Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

Preparation of Short Cytosine-Modified Oligonucleotides by Nicking Enzyme Amplification Reaction

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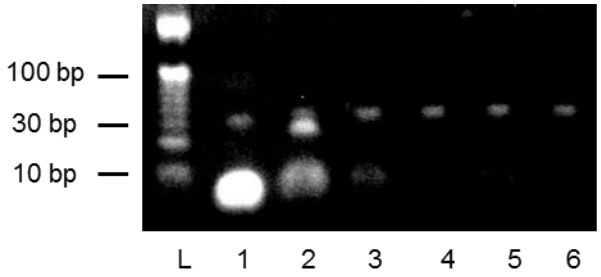
Contents:

1. Optimization of the NEAR procedure

1.1 Enzymes

The use of nicking enzyme Nt.BstNBI was taken over from the original protocol.[1] Regarding the DNA polymerase, several polymerases were tested: Vent(exo-), KOD XL, Deep Vent(exo-), Pwo, Phusion, and DyNAzyme. All these polymerases have been successfully used for the incorporation of modified dNTPs.[2] The best results were obtained with Vent(exo-) (Figure S1).

a)



b)

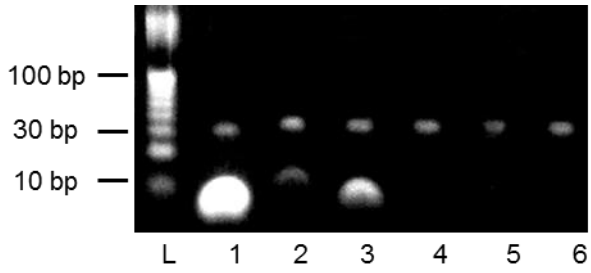
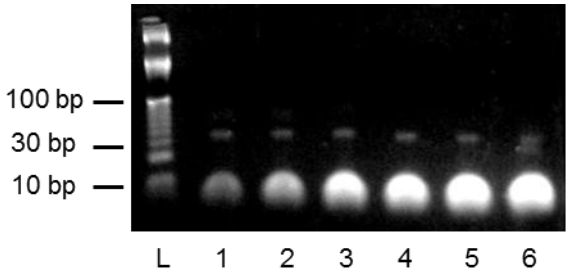


Figure S1. Screening of various DNA polymerases. a) Positive control (natural dNTPs), b) incorporation of modified dCTP (**dC^{NH2}TP**, dATP, dGTP, dTTP). L = DNA ladder; lane 1: Vent(exo-), lane 2: KOD XL; lane 3: Deep Vent(exo-); lane 4: Pwo; lane 5: Phusion; lane 6: DyNAzyme. Except for the DNA polymerase, the standard reaction conditions were used (template **Nick1**).

1.2 Incubation time

The optimal reaction time was determined by kinetic experiments (Figure S2). The reactions were incubated for 30 min, 1 h, 2 h, 3 h, 5 h and 7 h, respectively. The reaction time of 3 hours appears to be optimal, since with longer reaction times more non-desirable background products are obtained (results from HPLC and MALDI-TOF). These non-desirable products contain mainly the correct oligonucleotide extended with an additional adenosine or thymidine at the 5' end.

a)



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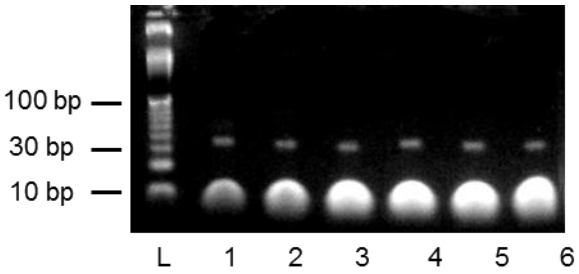


Figure S2. Optimization of the reaction time. a) Positive control (natural dNTPs), b) incorporation of modified dCTP (**dC^{NH2}TP**, dATP, dGTP, dTTP). L = DNA ladder; lane 1: 30 min; lane 2: 1 h; lane 3: 2 h; lane 4: 3 h; lane 5: 5 h; lane 6: 7 h. Except for the reaction time, the standard reaction conditions were used (template **Nick1**).

1.3 Amount of dNTPs

In the original protocol, Van Ness used 400 µM dNTPs.[1] Standard EXPAR protocols use 250 µM natural dNTPs.[3,4,5] However, we found 125 µM natural dNTPs to be sufficient for the linear nicking enzyme reaction, sometimes even more favourable, since in some cases the DNA polymerase tends to add an additional base (mainly adenosine) to the 3' end. The higher is the concentration of dNTPs in the solution, the higher proportion of the adenosine-extended product is obtained. We also found it better to use a higher concentration of the modified dNTP over the non-modified dNTPs (1.5-fold excess); this approach has already been successfully applied for some PCR experiments with functionalized dNTPs.[2c]

1.4 Final optimized conditions

A comparison of the original NEAR conditions used by Van Ness[1] and our optimized conditions is shown in Table S1. An agarose gel comparing these conditions is shown in Figure S3.

Table S1. Comparison of the NEAR conditions described by Van Ness in the original protocol[1] and our optimized conditions for the preparation of ssON.

	Literature[1]	Optimized positive	Optimized modifications
Template	0.05 µM	0.125 µM	0.125 µM
Primer	0.05 µM	0.125 µM	0.125 µM
dNTPs	400 µM	125 µM	125/187.5[a] µM
Nt.BstNBI	0.4 U/µL	0.9 U/µL	0.6 U/µL
Vent(exo-)	0.05 U/µL	0.075 U/µL	0.15 U/µL
ThermoPol Buffer	1×	1×	1×
NEBuffer 3	0.5×	0.5×	0.5×

[a] Natural dNTPs 125 µM, modified dNTP 187.5 µM.

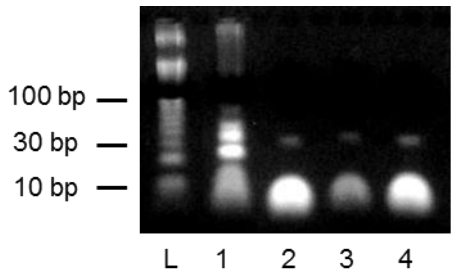


Figure S3. Comparison of the reaction performed according to the original literature conditions and according to our optimized procedure (referred to as standard reaction conditions). L = DNA ladder; lane 1: positive control, literature conditions; lane 2: positive control, standard reaction conditions; lane 3: **dC^{NH2}TP**, dATP, dGTP, dTTP, literature conditions; lane 4: **dC^{NH2}TP**, dATP, dGTP, dTTP, standard reaction conditions (template **Nick1**).

2. Product isolation

To isolate the product from the crude reaction mixture, a standard procedure of phenol-chloroform extraction followed by ethanol precipitation was first employed. While this protocol worked well for the isolation of non-modified oligonucleotides and oligonucleotides bearing only one modification, all attempts to isolate more heavily substituted oligonucleotides failed due to high lipophilicity of the products, which were extracted into the chloroform phase (Figure S4). Therefore, a new isolation protocol was developed. The reaction mixture was first concentrated on a vacuum concentrator to afford about 100 µL of viscous liquid, which was directly injected onto an HPLC column. This approach worked well for the semi-preparative experiments when the contents of one reaction vial were separated (approx. 2 nmol of isolated product). For the work-up of the preparative reaction, which proceeded in 20 separate vials, the concentration on vacuum concentrator would lead to 2 mL of viscous solution and would require many parallel HPLC separations. To make the methodology more robust, we decided to optimize the ethanol-precipitation protocol. Prior phenol-chloroform extraction is essential to remove the enzyme from the reaction mixture. We decided to precipitate directly without the extraction, because even if the enzyme is co-precipitated, it can be easily separated by HPLC. The precipitation was done in the presence of ammonium acetate or sodium acetate, from which the latter afforded much higher yields. The pellet was not washed after the precipitation in order to minimize the loss of the product. The pellets from the 20 reaction vials were dissolved in a minimum volume of water, the solutions were combined and purified by HPLC.

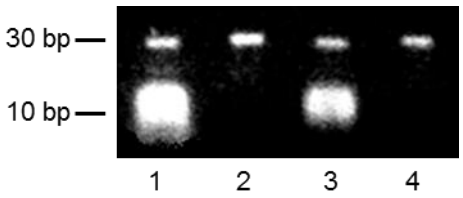


Figure S4. Agarose gel electrophoresis of the reaction mixture before and after extraction. Lane 1: NEAR product **ON2 C^{FT}**, before extraction; lane 2: NEAR product **ON2 C^{FT}**, after extraction; lane 3: NEAR product **ON2 C^{NH2}**, before extraction; lane 4: NEAR **ON2 C^{NH2}**, after extraction.

3. Incorporation of *m*-aminophenyl-modified dNTPs

The incorporation of *m*-aminophenyl-modified dNTPs (**dA^{NH2}TP**, **dC^{NH2}TP**, **dG^{NH2}TP**, **dU^{NH2}TP**) together with the three remaining natural dNTPs was studied using templates **Nick2** and **Nick3** (Figure S5). Both the incorporation of **dC^{NH2}TP** and **dU^{NH2}TP** (accompanied with the remaining three non-modified dNTPs) led to the desired product, with **dC^{NH2}TP** affording much higher yields. When **dG^{NH2}TP** was used, no product was observed. The attempts to incorporate **dA^{NH2}TP** under the optimized conditions also failed. However, the desired product was obtained when the amount of all dNTPs was lowered (to 100 µM) and the reaction time shortened (to 2 h) (Figure S6).

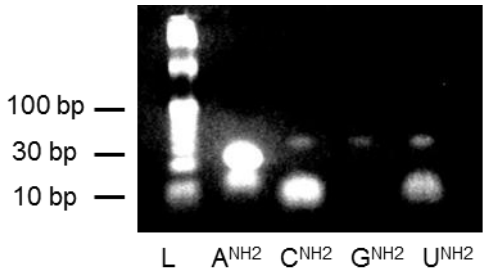


Figure S5. Incorporation of *m*-aminophenyl-modified dNTPs by NEAR. L = DNA ladder; lane 1 (A^{NH2}): **dA^{NH2}TP**, dCTP, dGTP, dTTP; lane 2 (C^{NH2}): dATP, **dC^{NH2}TP**, dGTP, dTTP; lane 3 (G^{NH2}): dATP, dCTP, **dG^{NH2}TP**, dTTP; lane 4 (U^{NH2}): dATP, dCTP, dGTP, **dU^{NH2}TP**. The standard reaction conditions were used (template **Nick2** for **C^{NH2}**, **G^{NH2}** and **U^{NH2}**; template **Nick3** for **A^{NH2}**).

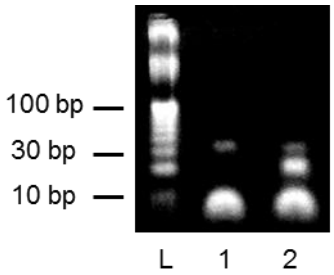


Figure S6. Optimized incorporation of **dA^{NH2}TP**. 100 µM dNTPs. L = DNA ladder; lane 1: incubation 2 h; lane 2: incubation 3 h (template **Nick3**).

4. NEAR with various-length templates

A series of NEAR experiments with various-length templates (Table S2) was done to find the range of ON lengths preparable by NEAR. Standard reaction conditions were used in all the experiments (see the Experimental). The reactions were done on an analytical scale (50 µL) and analyzed by agarose gel electrophoresis and MALDI-TOF spectrometry (Table S3, Figure S7).

Table S2. Sequences of templates, primers and products.

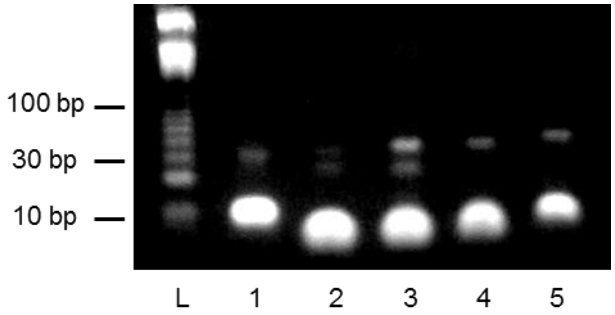
Template	Sequence	Primer	Product
Nick4	5´-CAGACTGTTAcgagGACTCACTAGATCGG-3´	**PrimNick1**	10-mer
Nick2	5´-TCAGCGTAGACTcgagGACTCACTAGATCGG-3´	**PrimNick1**	12-mer
Nick5	5´-TCAGACTGTAGACTcgagGACTCACTAGATCGG-3´	**PrimNick1**	14-mer
Nick6	5´-TGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3´	**PrimNick1**	16-mer
Nick7	5´-TGAAACCTACGACTGGAACTgagcGACTCACTAGATCGG-3´	**PrimNick2**	20-mer
Primer	Sequence		
PrimNick1	5´-CCGATCTAGTGAGTCctcg-3´		
PrimNick2	5´-CCGATCTAGTGAGTCgctc-3´		
Product	Sequence		
ON4	5´-P-TAA**C**AGT**C**TG-3´		
ON2	5´-P-AGT**C**TA**C**G**C**TGA-3´		
ON5	5´-P-AGT**C**TA**C**AGT**C**TGA-3´		
ON6	5´-P-TAG**C**TGA**C**ATG**C**TG**C**A-3´		
ON7	5´-P-AGTT**CC**AGT**C**GTAGGTTT**C**A-3´		

Lower case: spacer bases, underlined: nicking enzyme site or its complement, bold: position of the modification in the product, P: phosphate group.

Table S3. NEAR with various-length templates – results.

Product	Length	Number of cytidines	M(calc)	M(found) $[M+H]^+$
ON4 C (+)	10 nt	2	3107.0 Da	3108.1 Da
ON2 C (+)	12 nt	3	3725.4 Da	3726.3 Da
ON5 C (+)	14 nt	3	4342.8 Da	4343.6 Da
ON6 C (+)	16 nt	4	4961.2 Da	4962.2 Da
ON7 C (+)	20 nt	4	6203.0 Da	6204.1 Da
ON4 C^{NH2}	10 nt	2	3289.2 Da	3290.1 Da
ON2 C^{NH2}	12 nt	3	3998.7 Da	3999.7 Da
ON5 C^{NH2}	14 nt	3	4616.2 Da	4617.1 Da
ON6 C^{NH2}	16 nt	4	5325.7 Da	5326.3 Da
ON7 C^{NH2}	20 nt	4	6567.5 Da	6568.4 Da

a)



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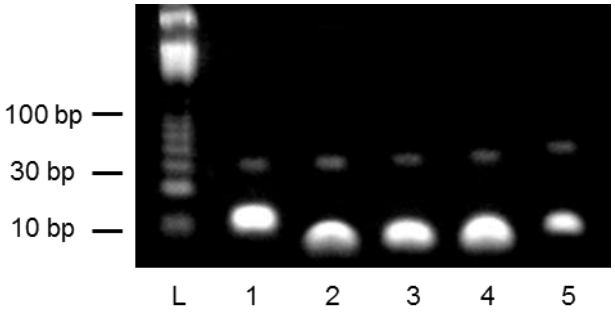

Figure S7. Agarose gel electrophoresis of various-length NEAR products. a) Positive control (natural dNTPs), b) incorporation of modified dCTP (**dC^{NH2}TP**, dATP, dGTP, dTTP). L = DNA ladder; lane 1: **ON4**; lane 2: **ON2**; lane 3: **ON5**; lane 4: **ON6**; lane 5: **ON7**. Standard reaction conditions were used.

5. PEX using ON2 C^{NH2} as primer

The use of modified NEAR products as primers in PEX and PCR experiments is a simple way to introduce modifications into the primer part of the target oligonucleotides. Furthermore, such approach enables the preparation of oligonucleotides with two different modifications on one nucleobase, e.g. on cytidine as shown in the following experiment.

To prove the principle, we designed a simple PEX experiment with a 36-mer biotinylated template **Nick2PEX** and 12-mer **ON2 C^{NH2}** primer (Table S4). Either natural bases (positive control), or a combination of natural bases with modified dCTP (**$dC^{E}TP$**, dATP, dGTP, dTTP) were used. After the PEX, the strands were separated using streptavidine-coated magnetic beads. The product was analysed by agarose gel electrophoresis, UV spectroscopy and MALDI-TOF spectrometry (Figure S8, S9).

Table S4. Sequences of the template, primer and product used in NEAR & PEX experiment.

