Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Traceless enzymatic synthesis of monodispersed hypermodified oligodeoxyribonucleotide polymers from RNA templates

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1) Experimental section

1.1 General remarks

All PAGEs were analyzed by FAM and Cy5 fluorescence imaging using Typhoon FLA 9500 (GE Healthcare). The MALDI-TOF spectra of modified ONs were measured using UltrafleXtreme MALDI-TOF/TOF (Bruker) mass spectrometer with 1 kHz smartbeam II laser technology. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ ammonium tartrate in ratio 9/1/1. The matrix (1 µL) was applied to the target (ground steel) and dried at room temperature. ESI spectra of modified ONs were measured using Xevo G2-XS QTof (Waters) mass spectrometer in 10 mM NH_4OAc buffer pH 9. Concentrations of modified ONs were determined using NanoDrop1000 (ThermoScientific). Synthetic ONs (5´-Cy5-labelled RNA templates, 5´-6-FAM-labelled primers, Table S1) were purchased from Generi Biotech. Natural dNTPs and RNase AT1 were purchased from ThermoFisher Scientific. Set of modified **dN^{ER}TP**s (Figure S1) were prepared according to published procedures.[1] KOD XL DNA polymerase was purchased

from Merck. Vent (exo-), *Bst* Large Fragment, *Bst* 2.0, *Bst* 3.0 DNA polymerases; AMV, Warmstart RTx, M-MuLV and ProtoScript II reverse transcriptases; RNase H and Terminal transferase (TdT) were all purchased from New England Biolabs. ddA^{Cy5}TP (NU-1612-CY) and ddU^{FAM}TP (NU-1619-5FM) were purchased from Jena Bioscience. Mili-Q water was used for all experiments. PAGE stop solution contained: 95% [v/v] formamide, 0.5 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] SDS and Milli-Q water. All reactions were separated on 12.5% PAGE (acrylamide/bisacrylamide 19:1, 25% urea) in 1X TBE buffer (25 mA, 1 h) without preheating. Hypermodified DNA (Table S2) were purified by C18 column (Waters X Bridge BEH C18 2.5 µm, 4.6 x 150 mm) heated by SICO-300 column oven (SISw) or by MicroSpin G-25 or G-50 (Cytiva illustra), Zeba (ThermoFisher Scientific), Mini Quick Spin Oligo or DNA (Roche) and Micro Bio-Spin P6 or P30 (Bio-Rad) spin columns. HPLC mobile phase pH was adjusted using InoLab pH 720. Other chemicals were of analytical grade.

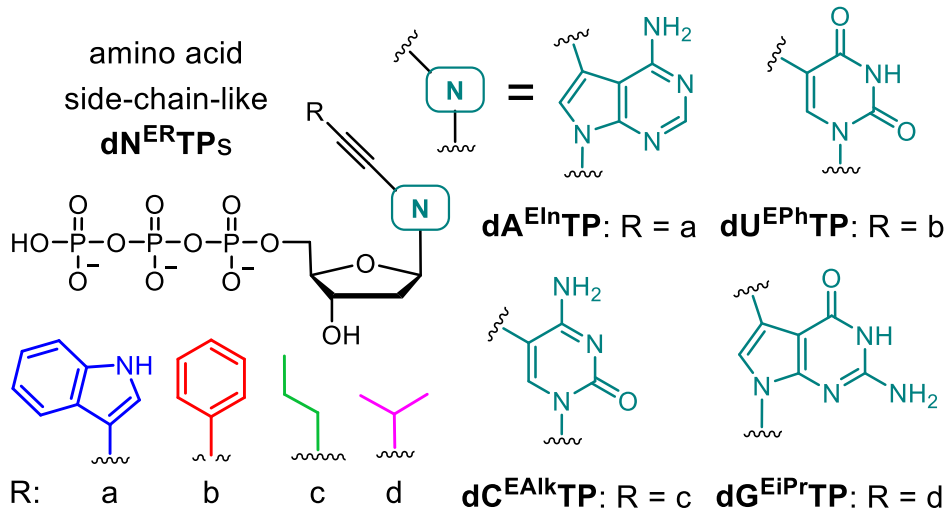


Figure S1. Chemical structures of modified **dN^{ER}TP**s.

Table S1. List of synthetic RNAs and DNAs used in this study

Title	Size [nt]	Sequence (5´→3´)
35RNA[a]	35	[rC][rA][rG][rU][rC][rU][rA][rG][rC][rA][rU][rG][rA][rG][rC][rU][rC][rA][rG][rU]<u>[rC][rC][rC][rA][rU][rG][rC][rC][rG][rC][rC][rC][rA][rU][rG]</u>
35RNAα[a]	35	[rA][rA][rC][rA][rA][rA][rG][rC][rU][rG][rU][rA][rG][rU][rA][rG][rU][rA][rU][rU]<u>[rC][rC][rC][rA][rU][rG][rC][rC][rG][rC][rC][rC][rA][rU][rG]</u>
35RNAβ[a]	35	[rU][rG][rU][rU][rU][rC][rG][rA][rC][rG][rG][rU][rU][rC][rA][rC][rU][rU][rA][rU]<u>[rC][rC][rC][rA][rU][rG][rC][rC][rG][rC][rC][rC][rA][rU][rG]</u>

35RNAγ[a]	35	[rA][rG][rC][rU][rG][rG][rG][rG][rU][rU][rC][rG][rU][rG][rU][rC][rG][rG][rU][rA]<u>[rC][rC][rC][rA][rU][rG][rC][rC][rG][rC][rC][rC][rA][rU][rG]</u>
35RNAδ[a]	35	[rU][rU][rC][rU][rC][rC][rU][rA][rC][rG][rG][rC][rG][rG][rG][rC][rU][rC][rC][rG]<u>[rC][rC][rC][rA][rU][rG][rC][rC][rG][rC][rC][rC][rA][rU][rG]</u>
60RNA[a]	60	[rA][rC][rA][rU][rC][rA][rU][rG][rA][rG][rA][rG][rA][rC][rA][rU][rC][rG][rC][rC][rU][rA][rG][rC][rA][rU][rG][rA][rG][rC][rU][rC][rA][rG][rU]<u>[rA][rA][rG][rG][rA][rA][rU][rA][rC][rA][rG][rG][rU][rA][rU][rU][rU][rU][rG][rU][rC][rC][rU][rU][rG]</u>
P15-I[b]	15	CATGGGCGGCATGGG
P15-II[b]	15	CATGGGCGGCATGG[rG]
P15-III[b]	15	CATGGG[rC]GGCATGG[rG]
P15-IV[b]	15	[rC][rA][rU][rG][rG][rG][rC][rG][rG][rC][rA][rU][rG][rG][rG]
P15-V[b]	15	[rC][rA][rU][rG][rG][rG][rC][rG][rG][rC][rA][rU][rG][rG]G
P25-I[b]	25	CAAGGACAAAATACCTGTATTCCTT

[a] 5´-Cy5; [b] 5´-6-FAM; [rN] ribonucleotide; primer regions underlined.

Table S2. List of enzymatically synthesized (hypermodified) DNA products

Title	Sequence (5´→3´)
35NA^{I}[a]	<u>CATGGGCGGCATGGG</u>ACTGAGCTCATGCTAGACTG
35ON^{I}	<u>CATGGGCGGCATGGG</u>ACTGAGCTCATGCTAGACTG
35NA^{I}_N^{ER}[a]	<u>CATGGGCGGCATGGG</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}
35ON^{I}_N^{ER}	<u>CATGGGCGGCATGGG</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}
35ON^{I}α_N^{ER}	<u>CATGGGCGGCATGGG</u>A^{EIn}A^{EIn}U^{EPh}A^{EIn}C^{EAlk}U^{EPh}A^{EIn}C^{EAlk}U^{EPh}A^{EIn}C^{EAlk}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}U^{EPh}U^{EPh}G^{EiPr}U^{EPh}U^{EPh}
35ON^{I}β_N^{ER}	<u>CATGGGCGGCATGGG</u>A^{EIn}U^{EPh}A^{EIn}A^{EIn}G^{EiPr}U^{EPh}G^{EiPr}A^{EIn}A^{EIn}C^{EAlk}C^{EAlk}G^{EiPr}U^{EPh}C^{EAlk}G^{EiPr}A^{EIn}A^{EIn}A^{EIn}C^{EAlk}A^{EIn}
35ON^{I}γ_N^{ER}	<u>CATGGGCGGCATGGG</u>U^{EPh}A^{EIn}C^{EAlk}C^{EAlk}G^{EiPr}A^{EIn}C^{EAlk}A^{EIn}C^{EAlk}G^{EiPr}A^{EIn}A^{EIn}C^{EAlk}C^{EAlk}C^{EAlk}C^{EAlk}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}
35ON^{I}δ_N^{ER}	<u>CATGGGCGGCATGGG</u>C^{EAlk}G^{EiPr}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}C^{EAlk}C^{EAlk}G^{EiPr}C^{EAlk}C^{EAlk}G^{EiPr}U^{EPh}A^{EIn}G^{EiPr}G^{EiPr}A^{EIn}G^{EiPr}A^{EIn}A^{EIn}
35NA^{II}_N^{ER}[a]	<u>CATGGGCGGCATGG[rG]</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}
35ON^{II}_N^{ER}	<u>CATGGGCGGCATGG[rG]</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}
36ON^{II}_N^{ER}	<u>CATGGGCGGCATGG[rG]</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{Cy5}
35NA^{III}_N^{ER}[a]	<u>CATGGG[rC]GGCATGG[rG]</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}
35ON^{III}_N^{ER}	<u>CATGGG[rC]GGCATGG[rG]</u>A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}
35ON^{III}α_N^{ER}	<u>CATGGG[rC]GGCATGG[rG]</u>A^{EIn}A^{EIn}U^{EPh}A^{EIn}C^{EAlk}U^{EPh}A^{EIn}C^{EAlk}U^{EPh}A^{EIn}

