Molekula Deutschland Ltd and 5-formylthiophene-2--boronic acid from Frontier Scientific. NMR spectra were measured on Bruker Avance 500 at 500 MHz for ^{1}H, 200 MHz for ^{31}P and 125.7 MHz for ^{13}C, in 50mM phosphate buffer (in D_2O) at pD 7.1 (reference to dioxane as internal standard, δ_H = 3.75 ppm, δ_C = 67.19 ppm). ^{31}P NMR spectra were referenced to the phosphate buffer signal (δ = 2.35 ppm). Chemical shifts are given in ppm (δ scale), coupling constants (*J*) in Hz. Mass spectra were measured by ESI. High resolution mass spectra were measured on a LTQ Orbitrap XL (Hermo Fischer Scientific) spectrometer using ESI ionization technique.

2'-Deoxy-7-(ferrocene-1-yl-ethynyl)-7-deazaguanosine 5'-O-triphosphate tris(triethylammonium) salt (**dG^{Fc}TP**)

A water/acetonitrile mixture 2:1 (1 mL) and Et_3N (58 µL, 0.416 mmol, 8 equiv.) were added to an argon-purged flask containing 2'-deoxy-7-iodo-7-deazaguanosine triphosphate (**dG^{I}TP**) (36 mg, 0.052 mmol), ethynylferrocene, (16 mg, 0.076 mmol, 1.5 equiv.), and CuI (1 mg, 0.005 mmol, 10 mol%). In a separate flask, $Pd(OAc)_2$ (0.6 mg, 0.0025 mmol, 5 mol%) and TPPTS (7.5 mg, 0.013 mmol, 5 equiv. to Pd) were combined under argon and a mixture of water/acetonitrile 2:1 (0.5 mL) was added. After dissolution of the solids, the catalyst solution was added to the reaction mixture through a septum and the mixture was stirred at 70 °C for 1 h. The reaction mixture was concentrated on a rotatory evaporator and the product was isolated by semi-preparative HPLC on a C18 column with a linear gradient of 0.1 M TEAB in H_2O to 0.1 M TEAB in H_2O/MeOH 1:1 as eluent. Several co-distillations with water, followed by freeze-drying from water gave **dG^{Fc}TP** as a yellow powder (10 mg, 25 %).

^{1}H NMR (500 MHz, D_2O, pH = 7.1, phosphate buffer, $ref_{dioxane}$ = 3.75 ppm): 1.27 (bs, 32H, C*H*$_3$CH$_2$N); 2.41 (ddd, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.1, H-2´b); 2.66 (ddd, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 6.3, H-2´a); 3.19 (bs, 24H, CH$_3$C*H*$_2$N); 4.15 (bm, 2H, H-5´); 4.21 (bm, 1H, H-4´); 4.37 (bs, 5H, cp); 4.59 (bs, 2H, cp); 4.72 (bm, 1H, H-3´); 4.83 (bs, 2H, cp); 6.41 (dd, 1H, $J_{1'2'}$ = 8.1, 6.2, H-1´); 7.32 (s, 1H, H-6). ^{13}C NMR (151 MHz, D_2O, pH = 7.1, phosphate buffer, $ref_{dioxane}$ = 69.3 ppm): 10.95 (*C*H$_3$CH$_2$N); 40.89 (CH$_2$-2´); 49.34 (CH$_3$*C*H$_2$N); 61.70 (C-cp); 68.31 (d, $J_{C,P}$ = 6, CH$_2$-5´); 71.99, 72.77 and 73.80 (CH-cp); 74.12 (CH-3´); 81.11 (*C*≡C-fer); 85.76 (CH-1´); 87.85 (d, $J_{C,P}$ = 8, CH-4´); 92.94 (C≡*C*-fer); 102.27 and 102.88 (C-4a,5); 125.54 (CH-6); 153.56 (C-7a); 156.08 and 163.63 (C-2,4). ^{31}P (^{1}H dec.)

NMR (202.3 MHz, D_2O, pH = 7.1, phosphate buffer, ref_{H3PO4} = 0 ppm): -21.22 (t, J = 19.4, P_β); -10.23 (d, J = 19.4, P_α); -6.20 (d, J = 19.4, P_γ). MS (ESI$^-$): *m/z*: 800.9 $[M + Na]^-$, 778.9 $[M]^-$, 655.0 $[M + 2H – Na – HPO_3Na]^-$, 633.0 $[M + 3H – 2Na – HPO_3Na]^-$, 553.0 $[M + 3H – Na – 2HPO_3Na]^-$. HRMS (ESI$^-$): *m/z* $[M + Na]^+$ calculated for $C_{23}H_{21}O_{13}N_4FeNa_3P_3$: 778.93660; found 778.93694.

2'-Deoxy-7-(5-formylthiophene-2-yl)-7-deazaguanosine 5'-O-triphosphate trisodium salt (**dG^{FT}TP**)

A water/acetonitrile mixture 2:1 (1 mL) was added through a septum to an argon-purged vial containing 2'-deoxy-7-iodo-7-deazaguanosine triphosphate (**dG^{I}TP**) (20 mg, 0.030 mmol), 5-formylthiophene-2-boronic acid (28 mg, 0.180 mmol, 6 equiv.) and Cs_2CO_3 (49 mg, 0.150 mmol, 5 equiv.). In a separate flask, $Pd(OAc)_2$ (1.0 mg, 15 mol%) and TPPTS (13 mg, 0.023 mmol, 5 equiv. to Pd) were combined under argon atmosphere and a mixture of water/acetonitrile 2:1 (0.5 mL) was added. After dissolution, the catalyst solution was added to the reaction mixture through a septum and the resulting mixture was stirred at 100 °C for 1 h. The reaction mixture was then extracted with chloroform (2 × 4 mL). The aqueous layer containing the product was concentrated on a rotatory evaporator and the product was isolated by semi-preparative HPLC on a C18 column with a linear gradient of 0.1 M TEAB in H_2O to 0.1 M TEAB in H_2O/MeOH 1:1 as eluent. Several co-distillations with water and the conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle), followed by freeze-drying from water gave **dG^{FT}TP** as a yellowish powder (9 mg, 49 %).

^{1}H NMR (499.8 MHz, D_2O, pD = 7.1, phosphate buffer, $ref_{dioxane}$ = 3.75 ppm): 2.42 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 3.1, H-2'b); 2.67 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 6.4, H-2'a); 4.16 (bm, 2H, H-5'); 4.22 (bm, 1H, H-4'); 4.73 (bddd, 1H, $J_{3',2'}$ = 6.4, 3.1, $J_{3',4'}$ = 2.7, H-3'); 6.33 (dd, 1H, $J_{1',2'}$ = 8.1, 6.1, H-1'); 7.50 (s, 1H, H-6); 7.66 (d, 1H, $J_{3,4}$ = 4.1, H-3-thienyl); 7.82 (d, 1H, $J_{4,3}$ = 4.1, H-4-thienyl); 9.65 (s, 1H, CHO). ^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, $ref_{dioxane}$ = 69.3 ppm): 40.96 (CH_2-2'); 68.39 (d, $J_{C,P}$ = 5.1, CH_2-5'); 73.94 (CH-3'); 85.86 (CH-1'); 87.82 (d, $J_{C,P}$ = 8.6, CH-4'); 100.27 (C-4a); 115.97 (C-5); 121.24 (CH-6); 129.58 (CH-3-thienyl); 142.03 (C-5-thienyl); 143.40 (CH-4-thienyl); 151.17 (C-2-thienyl); 154.88 (C-7a); 155.72 (C-2); 163.13 (C-4); 188.98 (CHO). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, pD = 7.1, phosphate buffer, $ref_{phosphate\ buffer}$ = 2.35 ppm): -21.02 (bdd, J = 19.2, 17.4, P_β); -10.02 (d, J = 19.2, P_α); -6.89 (bd, J = 19.2, P_γ). IR: 3437, 3178, 2954, 1663, 1543, 1506, 1453, 1242, 1131, 1093, 984, 908 cm^{-1}. MS (ESI$^-$): *m/z (%)*: 615.0 (10)

$[M]^-$, 557.0 (45) $[M + 2H – Na – HPO_3Na]^-$, 535.1 (100) $[M + 3H – 2Na – HPO_3Na]^-$. HRMS ($ESI^-$): *m/z* $[M – H]^-$ calculated for $C_{16}H_{18}O_{14}N_4P_3S$: 614.9759; found 614.9759. UV/Vis (H_2O, 100 μM) λ_{max} (ε) = 378 (17390), λ (ε) = 269 (17180), λ (ε) = 232 (20820).

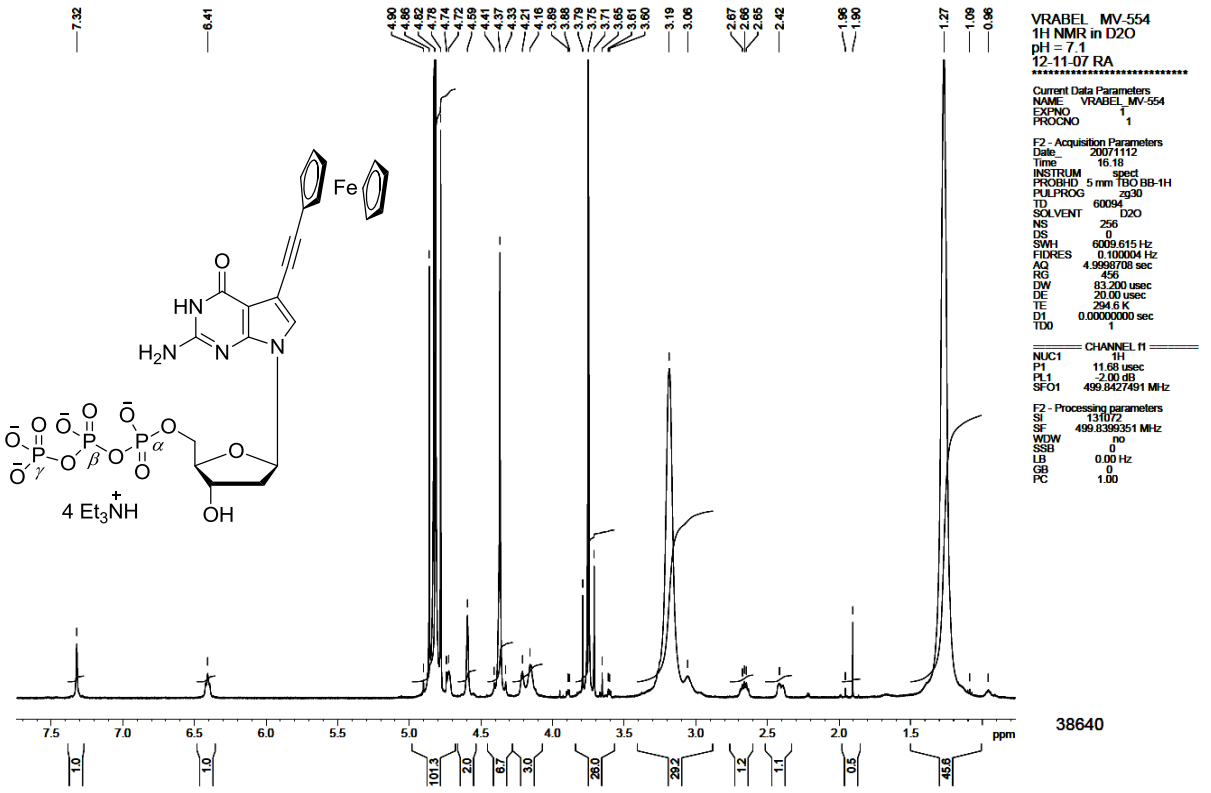
2'-Deoxy-5-(5-formylthiophene-2-yl)uridine 5'-O-triphosphate trisodium salt (**$dU^{FT}TP$**)

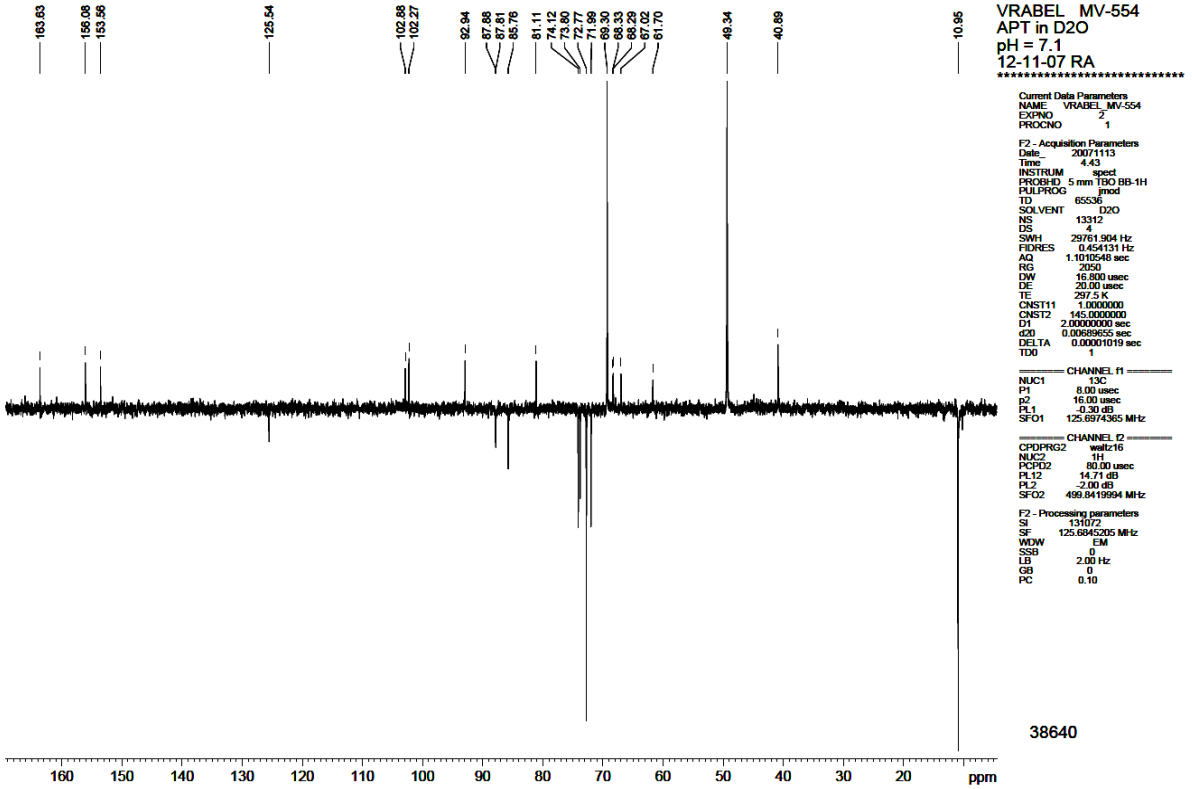
A water/acetonitrile mixture 2:1 (1 mL) was added through a septum to an argon-purged vial containing 2'-deoxy-5-iodouridine triphosphate (**$dU^{I}TP$**) (20 mg, 0.030 mmol), 5-formylthiophene-2-boronic acid (28 mg, 0.180 mmol, 6 equiv.) and Cs_2CO_3 (49 mg, 0.150 mmol, 5 equiv.). In a separate flask, $Pd(OAc)_2$ (0.7 mg, 10 mol%) and TPPTS (9 mg, 0.015 mmol, 5 equiv. to Pd) were combined under argon atmosphere and a mixture of water/acetonitrile 2:1 (0.5 mL) was added. After dissolution, the catalyst solution was added to the reaction mixture through a septum and the resulting mixture was stirred at 90 °C for 1 hour. The resulting mixture was then extracted with chloroform (2 × 4 mL). The aqueous layer containing the product was concentrated on a rotatory evaporator and the product was isolated by semi-preparative HPLC on a C18 column with the use of linear gradient of 0.1 M TEAB in H_2O to 0.1 M TEAB in H_2O/MeOH 1:1 as eluent. Several co-distillations with water and the conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle), followed by freeze--drying from water gave **$dU^{FT}TP$** as a light-yellow powder (8 mg, 40 %).

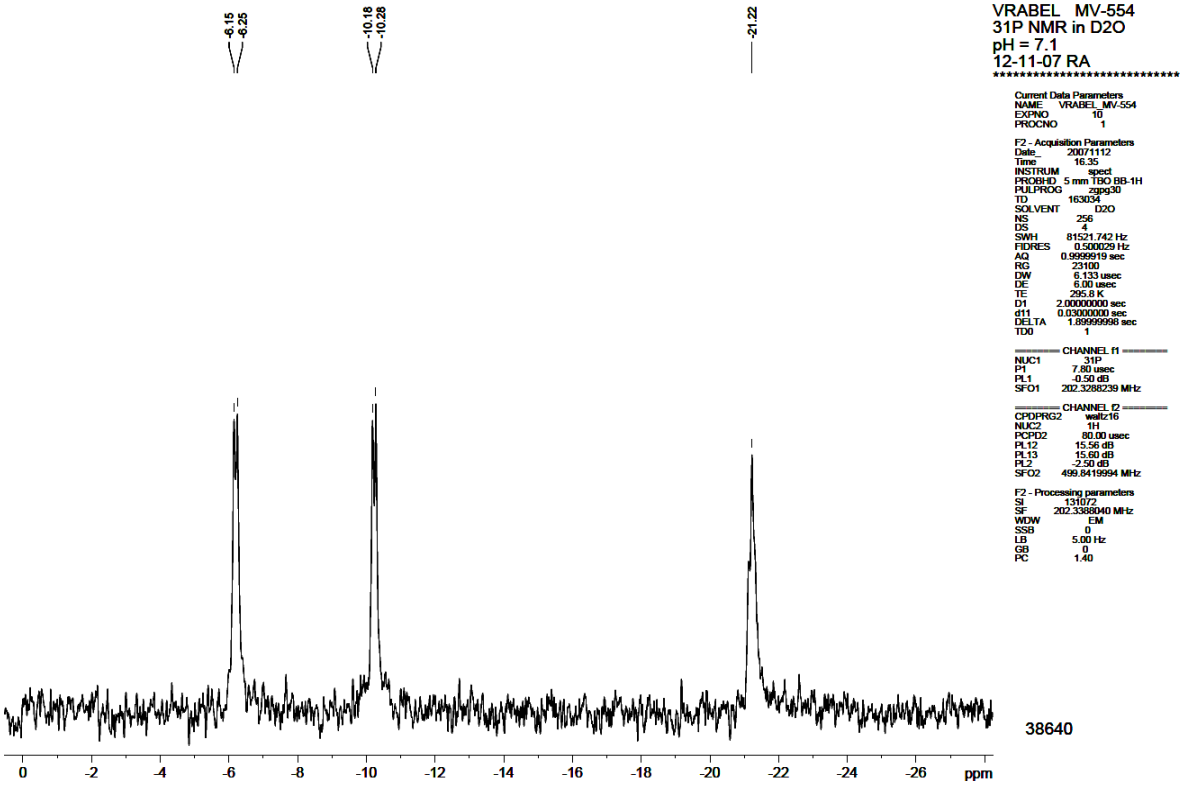
^{1}H NMR (499.8 MHz, D_2O, pD = 7.1, phosphate buffer, $ref_{dioxane}$ = 3.75 ppm): 2.47 (m, 2H, H-2'b); 4.25-4.33 (bm, 3H, H-4',5'); 4.71 (m, 1H, H-3'); 6.36 (t, 1H, $J_{1'2'}$ = 6.8, H-1'); 7.72 (d, 1H, $J_{3,4}$ = 4.2, H-3-thienyl); 8.02 (d, 1H, $J_{4,3}$ = 4.2, H-4-thienyl); 8.47 (s, 1H, H-6); 9.79 (s, 1H, CHO). ^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, $ref_{dioxane}$ = 69.3 ppm): 42.06 (CH_2-2′); 68.13 (d, $J_{C,P}$ = 5.6, CH_2-5′); 73.35 (CH-3′); 88.83 (d, $J_{C,P}$ = 8.8, CH-4′); 89.93 (CH-1′); 111.55 (C-5); 128.15 (CH-3-thienyl); 141.70 (CH-6); 142.47 (CH-4-thienyl); 144.04 (C-5-thienyl); 147.23 (C-2-thienyl); 153.27 (C-2); 165.88 (C-4); 189.92 (CHO). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, pD = 7.1, phosphate buffer, $ref_{phosphate\ buffer}$ = 2.35 ppm): -21.19 (bdd, *J* = 22.8, 18.2, P_β); -10.37 (d, *J* = 18.2, P_α); -6.98 (bd, *J* = 22.8, P_γ). IR: 3424, 2923, 2854, 1704, 1654, 1523, 1457, 1241, 1127, 1092, 989, 911 cm^{-1}. MS (ESI^+): *m/z (%)*: 666.9 (60) $[M + Na]^+$, 689.0 (100) $[M + 2Na]^+$. HRMS (ESI^+): *m/z* $[M + Na]^+$ calculated for $C_{14}H_{14}O_{15}N_2Na_4P_3S$: 666.8913; found 666.8918. UV/Vis (H_2O, 100μM) λ_{max} (ε) = 352 (16090), λ (ε) = 291 (7190).