Template	**Nick2PEX**	5´-bio-ACACTGTCGAAGCATCTGTCGTGA*TCAGCGTAGACT*-3´
Primer	**ON2 C^{NH2}**	5´-P-AGT**C**TA**C**G**C**TGA-3´
Product	**ON8**	5´-P-AGT**C**TA**C**G**C**TGAT**C**A**C**GA**C**AGATG**C**TT**C**GA**C**AGTGT-3´

Italics: segments forming duplex with the primer, bold: position of the modification in the product, P: phosphate group, bio: biotin.

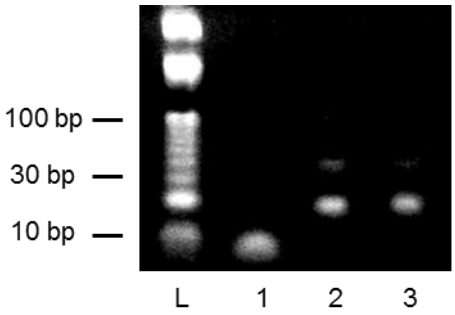

Figure S8. Agarose gel electrophoresis of PEX primer and products. L = DNA ladder; lane 1: primer **ON2 C^{NH2}**; lane 2: PEX product – positive control (natural dNTPs); lane 3: PEX product – incorporation of modified dCTP (**$dC^{E}TP$**, dATP, dGTP, dTTP).

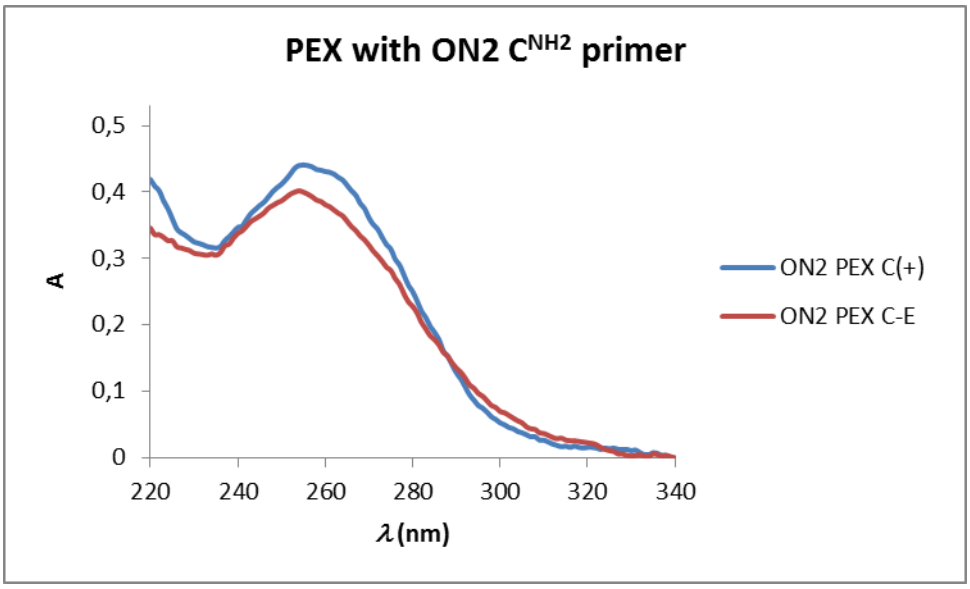

Figure S9. UV spectra of the PEX products. Aqueous solutions, **ON2 PEX C(+)** 1.3 µM and **ON2 PEX C^{E}** 1.1 µM.

6. Comparison of NEAR with magnetoseparation

To prepare medium-length single-stranded oligonucleotides (usually above 30 nt), PEX followed by magnetoseparation of DNA strands is generally used.[2c] This procedure is not directly comparable with NEAR, as each method can be used for the preparation of different--length ONs (> 20 nt in magnetoseparation, < 20 nt in NEAR). Nevertheless, to get at least rough comparison, a magnetoseparation experiment leading to a 31-mer product ON (Table S5) was done on the same scale as NEAR (750 µL). The concentration of the isolated product was calculated from the absorbance at 260 nm (Figure S10) and extinction coefficient obtained in an online calculator by IDT Biophysics.[6] The product was analyzed by MALDI--TOF mass spectrometry.

The magnetoseparation experiment afforded 1.3 nmol of a 31-mer ON. This is approximately one half of the yield of the nicking enzyme reaction. In NEAR, the use of rather costly streptavidine-coated magnetic beads is avoided. Furthermore, NEAR requires only catalytic amount of both template and primer. These factors make NEAR fully complementary, or even better method for the preparation of ssONs.

Table S5. Sequences of the template, primer and product used in PEX & magnetoseparation experiment.

Template	**TempPEX**	5´-bio-CTAGCATGAGCTCAGT*CCCATGCCGCCCATG*-3´
Primer	**PrimPEX**	5´-CATGGGCGGCATGGG-3´
Product	**ON9**	5´-CATGGGCGGCATGGGACTGAGCTCATGCTAG-3´

Italics: segment forming duplex with the primer.

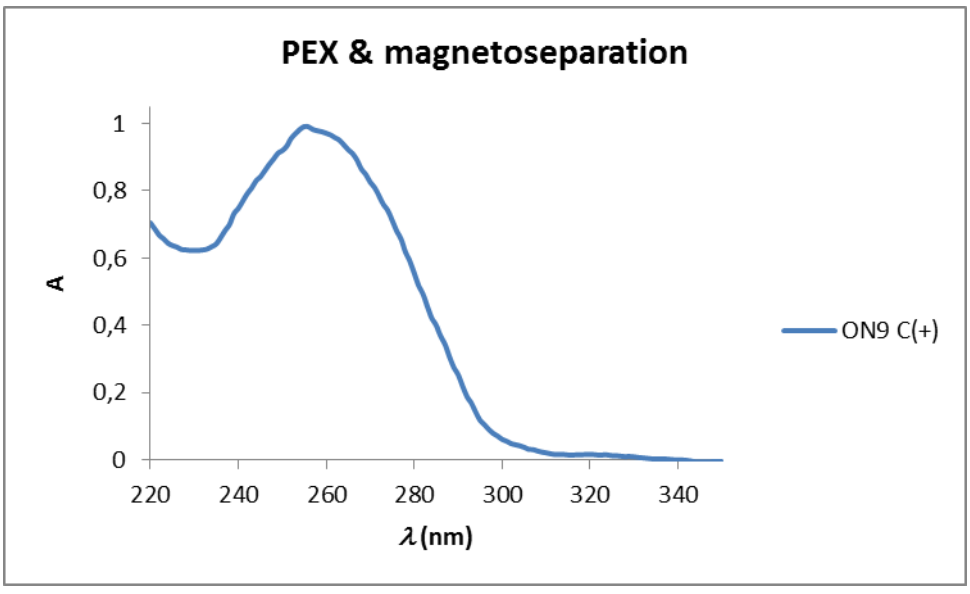

Figure S10. UV spectrum of the PEX product **ON9 C(+)** isolated by magnetoseparation. Aqueous solution, 3.3 µM, total 1.3 nmol.

7. Experimental part

Synthetic oligonucleotides (primers **PrimNick1**, **PrimNick2**, and **PrimPEX**; NEAR templates **Nick1**, **Nick2**, **Nick3**, **Nick4**, **Nick5**, **Nick6**, and **Nick7**; biotinylated PEX templates **Nick2PEX** and **TempPEX**; for sequences see Table S6) were purchased from Sigma-Aldrich. DNA polymerases Vent(exo-), Nt.BstNBI, Deep Vent(exo-) as well as natural nucleoside triphosphates (dATP, dCTP, dGTP, dTTP) were purchased from New England Biolabs, Phusion and DyNAzyme II polymerases from Finnzymes, KOD XL polymerase from Merck, and Pwo polymerase from Peqlab. Streptavidine-coated magnetic beads Mag-Prep P25 were obtained from Merck. Acetonitrile for HPLC and TEAA buffer were purchased from Sigma-Aldrich. All solutions for the nicking enzyme amplification reaction were prepared in PCR Ultra H_2O (Top-Bio, Czech Republic).

Synthesis and characterization data for 5-(3-aminophenyl)-2'-deoxycytidine 5'-*O*-triphosphate (**dC^{NH2}TP**)[2b], 2'-deoxy-5-(3-nitrophenyl)cytidine 5'-*O*-triphosphate (**dC^{NO2}TP**)[2b], 2'-deoxy--5-(5-formylthiophene-2-yl)cytidine 5'-*O*-triphosphate (**dC^{FT}TP**)[2c], 5-ethynyl-2'--deoxycytidine 5'-*O*-triphosphate (**dC^{E}TP**)[7], 7-(3-aminophenyl)-2'-deoxy-7-deazaadenosine 5'-*O*-triphosphate (**dA^{NH2}TP**)[2b], 5-(3-aminophenyl)-2'-deoxyuridine 5'-*O*-triphosphate (**dU^{NH2}TP**)[2b], 7-(3-aminophenyl)-2'-deoxy-7-deazaguanosine 5'-*O*-triphosphate (**dG^{NH2}TP**)[8] were reported previously.

Samples were concentrated on CentriVap Vacuum Concentrator System (Labconco). Mass spectra of the prepared ONs were measured by MALDI-TOF, on Reflex IV (Bruker Daltonics, Germany) with nitrogen UV laser (337 nm). UV spectra were measured on Varian CARY 100 Bio spectrophotometer and on NanoDrop1000 (ThermoScientific).

Table S6. Sequences for all commercial template and primer oligonucleotides.

NEAR Templates	
Nick1	5´-TCAACTCATGACcgagGACTCACTAGATCGG-3´
Nick2	5´-TCAGCGTAGACTcgagGACTCACTAGATCGG-3´
Nick3	5´-TCGACTGGACTAcgagGACTCACTAGATCGG-3´
Nick4	5´-CAGACTGTTAcgagGACTCACTAGATCGG-3´
Nick5	5´-TCAGACTGTAGACTcgagGACTCACTAGATCGG-3´
Nick6	5´-TGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3´
Nick7	5´-TGAAACCTACGACTGGAACTgagcGACTCACTAGATCGG-3´
Primers	
PrimNick1	5´-CCGATCTAGTGAGTCctcg-3´
PrimNick2	5´-CCGATCTAGTGAGTCgctc-3´
PrimPEX	5´-CATGGGCGGCATGGG-3´
PEX Templates	
Nick2PEX	5´-bio-ACACTGTCGAAGCATCTGTCGTGA*TCAGCGTAGACT*-3´
TempPEX	5´-bio-CTAGCATGAGCTCAGT*CCCATGCCGCCCATG*-3´

Lower case: spacer bases, underlined: nicking enzyme site or its complement, italics: segments forming duplex with the primer (shown only for PEX templates), bio: biotin.

General procedure

The reaction mixtures were prepared separately at 4 °C as Mix A and Mix B. Mix A consisted of NEBuffer 3, template, primer and dNTPs. Mix B consisted of ThermoPol buffer, water, the nicking endonuclease Nt.BstNBI and the DNA polymerase Vent(exo-). The reaction mixture contained the template (0.125 µM), primer (0.125 µM), modified dN^XTP (187.5 µM), natural dNTPs (125 µM), 1× ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl /pH 8.8/, 0.1% Triton-X-100, and 2 mM $MgSO_4$), and 0.5× NEBuffer 3 (50 mM NaCl, 25 mM Tris-HCl /pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT). The amounts of enzymes depend on whether only natural dNTPs or modified dN^XTP together with the three remaining natural dNTPs were incorporated, being 0.075 U/µL Vent(exo-), 0.9 U/µL Nt.BstNBI and 0.15 U/µL Vent(exo-), 0.6 U/µL Nt.BstNBI, respectively. Mix A and B were heated separately until the temperature reached 55 °C. After this pre-heating period, Mix A was transferred to Mix B, the resulting mixture was mixed thoroughly and incubated at 55 °C for 3 hours. The reaction was stopped by cooling to 4 °C.

NEAR on analytical scale

The analytical reactions were performed according to the general procedure in a volume of 50 µL. Mix A and B were preheated for 2 min. The products were analyzed by agarose gel electrophoresis using 4% agarose gels. Samples were prepared by mixing 2 µL of TrackIt Cyan/Orange loading buffer (Invitrogen) and 10 µL of the reaction mixture or 10 bp DNA

ladder (Invitrogen). The gel was run for 45 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum). For mass spectrometry analysis the excess nucleotides and buffers were removed by the filtration on Illustra MicroSpin G-25 columns (GE Healthcare). The MALDI spectra were measured in reflectron mode by dried droplet technique.

NEAR on semi-preparative scale

The semi-preparative reactions were performed according to the general procedure in a volume of 750 µL. Mix A and B were preheated for 5 min. After the reaction was stopped, the solution was concentrated on a vacuum concentrator to approximately 100 µL. The viscous concentrate was injected on HPLC XBridge OST C_{18} Column (Waters; 2.5 µm particle size, 4.6 mm × 50 mm) and separated using a gradient of triethylammonium acetate (TEAA) and acetonitrile and flow rate 1 mL/min. Mobile phase A corresponds to 0.1M TEAA in HPLC--grade water, mobile phase B to acetonitrile/0.1M TEAA in HPLC-grade water 20/80 (v/v). The gradient started with 80 % mobile phase A and 20 % mobile phase B, going linearly to 40 % mobile phase B in 10 min, then to 60 % mobile phase B in 30 min and then to 100 % mobile phase B in 40 min. The fractions containing the product were evaporated on a vacuum concentrator. The residue was diluted with known volume of water and a UV spectrum was measured. The concentration of the product was calculated from the absorbance at 260 nm and extinction coefficient obtained from an online calculator by IDT Biophysics.[6] The products were analyzed by MALDI-TOF mass spectrometry.

NEAR on preparative scale

The reaction was set according to the general procedure to 20 separate vials, each containing 750 µL of the reaction mixture. Mix A and B were preheated for 5 min. The vials were incubated at 55 °C for 3 hours. The reaction was stopped by cooling to 4 °C. The reaction mixtures were divided into 250 µL aliquots. To each aliquot, 25 µL o 3M sodium acetate and 750 µL of 99.8% ethanol were added. The resulting mixtures were mixed thoroughly and kept at -18 °C overnight. Next, the samples were centrifuged at 13 400 rpm for 30 min. The solutions were pipetted off and the pellets were dried at room temperature for 30 min. The dry pellets were dissolved in 20 µL of water (HPLC grade). These solutions were combined and the resulting solution was concentrated to 100 µL. The concentrate was injected on HPLC XBridge OST C_{18} Column (Waters; 2.5 µm particle size, 4.6 mm × 50 mm) and separated

using a gradient of triethylammonium acetate (TEAA) and acetonitrile and flow rate 1 mL/min. Mobile phase A corresponds to 0.1M TEAA in HPLC-grade water, mobile phase B to acetonitrile/0.1M TEAA in HPLC-grade water 20/80 (v/v). The gradient started with 80 % mobile phase A and 20 % mobile phase B, going linearly to 40 % mobile phase B in 10 min, then to 60 % mobile phase B in 30 min and then to 100 % mobile phase B in 40 min. The fractions containing the product were evaporated on a vacuum concentrator. The residue was diluted with known volume of water and a UV spectrum was measured. The concentration of the product was calculated from the absorbance at 260 nm and extinction coefficient obtained from an online calculator by IDT Biophysics.[6] The product was analyzed by MALDI-TOF mass spectrometry.

Magnetoseparation procedure

The reaction mixture (750 μL) contained Vent(exo-) DNA polymerase (2 U/μL, 30 μL), natural dNTPs (4 mM, 37.5 μL), primer **PrimPEX** (100 μM, 15 μL), and 5´-biotinylated template **TempPEX** (100 μM, 15 μL) in ThermoPol reaction buffer (75 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler. The reaction was stopped by cooling to 4 °C.

Streptavidine-coated magnetic particles (MagPrep P-25, Merck, 1 mL) were washed with 0.3 M NaCl in 10 mM Tris, pH = 7.5 (3 × 1 mL). The PEX solution and 2.5 M NaCl (2.4 μL) were added. The mixture was incubated for 30 min at 20 °C and 1200 rpm. After the incubation, the magnetic beads were collected on a magnet (DynaMag^{TM}-2, Invitrogen) and the solution was discarded. The beads were washed successively with 1 × PBS (3 × 1 mL), 0.3 M NaCl in 10 mM Tris, pH = 7.5 (3 × 1 mL), and water (3 × 1 mL). Then water (200 μL) was added and the sample was denatured for 2 min at 75 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The UV spectrum of the solution was measured on NanoDrop. The concentration of the product was calculated from the absorbance at 260 nm and extinction coefficient obtained from an online calculator by IDT Biophysics.[6] The product was analyzed by MALDI-TOF mass spectrometry.