	$C^{EAlk}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}U^{EPh}U^{EPh}G^{EiPr}U^{EPh}U^{EPh}$
35ON$^{III}\beta$_N^{ER}	CATGGG[rC]GGCATGG[rG]$A^{EIn}U^{EPh}A^{EIn}A^{EIn}G^{EiPr}U^{EPh}G^{EiPr}A^{EIn}A^{EIn}C^{EAlk}$ $C^{EAlk}G^{EiPr}U^{EPh}C^{EAlk}G^{EiPr}A^{EIn}A^{EIn}A^{EIn}C^{EAlk}A^{EIn}$
35ON$^{III}\gamma$_N^{ER}	CATGGG[rC]GGCATGG[rG]$U^{EPh}A^{EIn}C^{EAlk}C^{EAlk}G^{EiPr}A^{EIn}C^{EAlk}A^{EIn}C^{EAlk}G^{EiPr}$ $A^{EIn}A^{EIn}C^{EAlk}C^{EAlk}C^{EAlk}C^{EAlk}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}$
35ON$^{III}\delta$_N^{ER}	CATGGG[rC]GGCATGG[rG]$C^{EAlk}G^{EiPr}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}C^{EAlk}C^{EAlk}G^{EiPr}$ $C^{EAlk}C^{EAlk}G^{EiPr}U^{EPh}A^{EIn}G^{EiPr}G^{EiPr}A^{EIn}G^{EiPr}A^{EIn}A^{EIn}$
60NA^{I}_N^{ER}[a]	CAAGGACAAAATACCTGTATTCCTT$A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}$ $C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}G^{EiPr}C^{EAlk}G^{EiPr}A^{EIn}U^{EPh}G^{EiPr}U^{EPh}C^{EAlk}$ $U^{EPh}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}A^{EIn}U^{EPh}G^{EiPr}U^{EPh}$
60ON^{I}_N^{ER}	CAAGGACAAAATACCTGTATTCCTT$A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}$ $C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}G^{EiPr}C^{EAlk}G^{EiPr}A^{EIn}U^{EPh}G^{EiPr}U^{EPh}C^{EAlk}$ $U^{EPh}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}A^{EIn}U^{EPh}G^{EiPr}U^{EPh}$
20ON_N^{ER}	$A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}$ $C^{EAlk}U^{EPh}G^{EiPr}$
20ONα_N^{ER}	$A^{EIn}A^{EIn}U^{EPh}A^{EIn}C^{EAlk}U^{EPh}A^{EIn}C^{EAlk}U^{EPh}A^{EIn}C^{EAlk}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}U^{EPh}U^{EPh}$ $G^{EiPr}U^{EPh}U^{EPh}$
20ONβ_N^{ER}	$A^{EIn}U^{EPh}A^{EIn}A^{EIn}G^{EiPr}U^{EPh}G^{EiPr}A^{EIn}A^{EIn}C^{EAlk}C^{EAlk}G^{EiPr}U^{EPh}C^{EAlk}G^{EiPr}A^{EIn}A^{EIn}$ $A^{EIn}C^{EAlk}A^{EIn}$
20ONγ_N^{ER}	$U^{EPh}A^{EIn}C^{EAlk}C^{EAlk}G^{EiPr}A^{EIn}C^{EAlk}A^{EIn}C^{EAlk}G^{EiPr}A^{EIn}A^{EIn}C^{EAlk}C^{EAlk}C^{EAlk}C^{EAlk}A^{EIn}$ $G^{EiPr}C^{EAlk}U^{EPh}$
20ONδ_N^{ER}	$C^{EAlk}G^{EiPr}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}C^{EAlk}C^{EAlk}G^{EiPr}C^{EAlk}C^{EAlk}G^{EiPr}U^{EPh}A^{EIn}G^{EiPr}G^{EiPr}$ $A^{EIn}G^{EiPr}A^{EIn}A^{EIn}$
28ON_N^{ER}	GGCATGG[rG]$A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}$ $U^{EPh}A^{EIn}G^{EiPr}A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}$
21ON_N^{ER}	$A^{EIn}C^{EAlk}U^{EPh}G^{EiPr}A^{EIn}G^{EiPr}C^{EAlk}U^{EPh}C^{EAlk}A^{EIn}U^{EPh}G^{EiPr}C^{EAlk}U^{EPh}A^{EIn}G^{EiPr}A^{EIn}$ $C^{EAlk}U^{EPh}G^{EiPr}A^{Cy5}$

[a] NA = double-stranded RNA-DNA heteroduplex (complementary RNA strands are shown in Table S1); ON = single-stranded DNA; [rN] ribonucleotide; primer regions underlined.

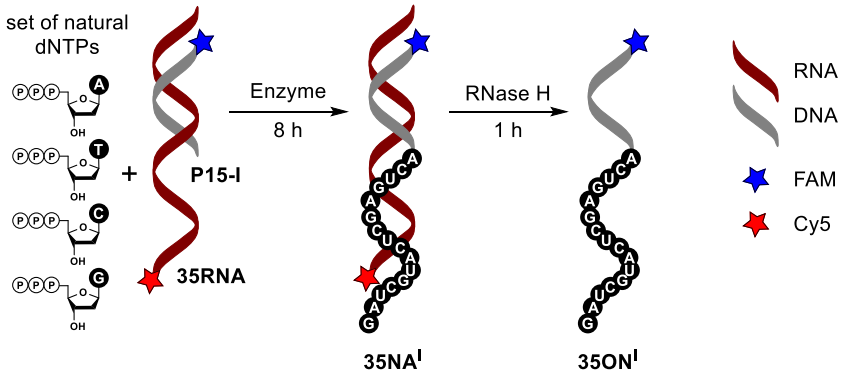
1.2 **Reverse transcription**

Method A: Reaction mixture (10 µL) contained Cy5-labelled RNA template (2.25 µM, 1 µL, Table S3), 5´-6-FAM-labelled primer (1.5 µM, 1 µL, Table S3), either natural dNTPs (1 µL, Table S4) or modified **dN^{ER}TP**s (1µL, Table S5), enzyme (Tables S4 and S5) and reaction buffer (10X, 1 µL) as supplied by the manufacturer. The reaction mixture was incubated for 8 h at enzyme-appropriate temperature (Tables S4 or S5) to achieve RNA-DNA hybrid and followed by incubation with RNase H (5 U) for 1 h at 37 °C to achieve ssON (Table S3). RNase H buffer supplied by the manufacturer was not used. Reaction

was stopped by cooling down to 4 °C and followed by addition of PAGE stop solution (11 µL). Samples were analyzed by PAGE using FAM and Cy5 fluorescence imaging (Figures S2 and S4). Control reactions without RNA template, *Bst* 3.0 DNA polymerase or dNTPs / **dN^{ER}TP**s were also performed (Figures S3, S5 and S6).

Table S3. Combination of RNA template and DNA primer used in reverse transcription

RNA template	DNA primer	Nucleotides	RNA-DNA hybrid	ssON
35RNA	P15-I	dNTPs	**35NA^{I}**	**35ON^{I}**
35RNA	P15-I	**dN^{ER}TP**s	**35NA^{I}_N^{ER}**	**35ON^{I}_N^{ER}**
60RNA	P25-I	**dN^{ER}TP**s	**60NA^{I}_N^{ER}**	**60ON^{I}_N^{ER}**

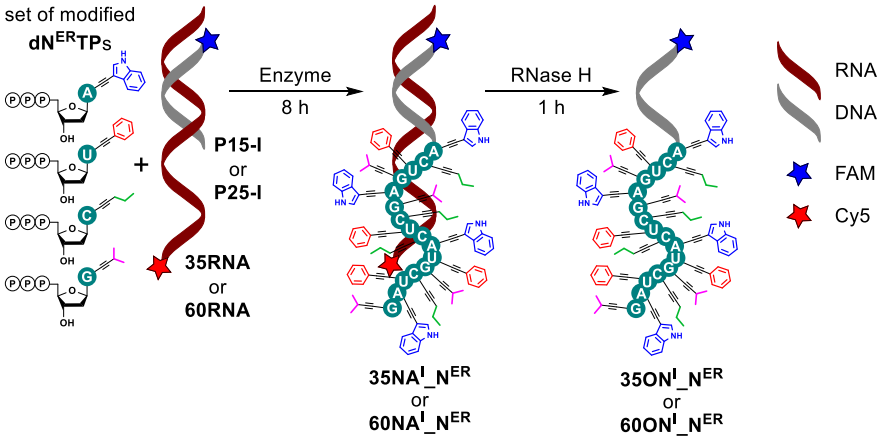


Scheme S1. Reverse transcription with natural dNTPs followed by RNA template digestion.