NMR Spectra

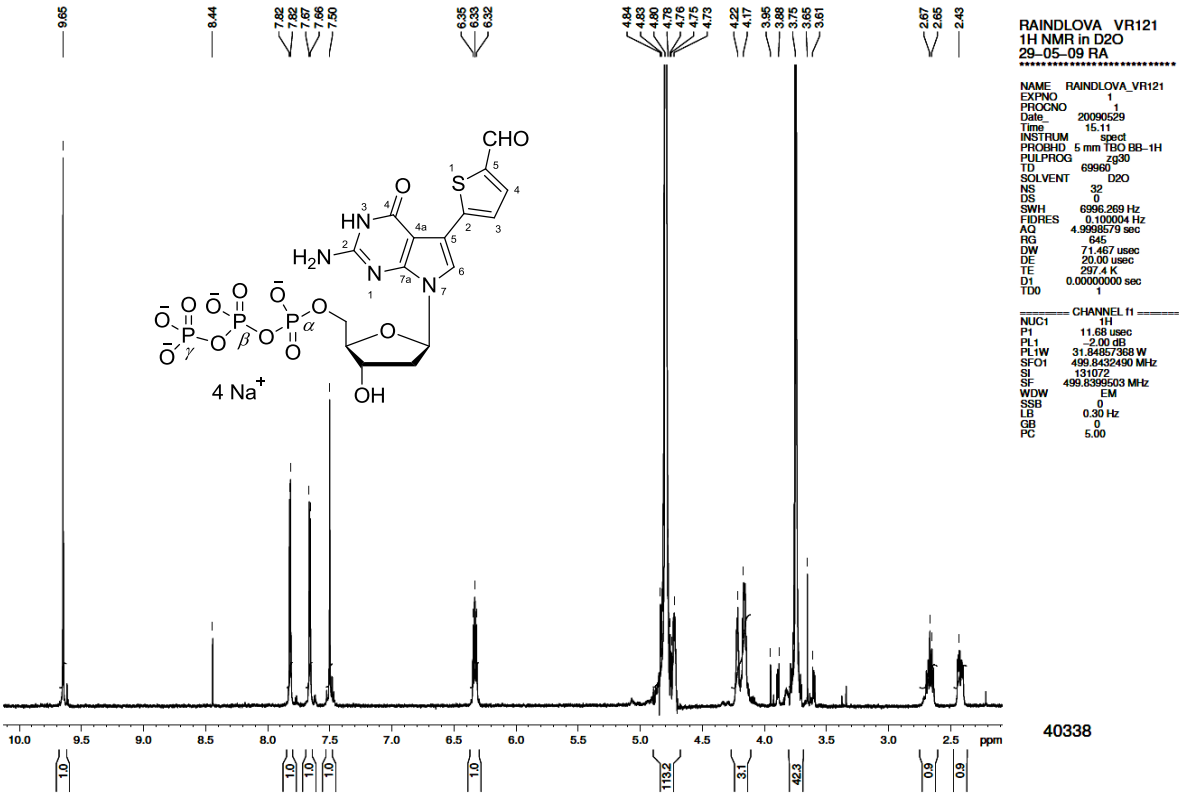
NMR spectra of compoud **dG^{Fc}TP**

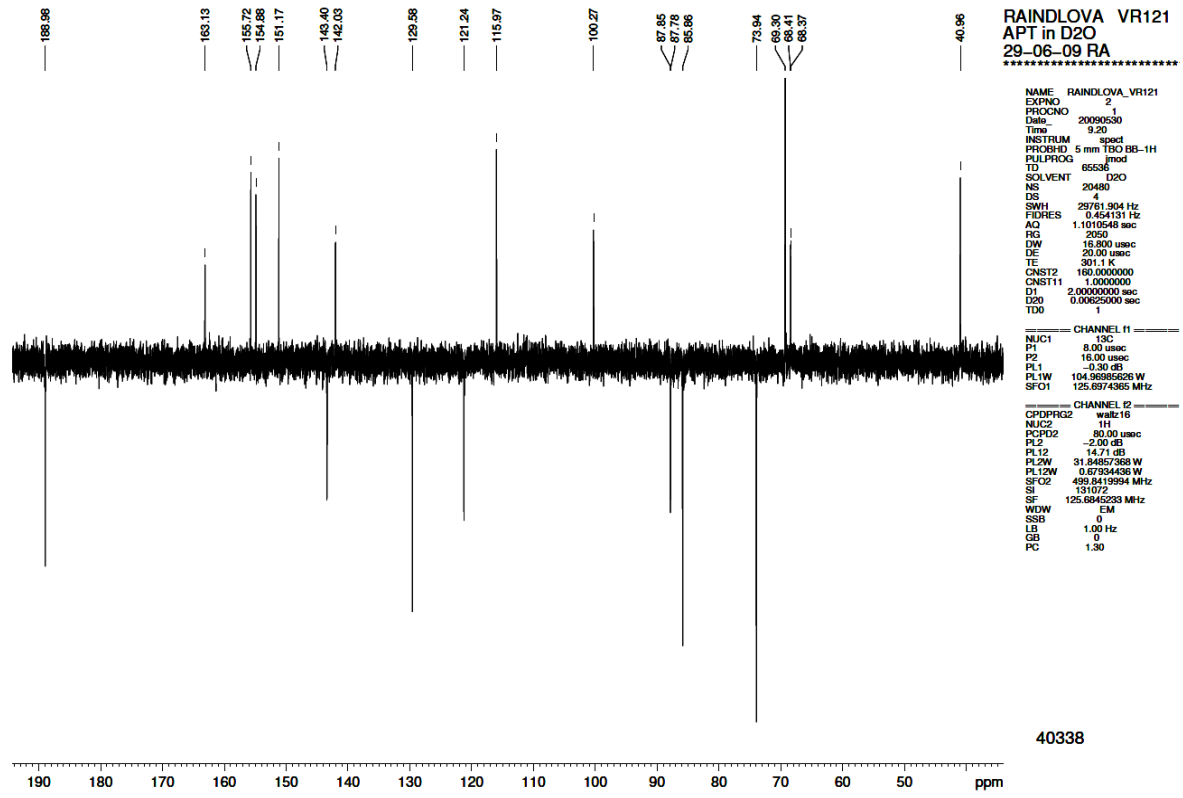


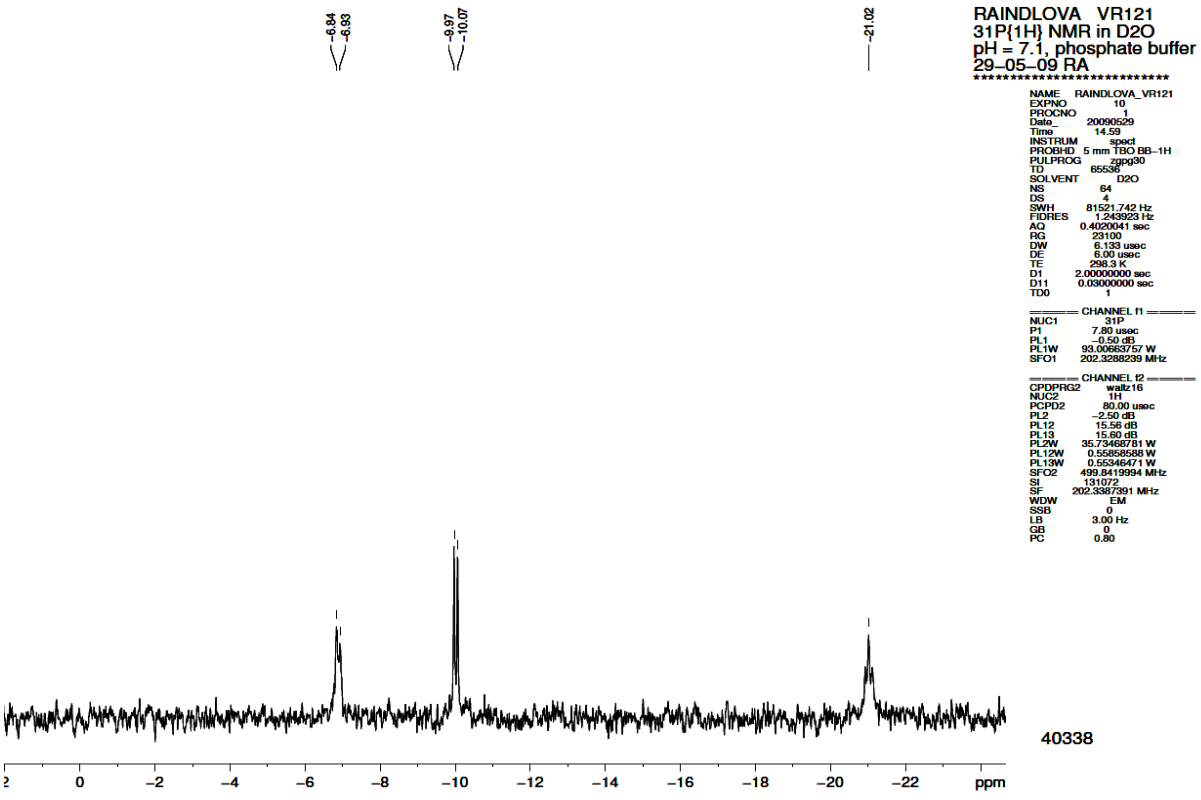



-6.15
-6.25
-10.18
-10.28
-21.22
VRABEL MV-554
31P NMR in D2O
pH = 7.1
12-11-07 RA
****
Current Data Parameters
NAME VRABEL_MV-554
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20071112
Time 16.35
INSTRUM spect
PROBHD 5 mm TBO BB-1H
PULPROG zgpg30
TD 163034
SOLVENT D2O
NS 256
DS 4
SWH 81521.742 Hz
FIDRES 0.500029 Hz
AQ 0.9998919 sec
RG 23100
DW 6.133 usec
DE 6.00 usec
TE 295.8 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1
CHANNEL f1
NUC1 31P
P1 7.80 usec
PL1 -0.50 dB
SFO1 202.3288239 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL12 15.56 dB
PL13 15.60 dB
PL2 -2.50 dB
SFO2 499.8419994 MHz
F2 - Processing parameters
SI 131072
SF 202.3388040 MHz
WDW EM
SSB 0
LB 5.00 Hz
GB 0
PC 1.40
38640
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
ppm

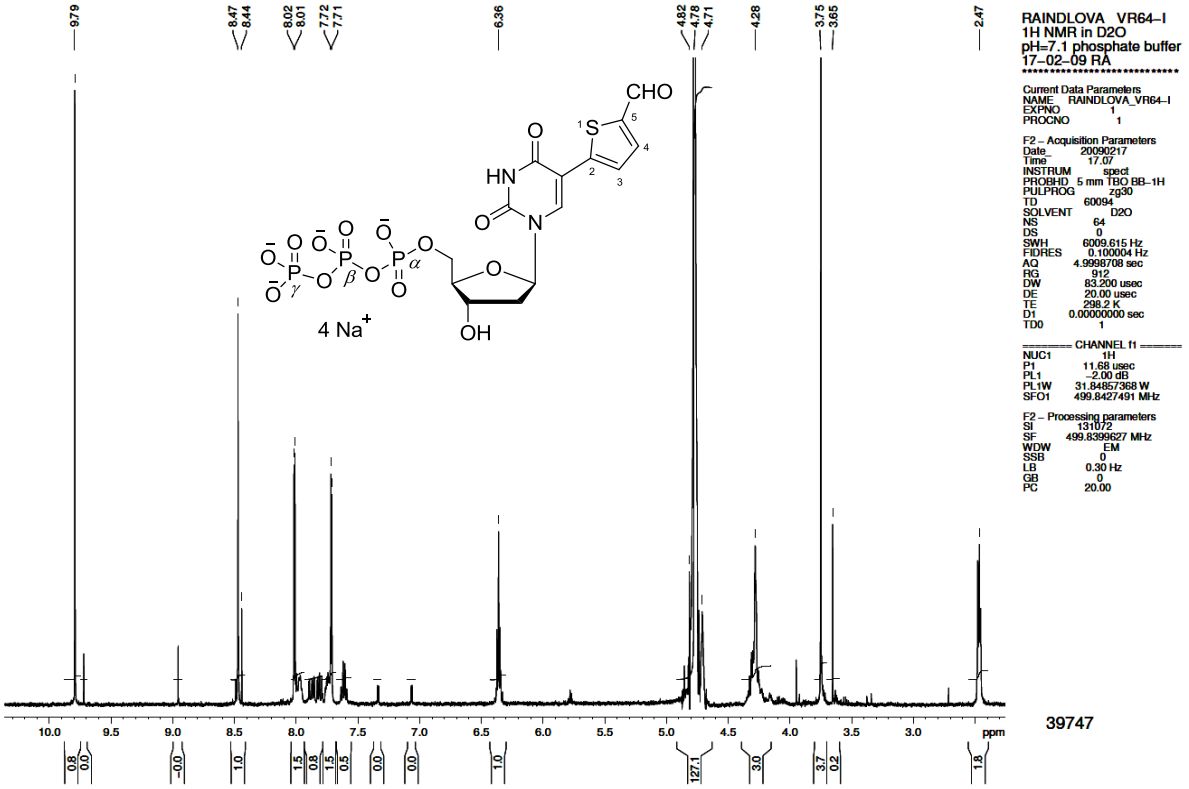
NMR spectra of compound **dG^{FT}TP**

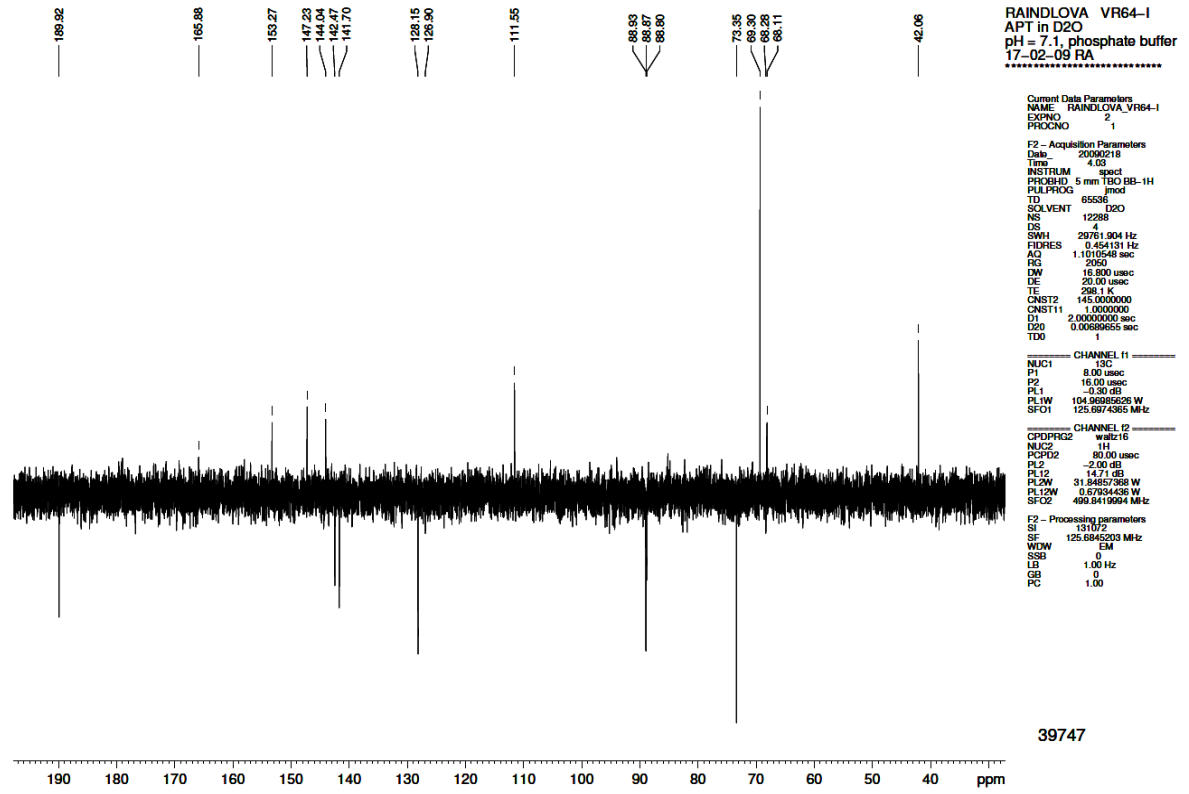


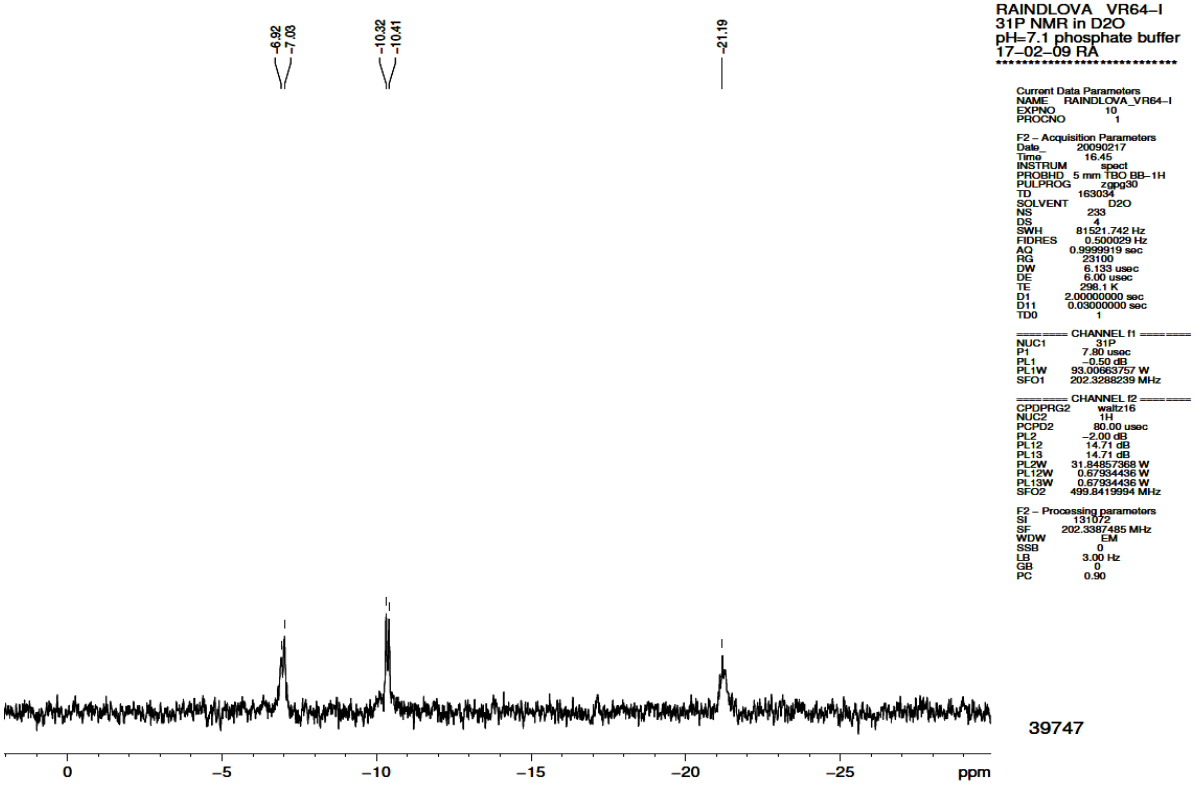



-6.84
-6.93
-9.97
-10.07
-21.02
RAINDLOVA VR121
31P{1H} NMR in D2O
pH = 7.1, phosphate buffer
29–05–09 RA
****
NAME RAINDLOVA_VR121
EXPNO 10
PROCNO 1
Date_ 20090529
Time 14.59
INSTRUM spect
PROBHD 5 mm TBO BB–1H
PULPROG zgpg30
TD 65536
SOLVENT D2O
NS 64
DS 4
SWH 81521.742 Hz
FIDRES 1.243923 Hz
AQ 0.4020041 sec
RG 23100
DW 6.133 usec
DE 6.00 usec
TE 298.3 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
CHANNEL f1
NUC1 31P
P1 7.80 usec
PL1 –0.50 dB
PL1W 93.00663757 W
SFO1 202.3288239 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 –2.50 dB
PL12 15.56 dB
PL13 15.80 dB
PL2W 35.73468781 W
PL12W 0.55858588 W
PL13W 0.55346471 W
SFO2 499.8419994 MHz
SI 131072
SF 202.3387391 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 0.80
40338
2
0
–2
–4
–6
–8
–10
–12
–14
–16
–18
–20
–22
ppm

NMR spectra of compound **dU^{FT}TP**






-6.92
-7.03
-10.32
-10.41
-21.19
RAINDLOVA VR64–I
31P NMR in D2O
pH=7.1 phosphate buffer
17–02–09 RA
Current Data Parameters
NAME RAINDLOVA_VR64–I
EXPNO 10
PROCNO 1
F2 – Acquisition Parameters
Date_ 20090217
Time 16.45
INSTRUM spect
PROBHD 5 mm TBO BB–1H
PULPROG zgpg30
TD 163034
SOLVENT D2O
NS 233
DS 4
SWH 81521.742 Hz
FIDRES 0.500029 Hz
AQ 0.9999919 sec
RG 23100
DW 6.133 usec
DE 6.00 usec
TE 298.1 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 31P
P1 7.80 usec
PL1 –0.50 dB
PL1W 93.00663757 W
SFO1 202.3288239 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 –2.00 dB
PL12 14.71 dB
PL13 14.71 dB
PL2W 31.84957368 W
PL12W 0.67934436 W
PL13W 0.67934436 W
SFO2 499.8419994 MHz
F2 – Processing parameters
SI 131072
SF 202.3387485 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 0.90
39747
0
–5
–10
–15
–20
–25
ppm

UV Spectra

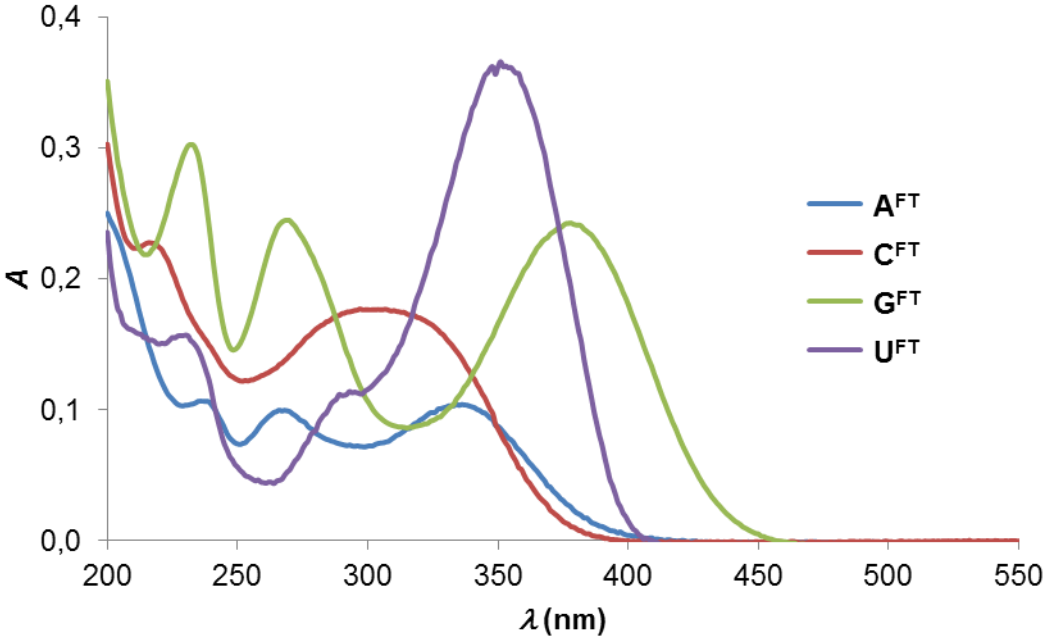


Figure S5. UV-VIS spectra of formylthienyl-modified nucleoside triphosphates ($\mathbf{X}^{\mathbf{FT}}$) (20 µM aqueous solutions) in water: $\mathbf{A}^{\mathbf{FT}}$ (λ_{abs} = 334 nm, ε = 5 200 L mol^{-1} cm^{-1}), $\mathbf{C}^{\mathbf{FT}}$ (λ_{abs} = 297 nm, ε = 8 800 L mol^{-1} cm^{-1}), $\mathbf{G}^{\mathbf{FT}}$ (λ_{abs} = 378 nm, ε = 12 100 L mol^{-1} cm^{-1}), $\mathbf{U}^{\mathbf{FT}}$ (λ_{abs} = 351 nm, ε = 18 300 L mol^{-1} cm^{-1}).