*PEX using **ON2 C^{NH2}** as primer*

The reaction mixture (50 μL) contained Vent(exo-) DNA polymerase (2 U/μL, 2.5 μL), dNTPs (either all natural or 3 natural and 1 modified, 4 mM, 2.5 μL), primer (**ON2 C^{NH2}**, 10 μM, 10 μL), and 5´-biotinylated template (**Nick2PEX**, 10 μM, 10 μL) in ThermoPol

reaction buffer (5 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler. The reaction was stopped by cooling to 4 °C.

Streptavidine-coated magnetic particles (MagPrep P-25, Merck, 30 μL) were washed with 0.3 M NaCl in 10 mM Tris, pH = 7.5 (3 × 200 μL). The PEX solution and 2.5 M NaCl (0.4 μL) were added. The mixture was incubated for 30 min at 20 °C and 1200 rpm. After the incubation, the magnetic beads were collected on a magnet (DynaMag^{TM}-2, Invitrogen) and the solution was discarded. The beads were washed successively with 1 × PBS (3 × 200 μL), 0.3 M NaCl in 10 mM Tris, pH = 7.5 (3 × 200 μL), and water (3 × 100 μL). Then water (25 μL) was added and the sample was denatured for 2 min at 70 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The UV spectrum of the solution was measured on NanoDrop. The product was analyzed by MALDI--TOF mass spectrometry and on agarose gels.

Agarose gel electrophoresis was done on 4% agarose gels. Samples were prepared by mixing 1.6 μL of TrackIt Cyan/Orange loading buffer (Invitrogen) and 8 μL of the purified PEX product or 10 bp DNA ladder (Invitrogen). The gel was run for 45 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum).

MALDI-TOF experiments

The MALDI-TOF spectra were measured on Reflex IV (Bruker Daltonics, Germany) with nitrogen UV laser (337 nm). The measurements were done in reflectron mode by dried droplet technique, with the mass range up to 100 kDa and resolution > 25.000.The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in ratio 9/1/1. The sample (0.5 μL) and matrix (2 μL) were mixed on target by use of anchor-chip desk. The crystallized spots were washed once by 0.1% formic acid and once by water.

8. UV spectra

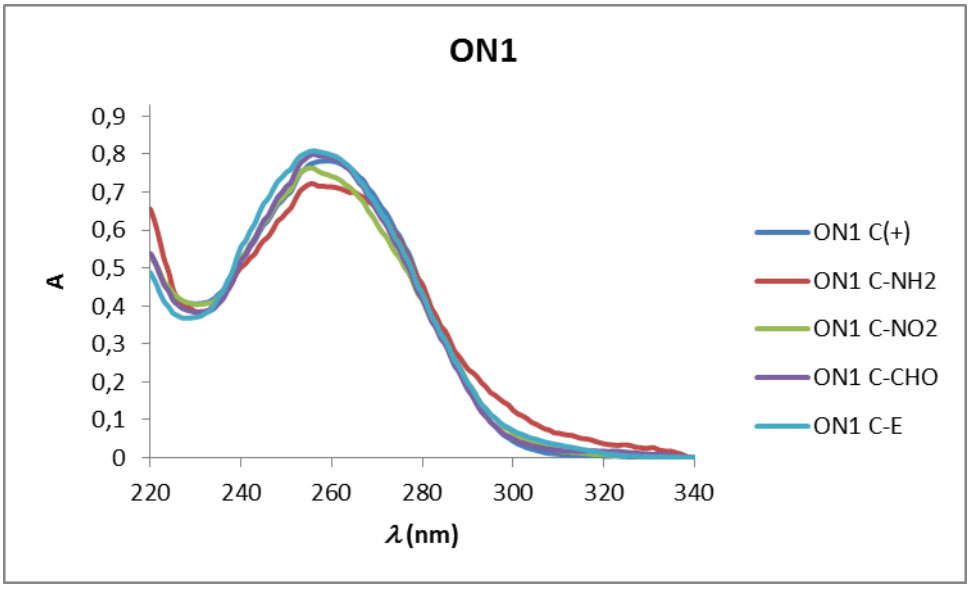

Figure S11. UV spectra of NEAR products **ON1**. Aqueous solutions: **C(+)** 6.4 µM, $\mathbf{C^{NH2}}$ 6.8 µM, $\mathbf{C^{NO2}}$ 6.0 µM, $\mathbf{C^{FT}}$ 6.4 µM, $\mathbf{C^{E}}$ 6.5 µM.

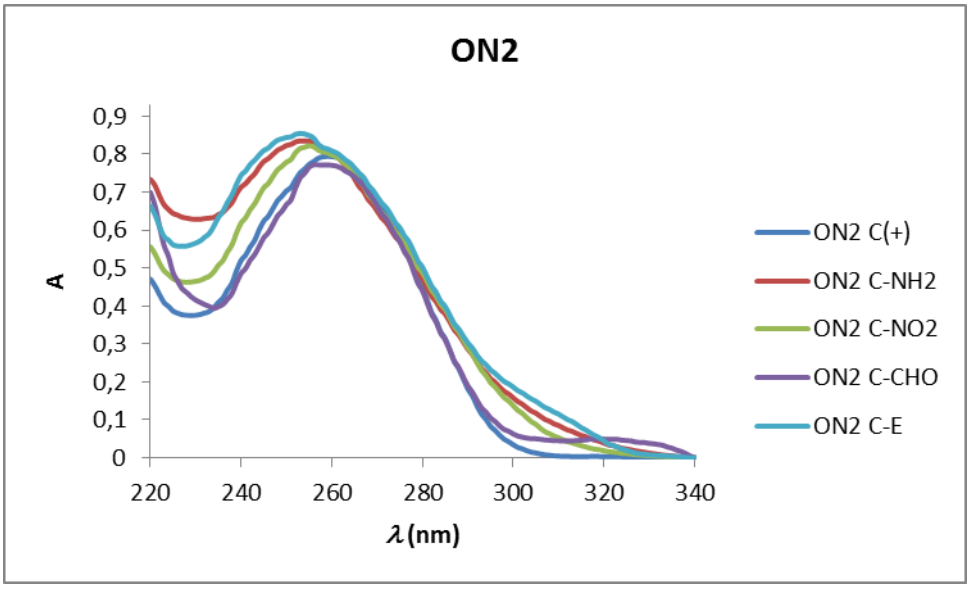

Figure S12. UV spectra of NEAR products **ON2**. Aqueous solutions: **C(+)** 6.8 µM, $\mathbf{C^{NH2}}$ 6.8 µM, $\mathbf{C^{NO2}}$ 6.8 µM, $\mathbf{C^{FT}}$ 6.6 µM, $\mathbf{C^{E}}$ 6.9 µM.

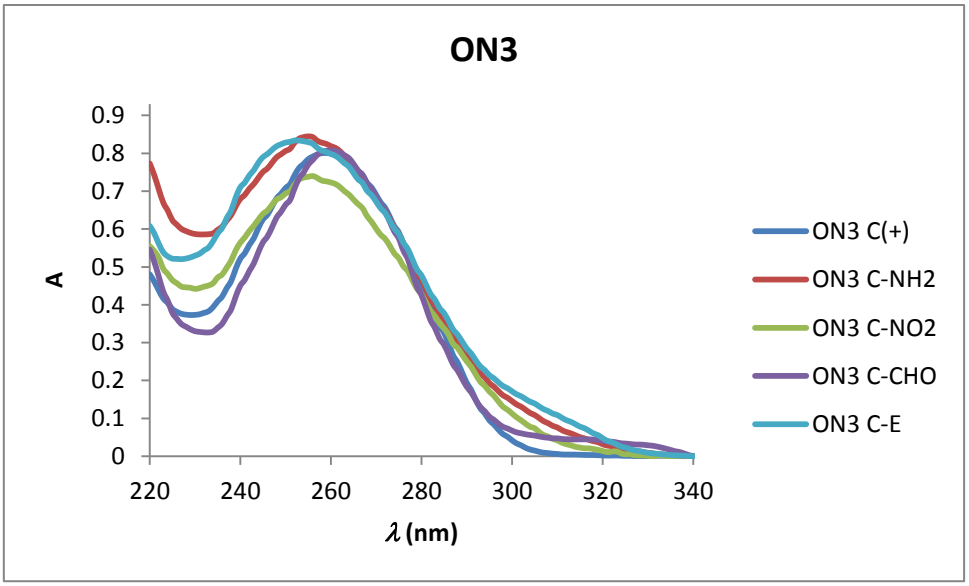

Figure S13. UV spectra of NEAR products **ON3**. Aqueous solutions: **C(+)** 6.8 µM, $\mathbf{C^{NH2}}$ 6.8 µM, $\mathbf{C^{NO2}}$ 6.0 µM, $\mathbf{C^{FT}}$ 6.7 µM, $\mathbf{C^{E}}$ 6.7 µM.

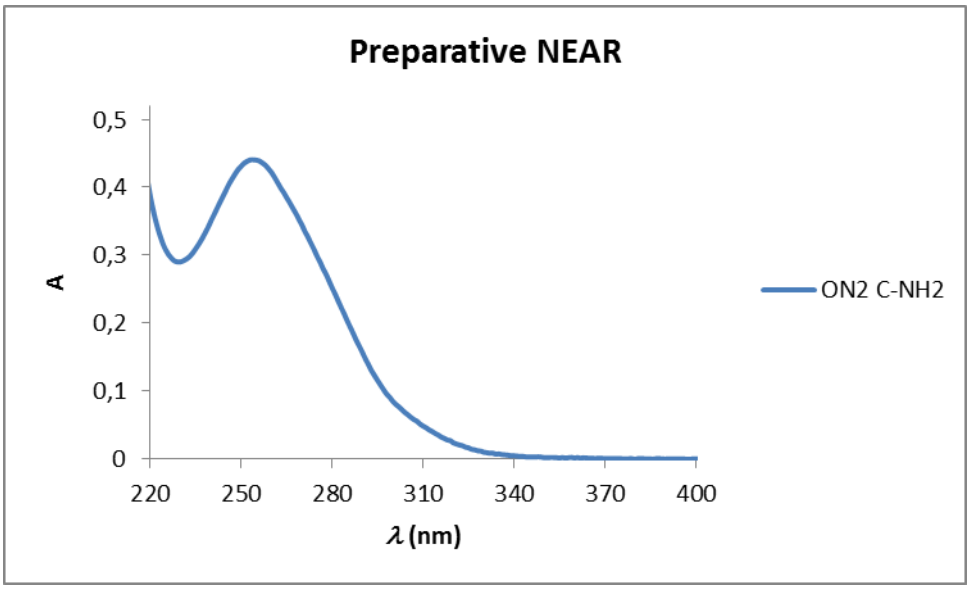

Figure S14. UV-VIS spectrum of the **ON2** $\mathbf{C^{NH2}}$ product of the preparative reaction. Aqueous solution, 3.9 µM, total 28 nmol.

9. MALDI spectra

General remarks:

Peaks at [M – 125] can be assigned to dethymination.

Peaks at [M + 313.2] can be assigned to product extended with adenosine.

Fragmentation of $\mathbf{C^{NO2}}$ modified products corresponds with literature data for mass spectra of polynitrocompounds to contain peaks at M – 16, M – 30 and M – 46 Da.[9]

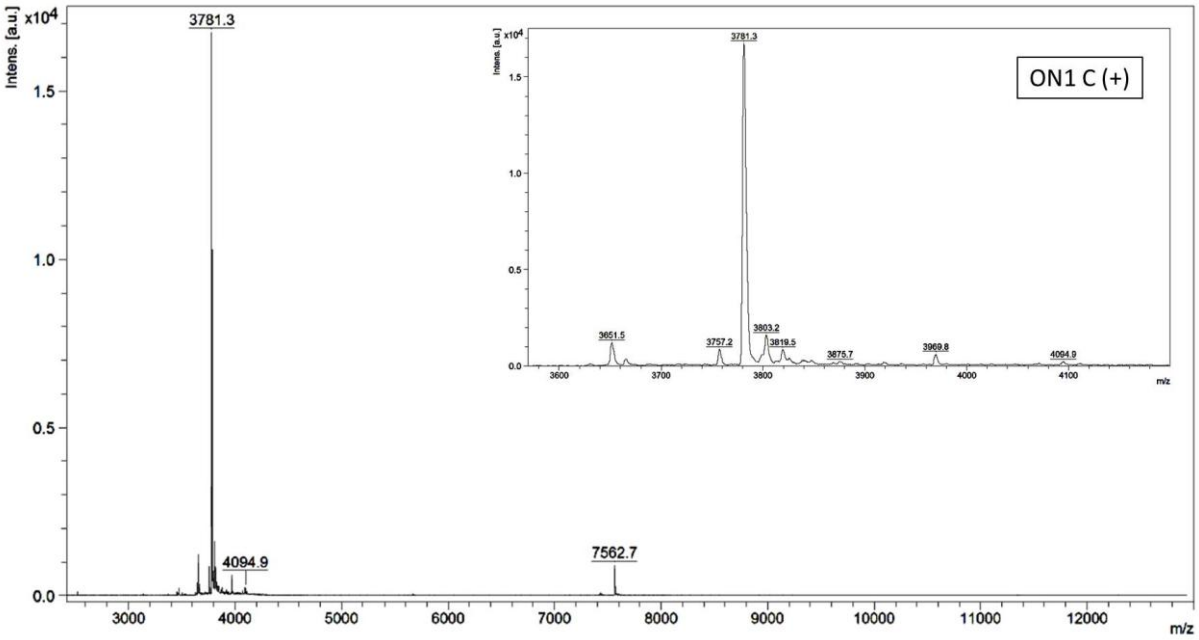

Figure S15. MALDI spectrum of **ON1 C(+)**. M (calc.) = 3780.5 Da, M (found) = 3781.3 Da ($[M+H]^+$).

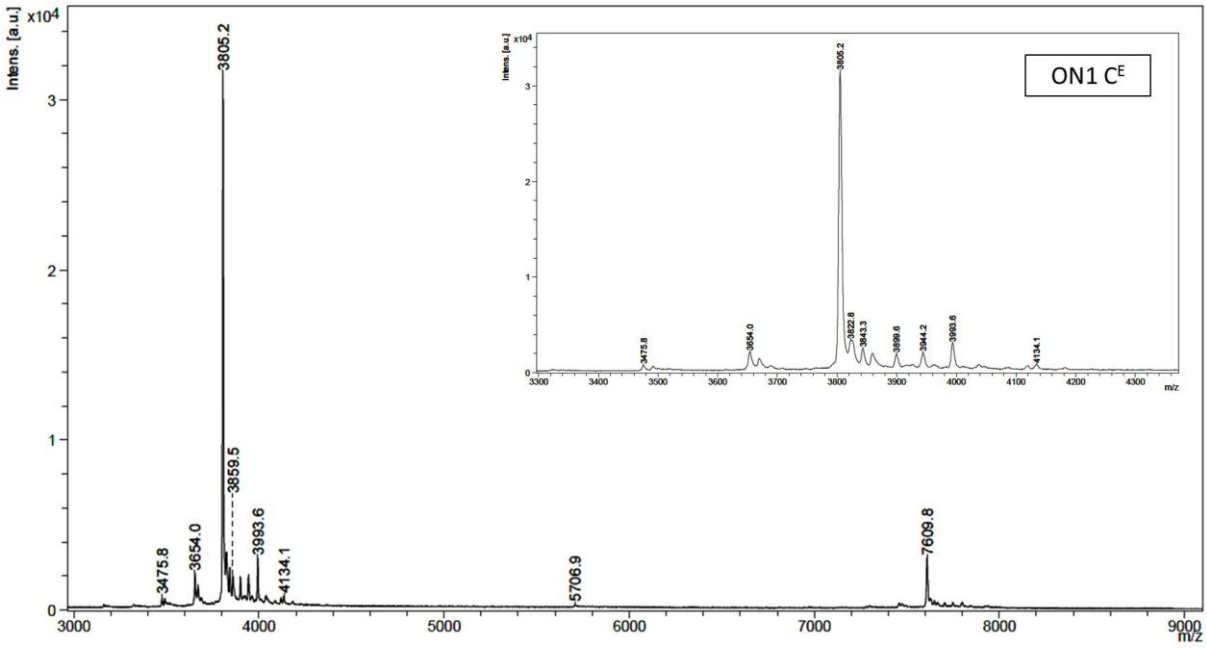

Figure S16. MALDI spectrum of **ON1 C^{E}**. M (calc.) = 3804.5 Da, M (found) = 3805.2 Da ($[M+H]^+$).