Table S4. Reaction conditions specifications for RT with natural dNTPs

Enzyme	Temperature [°C]	Enzyme [U]	c(dNTPs) [mM]	Figure S2 lanes
M-MuLV	42	40	0.05	1
ProtoScript II	42	100	0.1	2
AMV	42	10	2	3
Warmstart RTx	55	15	0.1	4
KOD XL	60	0.8-2.5[a]	0.1-4[a]	5
Vent (exo-)	60	10	4	6
Bst Large Fragment	65	8	0.5	7
Bst 2.0	65	4	0.1	8
Bst 3.0	65	4	0.1	9

[a] no conditions within this range worked.



Scheme S2. Reverse transcription with modified **dN^{ER}TP**s followed by RNA template digestion.

Table S5. Reaction conditions specifications for RT with modified **dN^{ER}TP**s

Enzyme	Temperature [°C]	Enzyme [U]	c(**dN^{ER}TP**s) [mM]		Figure S4 lanes
			35ON^{I}_N^{ER}	**60ON^{I}_N^{ER}**	
M-MuLV	42	40-120[a]	0.1-1[a]	NP	1
ProtoScript II	42	100-200[a]	0.5-2[a]	NP	2
AMV	42	5-10[a]	1-4[a]	NP	3
Warmstart RTx	55	7.5-15[a]	0.5-2[a]	NP	4
KOD XL	60	1.25	4	2	5
Vent (exo-)	60	6	2	1	6
Bst Large Fragment	65	8	2	1	7
Bst 2.0	65	4	2	4	8
Bst 3.0	65	4	2	6	9

[a] no conditions within this range worked; NP - reaction not performed.

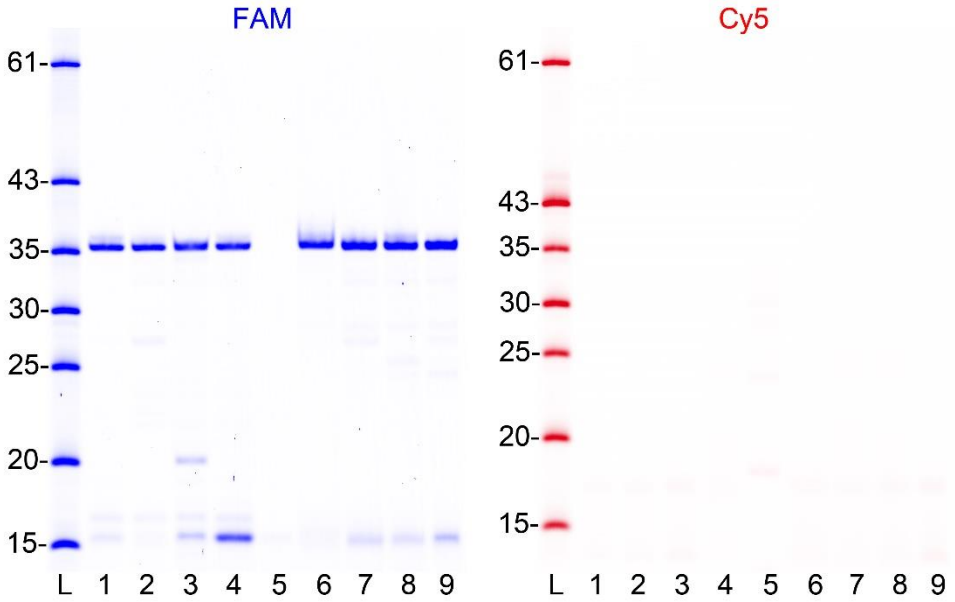


Figure S2. FAM (left) and Cy5 (right) scan of PAGE analysis of **35ON^{I}** after reverse transcription with various enzymes and RNA template digestion: (L) single-stranded ladder; (lanes 1) M-MuLV; (lanes 2) ProtoScript II; (lanes 3) AMV; (lanes 4) Warmstart RTx; (lanes 5) KOD XL; (lanes 6) Vent (exo-); (lanes 7) *Bst* Large Fragment; (lanes 8) *Bst* 2.0; (lanes 9) *Bst* 3.0.

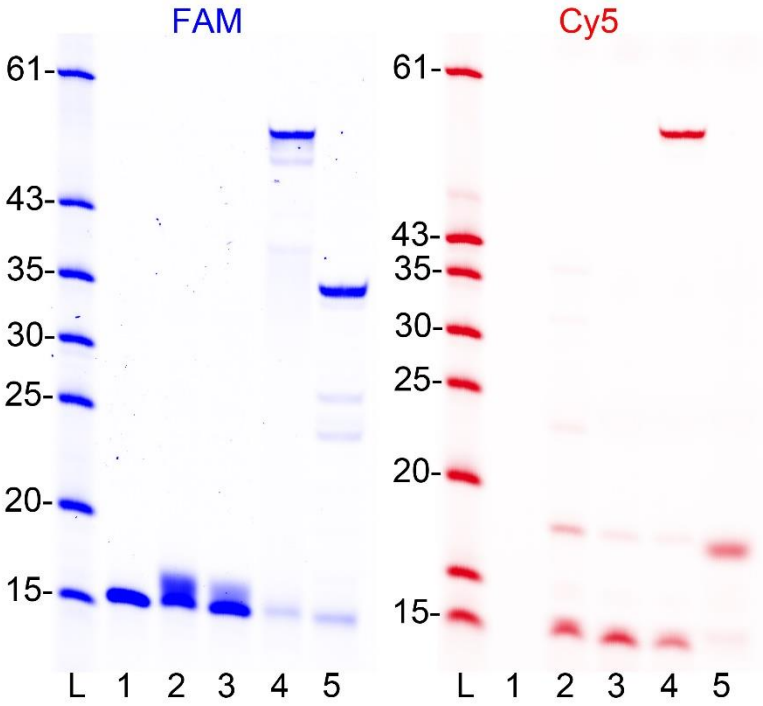


Figure S3. FAM (left) and Cy5 (right) scan of PAGE analysis of control reactions in synthesis of **35NA^{I}** and **35ON^{I}** according to Method A with *Bst* 3.0 DNA polymerase: (L) single-stranded ladder; (lanes 1) without 35RNA template; (lanes 2) without *Bst* 3.0 DNA polymerase; (lanes 3) without dNTPs; (lanes 4) **35NA^{I}**; (lanes 5) **35ON^{I}**.