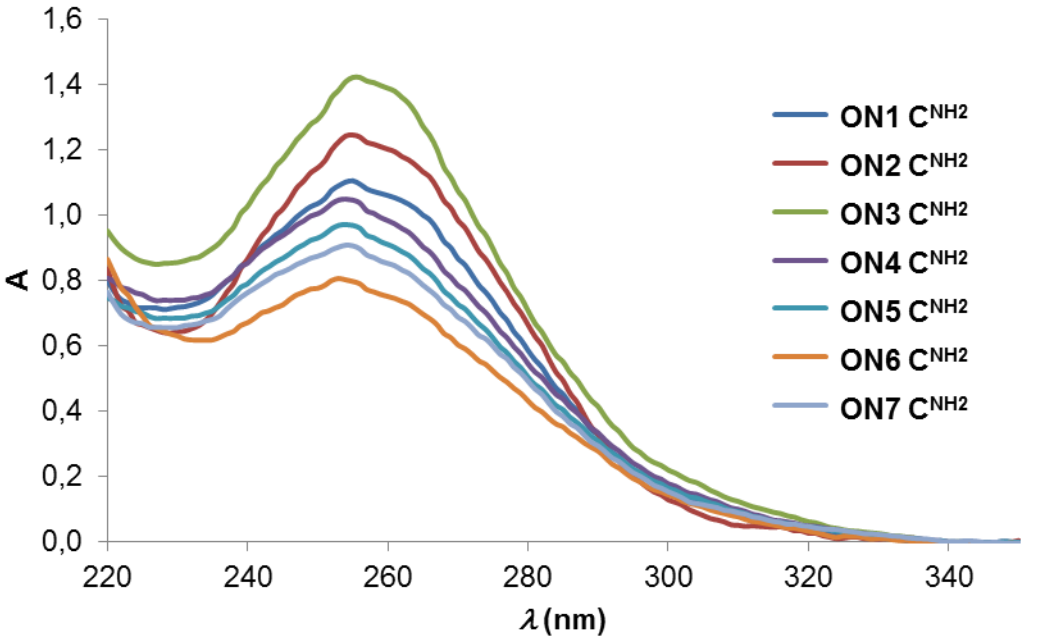


Figure S6. UV spectra of prepared **ON1-7** $\mathbf{C}^{\mathbf{NH2}}$ after HPLC purification. **ON1-3** $\mathbf{C}^{\mathbf{NH2}}$ (10 µM), **ON4-5** $\mathbf{C}^{\mathbf{NH2}}$ (6.5 µM), **ON6-7** $\mathbf{C}^{\mathbf{NH2}}$ (4 µM).

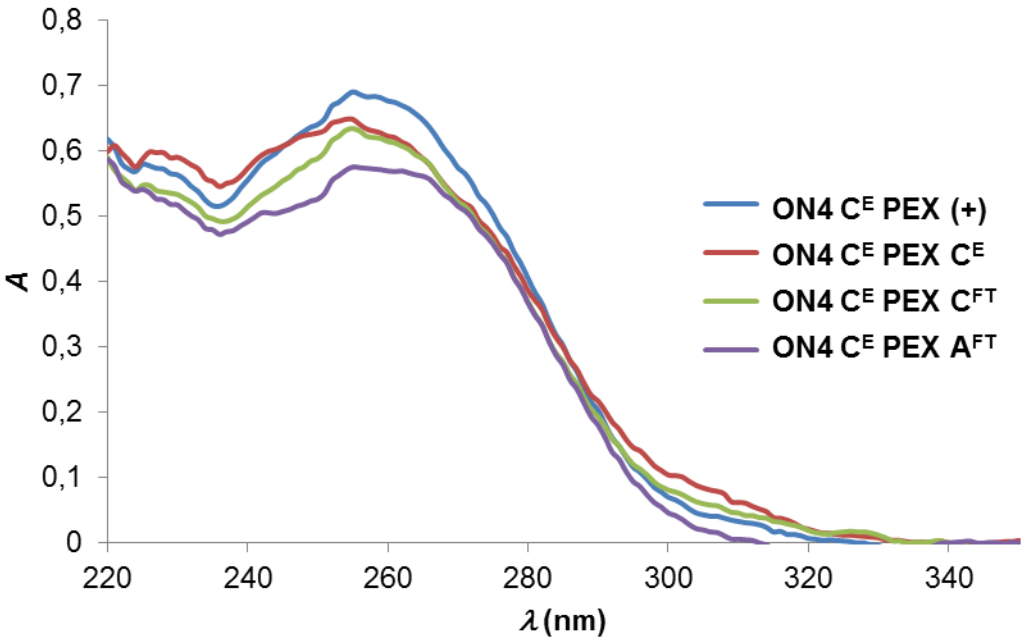


Figure S7. UV spectra of PEX products with ethynyl-modified primer **ON4 C^{E}**. Aqueous solutions, 2 µM.

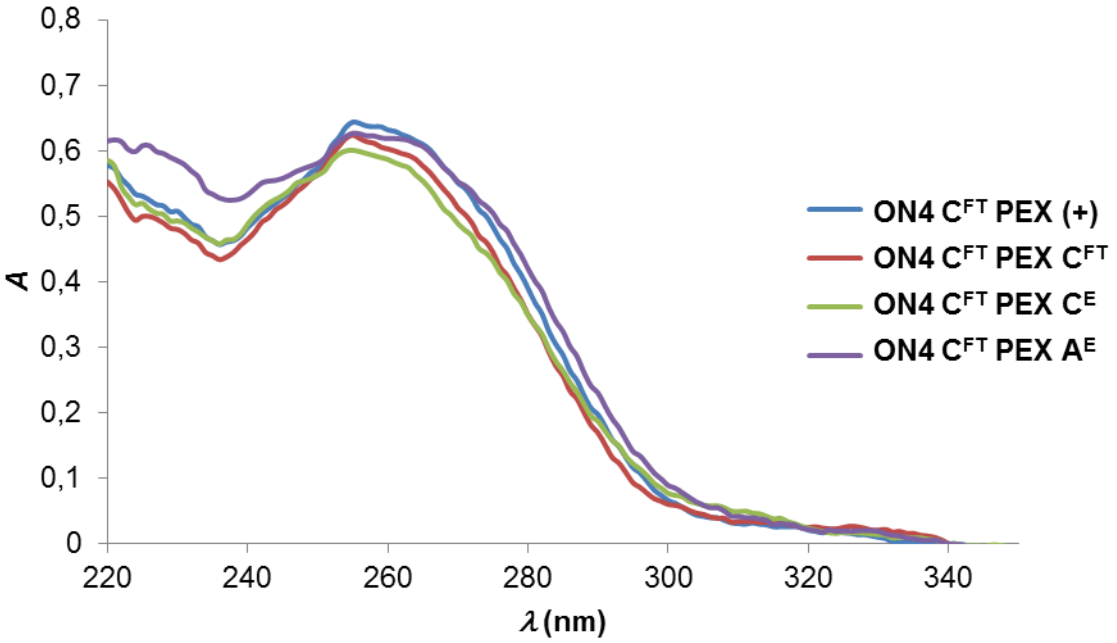


Figure S8. UV spectra of PEX products with formylthienyl-modified primer **ON4 C^{FT}**. Aqueous solutions, 2 µM.

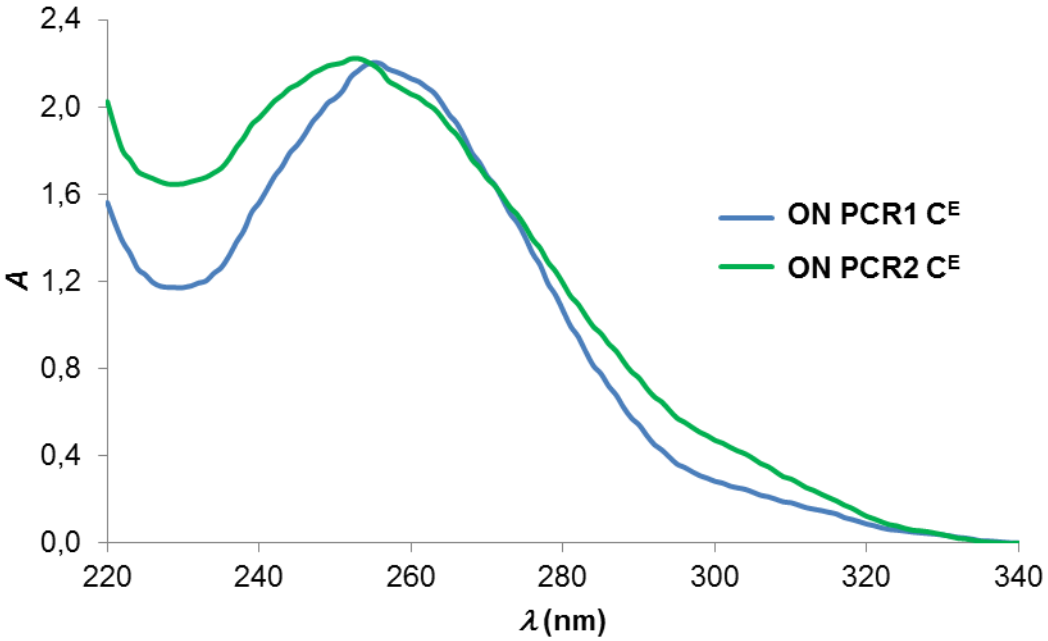


Figure S9. UV spectra of ethynyl-modified PCR primers **ON PCR1 C^{E}** and **ON PCR2 C^{E}**. Aqueous solutions, 10 μM.

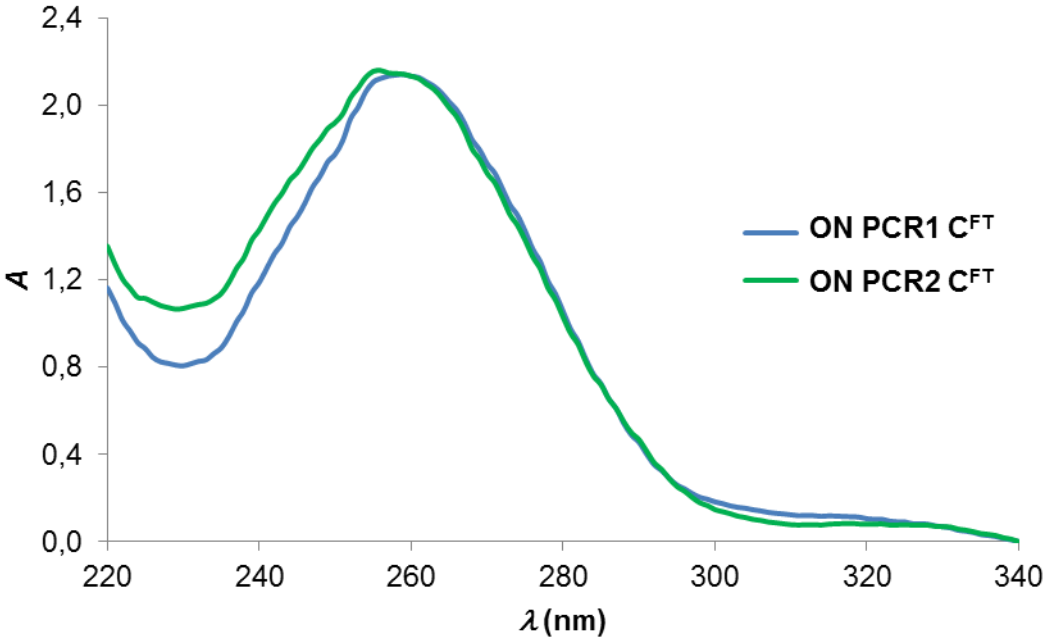


Figure S10. UV spectra of formylthienyl-modified PCR primers **ON PCR1 C^{FT}** and **ON PCR2 C^{FT}**. Aqueous solutions, 10 μM.

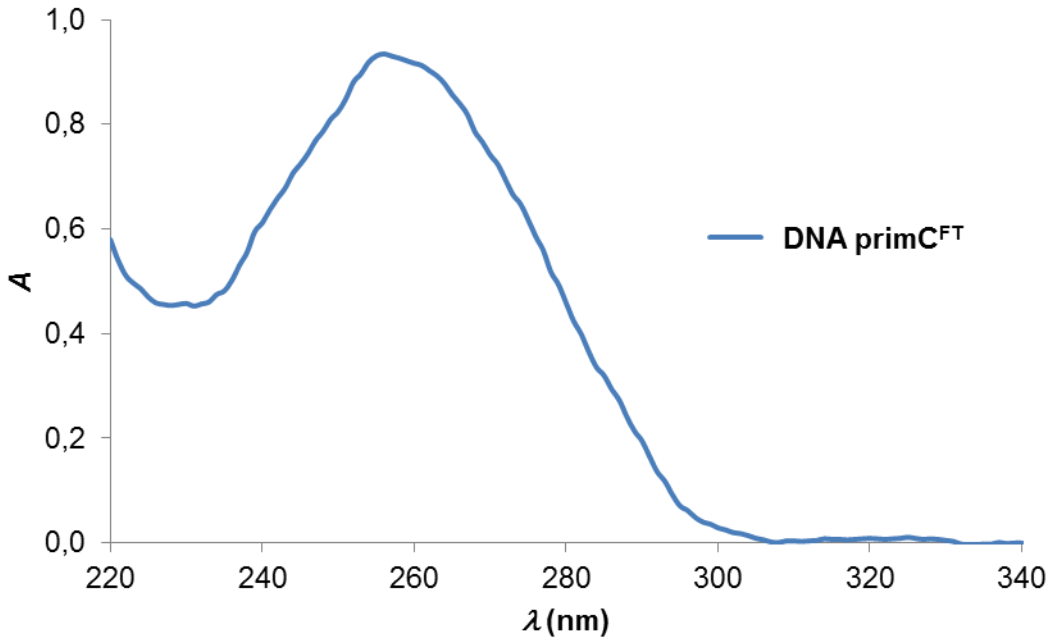


Figure S11. UV spectrum of PCR product with formylthienyl-modified primers. Aqueous solutions, 0.6 µM (46 ng/µL).

MALDI-TOF Spectra

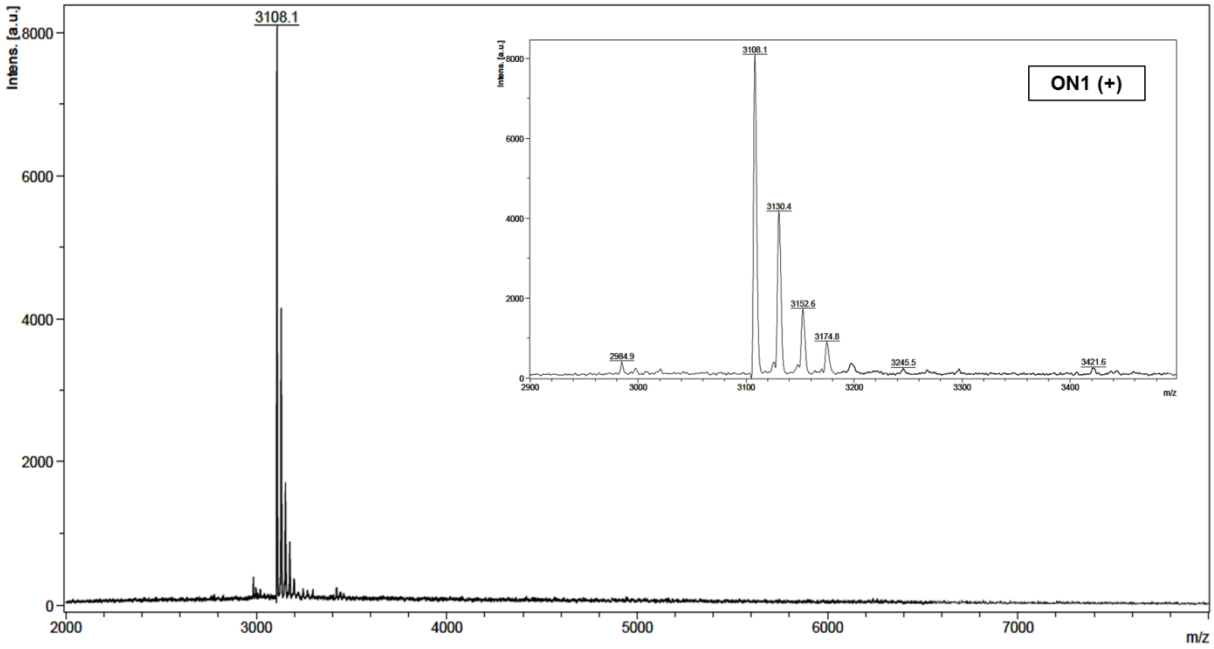


Figure S12. MALDI-TOF spectrum of **ON1 (+)**.

M (calc.) = 3 107.0 Da, M (found) = 3 108.1 Da ($[M+H]^+$).

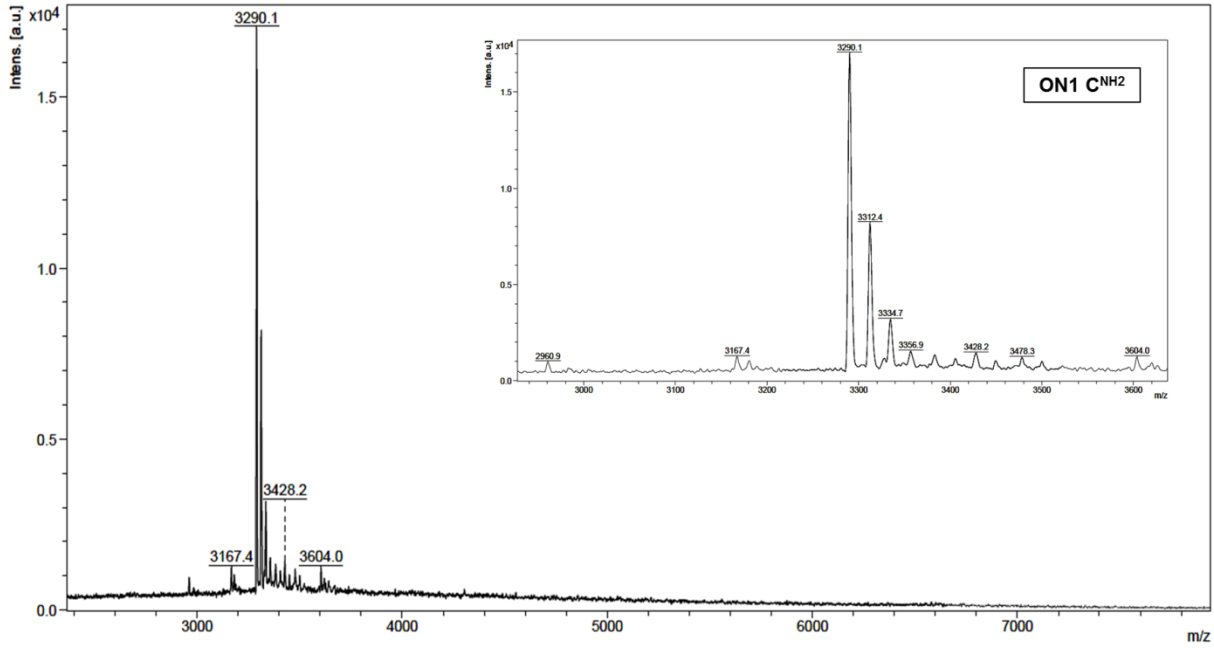


Figure S13. MALDI-TOF spectrum of **ON1 C^{NH2}**.

M (calc.) = 3 289.2 Da, M (found) = 3 290.1 Da ($[M+H]^+$).

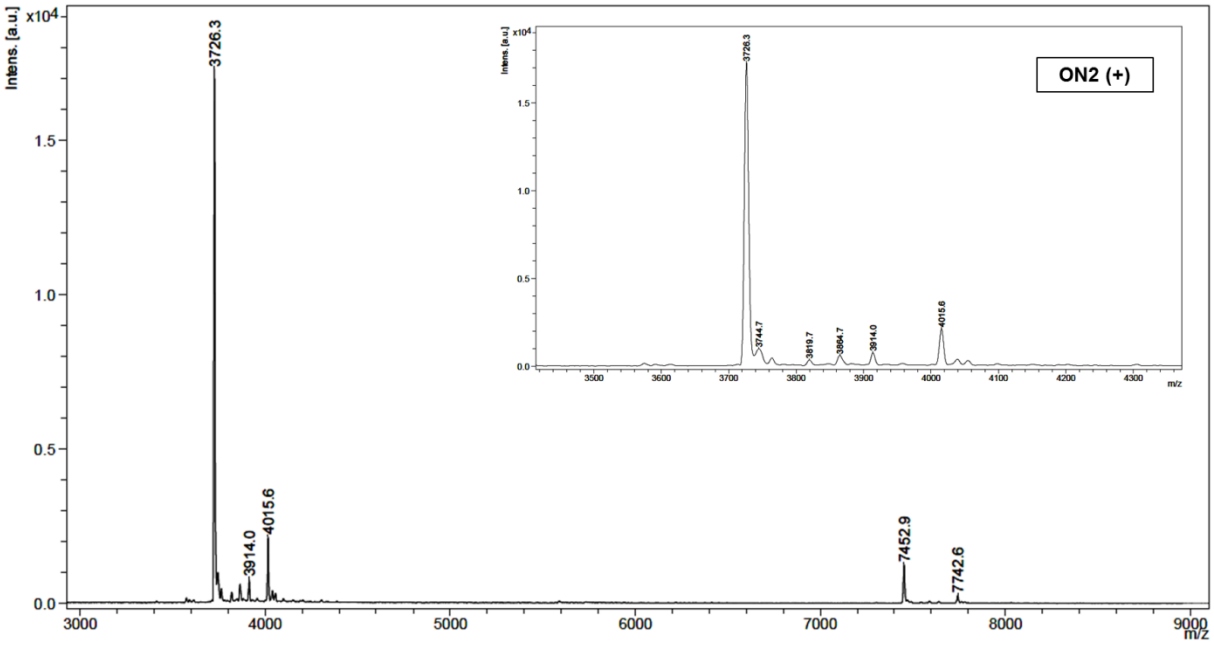


Figure S14. MALDI-TOF spectrum of **ON2 (+)**.

M (calc.) = 3 725.4 Da, M (found) = 3 726.3 Da ($[M+H]^+$).

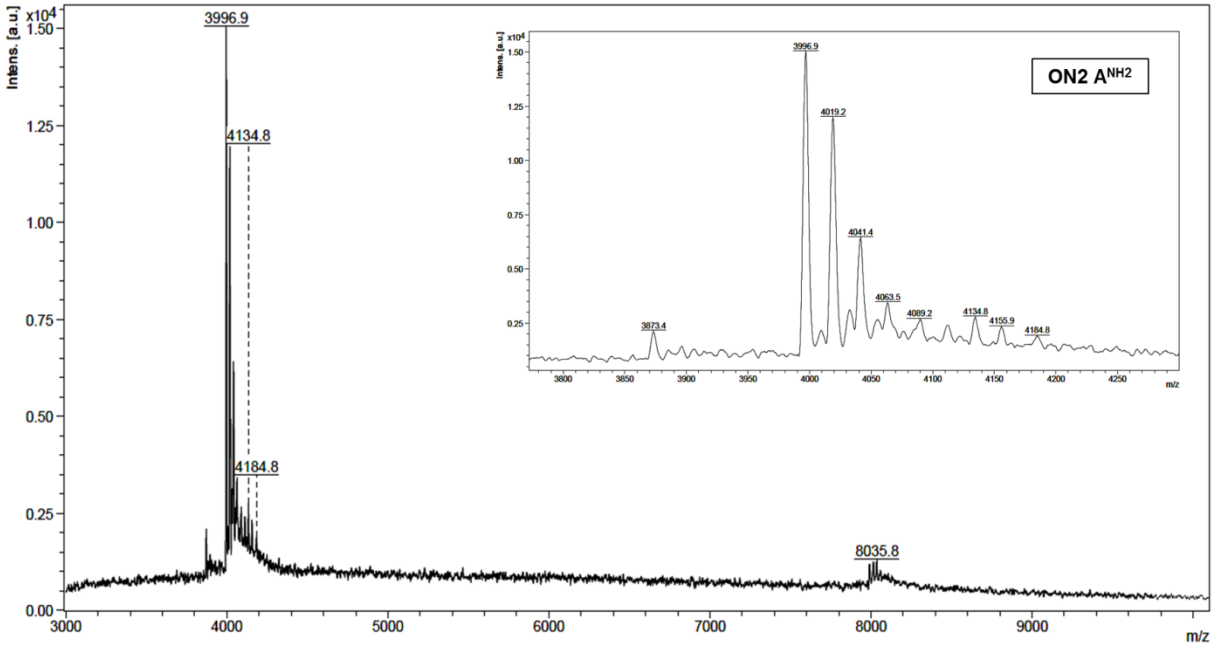


Figure S15. MALDI-TOF spectrum of **ON2 A^{NH2}**.