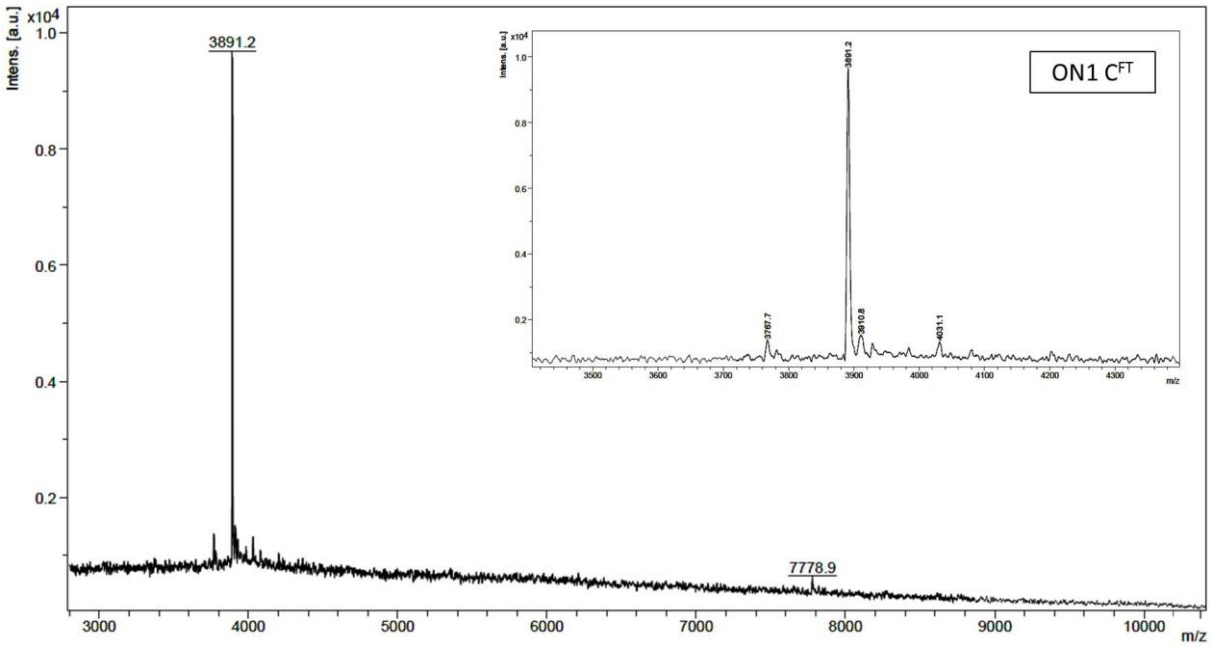

Figure S17. MALDI spectrum of **ON1 C^{FT}**. M (calc.) = 3890.6 Da, M (found) = 3891.2 Da ($[M+H]^+$).

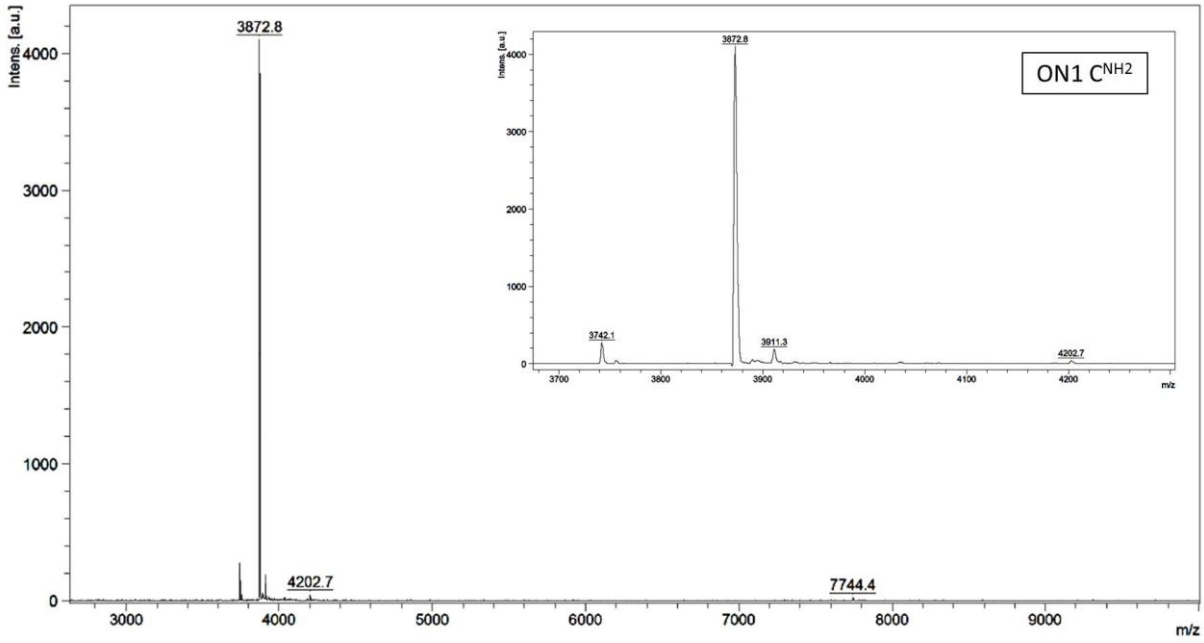

Figure S18. MALDI spectrum of **ON1 C^{NH2}**. M (calc.) = 3871.6 Da, M (found) = 3872.8 Da ($[M+H]^+$).

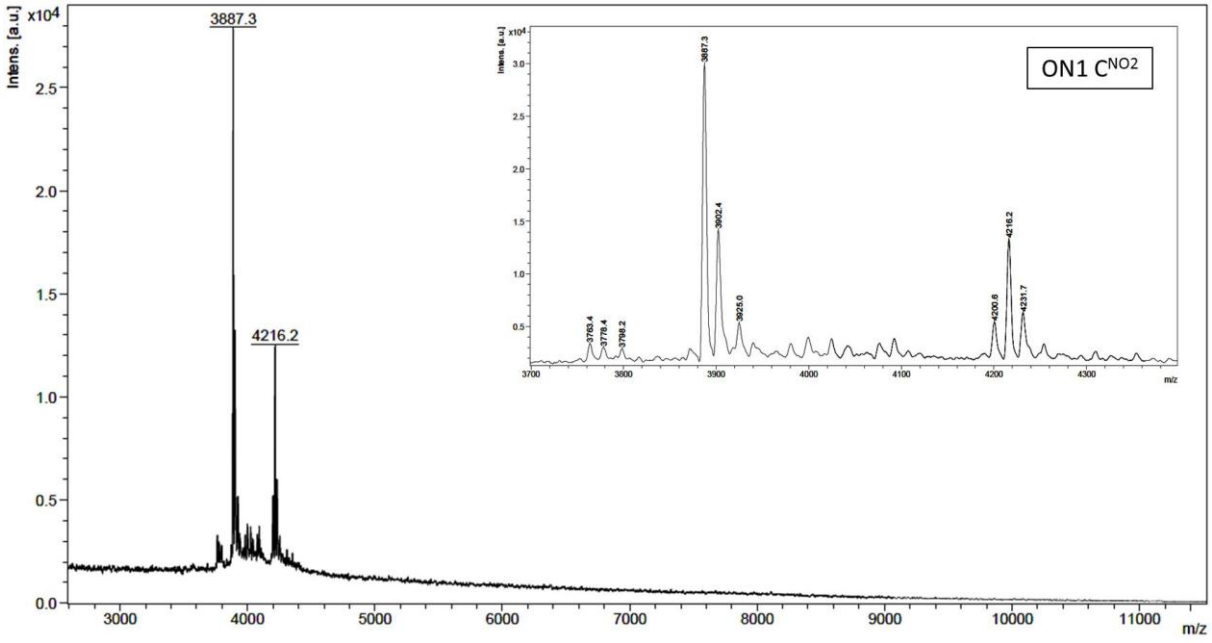

Figure S19. MALDI spectrum of **ON1 C^{NO2}**. M (calc.) = 3901.6 Da, M (found) = 3902.4 Da ($[M+H]^+$).

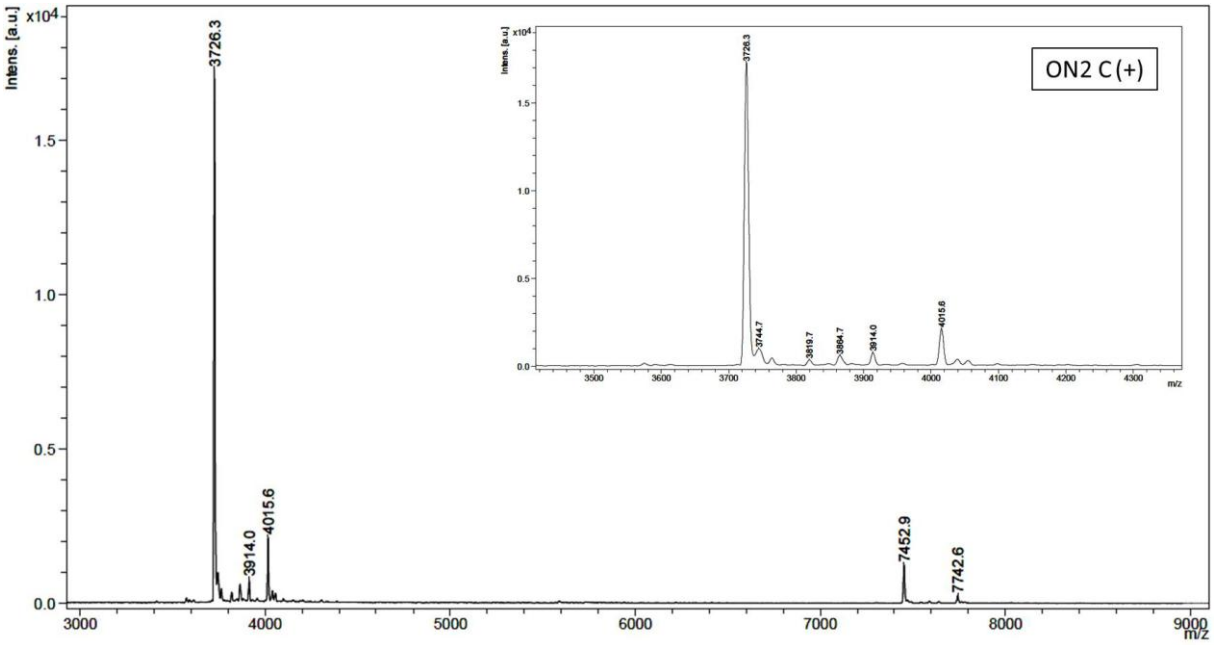

Figure S20. MALDI spectrum of **ON2 C(+)**. M (calc.) = 3725.4 Da, M (found) = 3726.3 Da ($[M+H]^+$).

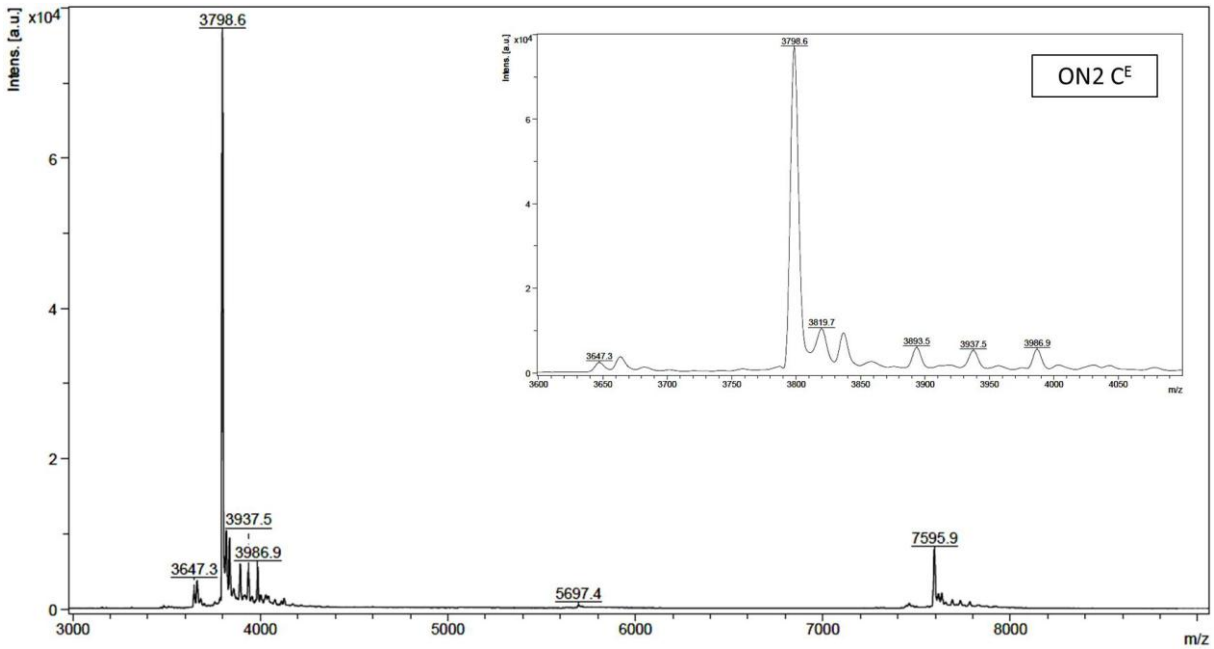

Figure S21. MALDI spectrum of **ON2 C^E**. M (calc.) = 3797.5 Da, M (found) = 3798.6 Da ($[M+H]^+$).

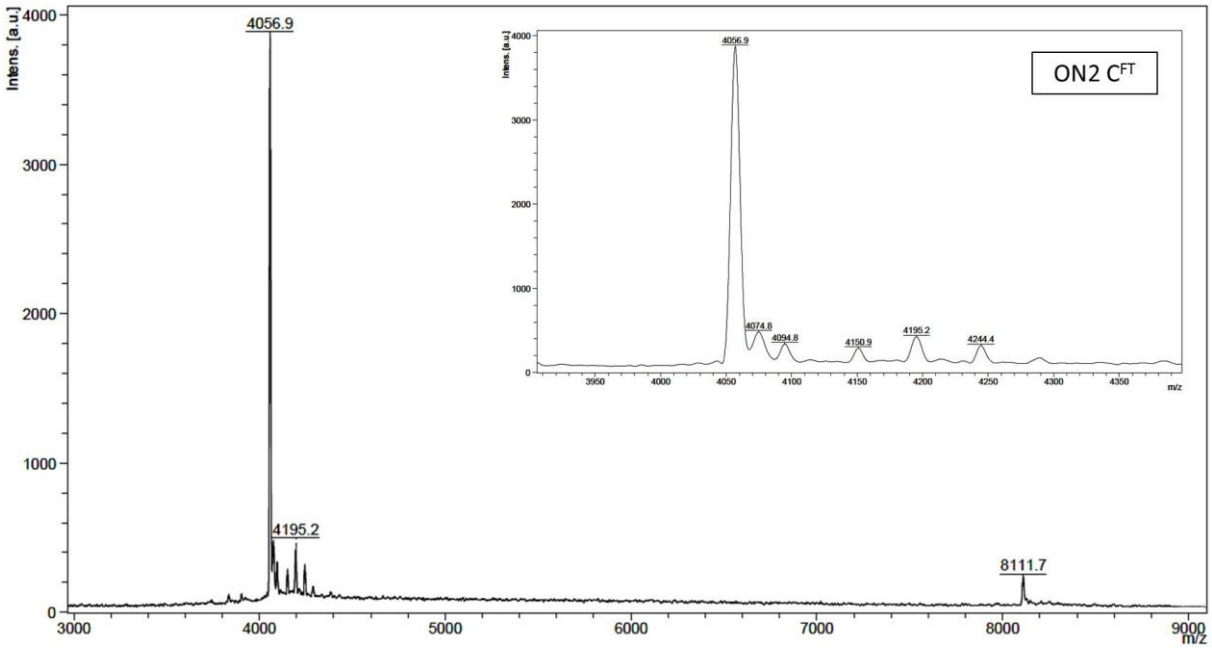

Figure S22. MALDI spectrum of **ON2 C^{FT}**. M (calc.) = 4055.8 Da, M (found) = 4056.9 Da ($[M+H]^+$).