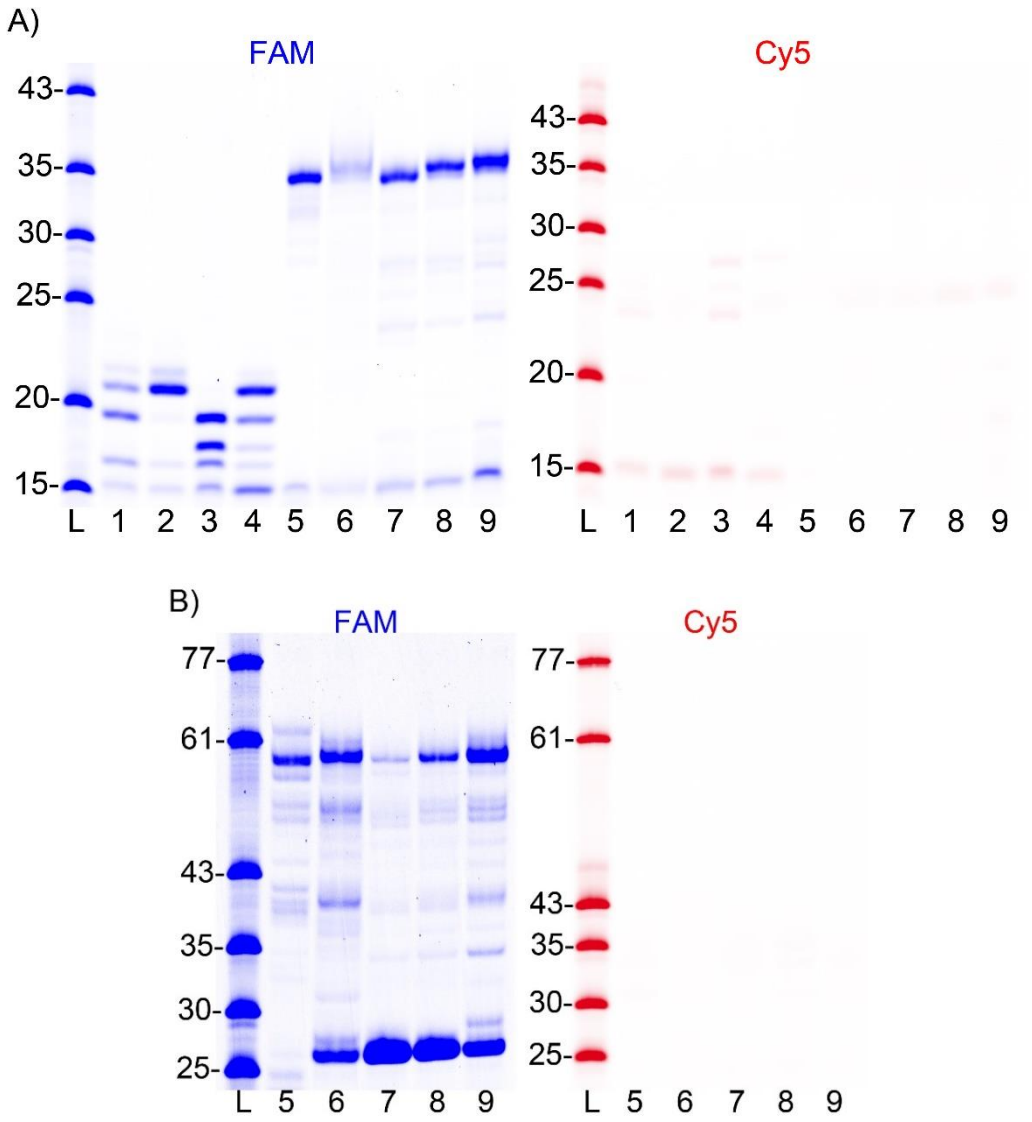


Figure S4. FAM (left) and Cy5 (right) scan of PAGE analysis of **35ON^{I}_N^{ER}** (A) and **60ON^{I}_N^{ER}** (B) after reverse transcription with various enzymes and RNA template digestion: (L) single-stranded ladder; (lanes 1) M-MuLV; (lanes 2) ProtoScript II; (lanes 3) AMV; (lanes 4) Warmstart RTx; (lanes 5) KOD XL; (lanes 6) Vent (exo-); (lanes 7) *Bst* Large Fragment; (lanes 8) *Bst* 2.0; (lanes 9) *Bst* 3.0.

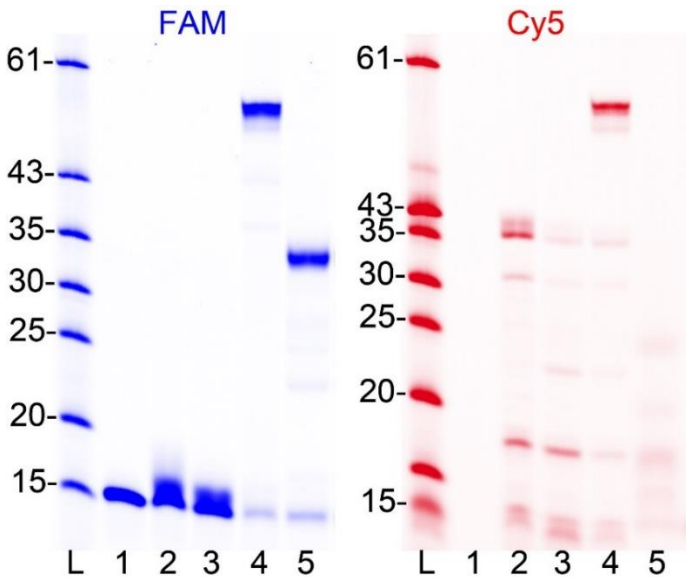


Figure S5. FAM (left) and Cy5 (right) scan of PAGE analysis of control reactions in synthesis of **35NA^{I}_N^{ER}** and **35ON^{I}_N^{ER}** according to Method A using *Bst* 3.0 DNA polymerase: (L) single-stranded ladder; (lanes 1) without 35RNA template; (lanes 2) without *Bst* 3.0 DNA polymerase; (lanes 3) without **dN^{ER}TPs**; (lanes 4) **35NA^{I}_N^{ER}**; (lanes 5) **35ON^{I}_N^{ER}**.

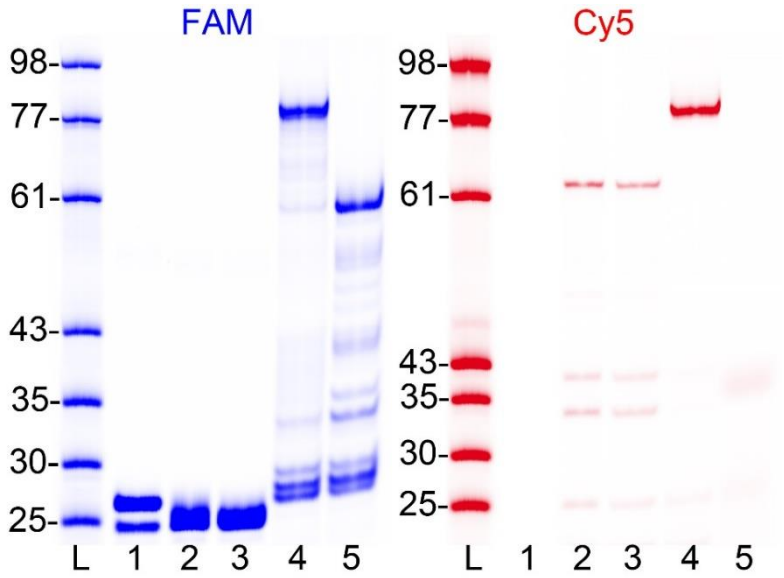


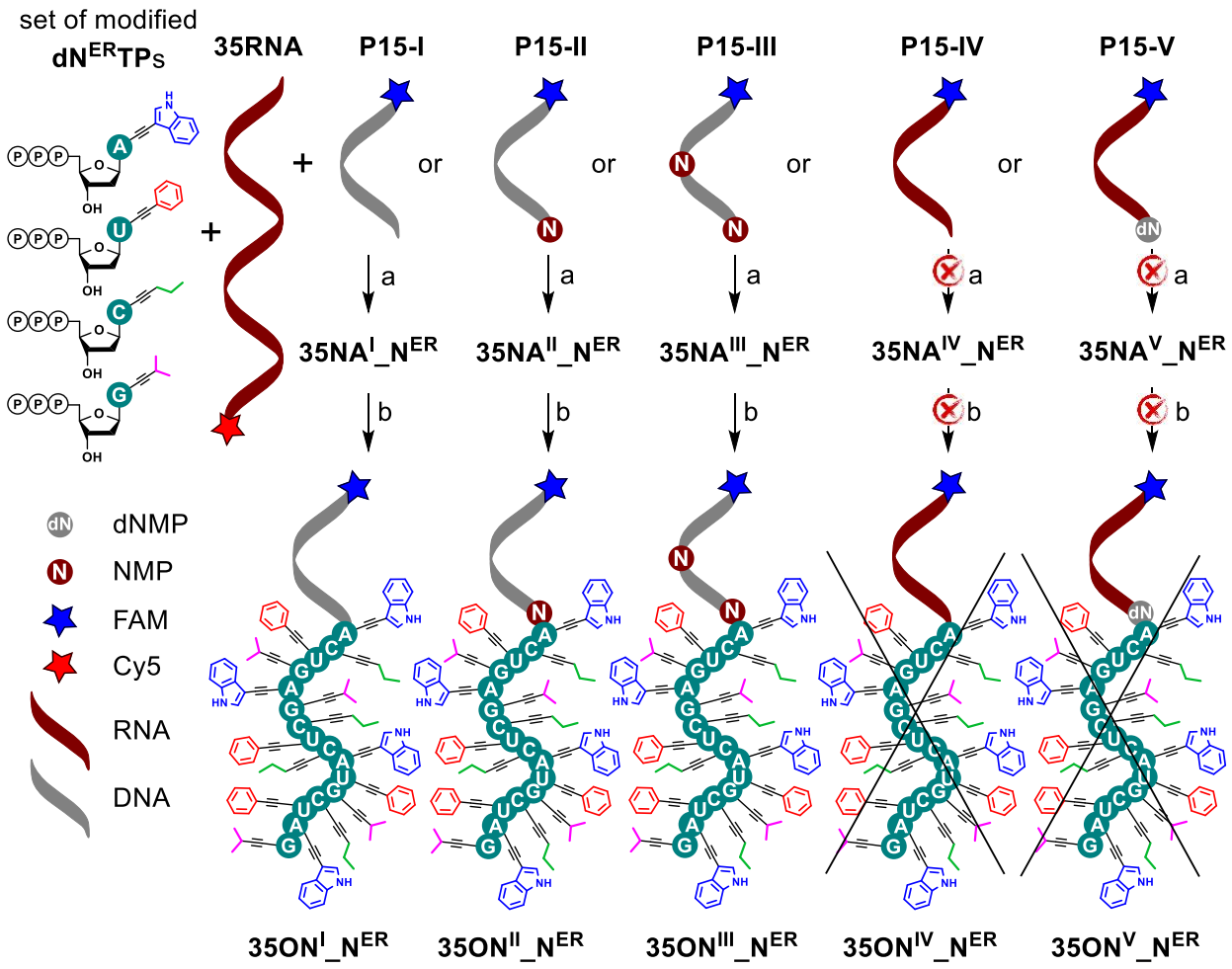
Figure S6. FAM (left) and Cy5 (right) scan of PAGE analysis of control reactions in synthesis of **60NA^{I}_N^{ER}** and **60ON^{I}_N^{ER}** according to Method A using *Bst* 3.0 DNA polymerase: (L) single-stranded ladder; (lanes 1) without 60RNA template; (lanes 2) without *Bst* 3.0 DNA polymerase; (lanes 3) without **dN^{ER}TPs**; (lanes 4) **60NA^{I}_N^{ER}**; (lanes 5) **60ON^{I}_N^{ER}**.

1.3 **Reverse transcription – various primers**

Reverse transcription with template and primer combinations showed in Table S6 were performed according to Method A described in Section 1.2 using only *Bst* 3.0 DNA polymerase (Scheme S3). PAGE analysis of these reactions is shown in Figure S7.