M (calc.) = 3 995.8 Da, M (found) = 3 996.9 Da ($[M+H]^+$).

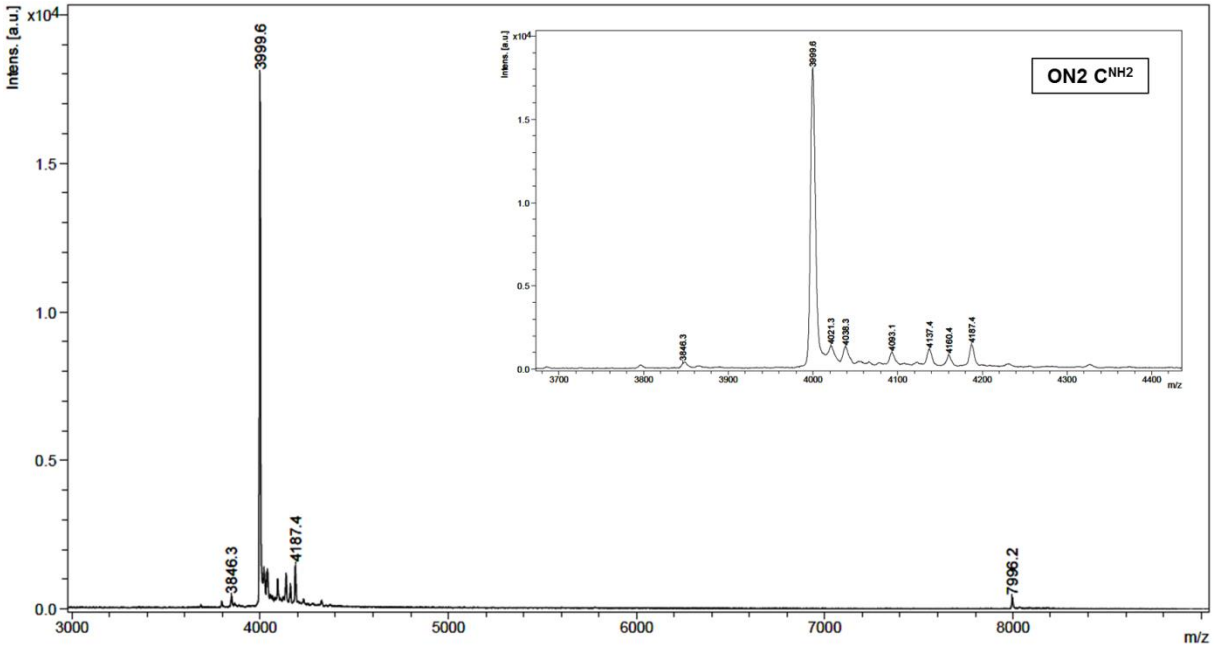


Figure S16. MALDI-TOF spectrum of **ON2 C^{NH2}**.

M (calc.) = 3 998.7 Da, M (found) = 3 999.6 Da ($[M+H]^+$).

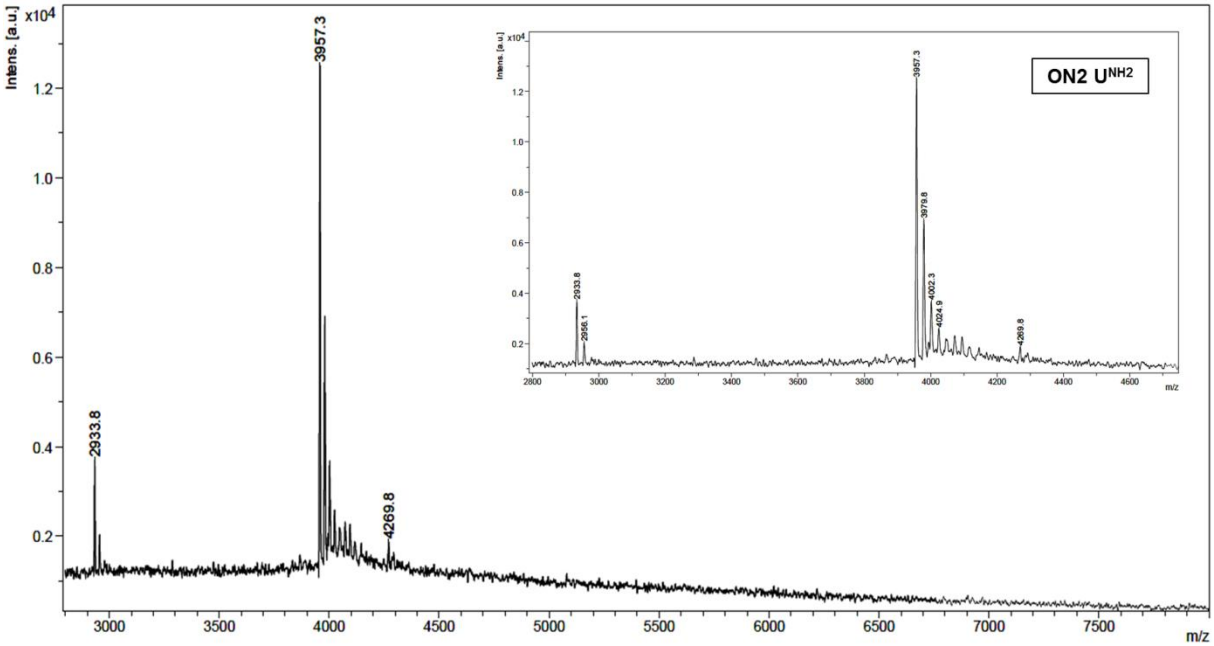


Figure S17. MALDI-TOF spectrum of **ON2 U^{NH2}**.

M (calc.) = 3 955.7 Da, M (found) = 3 957.3 Da ($[M+H]^+$).

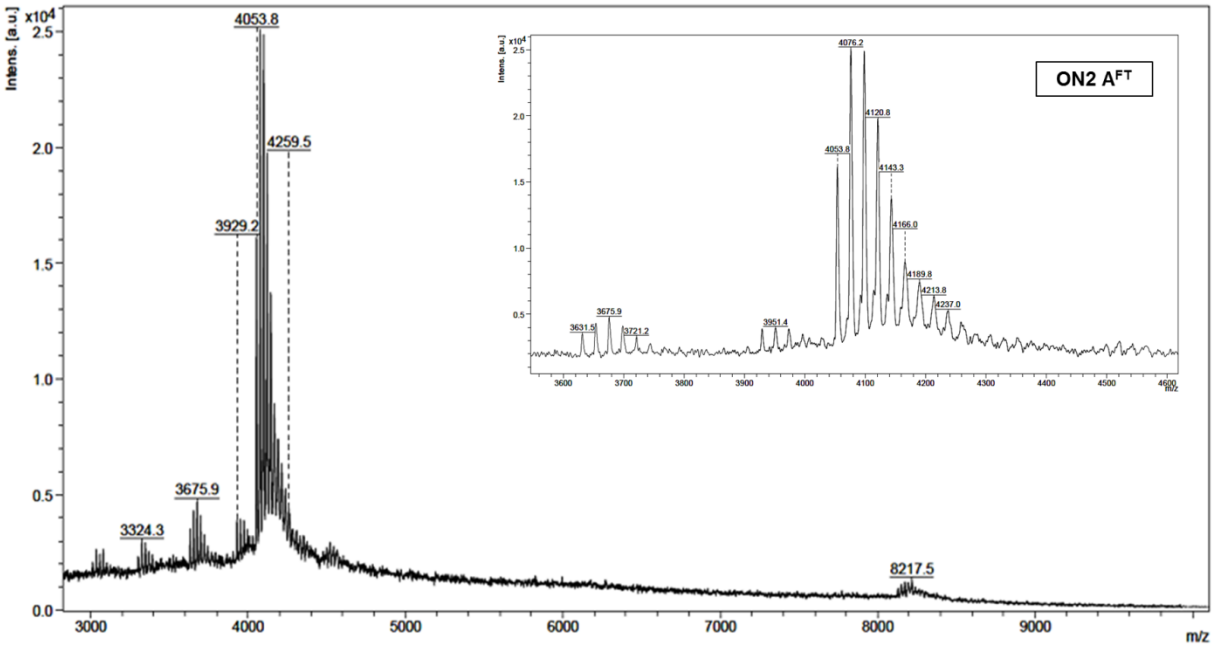


Figure S18. MALDI-TOF spectrum of **ON2 A^{FT}**.

M (calc.) = 4 052.8 Da, M (found) = 4 053.8 Da ($[M+H]^+$).

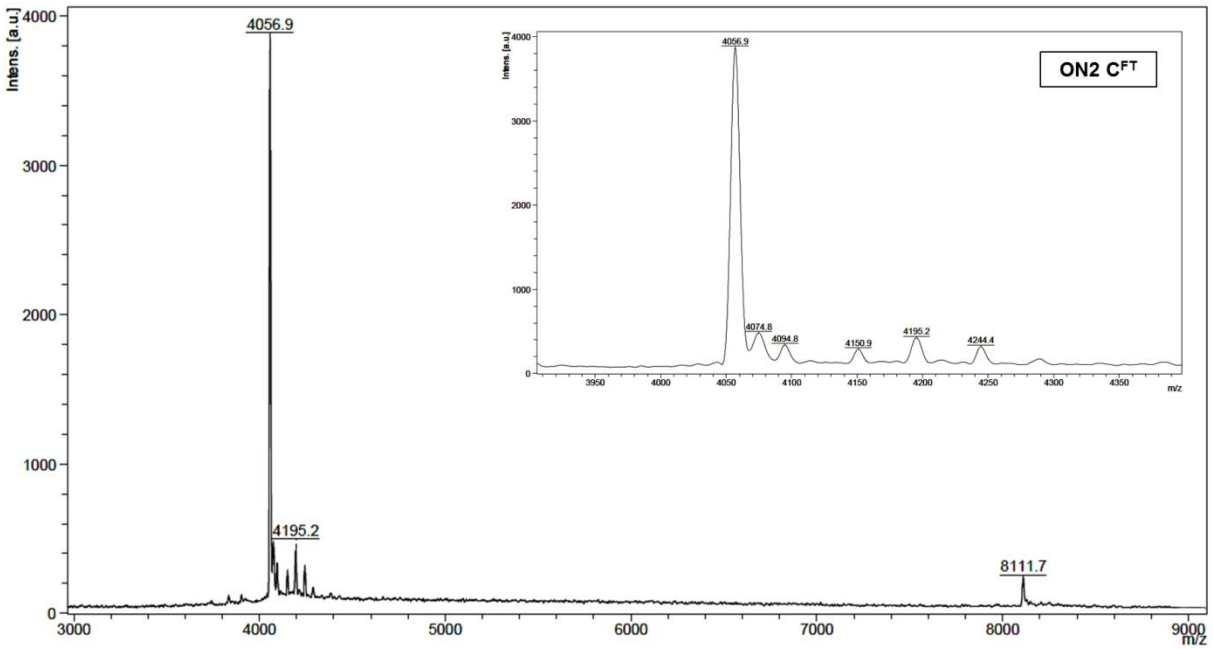


Figure S19. MALDI-TOF spectrum of **ON2 C^{FT}**.

M (calc.) = 4 055.8 Da, M (found) = 4 056.9 Da ($[M+H]^+$).

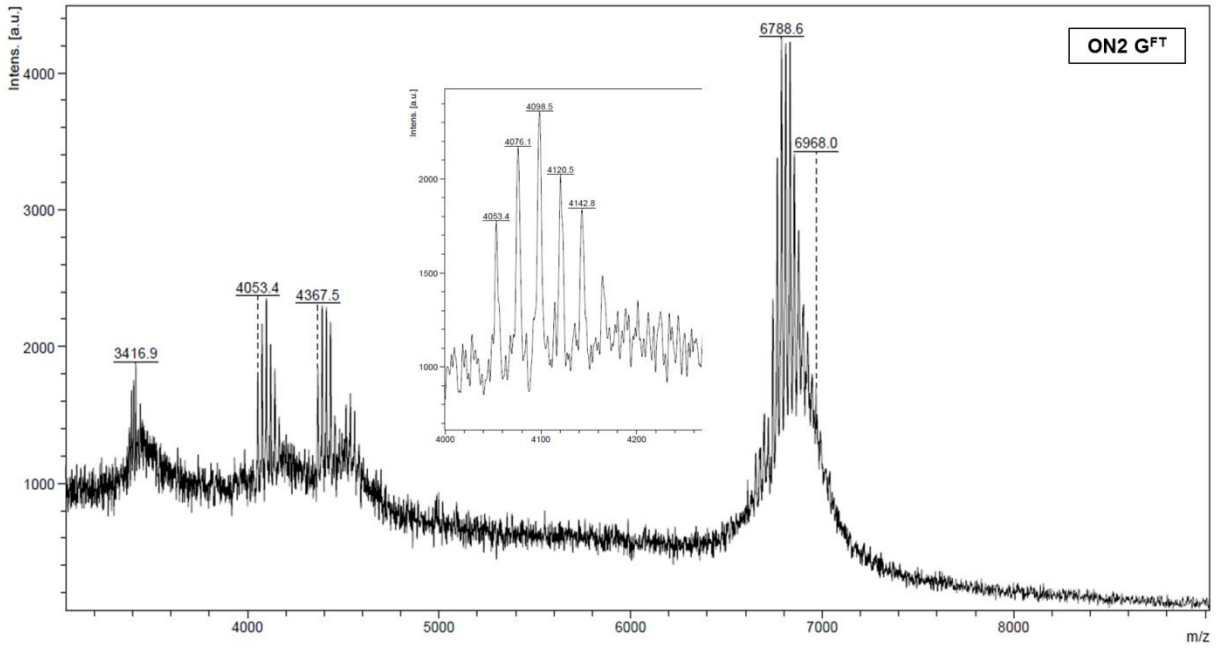


Figure S20. MALDI-TOF spectrum of **ON2 G^{FT}**.

M (calc.) = 4 052.8 Da, M (found) = 4 053.4 Da ($[M+H]^+$), 4 367.5 Da ($[M+A+H]^+$), 6 788.6 Da (?).

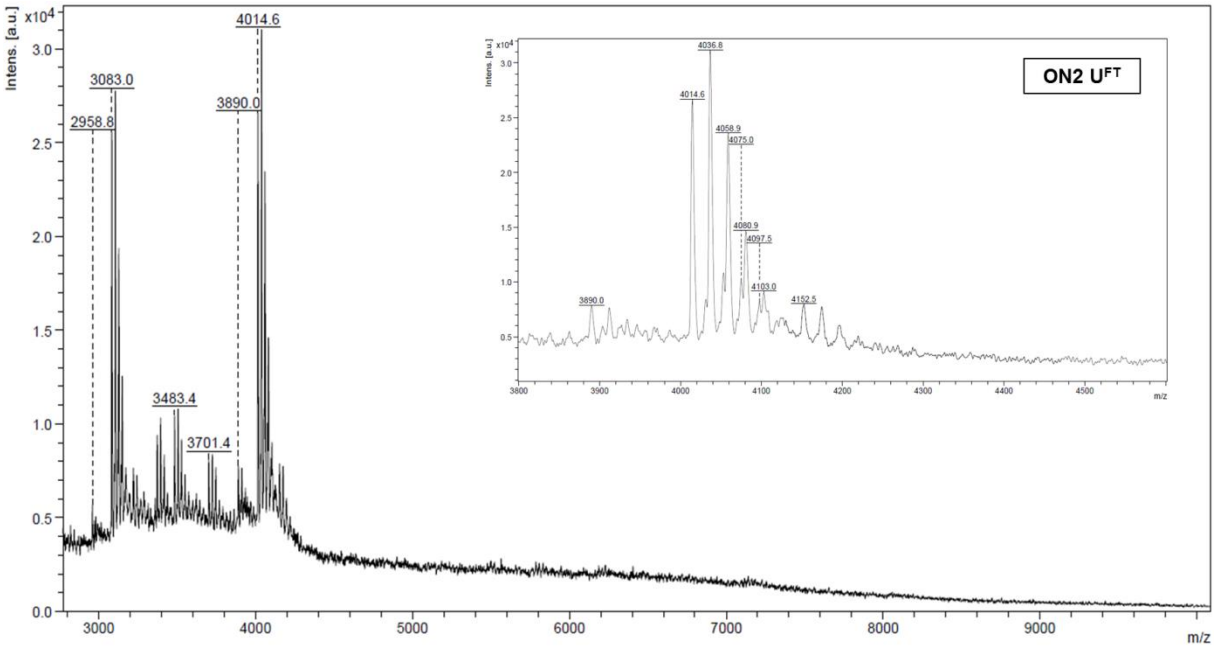


Figure S21. MALDI-TOF spectrum of **ON2 U^{FT}**.

M (calc.) = 4 013.7 Da, M (found) = 4 014.6 Da ($[M+H]^+$), 3 083.0 Da (9-mer, [M – C – G – A + H]$^+$).

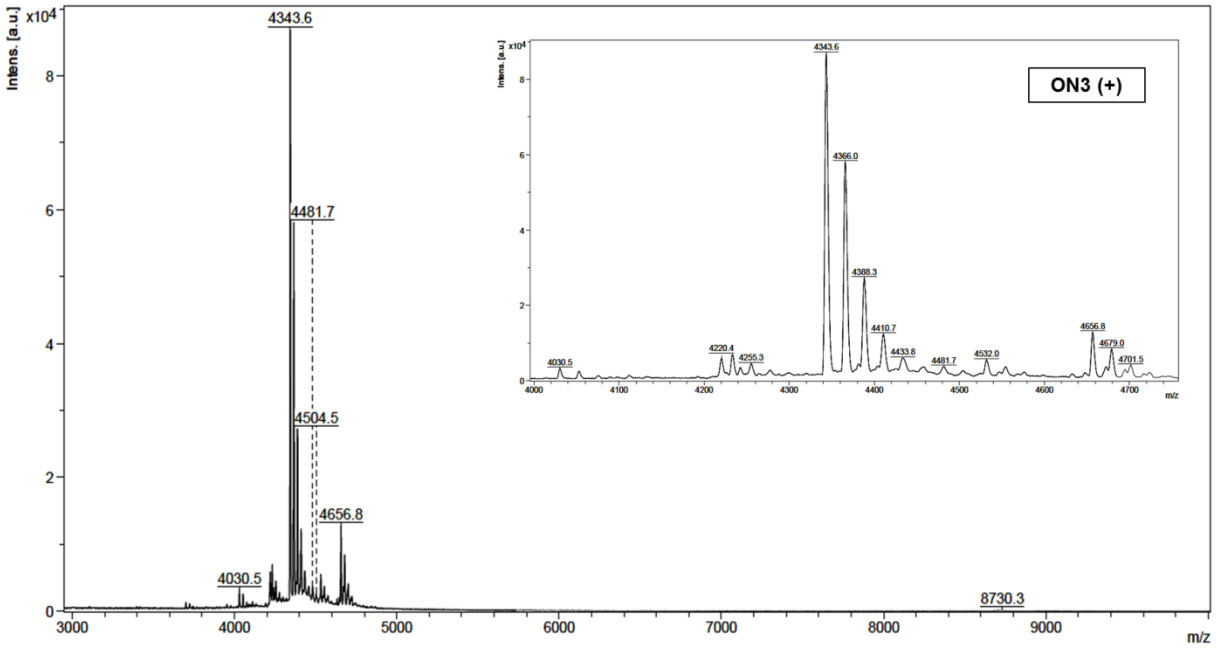


Figure S22. MALDI-TOF spectrum of **ON3 (+)**.

M (calc.) = 4 342.8 Da, M (found) = 4 343.6 Da ($[M+H]^+$).

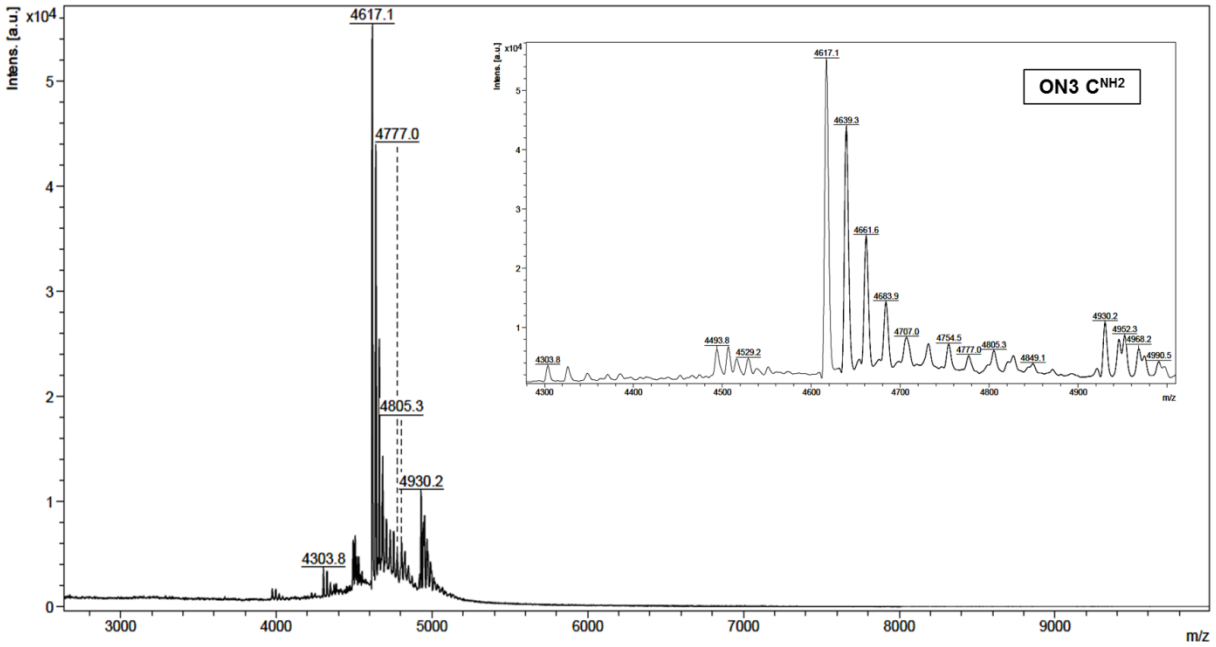


Figure S23. MALDI-TOF spectrum of **ON3 C^{NH2}**.

M (calc.) = 4 616.2 Da, M (found) = 4 617.1 Da ($[M+H]^+$).