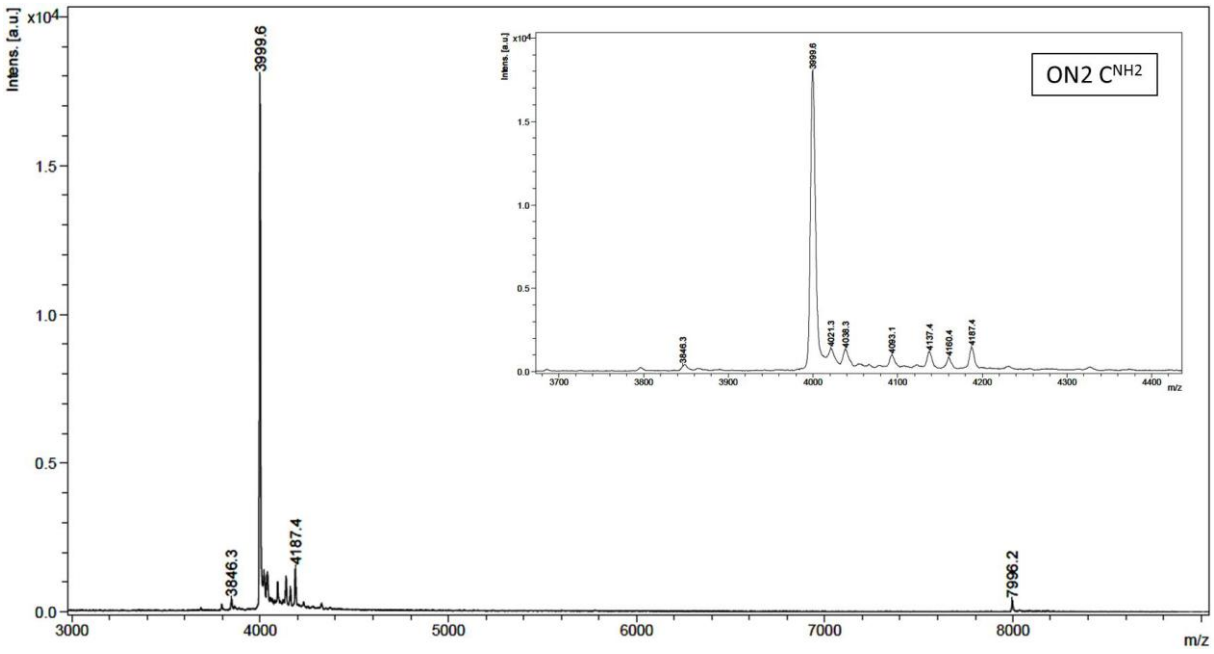

Figure S23. MALDI spectrum of **ON2 C^{NH2}**. M (calc.) = 3998.7 Da, M (found) = 3999.6 Da ($[M+H]^+$).

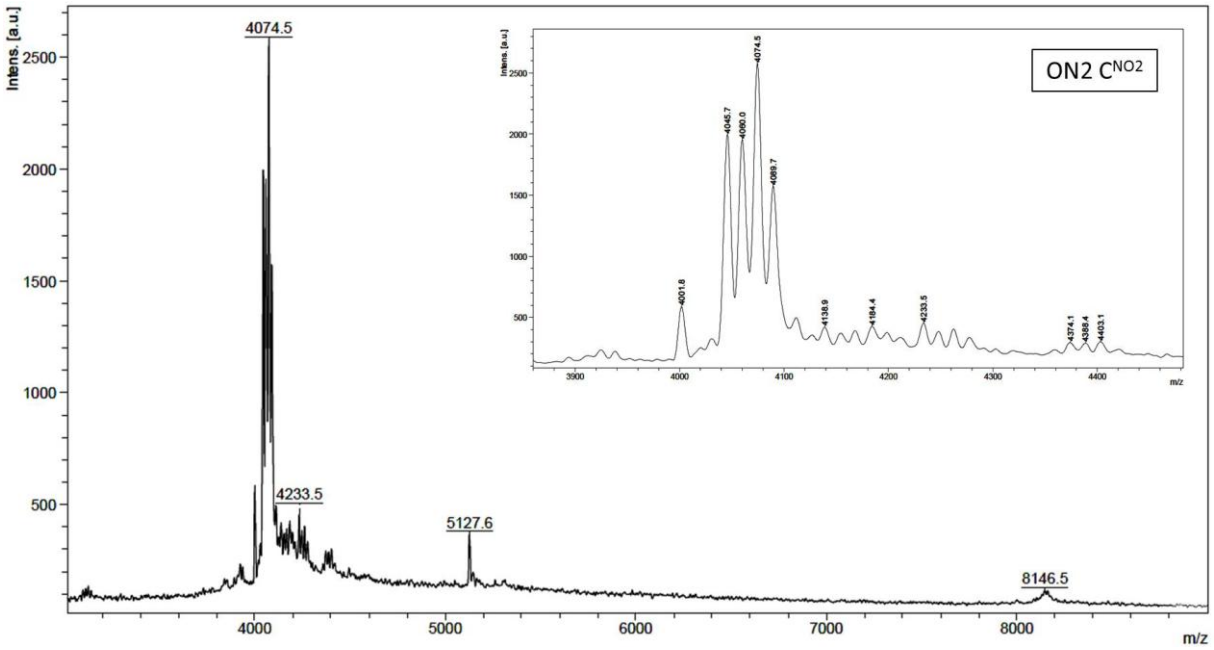

Figure S24. MALDI spectrum of **ON2 C^{NO2}**. M (calc.) = 4088.7 Da, M (found) = 4089.7 Da ($[M+H]^+$).

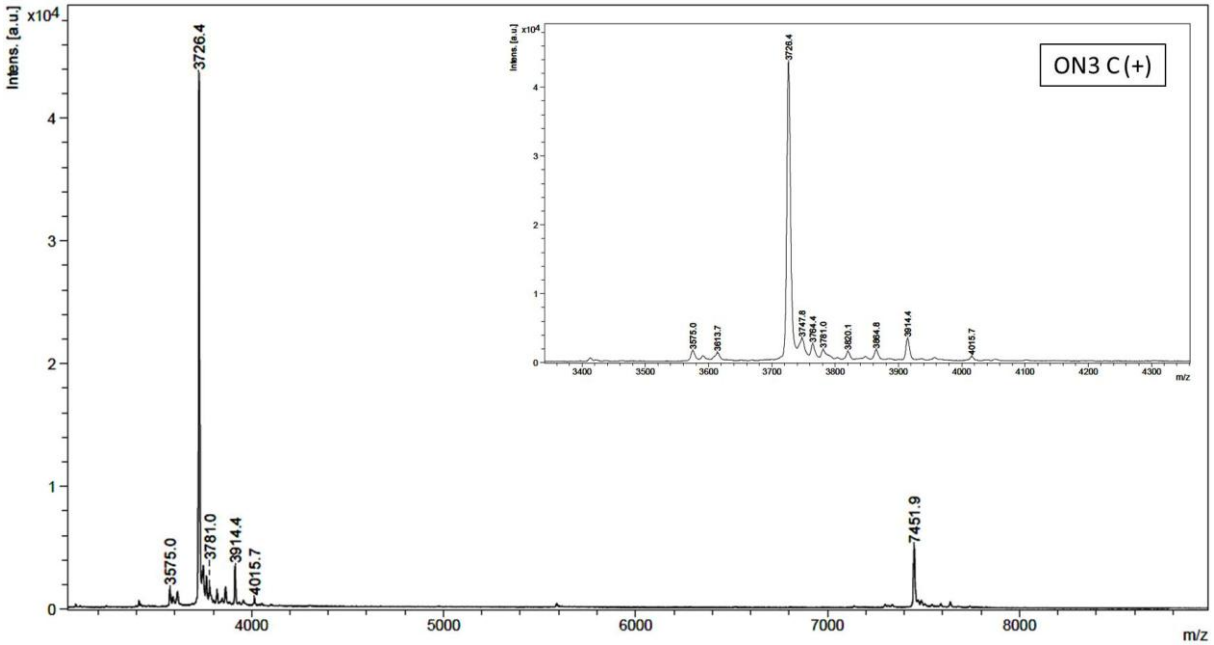

Figure S25. MALDI spectrum of **ON3 C(+)**. M (calc.) = 3725.4 Da, M (found) = 3726.4 Da ($[M+H]^+$).

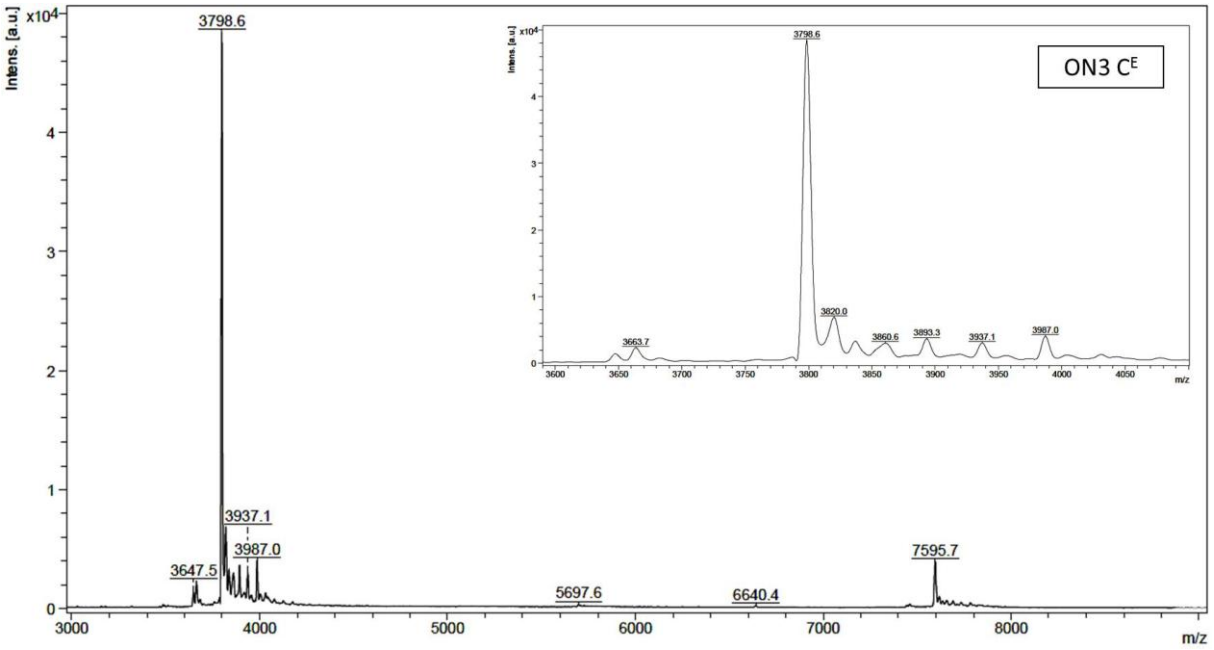

Figure S26. MALDI spectrum of **ON3 C^{E}**. M (calc.) = 3797.5 Da, M (found) = 3798.6 Da ($[M+H]^+$).

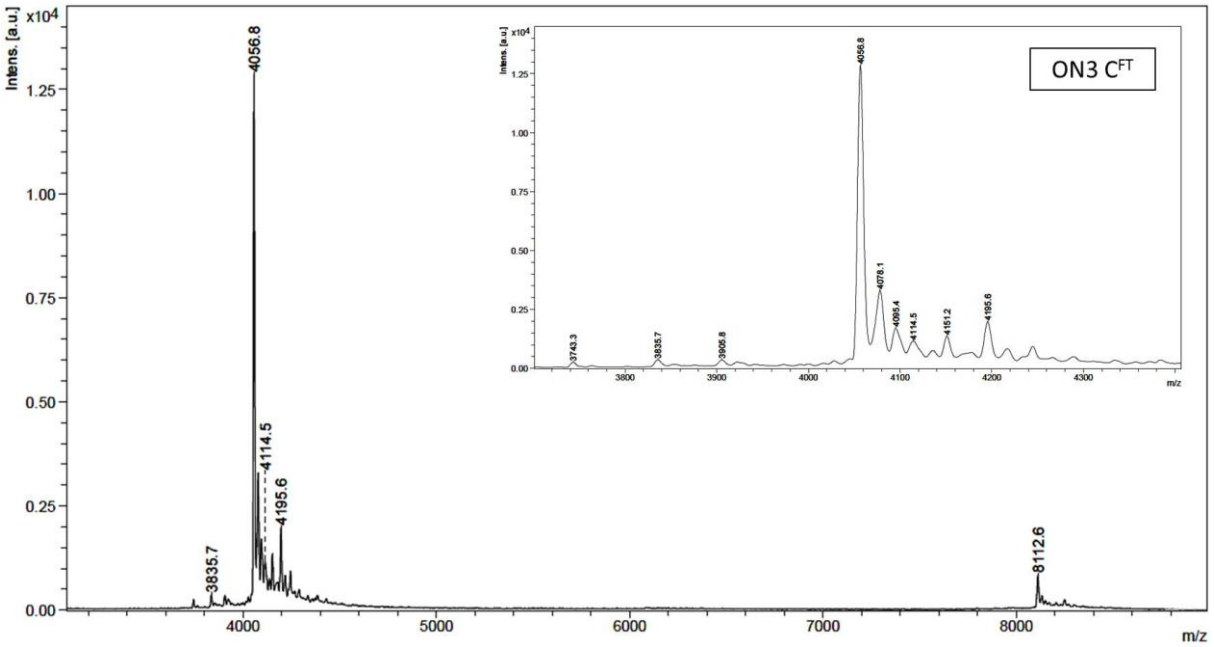

Figure S27. MALDI spectrum of **ON3 C^{FT}**. M (calc.) = 4055.8 Da, M (found) = 4056.8 Da ($[M+H]^+$).

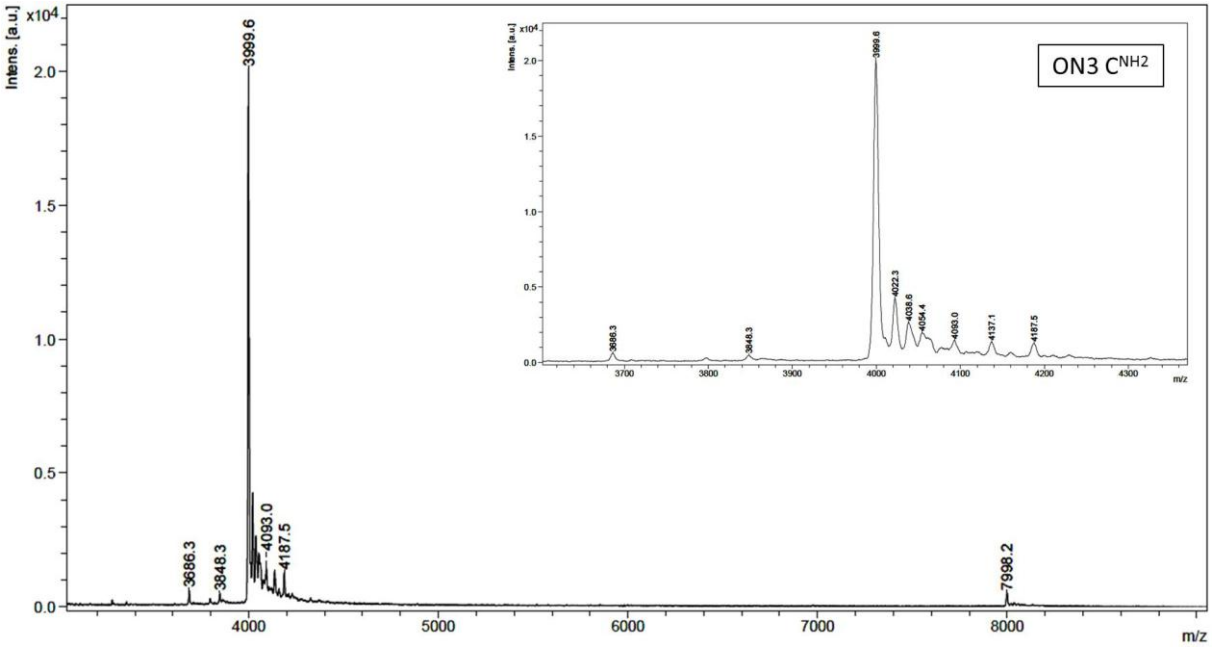

Figure S28. MALDI spectrum of **ON3 C^{NH2}**. M (calc.) = 3998.7 Da, M (found) = 3999.6 Da ($[M+H]^+$).