Table S6. Combination of RNA template and DNA primer used in reverse transcription with various primers

RNA template	DNA primer	Nucleotides	RNA-DNA hybrid	ssON
35RNA	P15-I	**dN^{ER}TP**s	**35NA^{I}_N^{ER}**	**35ON^{I}_N^{ER}**
35RNA	P15-II	**dN^{ER}TP**s	**35NA^{II}_N^{ER}**	**35ON^{II}_N^{ER}**
35RNA	P15-III	**dN^{ER}TP**s	**35NA^{III}_N^{ER}**	**35ON^{III}_N^{ER}**
35RNA	P15-IV	**dN^{ER}TP**s	**35NA^{IV}_N^{ER}**	**35ON^{IV}_N^{ER}**
35RNA	P15-V	**dN^{ER}TP**s	**35NA^{V}_N^{ER}**	**35ON^{V}_N^{ER}**



Scheme S3. Reverse transcriptions with modified **dN^{ER}TP**s and P15-I – P15-V primers. Conditions: a) *Bst* 3.0 DNA polymerase (4 U, 8 h, 65 °C); b) RNase H (5 U, 1 h, 37 °C).

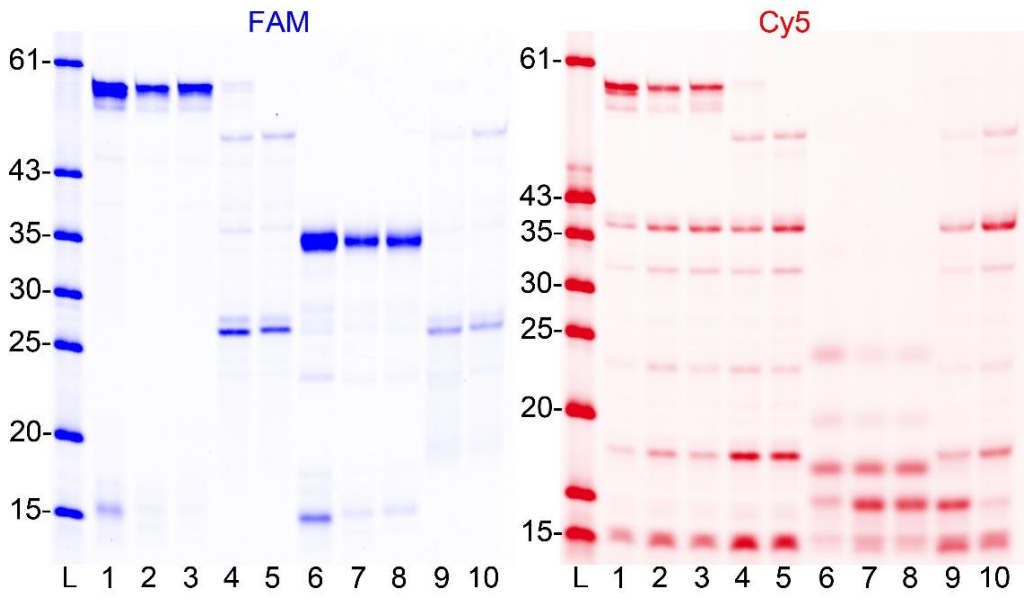


Figure S7. FAM (left) and Cy5 (right) scan of PAGE analysis of RT reactions performed with P15-I – P15-V primers not followed (lanes 1-5) or followed (lanes 6-10) by RNA template digestion with RNase H: (L) single-stranded ladder; (lanes 1) P15-I, **35NA^{I}_N^{ER}** product; (lanes 2) P15-II, **35NA^{II}_N^{ER}** product; (lanes 3) P15-III, **35NA^{III}_N^{ER}** product; (lanes 4) P15-IV, no product; (lanes 5) P15-V, no product; (lanes 6) P15-I, **35ON^{I}_N^{ER}** product; (lanes 7) P15-II, **35ON^{II}_N^{ER}** product; (lanes 8) P15-III, **35ON^{III}_N^{ER}** product; (lanes 9) P15-IV, product not formed; (lanes 10) P15-V, product not formed.

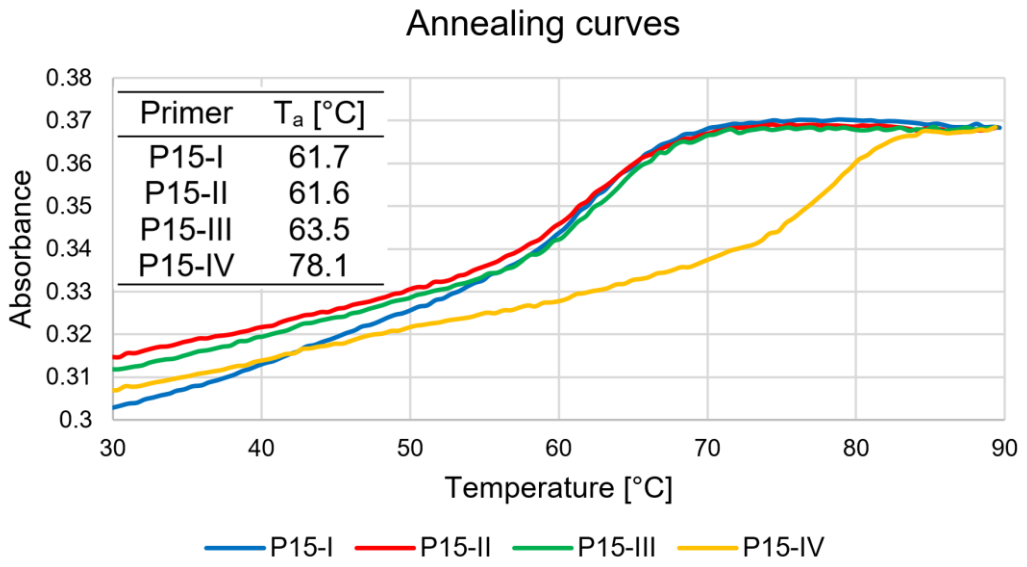
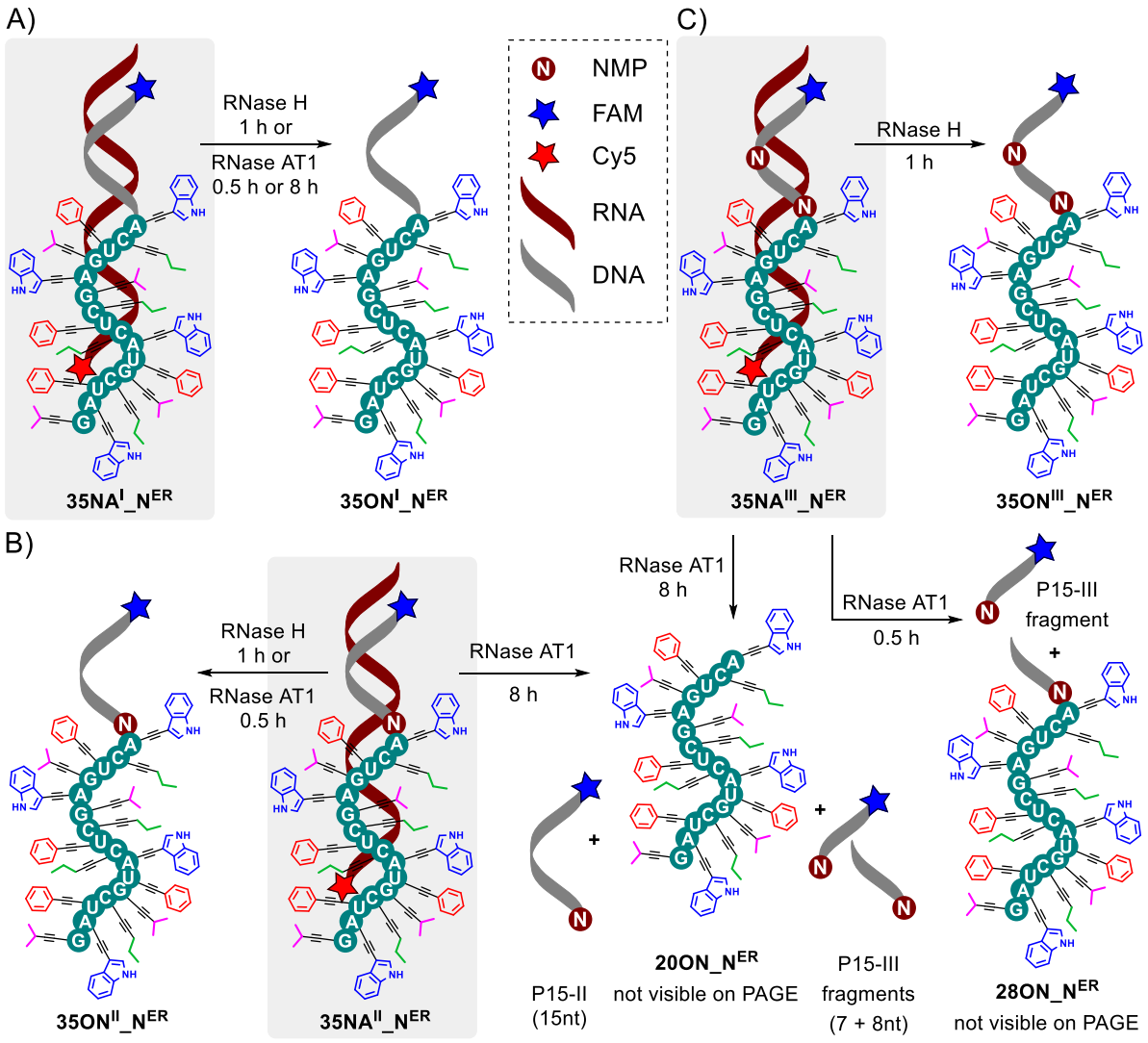


Figure S8. Annealing curves and calculated annealing temperatures (T_a) of 35RNA template with P15-I, P15-II, P15-III or P15-IV primers obtained from UV spectroscopy at 260 nm absorption. Measurements were carried out in TrisHCl buffer (10 mM, 1 mM EDTA, 65 mM NaCl, pH 8).