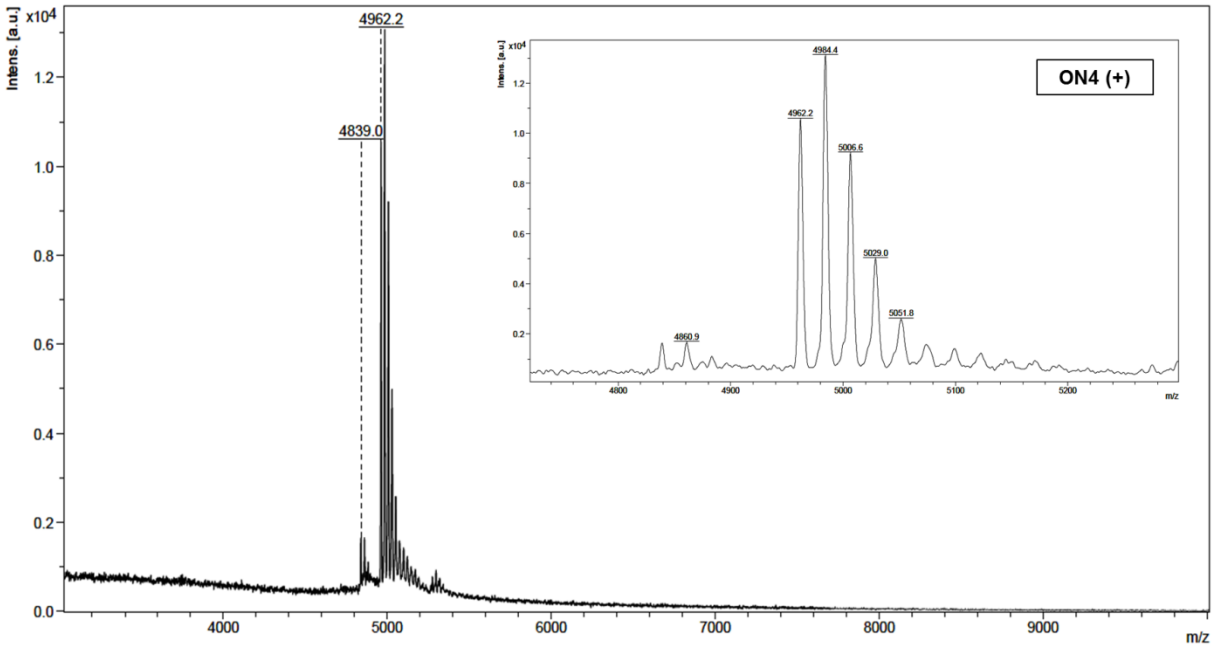


Figure S24. MALDI-TOF spectrum of **ON4 (+)**.

M (calc.) = 4 961.2 Da, M (found) = 4 962.2 Da ($[M+H]^+$).

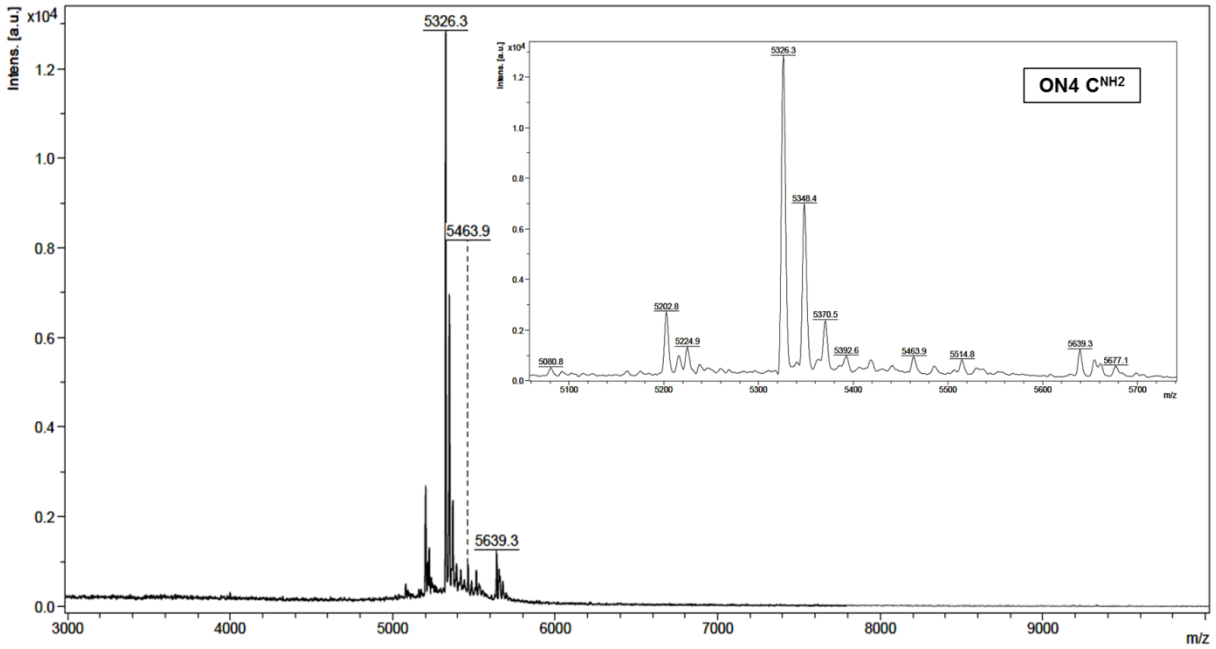


Figure S25. MALDI-TOF spectrum of **ON4 C^{NH2}**.

M (calc.) = 5 325.7 Da, M (found) = 5 326.3 Da ($[M+H]^+$).

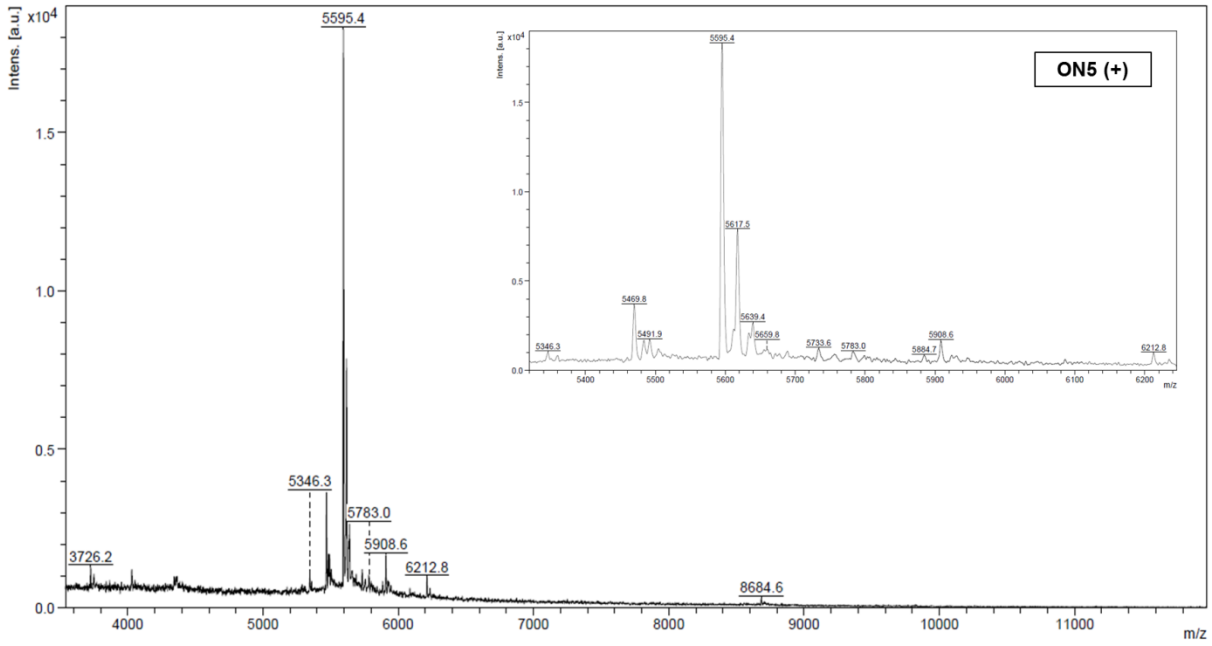


Figure S26. MALDI-TOF spectrum of **ON5 (+)**.

M (calc.) = 5 594.6 Da, M (found) = 5 595.4 Da ($[M+H]^+$).

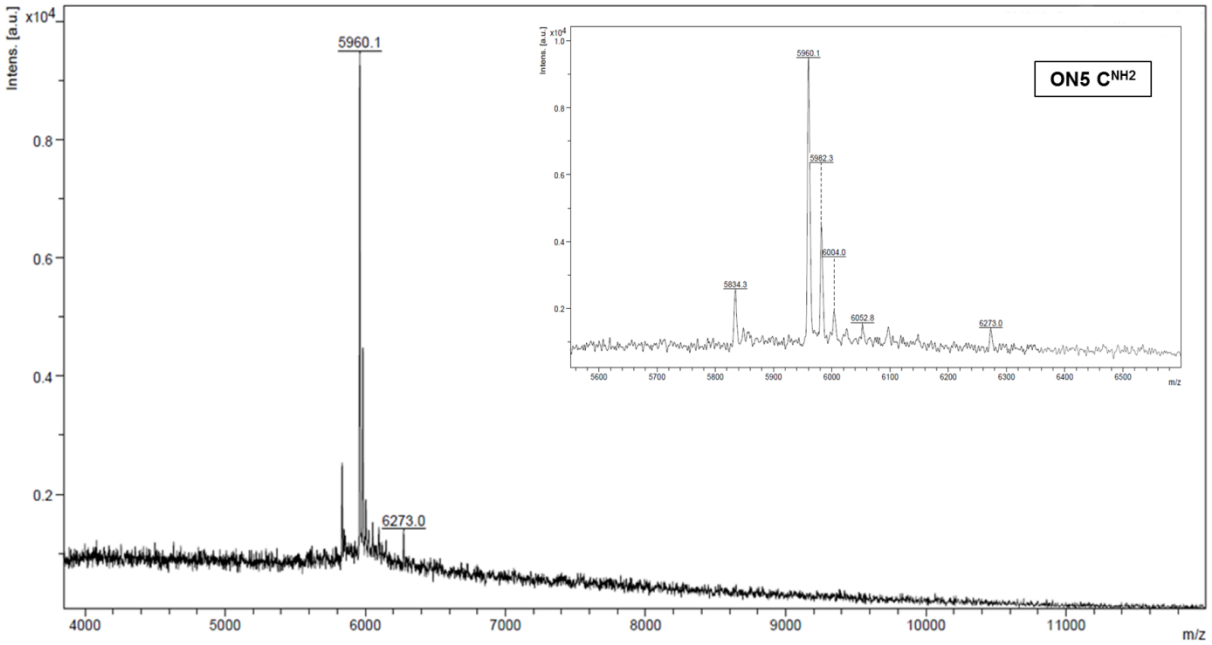


Figure S27. MALDI-TOF spectrum of **ON5 C^{NH2}**.

M (calc.) = 5 959.1 Da, M (found) = 5 960.1 Da ($[M+H]^+$).

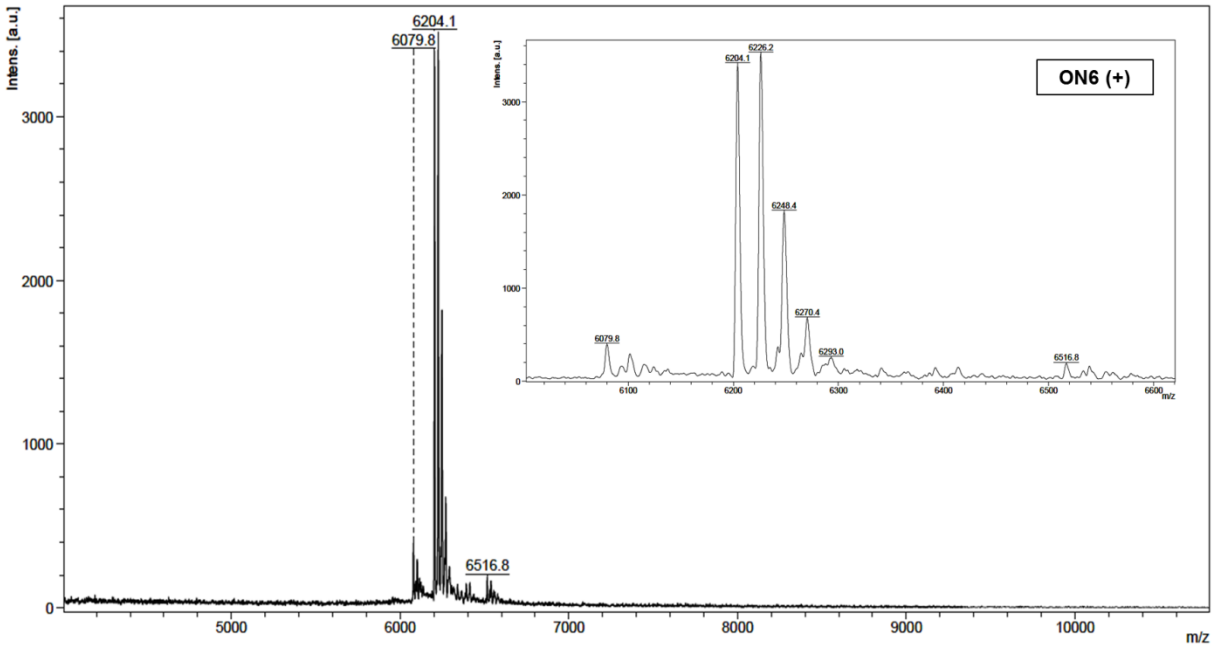


Figure S28. MALDI-TOF spectrum of **ON6 (+)**.

M (calc.) = 6 203.0 Da, M (found) = 6 204.1 Da ($[M+H]^+$).

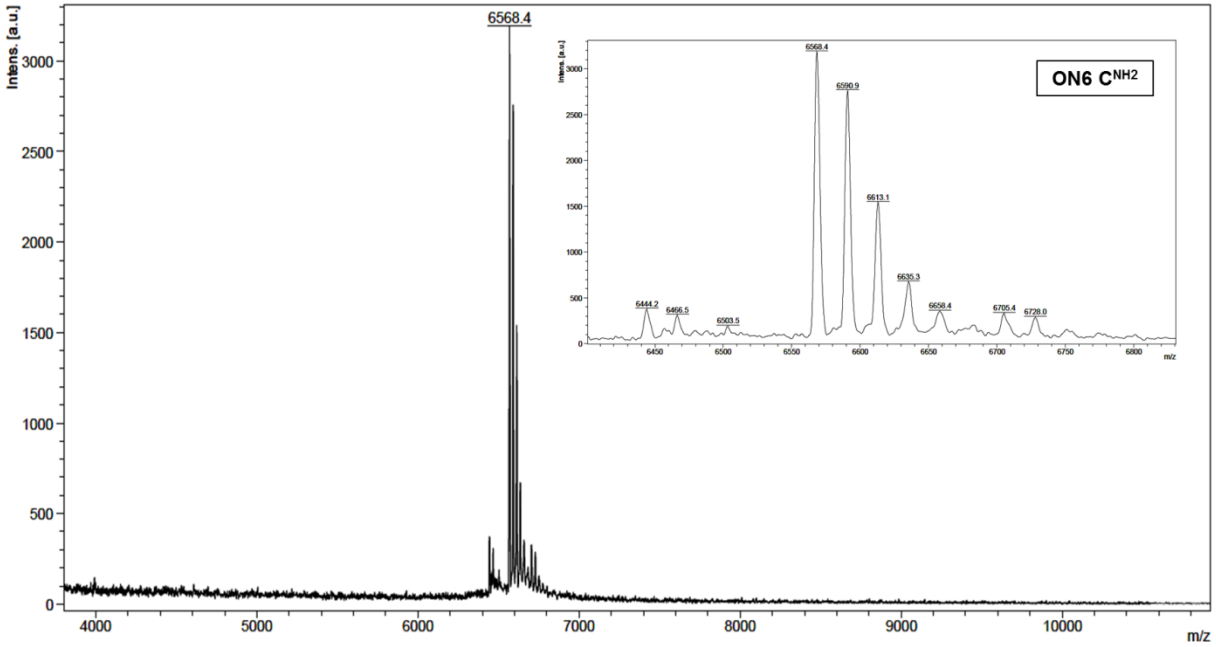


Figure S29. MALDI-TOF spectrum of **ON6 C^{NH2}**.

M (calc.) = 6 567.5 Da, M (found) = 6 568.4 Da ($[M+H]^+$).

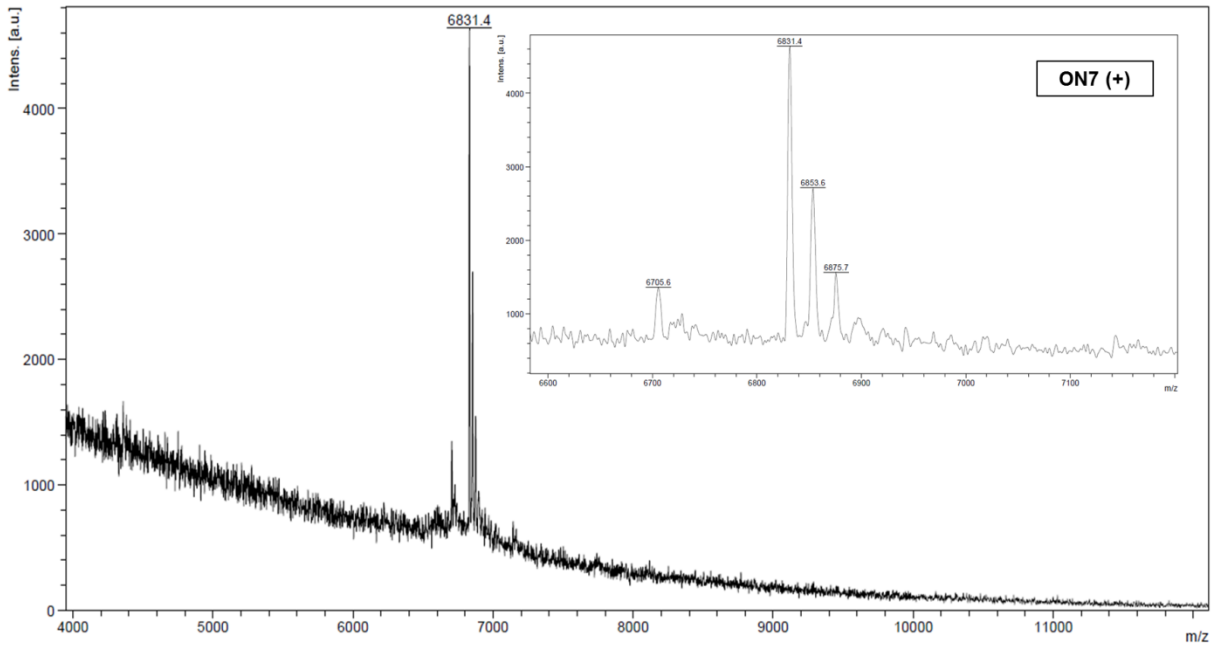


Figure S30. MALDI-TOF spectrum of **ON7 (+)**.

M (calc.) = 6 830.4 Da, M (found) = 6 831.4 Da ($[M+H]^+$).

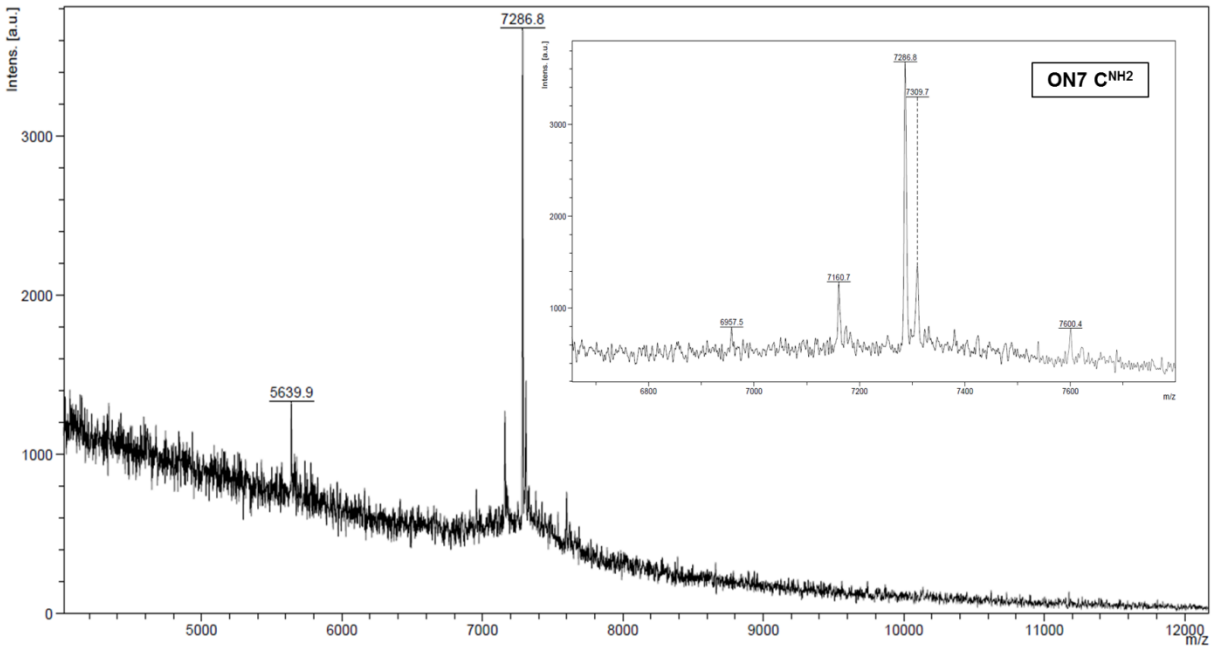


Figure S31. MALDI-TOF spectrum of **ON7 C^{NH2}**.

M (calc.) = 7 286.0 Da, M (found) = 7 286.8 Da ($[M+H]^+$).

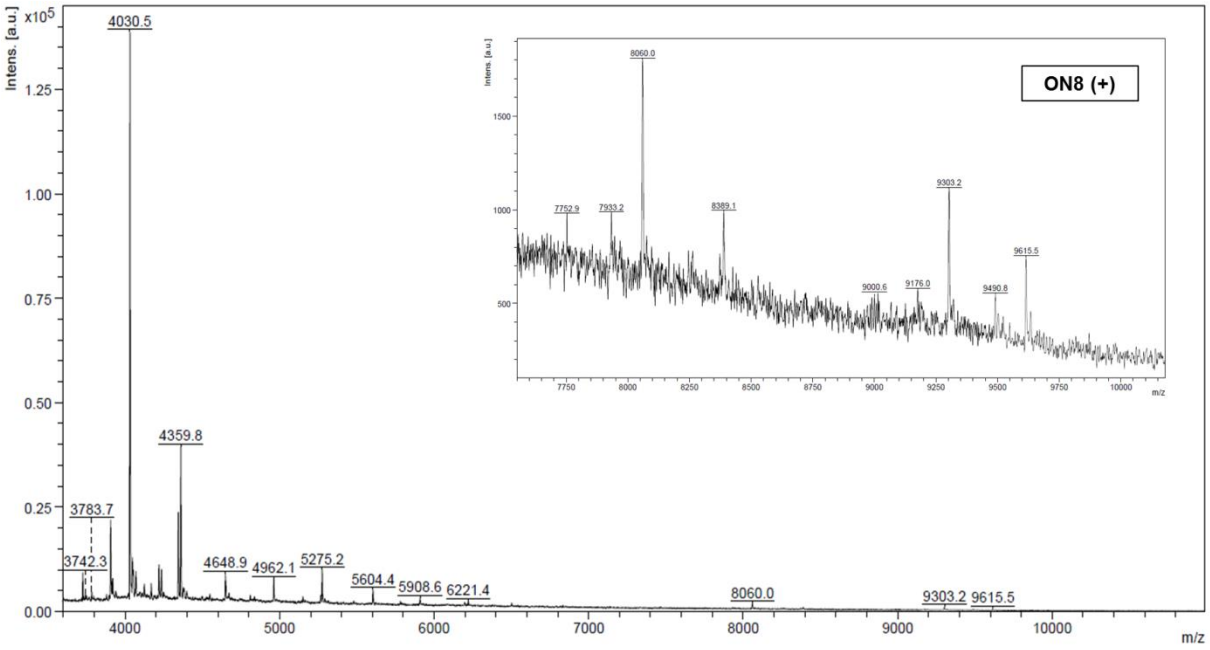


Figure S32. MALDI-TOF spectrum of **ON8 (+)**.