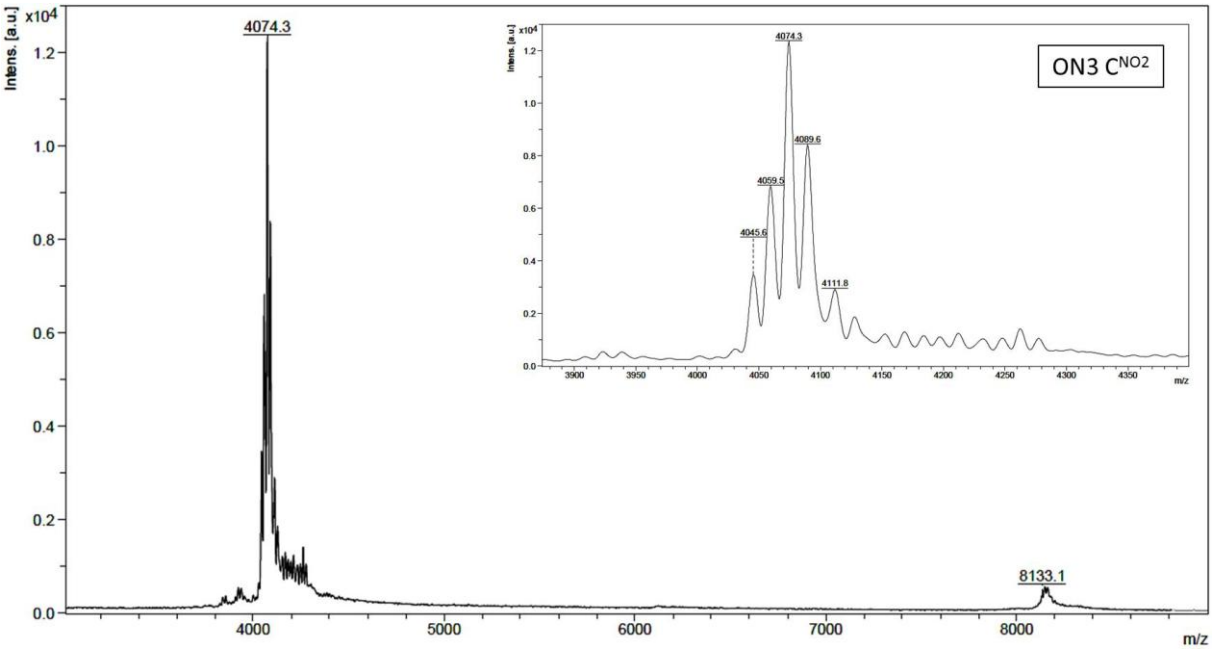

Figure S29. MALDI spectrum of **ON3 C^{NO2}**. M (calc.) = 4088.7 Da, M (found) = 4089.6 Da ($[M+H]^+$).

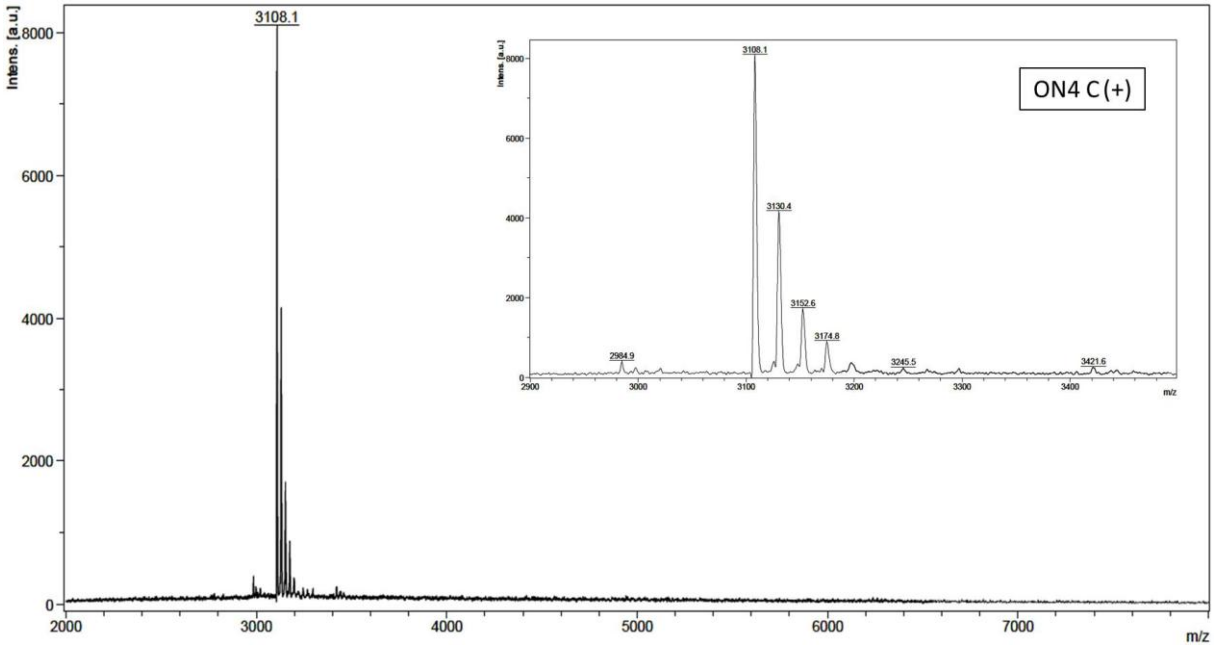

Figure S30. MALDI spectrum of **ON4 C(+)**. M (calc.) = 3107.0 Da, M (found) = 3108.1 Da ($[M+H]^+$).

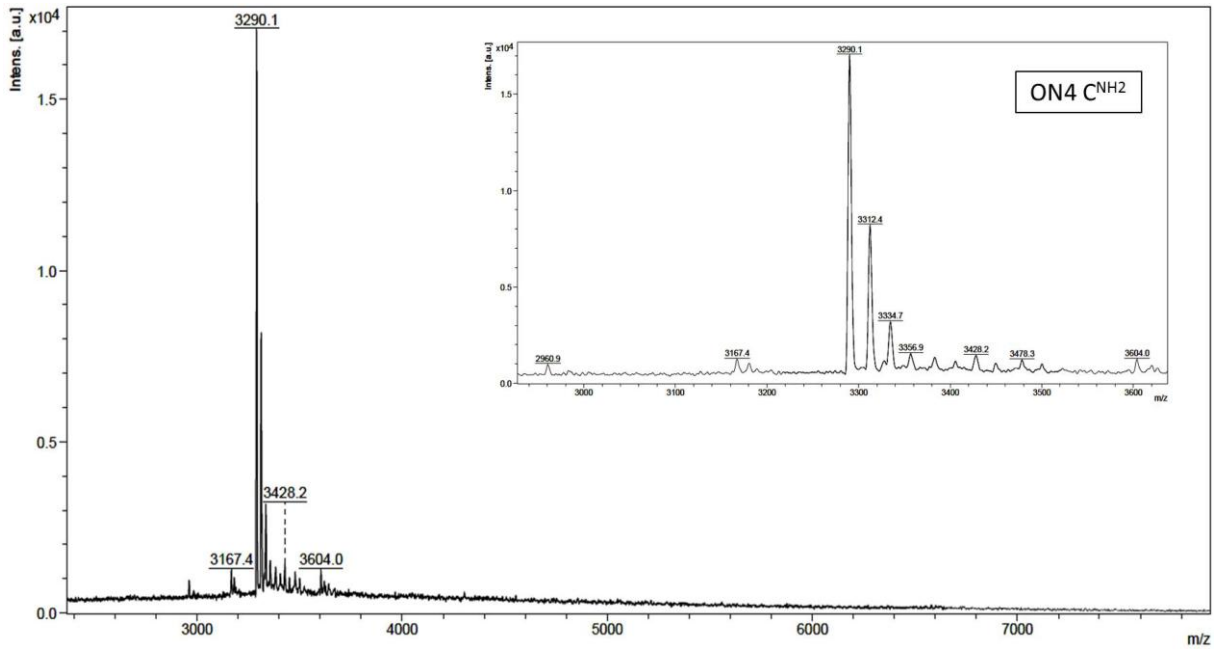

Figure S31. MALDI spectrum of **ON4 C^{NH2}**. M (calc.) = 3289.2 Da, M (found) = 3290.1 Da ($[M+H]^+$).

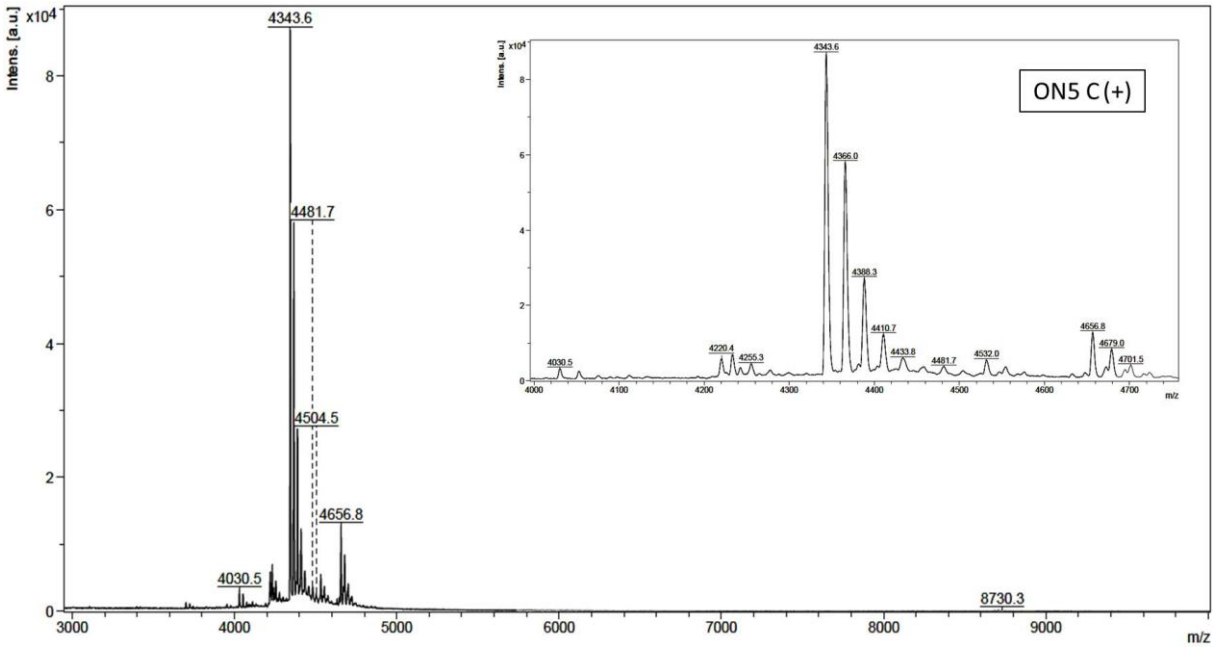

Figure S32. MALDI spectrum of **ON5 C(+)**. M (calc.) = 4342.8 Da, M (found) = 4343.6 Da ($[M+H]^+$).

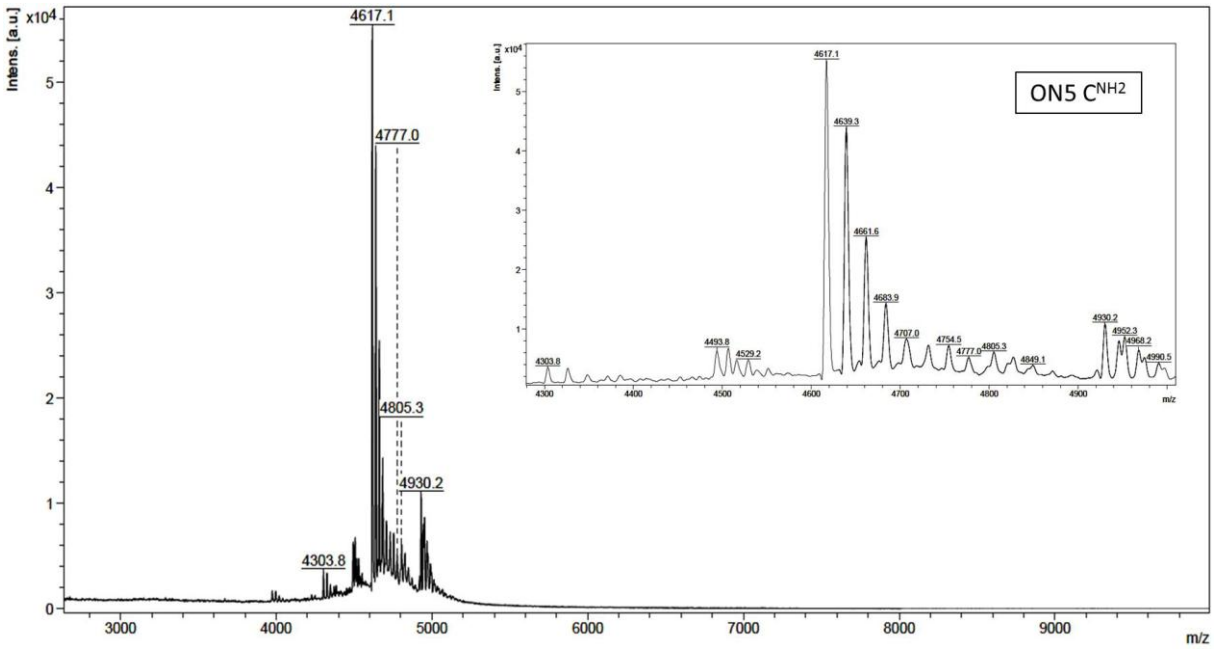

Figure S33. MALDI spectrum of **ON5 C^{NH2}**. M (calc.) = 4616.2 Da, M (found) = 4617.1 Da ($[M+H]^+$).

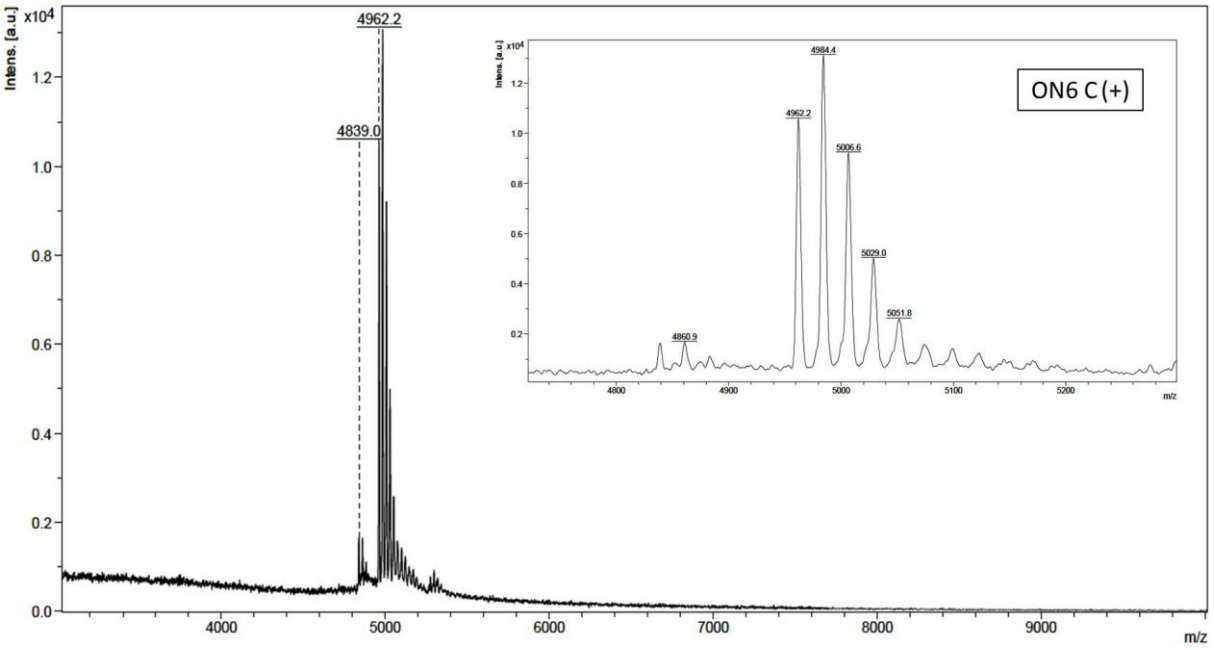

Figure S34. MALDI spectrum of **ON6 C(+)**. M (calc.) = 4961.2 Da, M (found) = 4962.2 Da ($[M+H]^+$).