1.4 **RNA digestion – RNase H vs. RNase AT1**

Hypermodified RNA-DNA heteroduplexes (**35NA^{I}_N^{ER}**, **35NA^{II}_N^{ER}** and **35NA^{III}_N^{ER}**) prepared according to Method A described in Section 1.2. using *Bst* 3.0 DNA polymerase and appropriate P15-I, P15-II or P15-III primer were incubated with RNase H (5 U) or RNase AT1 (5 U) for specific time (Scheme S4 and Table S7). PAGE analysis is shown in Figure S9.



Scheme S4. Overview of enzyme and time-dependent digestion of reverse transcription products **35NA^{I}_N^{ER}** (A), **35NA^{II}_N^{ER}** (B) or **35NA^{III}_N^{ER}** (C) using RNase H or RNase AT1.

Table S7. Overview of RNase H and AT1 time dependence

Template	Primer	RNA-DNA hybrid	RNA digestion		Digestion product	Figure S9 lanes
			RNase	Time [h]		
35RNA	P15-I	**35NA^{I}_N^{ER}** (Fig. S9, lanes 4)	H	1	**35ON^{I}_N^{ER}**	5
			AT1	0.5	**35ON^{I}_N^{ER}**	6
			AT1	8	**35ON^{I}_N^{ER}**	7
	P15-II	**35NA^{II}_N^{ER}** (Fig. S9, lanes 8)	H	1	**35ON^{II}_N^{ER}**	9
			AT1	0.5	**35ON^{II}_N^{ER}**	10
			AT1	8	**20ON_N^{ER}**	11
	P15-III	**35NA^{III}_N^{ER}** (Fig. S9, lanes 12)	H	1	**35ON^{III}_N^{ER}**	13
			AT1	0.5	**28ON_N^{ER}**	14
			AT1	8	**20ON_N^{ER}**	15

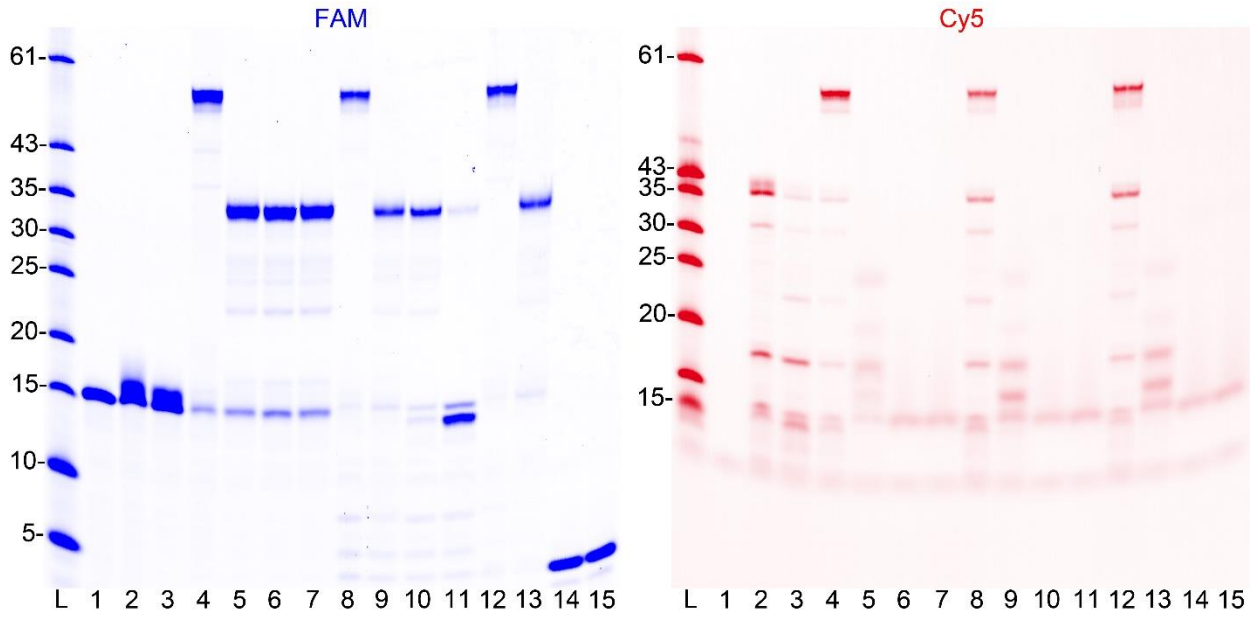


Figure S9. FAM (left) and Cy5 (right) scan of PAGE analysis after time-dependent digestion of **35NA^{I}_N^{ER}**, **35NA^{II}_N^{ER}** and **35NA^{III}_N^{ER}** using RNase H or RNase AT1: (L) single-stranded ladder; (lanes 1) control without 35RNA template; (lanes 2) control without *Bst* 3.0 DNA polymerase; (lanes 3) control without **dN^{ER}TP**s; (lanes 4) **35NA^{I}_N^{ER}**; (lanes 5-7) **35ON^{I}_N^{ER}**; (lanes 8) **35NA^{II}_N^{ER}**; (lanes 9-10) **35ON^{II}_N^{ER}**; (lanes 11) **20ON_N^{ER}**, not visible on PAGE; (lanes 12) **35NA^{III}_N^{ER}**; (lanes 13) **35ON^{III}_N^{ER}**; (lanes 14) **28ON_N^{ER}**, not visible on PAGE; (lanes 15) **20ON_N^{ER}**, not visible on PAGE.

1.5 **Reverse transcription – semi-preparative scale and HPLC purification**

Method B: Reaction mixture (10 µL) contained Cy5-labelled RNA template (20 µM, 2 µL, Table S8), 5´-6-FAM-labelled primer (20 µM, 3 µL, Table S8), modified **dN^{ER}TP**s (1 µL, Table S8), *Bst* 3.0 DNA polymerase (24 U) and reaction buffer (10X, 1 µL) as supplied by the manufacturer. The reaction mixture was incubated for 16 h at 65 °C to achieve modified RNA-DNA hybrid and followed by incubation with RNase H (5 U) or AT1 (5 U) for specific time according to desired ssON (Table S7). RNase H or AT1 buffer supplied by the manufacturer were not used. All reactions were stopped by cooling down to 4 °C and purified by HPLC (can be seen further in this section, Figures S11-15). PAGE analyses of **35NA^{I}_N^{ER}** and **35ON^{I}_N^{ER}** or **60NA^{I}_N^{ER}** and **60ON^{I}_N^{ER}** in semi-preparative scale before HPLC purification are shown in Figure S10.

HPLC method: Synthesis described in Method B was multiplied by 20 reactions. Resulting 200 µL was injected for HPLC separation using C18 column heated for 60°C with use of linear gradient starting from 0.1 M TEAB in H_2O to 0.1 M TEAB in H_2O/MeCN (4:1) in 1 h and to MeCN in 1 h. Buffer pH was adjusted to 7.4 by CO_2 (g). HPLC fractions were freeze-dried and applied for MALDI-TOF or ESI measurements (Section 2, Figures S19-24).