M (calc.) = 9 302.0 Da, M (found) = 9 303.2 Da ($[M+H]^+$), plus a mixture of shorter ONs (mainly 13-mer and 14-mer).

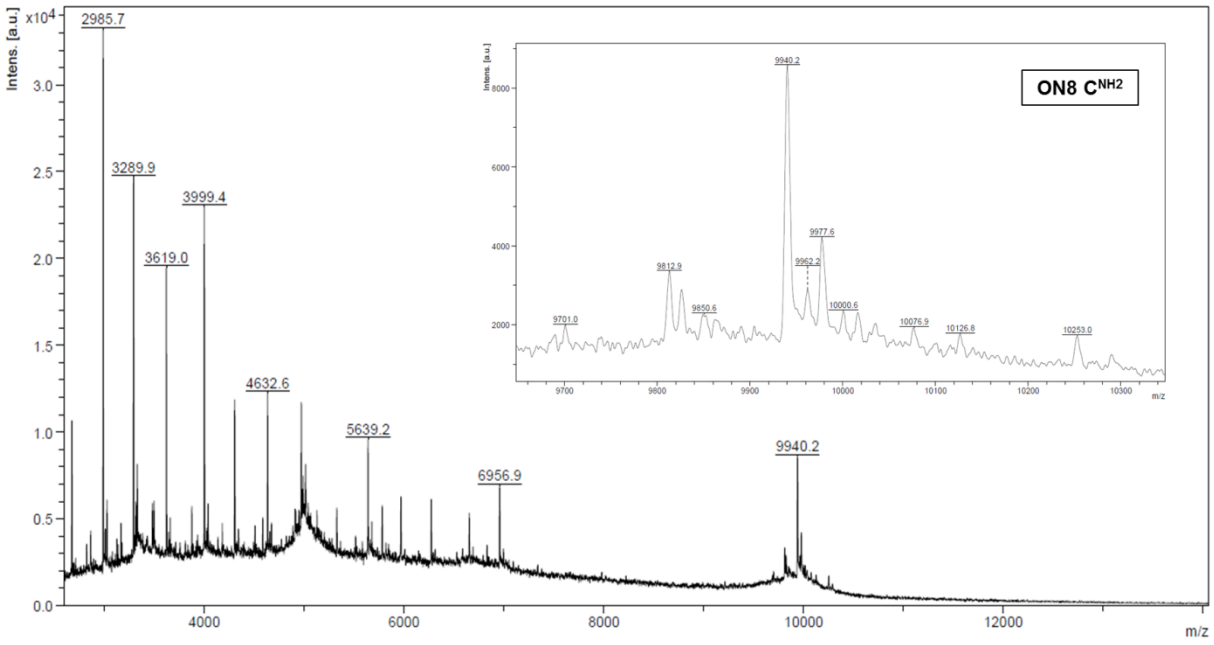


Figure S33. MALDI-TOF spectrum of **ON8 C^{NH2}**.

M (calc.) = 9 939.8 Da, M (found) = 9 940.2 Da ($[M+H]^+$), plus a mixture of shorter ONs.

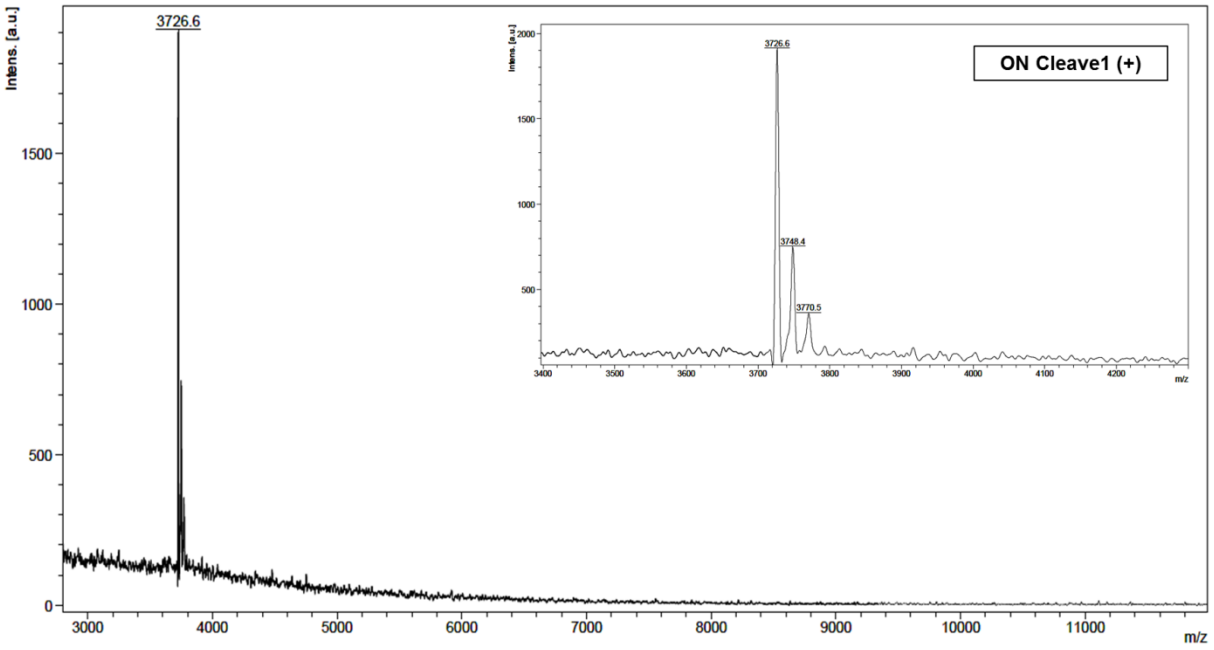


Figure S34. MALDI-TOF spectrum of **ON Cleave1 (+)**.

M (calc.) = 3 725.4 Da, M (found) = 3 726.6 Da ($[M+H]^+$).

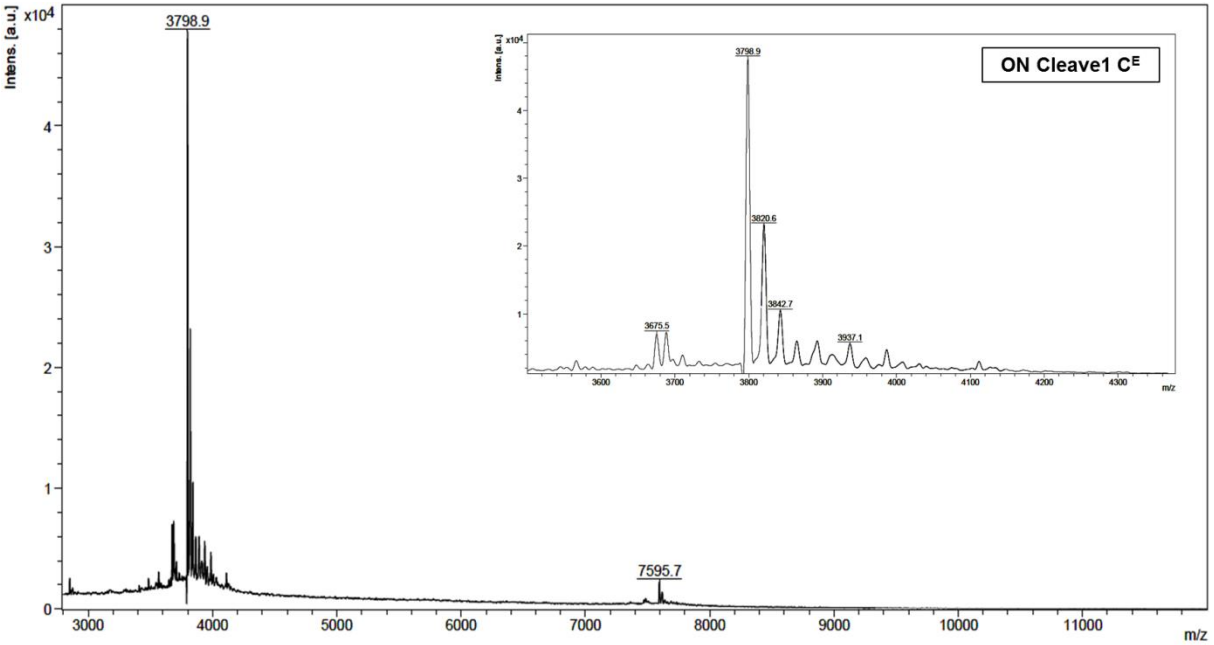


Figure S35. MALDI-TOF spectrum of **ON Cleave1 C^E**.

M (calc.) = 3 797.5 Da, M (found) = 3 798.9 Da ($[M+H]^+$).

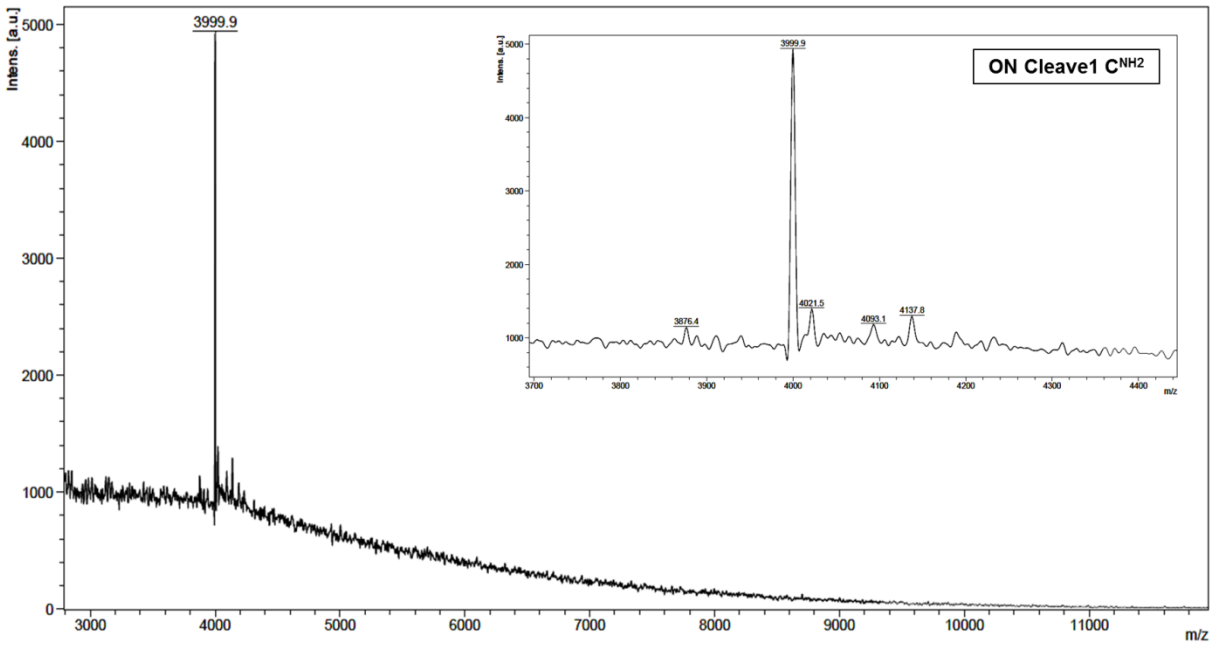


Figure S36. MALDI-TOF spectrum of **ON Cleave1 C^{NH2}**.

M (calc.) = 3 998.8 Da, M (found) = 3 999.9 Da ([M+H]$^+$).

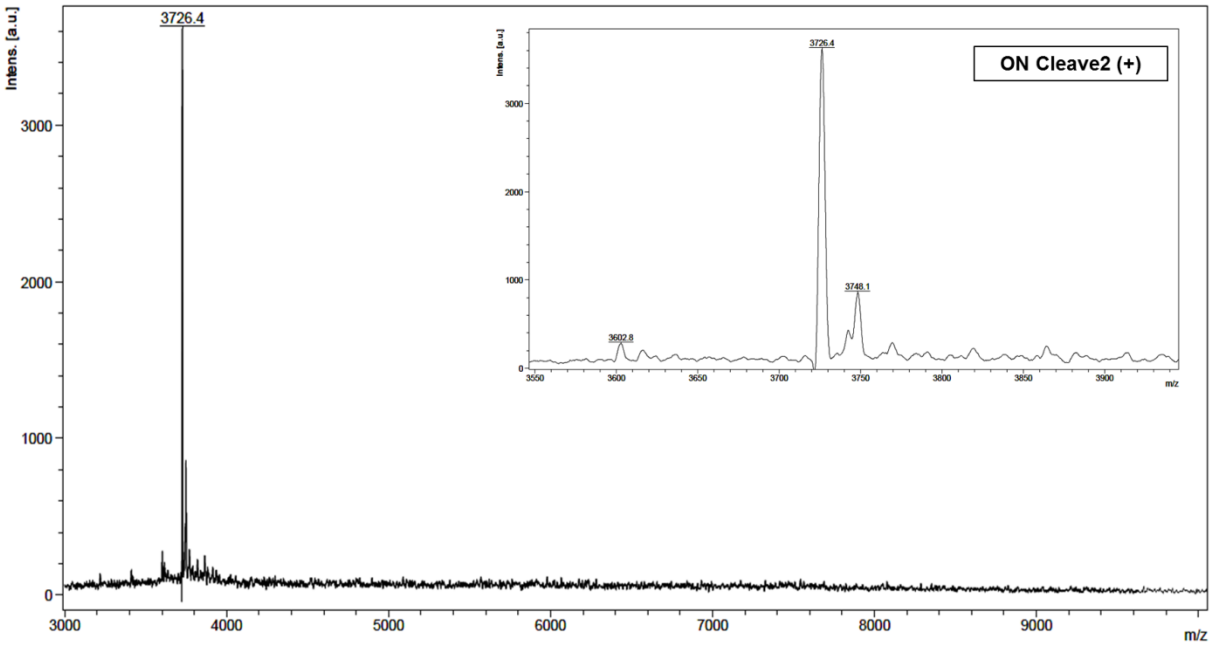


Figure S37. MALDI-TOF spectrum of **ON Cleave2 (+)**.

M (calc.) = 3 725.4 Da, M (found) = 3 726.4 ([M+H]$^+$).

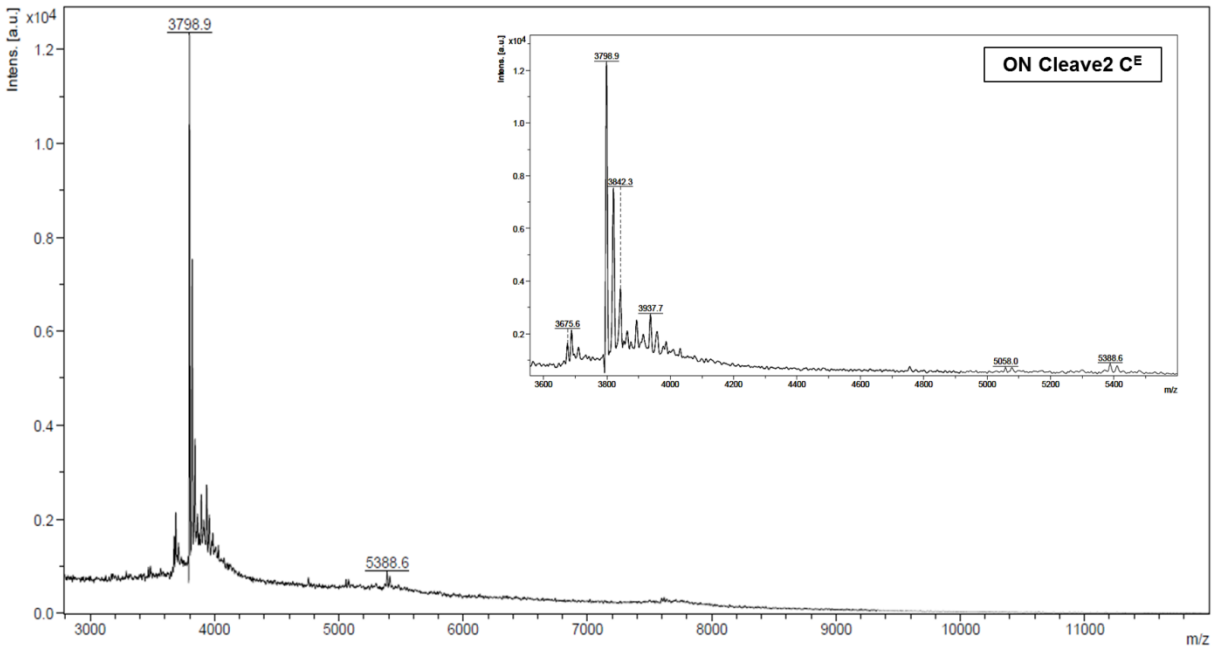


Figure S38. MALDI-TOF spectrum of **ON Cleave2 C^{E}**.

M (calc.) = 3 797.5 Da, M (found) = 3 798.8 Da ([M+H]$^{+}$).

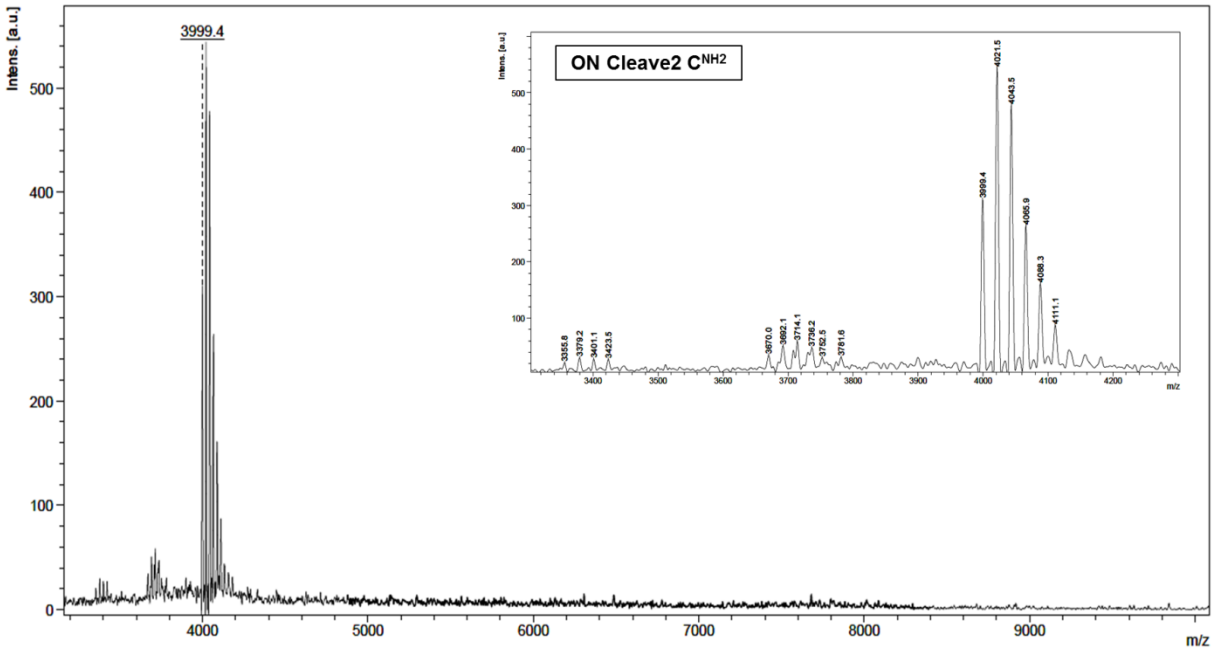


Figure S39. MALDI-TOF spectrum of **ON Cleave2 C^{NH2}**.

M (calc.) = 3 998.8 Da, M (found) = 3 999.4 Da ([M+H]$^{+}$).

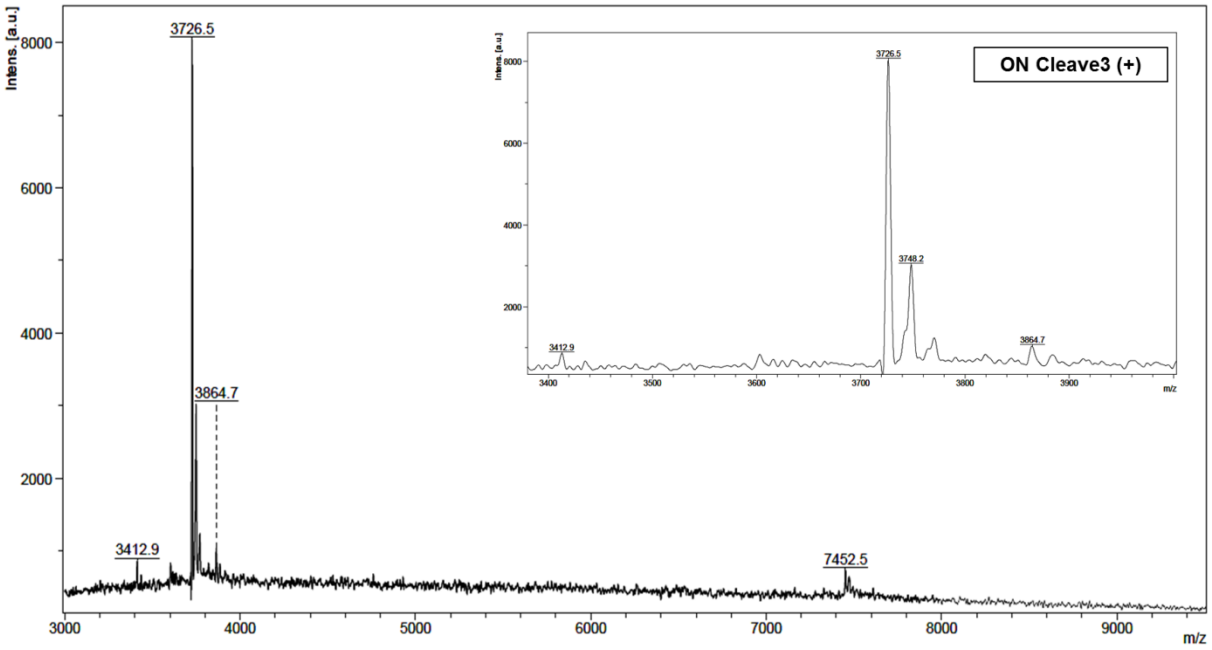


Figure S40. MALDI-TOF spectrum of **ON Cleave3 (+)**.

M (calc.) = 3 725.4 Da, M (found) = 3 726.5 Da ($[M+H]^+$).