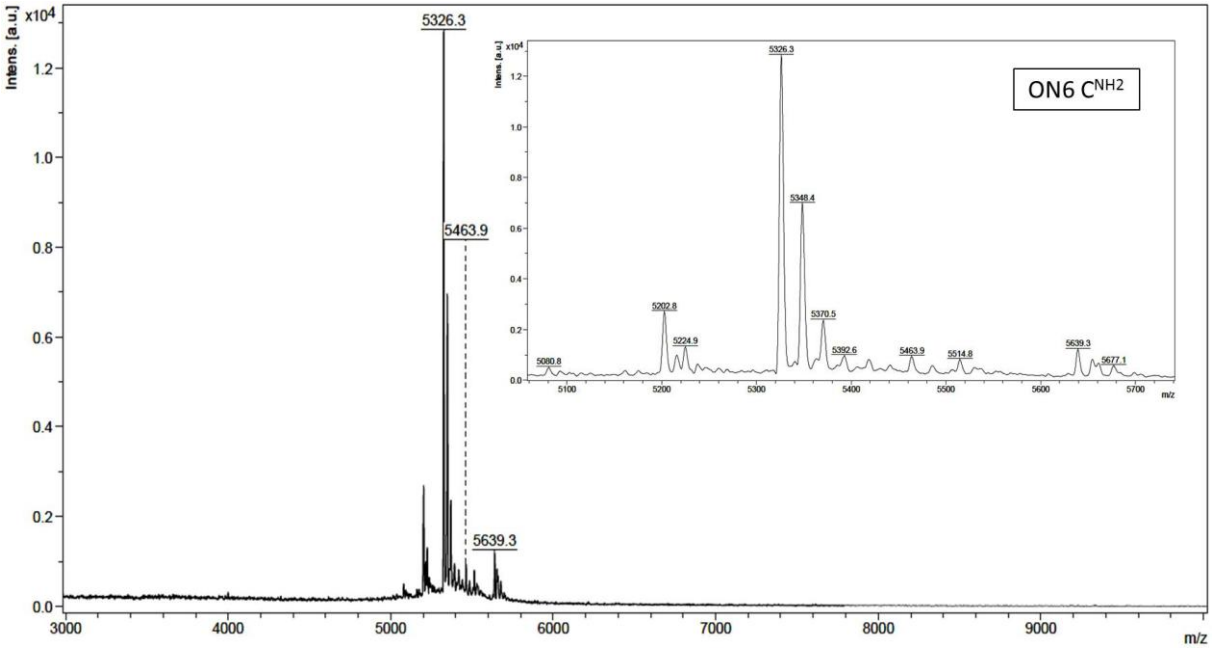

Figure S35. MALDI spectrum of **ON6 C^{NH2}**. M (calc.) = 5325.7 Da, M (found) = 5326.3 Da ($[M+H]^+$).

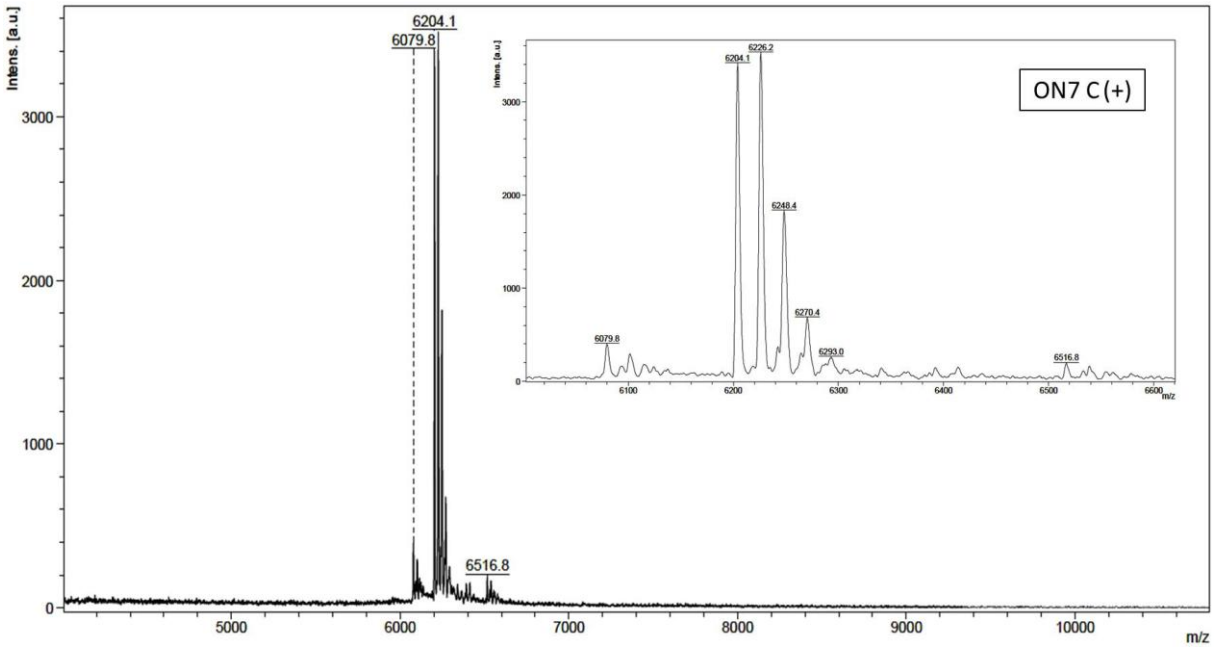

Figure S36. MALDI spectrum of **ON7 C(+)**. M (calc.) = 6203.0 Da, M (found) = 6204.1 Da ($[M+H]^+$).

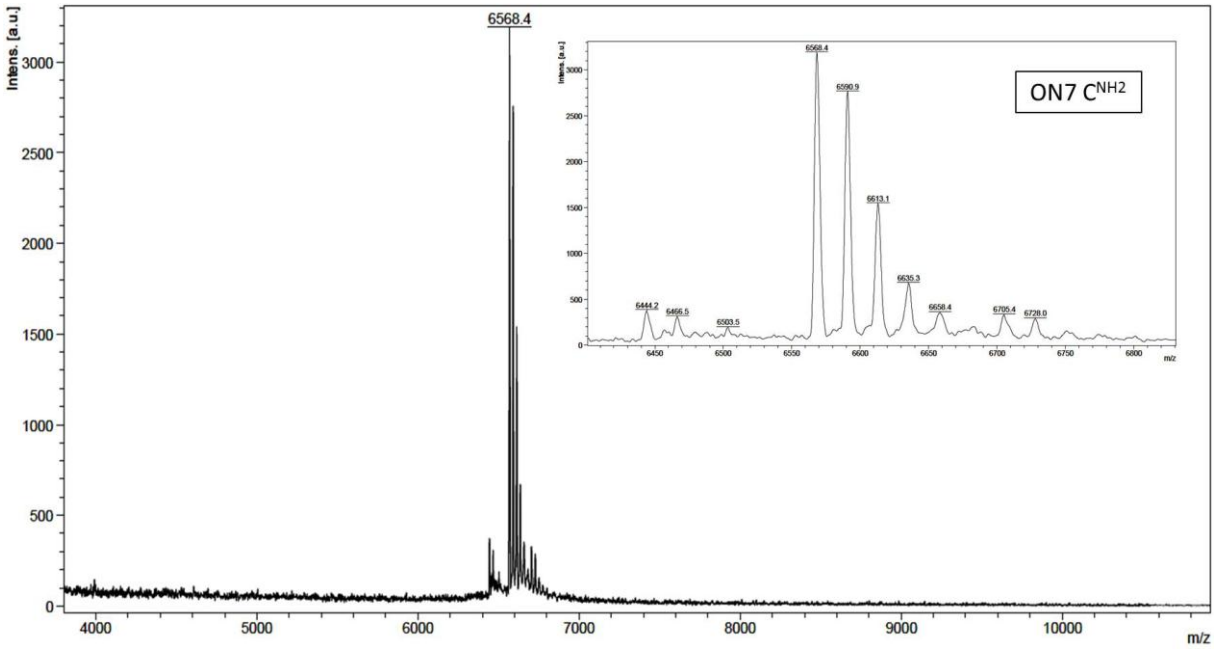

Figure S37. MALDI spectrum of **ON7 C^{NH2}**. M (calc.) = 6567.5 Da, M (found) = 6568.4 Da ($[M+H]^+$).

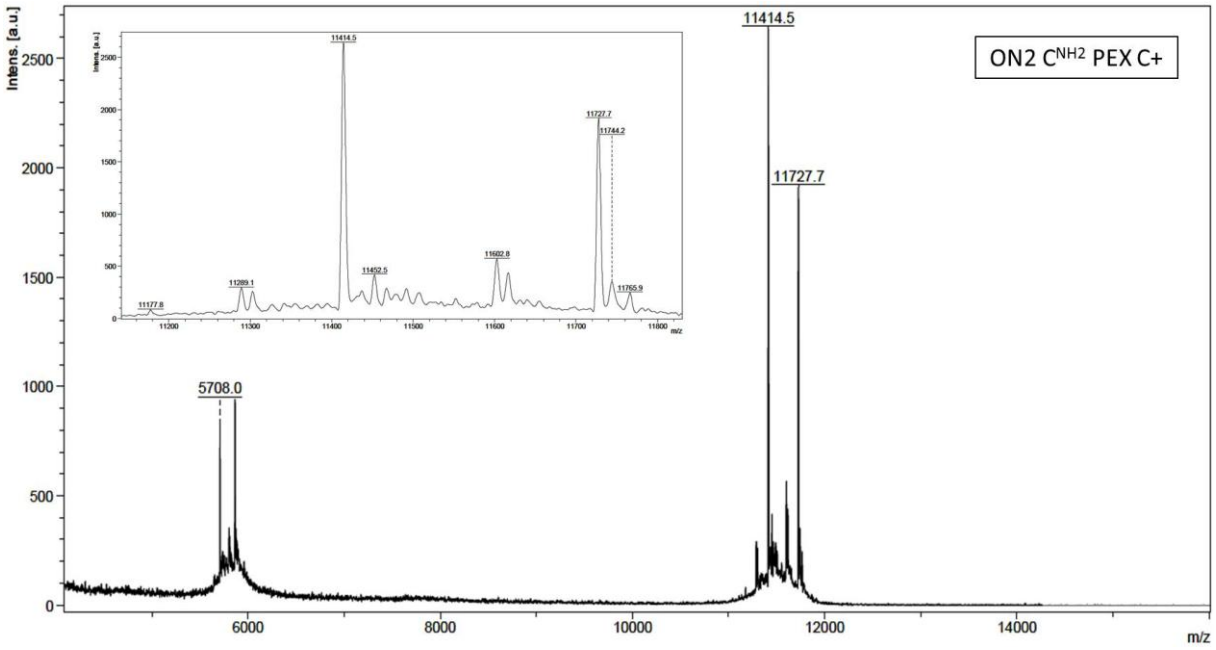

Figure S38. MALDI spectrum of **ON2 C^{NH2} PEX C(+)**. M (calc.) = 11 413.6 Da, M (found) = 11 414.5 Da ([M+H]$^+$).

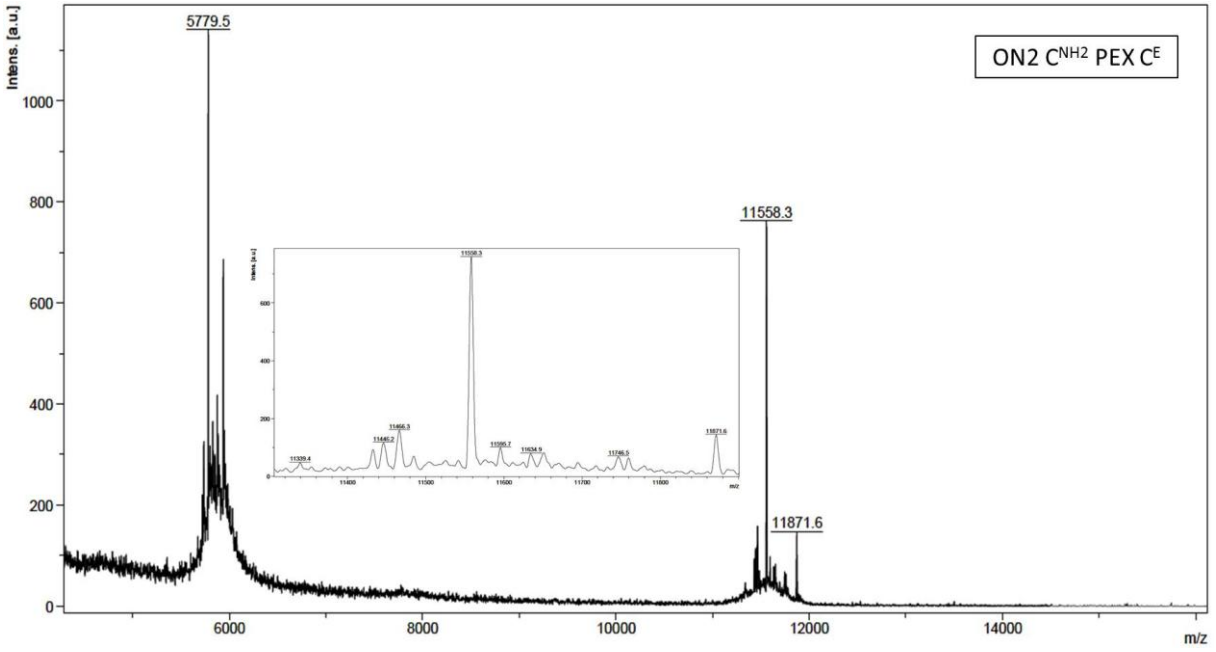

Figure S39. MALDI spectrum of **ON2 C^{NH2} PEX C^{E}**. M (calc.) = 11557.7 Da, M (found) = 11 558.3 Da ([M+H]$^+$).

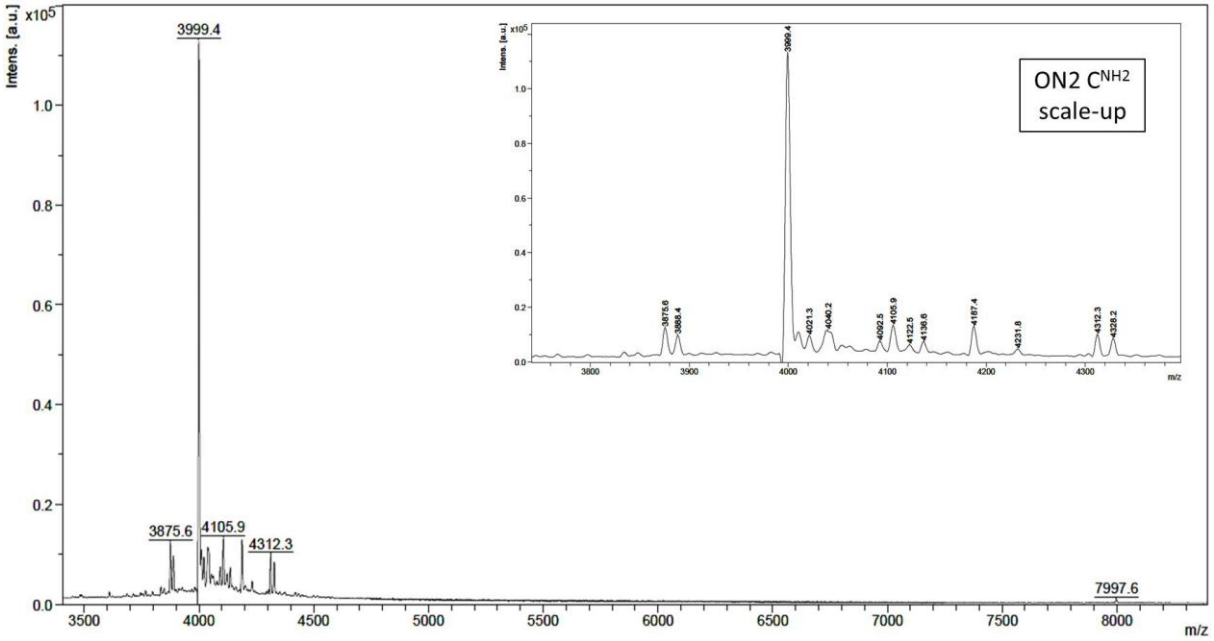

Figure S40. MALDI spectrum of **ON2 C^{NH2}** prepared on a preparative scale. M (calc.) = 3998.7 Da, M (found) = 3999.4 Da ($[M+H]^+$).