Table S8. Combination of RNA template and DNA primer used in semi-preparative reverse transcription

RNA template	DNA primer	**dN^{ER}TP**s [mM]	RNA-DNA hybrid	ssON	RNase
35RNA	P15-I	2 mM	**35NA^{I}_N^{ER}**	**35ON^{I}_N^{ER}**	H
35RNA	P15-II	2 mM	**35NA^{II}_N^{ER}**	**20ON_N^{ER}**	AT1
35RNA	P15-III	2 mM	**35NA^{III}_N^{ER}**	**28ON_N^{ER}**, **20ON_N^{ER}**	AT1
60RNA	P25-I	4 mM	**60NA^{I}_N^{ER}**	**60ON^{I}_N^{ER}**	H

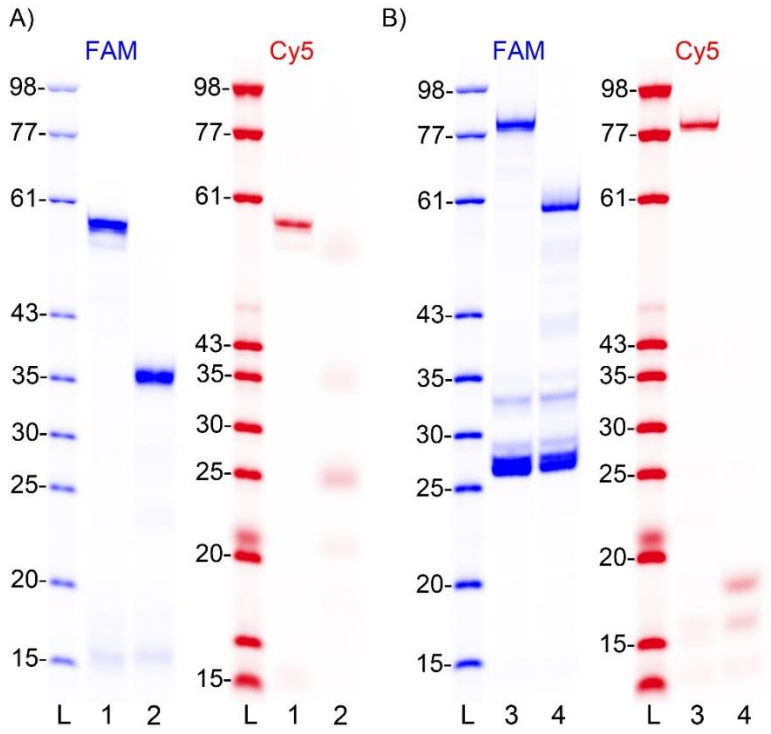


Figure S10. FAM (left) and Cy5 (right) scan of PAGE analyses of semi-preparative reverse transcription described by Method B for 35RNA (A) or 60RNA (B) template: (L) single-stranded ladder; (lanes 1) **35NA^{I}_N^{ER}**; (lanes 2) **35ON^{I}_N^{ER}**; (lanes 3) **60NA^{I}_N^{ER}**; (lanes 4) **60ON^{I}_N^{ER}**.

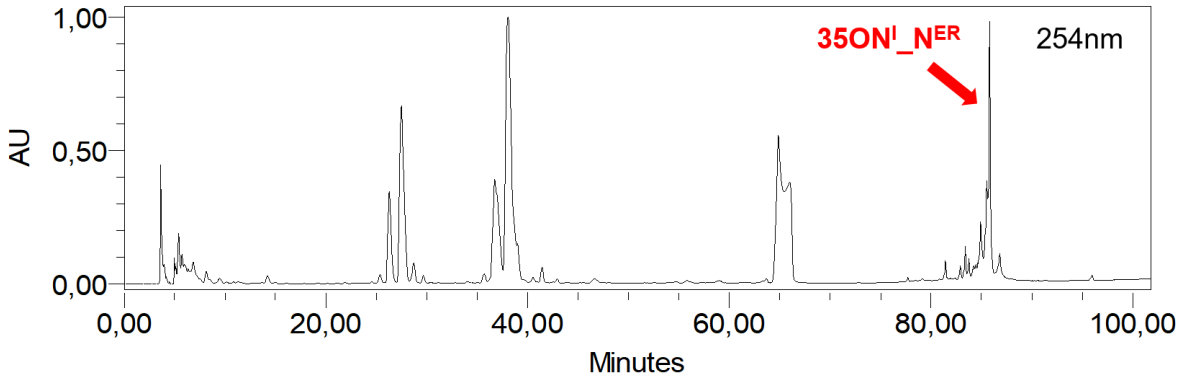


Figure S11. HPLC chromatogram with assigned fraction of FAM-labelled **35ON^{I}_N^{ER}** product, confirmed by MALDI-TOF (Figure S21) and ESI (Figure S22).

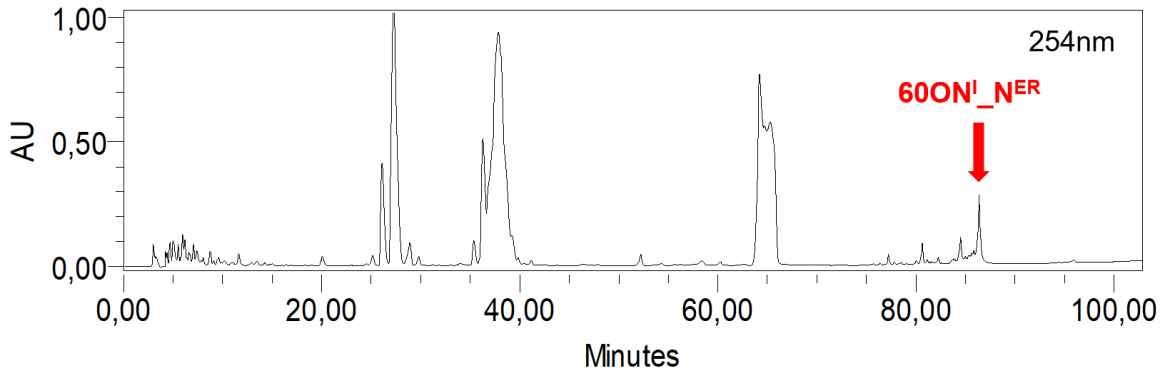


Figure S12. HPLC chromatogram with assigned fraction of FAM-labelled **60ON^{I}_N^{ER}** product, confirmed by ESI (Figure S23).

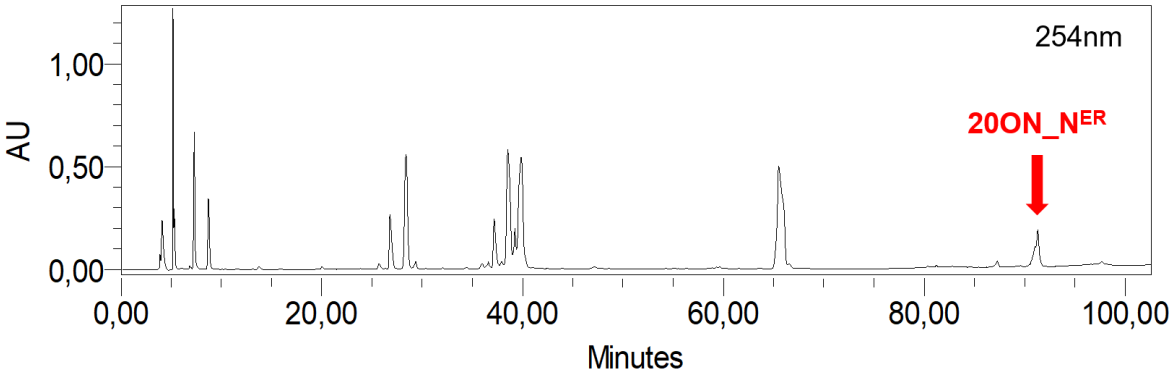


Figure S13. HPLC chromatogram with assigned fraction of non-labelled **20ON_N^{ER}** product after digestion of **35NA^{II}_N^{ER}** with RNase AT1, confirmed by ESI (Figure S24).

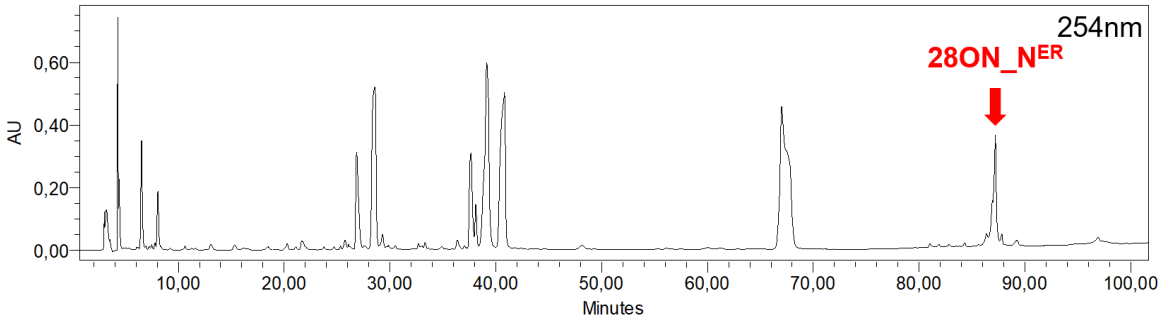


Figure S14. HPLC chromatogram with assigned fraction of non-labelled **28ON_N^{ER}** after digestion of **35NA^{III}_N^{ER}** with RNase AT1, confirmed by ESI (Figure S25).

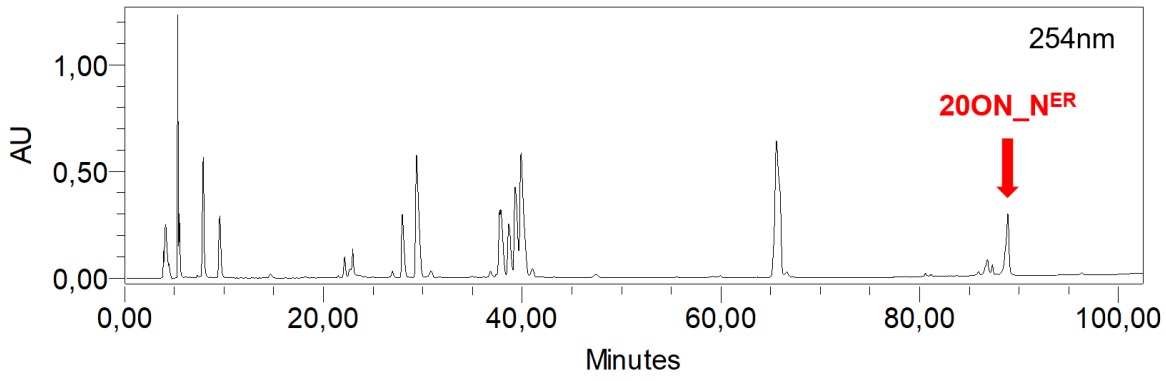


Figure S15. HPLC chromatogram with assigned fraction of non-labelled **20ON_N^{ER}** after digestion of **35NA^{III}_N^{ER}** with RNase AT1, confirmed by ESI (Figure S26).