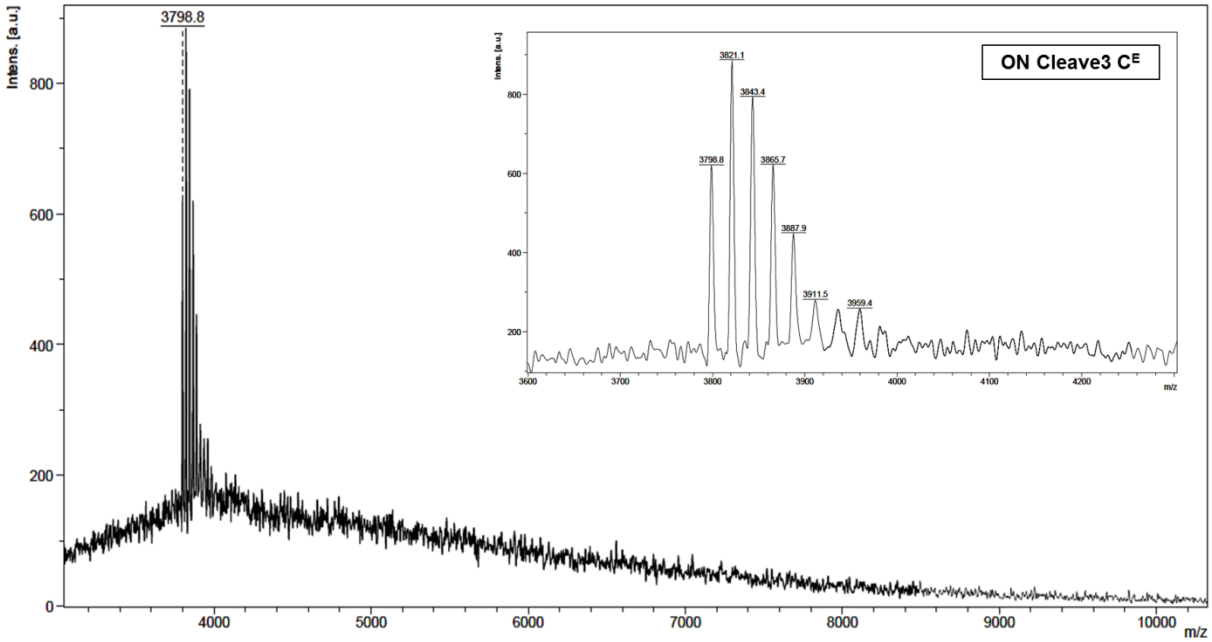


Figure S41. MALDI-TOF spectrum of **ON Cleave3 C^E**.

M (calc.) = 3 797.5 Da, M (found) = 3 798.8 ($[M+H]^+$).

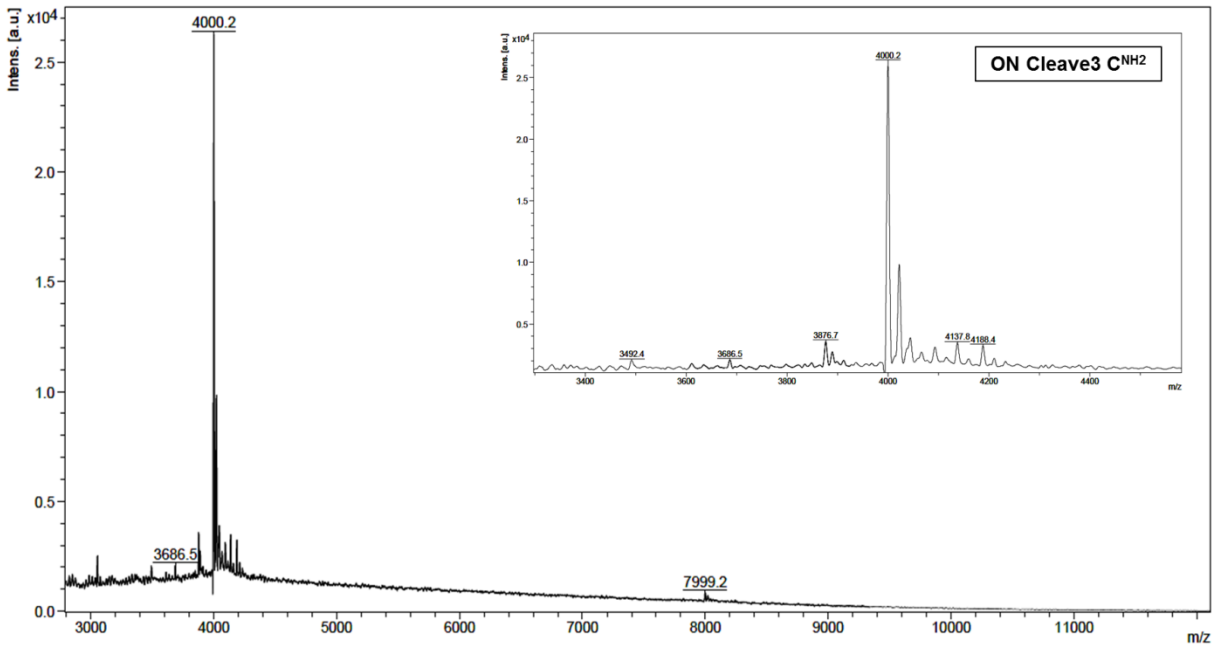


Figure S42. MALDI-TOF spectrum of **ON Cleave3 C^{NH2}**.

M (calc.) = 3 998.8 Da, M (found) = 4 000.2 Da ([M+H]$^+$).

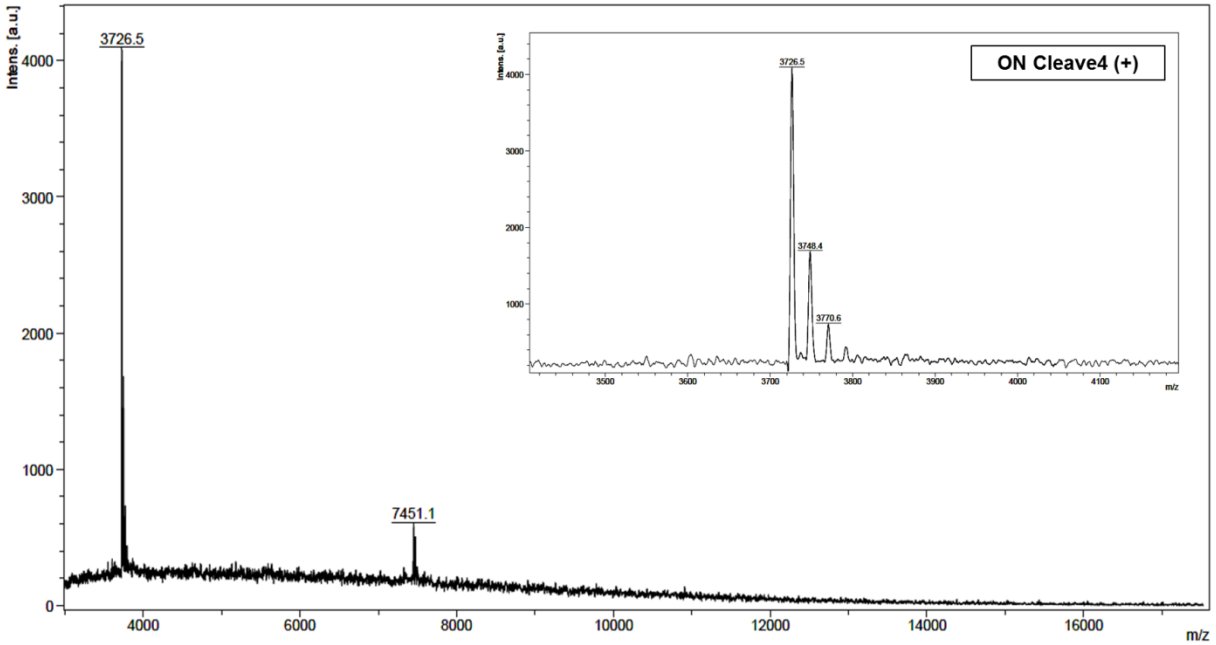


Figure S43. MALDI-TOF spectrum of **ON Cleave4 (+)**.

M (calc.) = 3 725.4 Da, M (found) = 3 726.5 Da ([M+H]$^+$).

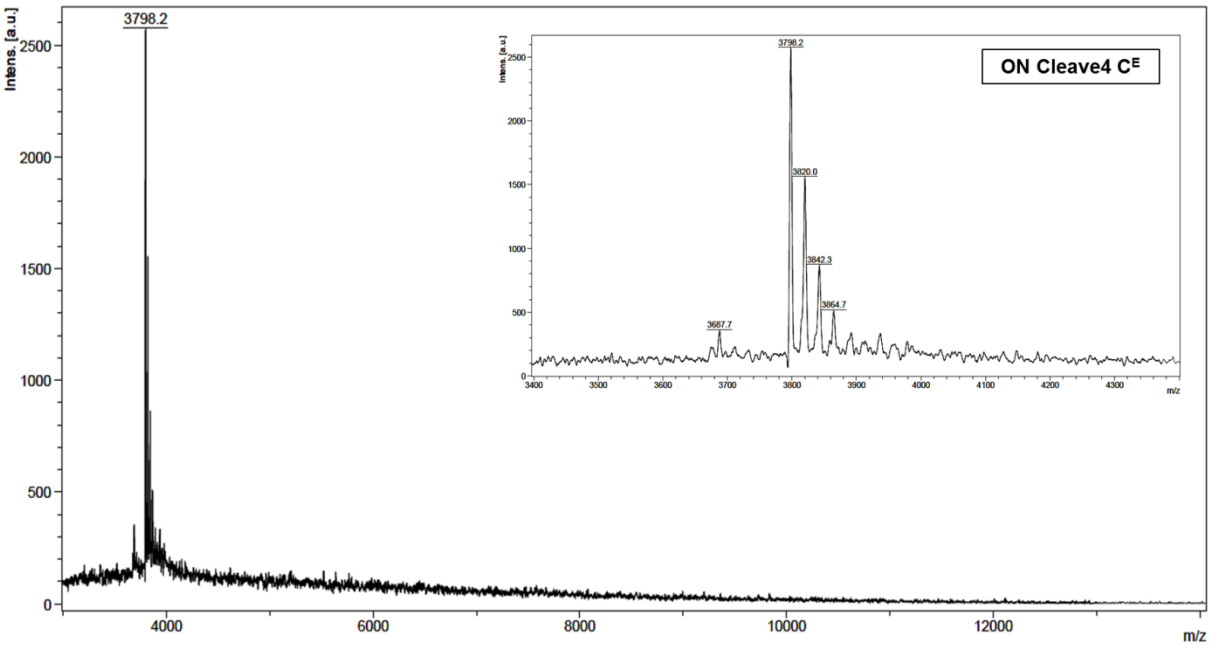


Figure S44. MALDI-TOF spectrum of **ON Cleave4 C^{E}**.

M (calc.) = 3 797.5 Da, M (found) = 3 798.2 Da ([M+H]$^{+}$).

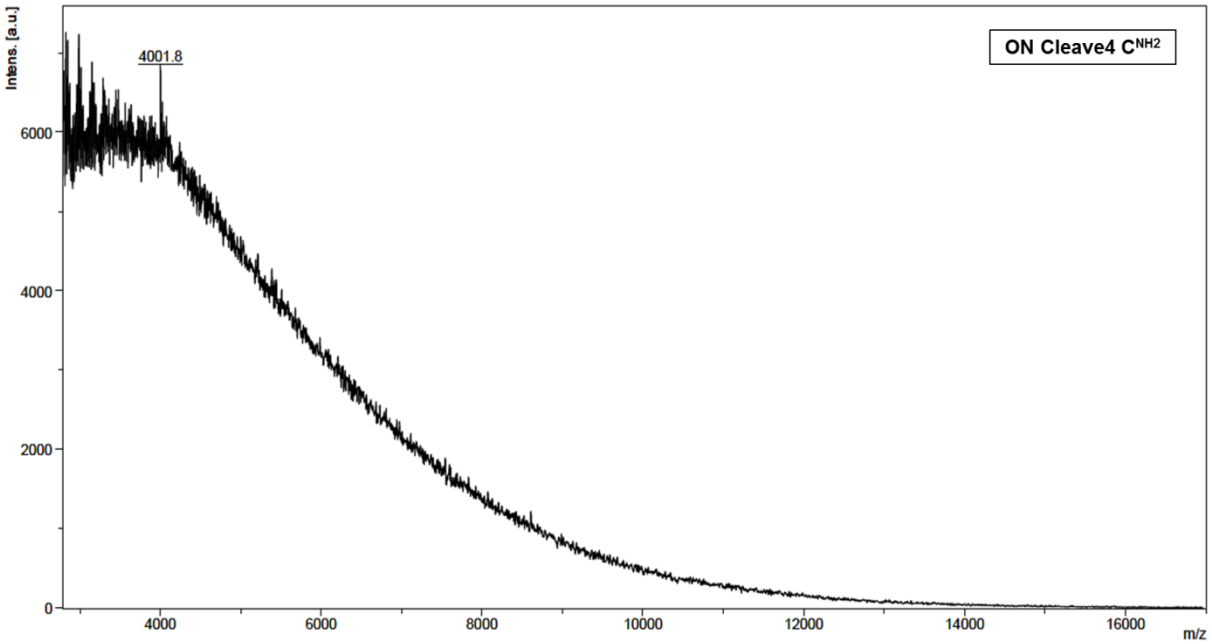


Figure S45. MALDI-TOF spectrum of **ON Cleave4 C^{NH2}**.

M (calc.) = 3 998.8 Da, M (found) = 4 001.8 Da ([M+H]$^{+}$).

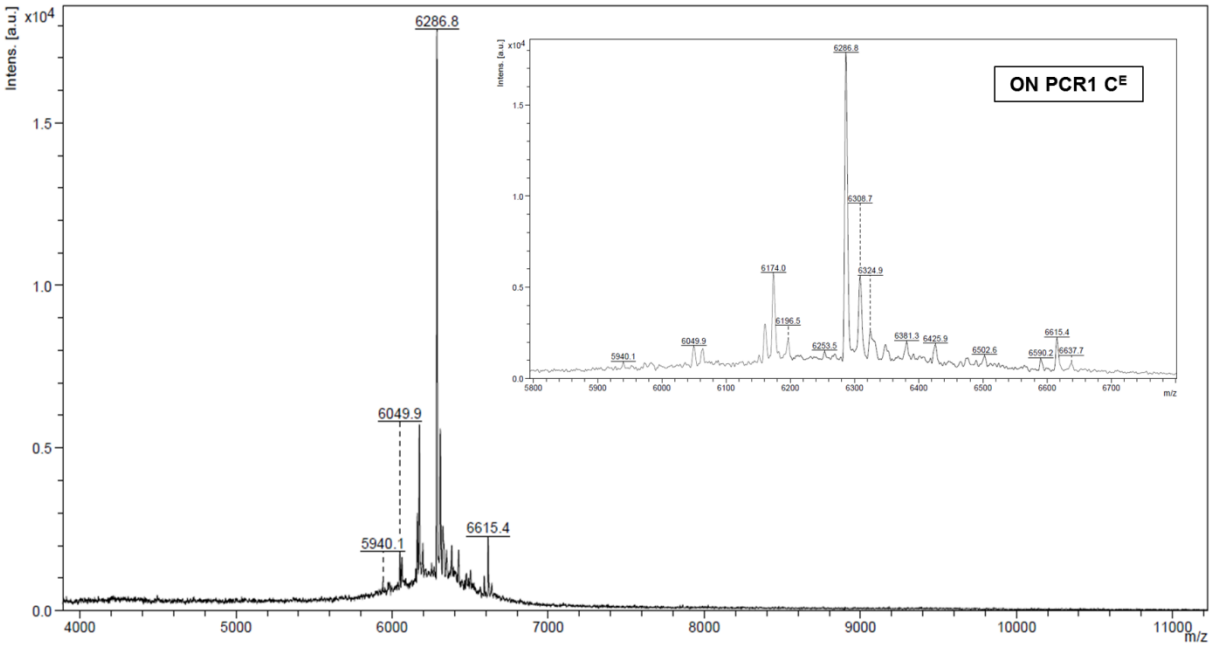


Figure S46. MALDI-TOF spectrum of **ON PCR1 C^{E}**.

M (calc.) = 6 285.1 Da, M (found) = 6 286.8 Da ([M+H]$^{+}$).

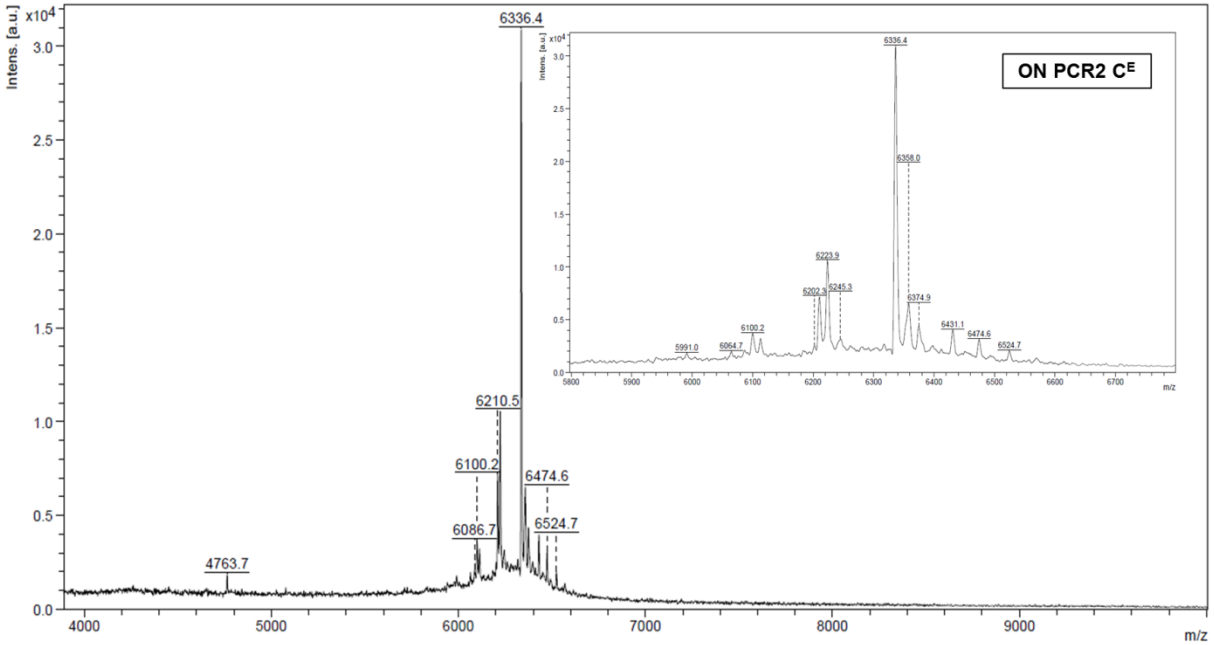


Figure S47. MALDI-TOF spectrum of **ON PCR2 C^{E}**.

M (calc.) = 6 335.2 Da, M (found) = 6 336.4 Da ([M+H]$^{+}$).

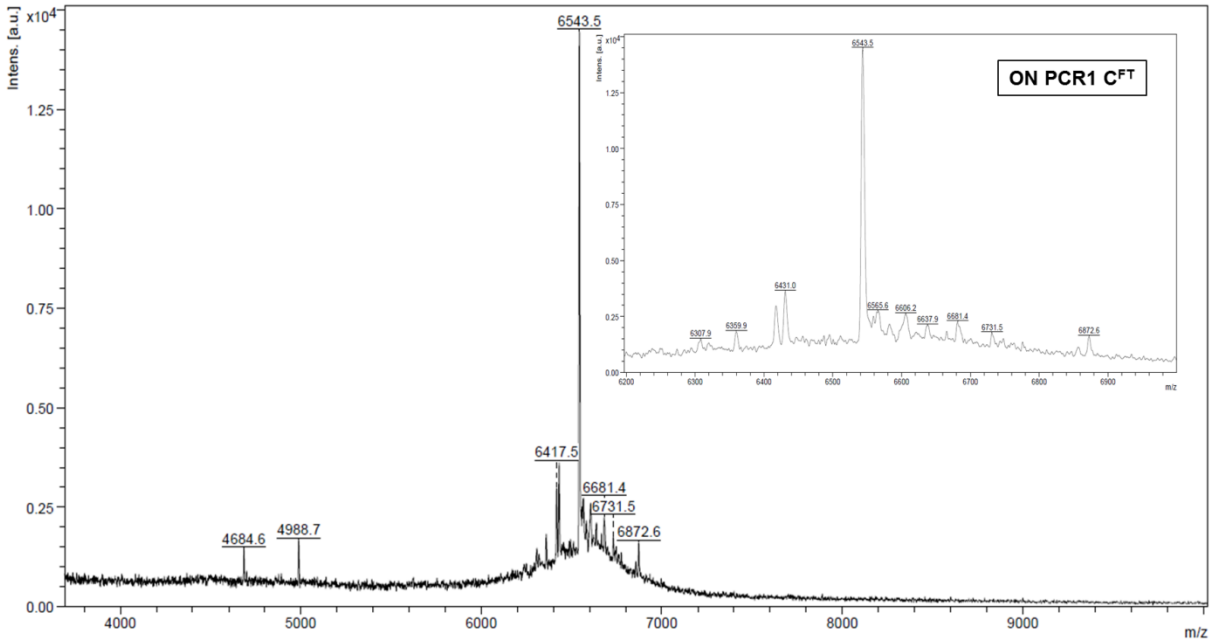


Figure S48. MALDI-TOF spectrum of **ON PCR1 C^{FT}**.

M (calc.) = 6 542.9 Da, M (found) = 6 543.5 Da ([M+H]$^+$).

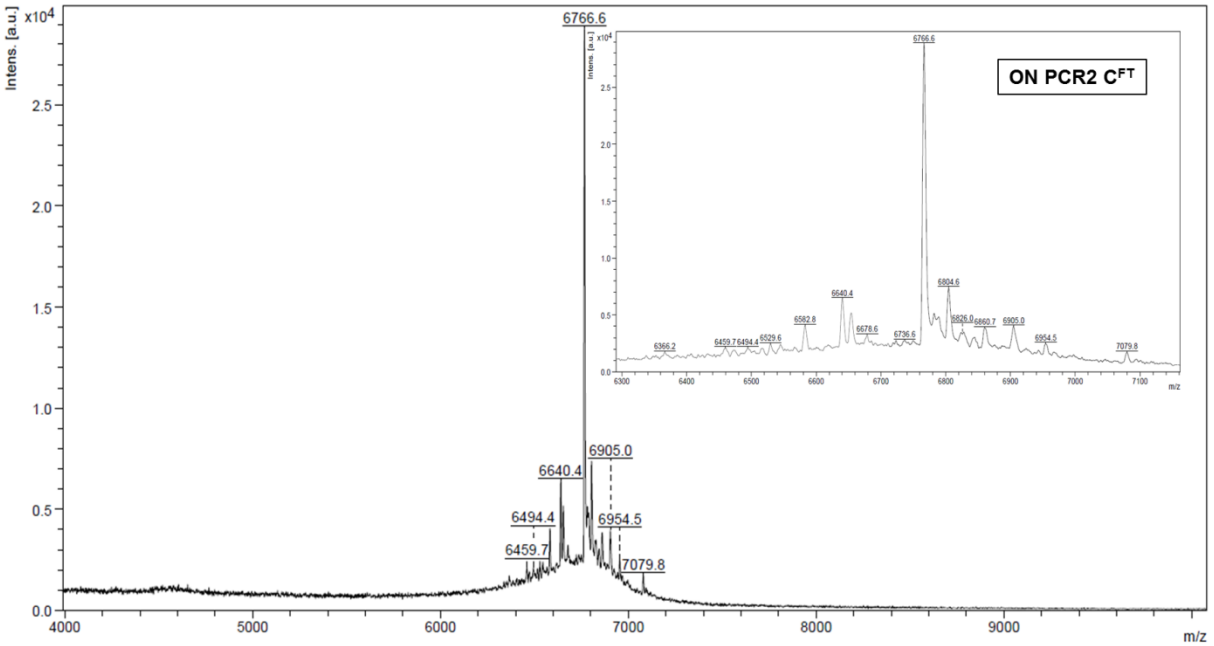


Figure S49. MALDI-TOF spectrum of **ON PCR2 C^{FT}**.