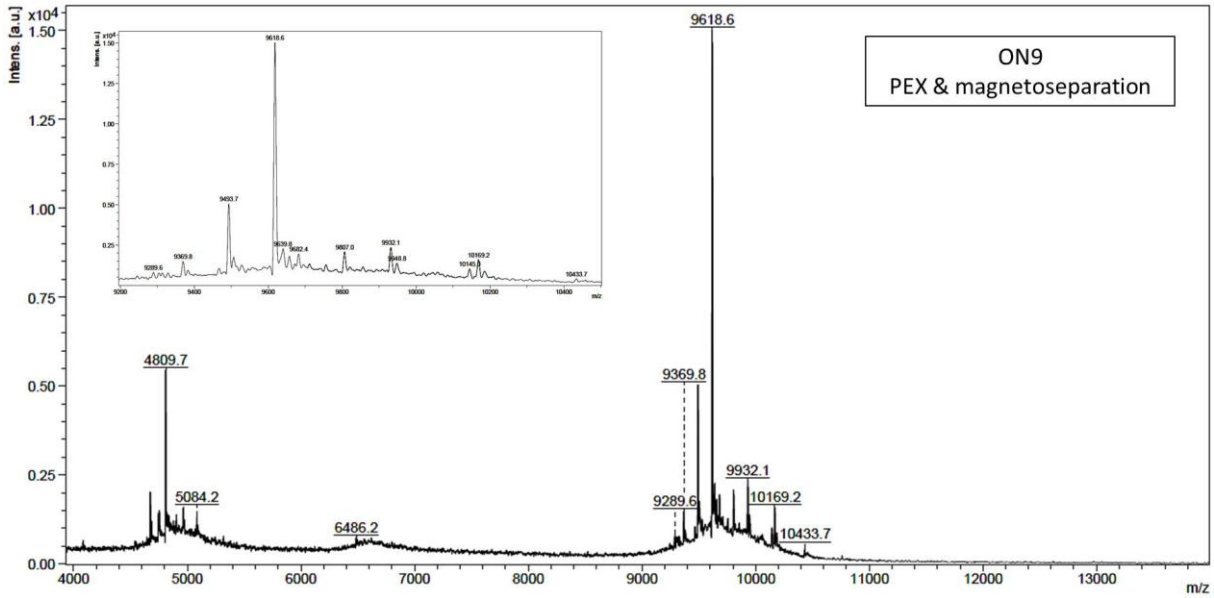

Figure S41. MALDI spectrum of PEX product **ON9** isolated by magnetoseparation. M (calc.) = 9617.3 Da, M (found) = 9618.6 Da ($[M+H]^+$).

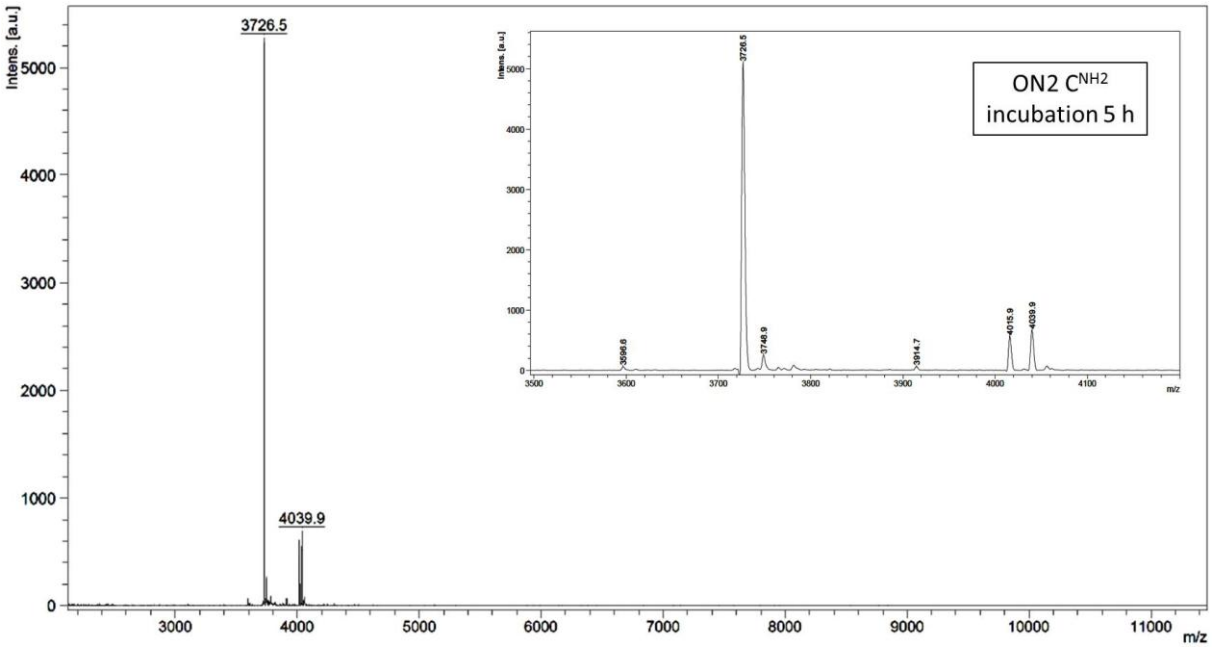

Figure S42. MALDI spectrum of the NEAR reaction incubated for 5 h.
Product **ON2 C(+)**: M (calc.) = 3725.4 Da, M (found) = 3726.5 Da ($[M+H]^+$).
Non-desired product **ON2 C(+)+A**: M (calc.) = 4038.7 Da, M (found) = 4039.9 Da ($[M+H]^+$).
Non-desired product **ON2 C(+)+C**: M (calc.) = 4014.6 Da, M (found) = 4015.9 Da ($[M+H]^+$).

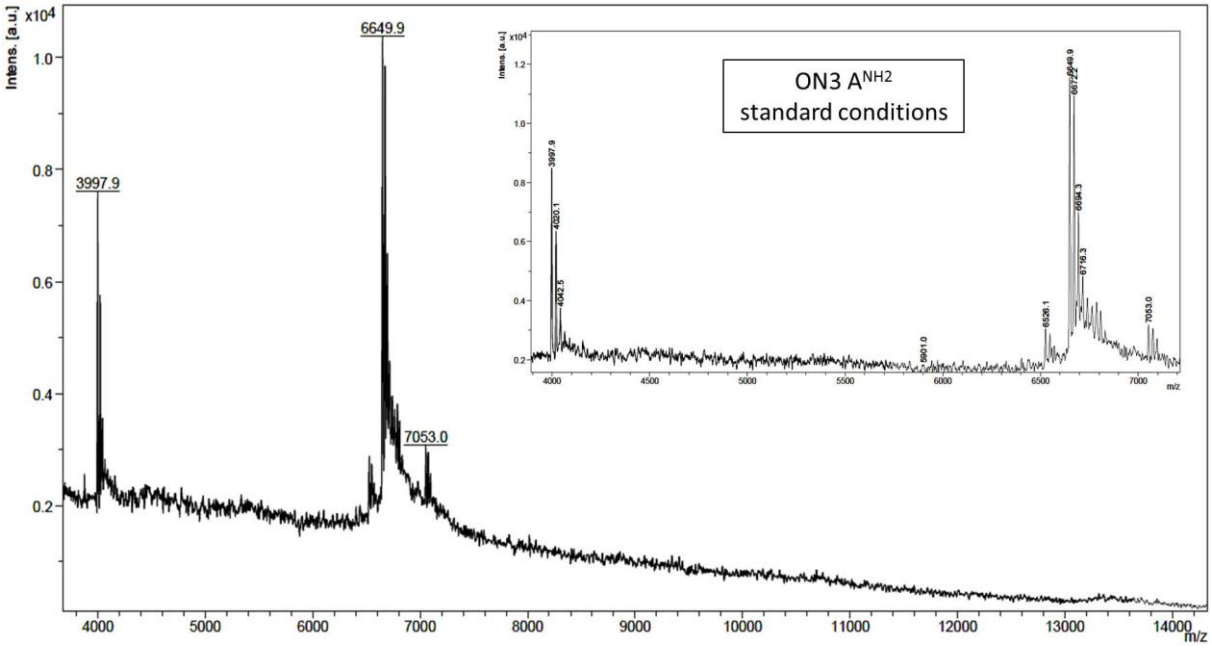

Figure S43. MALDI spectrum of the incorporation of $\mathbf{A^{NH2}}$ under standard reaction conditions. Product **ON3 $\mathbf{A^{NH2}}$**. M (calc.) = 3995.8 Da, M (found) = 3997.9 Da ($[M+H]^+$).

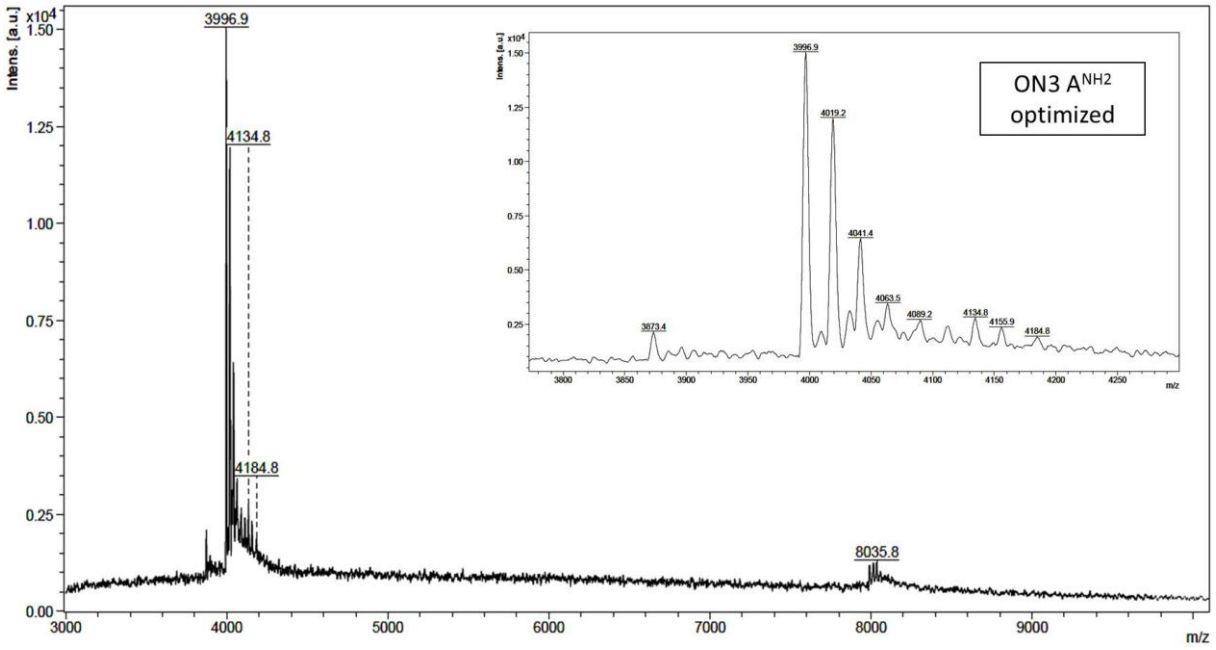

Figure S44. MALDI spectrum of the incorporation of $\mathbf{A^{NH2}}$ under optimized reaction conditions. Product **ON3** $\mathbf{A^{NH2}}$, 100 µM dNTPs, incubation time 2 h. M (calc.) = 3995.8 Da, M (found) = 3996.9 Da ($[M+H]^+$).

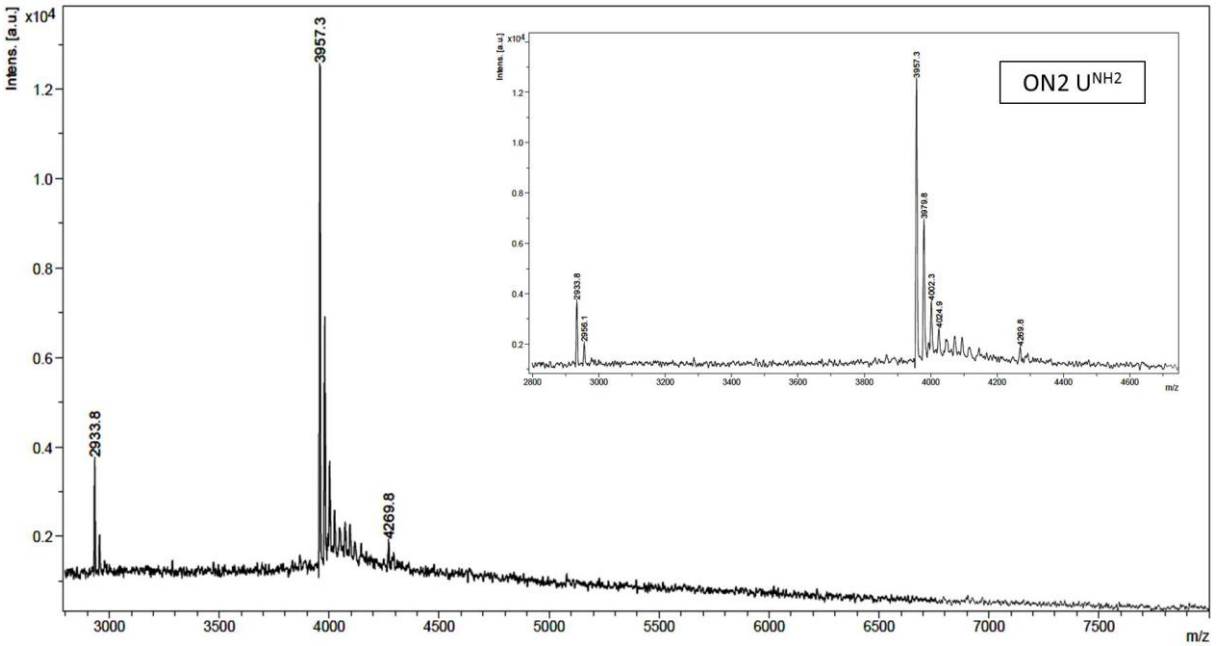

Figure S45. MALDI spectrum of the incorporation of $\mathbf{U^{NH2}}$ under standard reaction conditions. Product **ON2** $\mathbf{U^{NH2}}$. M (calc.) = 3955.7 Da, M (found) = 3957.3 Da ($[M+H]^+$).

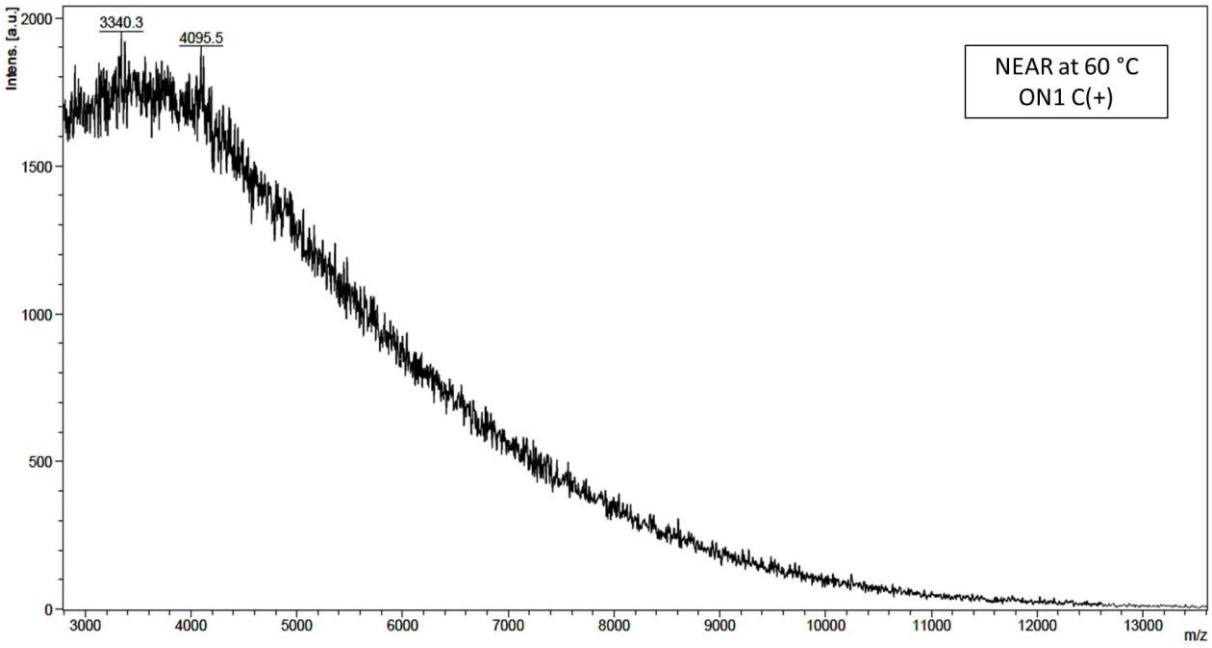

Figure S46. MALDI spectrum of the NEAR incubated at 60 °C. Template **Nick1**, natural dNTPs.

10. References

[1] J. Van Ness, L. K. Van Ness, D. J. Galas, *Proc. Nat. Ac. Sci.*, 2003, **100**, 4504–4509.

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