M (calc.) = 6 765.8 Da, M (found) = 6 766.6 Da ([M+H]$^+$).

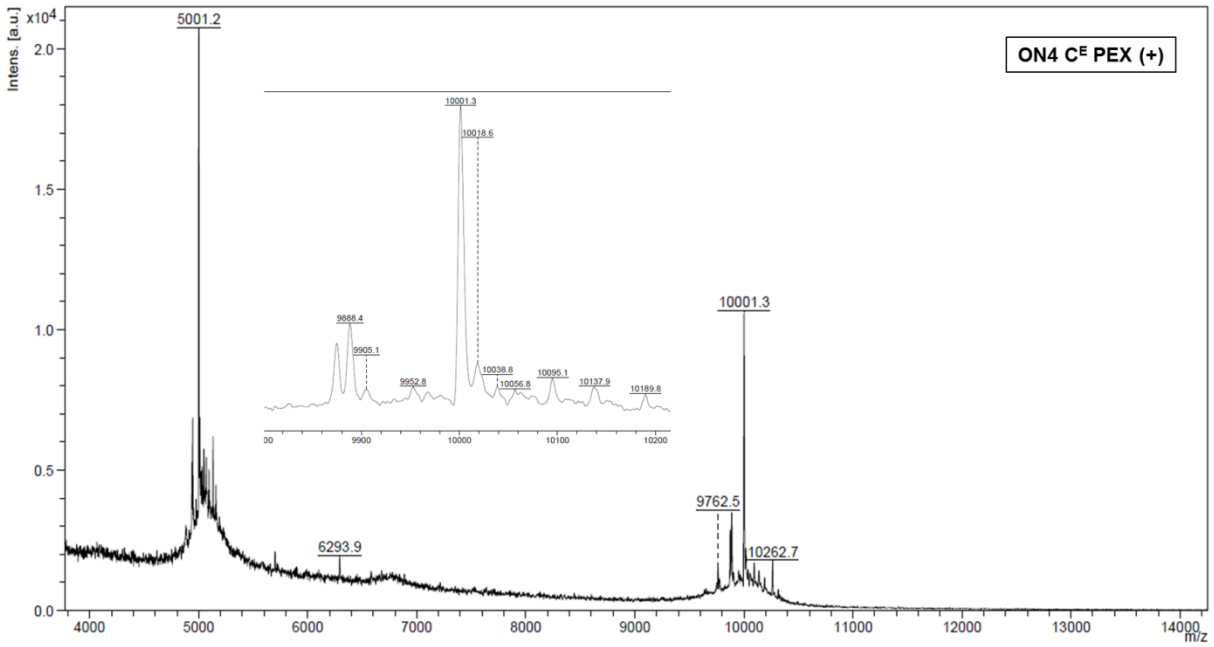


Figure S50. MALDI-TOF spectrum of **ON4 C^{E} PEX (+)**.

M (calc.) = 10 000.5 Da, M (found) = 10 001.3 Da ($[M+H]^+$).

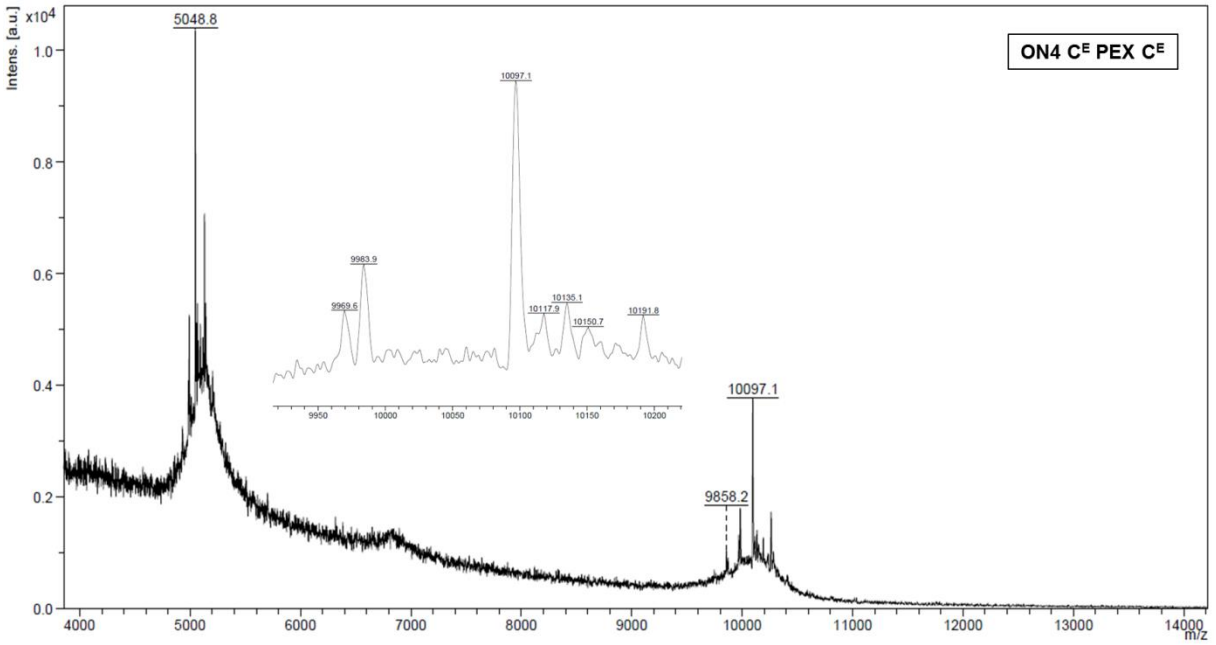


Figure S51. MALDI-TOF spectrum of **ON4 C^{E} PEX C^{E}**.

M (calc.) = 10 096.6 Da, M (found) = 10 097.1 Da ($[M+H]^+$).

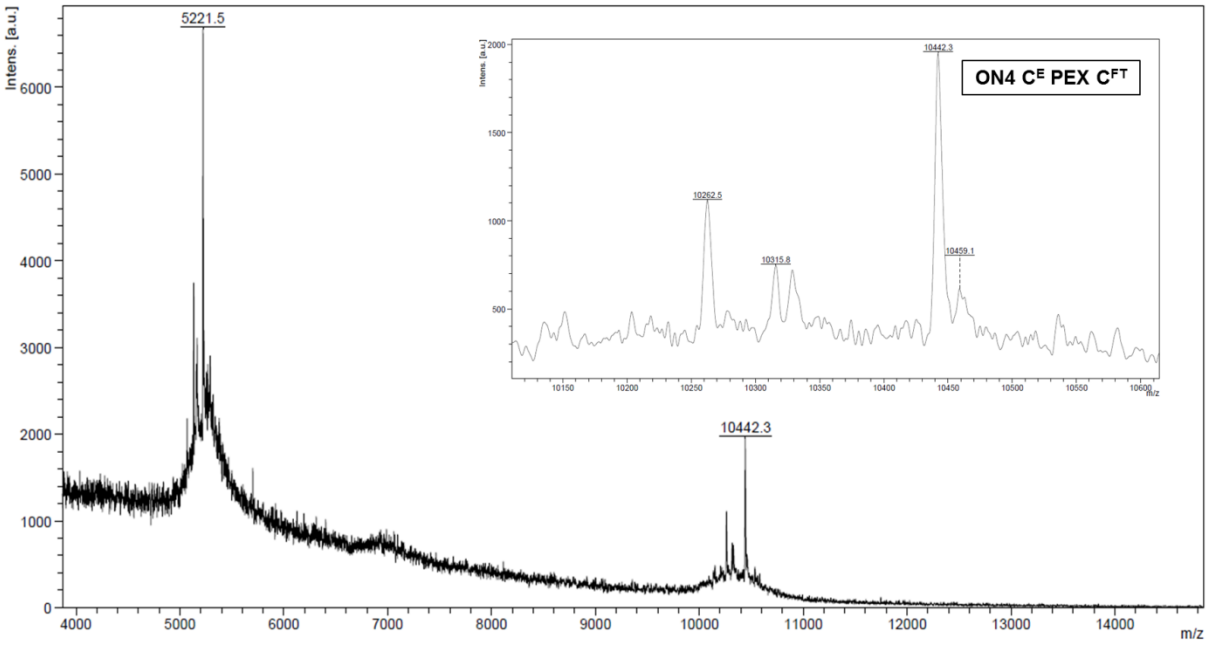


Figure S52. MALDI-TOF spectrum of **ON4 C^{E} PEX C^{FT}**.

M (calc.) = 10 441.1 Da, M (found) = 10 442.3 Da ($[M+H]^+$).

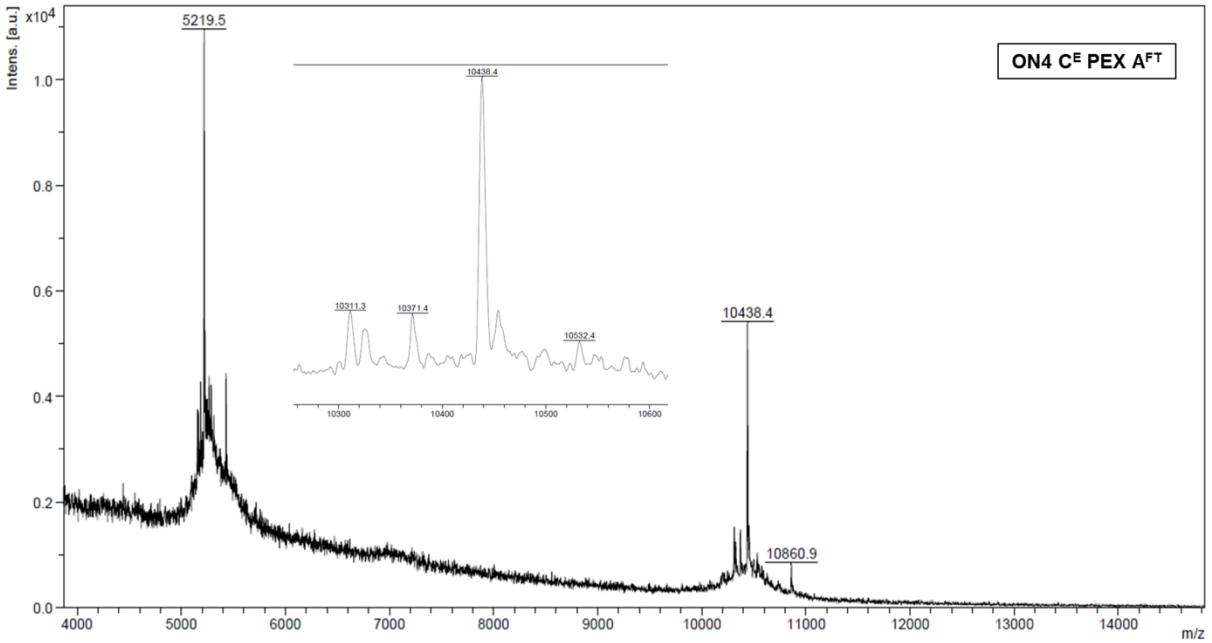


Figure S53. MALDI-TOF spectrum of **ON4 C^{E} PEX A^{FT}**.

M (calc.) = 10 437.1 Da, M (found) = 10 438.4 Da ($[M+H]^+$).

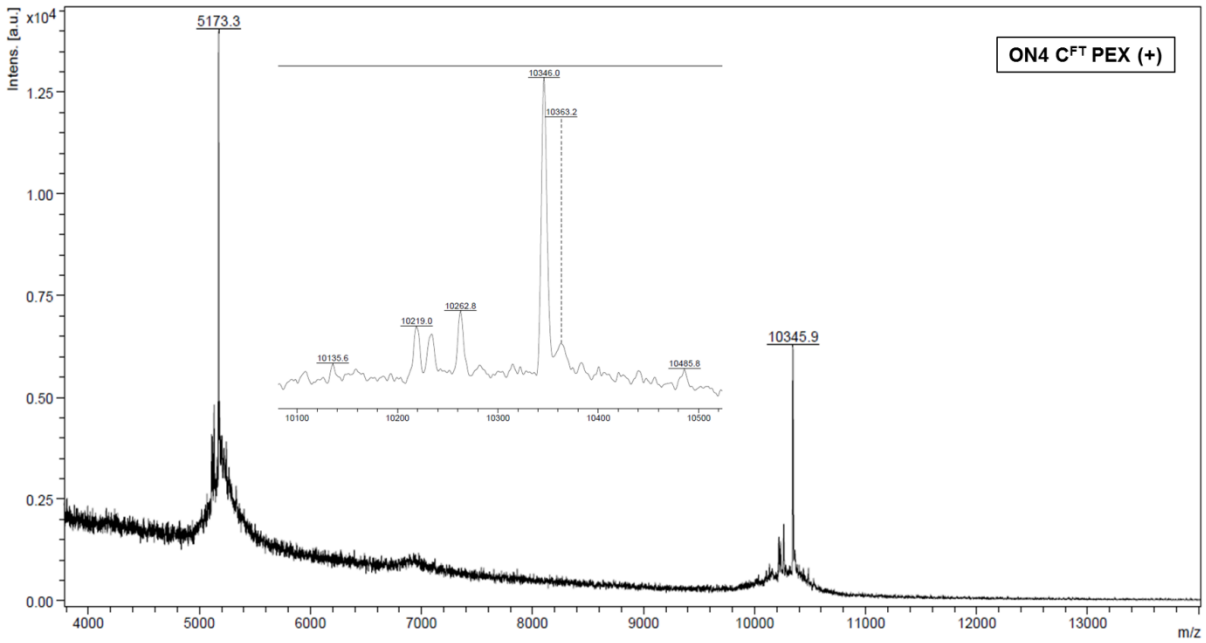


Figure S54. MALDI-TOF spectrum of **ON4 C^{FT} PEX (+)**.

M (calc.) = 10 345.0 Da, M (found) = 10 345.9 Da ($[M+H]^+$).

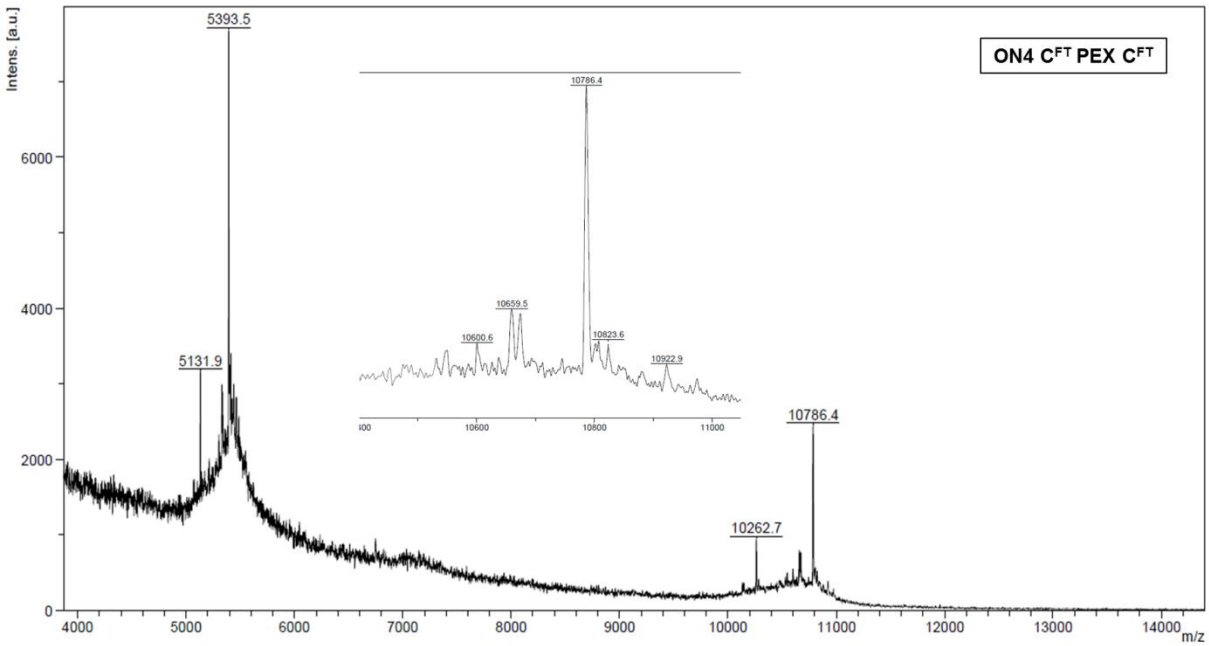


Figure S55. MALDI-TOF spectrum of **ON4 C^{FT} PEX C^{FT}**.

M (calc.) = 10 785.6 Da, M (found) = 10 786.4 Da ($[M+H]^+$).

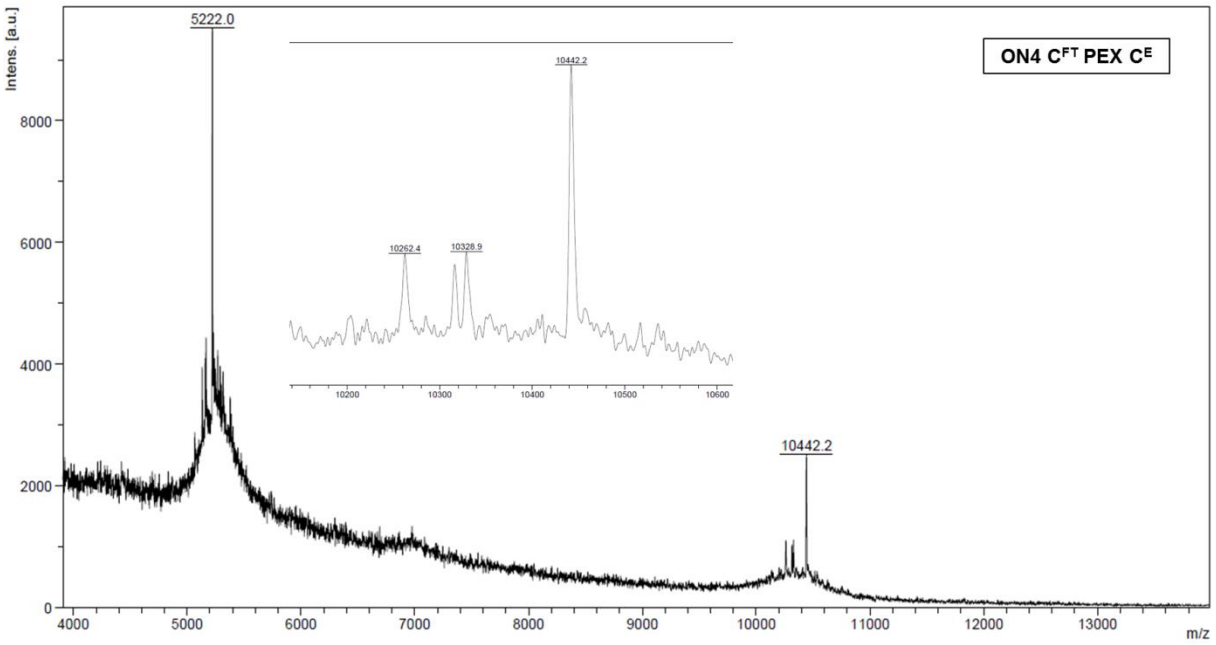


Figure S56. MALDI-TOF spectrum of **ON4 C^{FT} PEX C^{E}**.

M (calc.) = 10 441.1 Da, M (found) = 10 442.2 Da ($[M+H]^+$).

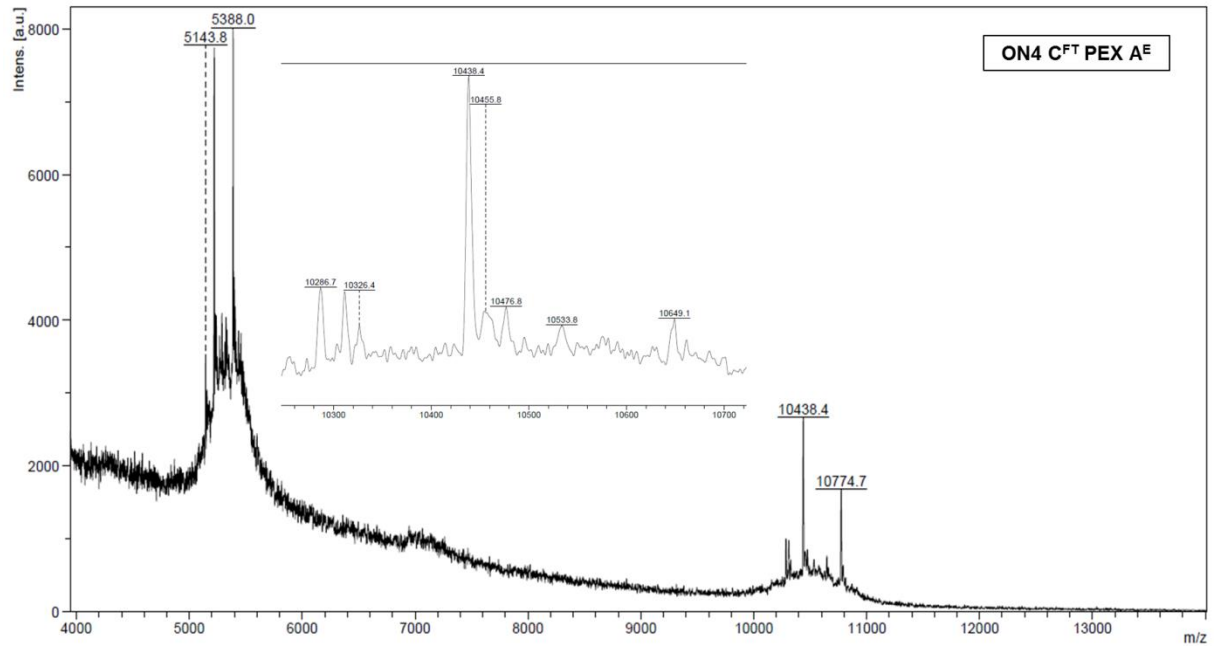


Figure S57. MALDI-TOF spectrum of **ON4 C^{FT} PEX A^{E}**.

M (calc.) = 10 437.1 Da, M (found) = 10 438.4 Da ($[M+H]^+$).

References

[S1] Ménová,P. and Hocek,M. (2012) Preparation of short cytosine-modified oligonucleotides by nicking enzyme amplification reaction. *Chem. Commun.*, **48**, 6921–6923.