1.6 Reverse transcription – spin column purification

Hypermodified **35ON^{I}_N^{ER}** and **20ON_N^{ER}** were prepared from **35NA^{I}_N^{ER}** and **35NA^{III}_N^{ER}**, respectively, using only RNase AT1 for RNA digestion according to procedure described in Section 1.4 and were purified by seven different spin columns (Table S9 and Figure S16). All purifications were performed using protocols provided by manufacturers. Hypermodified **35ON^{I}_N^{ER}** recovery and RNA template removal was calculated and is shown in Table S9. Since **20ON_N^{ER}** is not visible on PAGE, its recovery was not determined thus only removal of 7nt primer fragment was calculated. Furthermore, **20ON_N^{ER}** purified by Micro Bio-Spin P6 was confirmed by ESI (Figure S27).

Table S9. Spin column purification results

Spin column	**35ON^{I}_N^{ER}**		**20ON_N^{ER}**		Figure S16 lanes
	DNA recovery	RNA removal	P15-III fragment removal	RNA removal	
MicroSpin G-25	26%	100%	63%	100%	2
MicroSpin G-50	83%	100%	92%	100%	3
Zeba	85%	100%	14%	100%	4
Mini Quick Spin Oligo	63%	100%	38%	100%	5
Mini Quick Spin DNA	79%	100%	66%	100%	6
Micro Bio-Spin P6	77%	100%	37%	100%	7
Micro Bio-Spin P30	2%	100%	94%	100%	8

% calculated according to fluorescence signals using ImageJ quantification program.

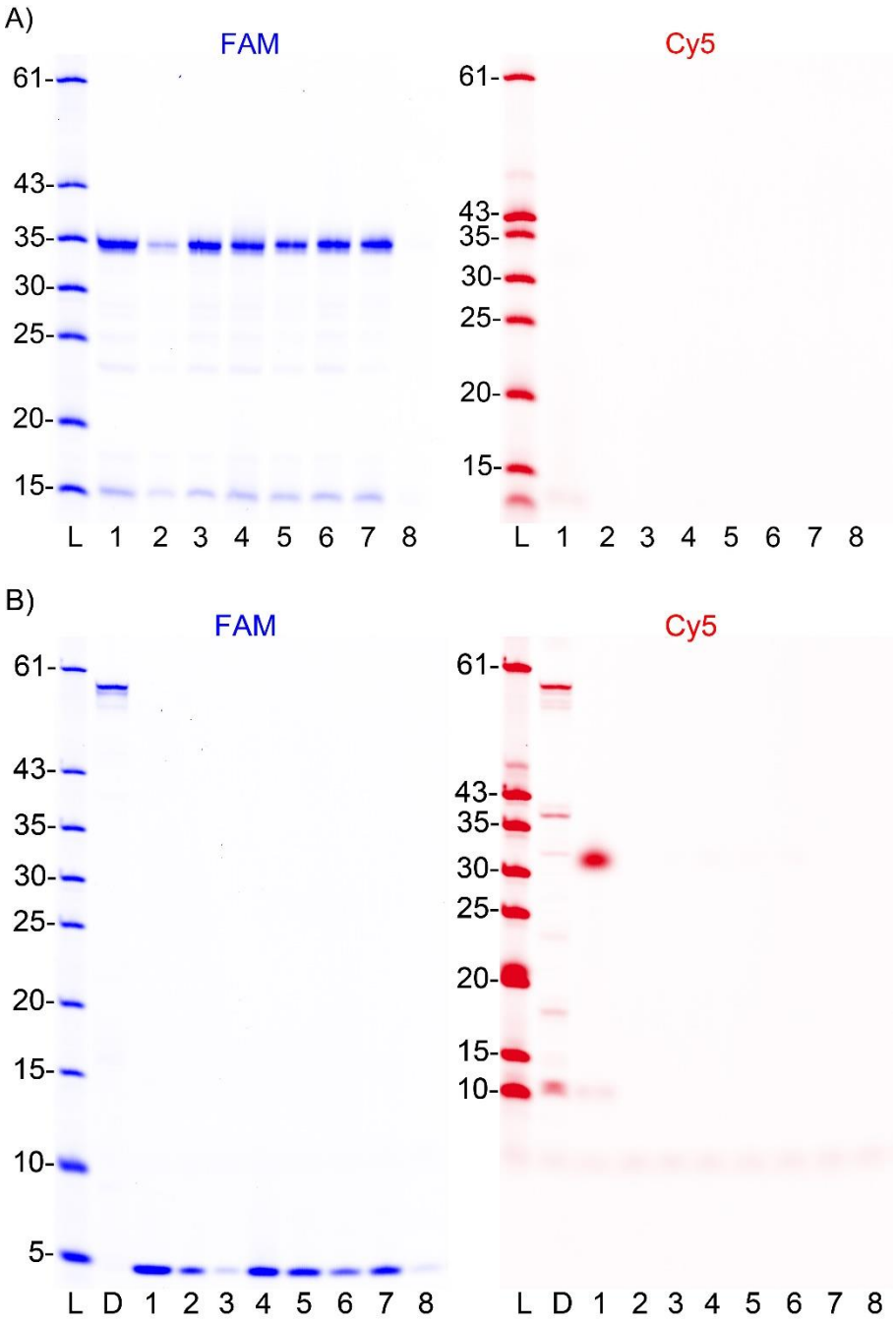
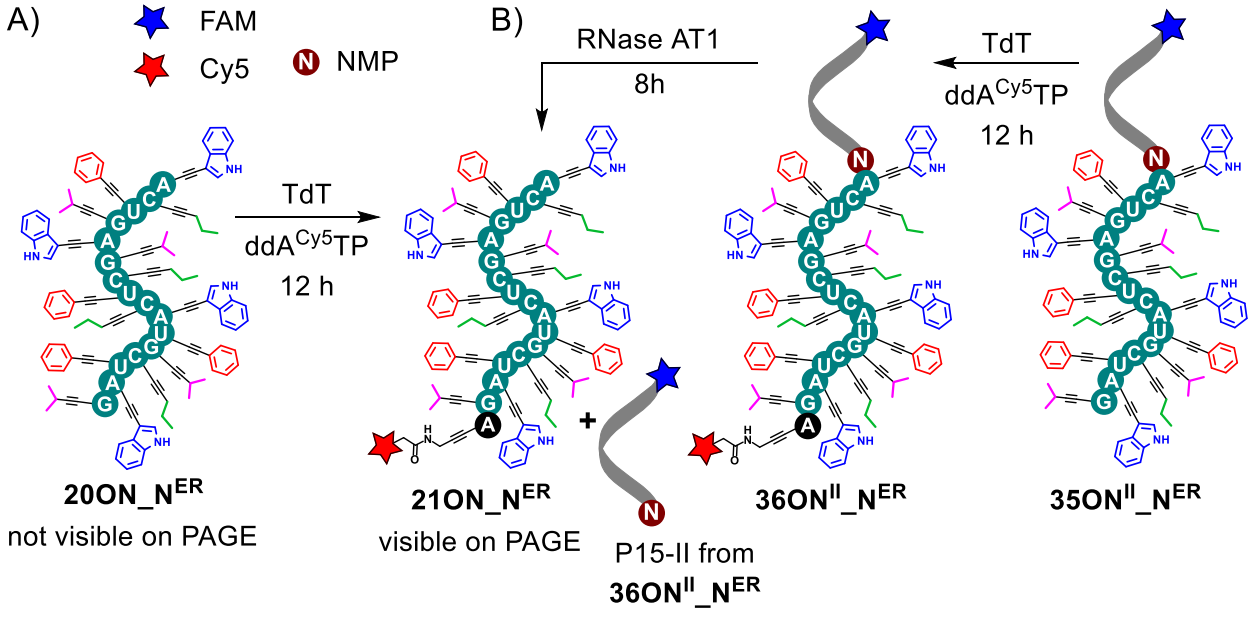


Figure S16. FAM (left) and Cy5 (right) scan of PAGE analyses of **35ON^{I}_N^{ER}** (A) and **20ON_N^{ER}** (B) after purification with various spin columns: (L) single-stranded ladder; (lanes D) **35NA^{III}_N^{ER}**; (lanes 1) not purified; (lanes 2) MicroSpin G-25; (lanes 3) MicroSpin G-50; (lanes 4) Zeba; (lanes 5) Mini Quick Spin Oligo; (lanes 6) Mini Quick Spin DNA; (lanes 7) Micro Bio-Spin P6; (lanes 8) Micro Bio-Spin P30.

1.7 **Non-templated labelling – Terminal transferase (TdT)**

Reaction conditions for oligonucleotide labelling using ddA^{Cy5}TP and TdT were first performed on FAM-labelled **35ON^{II}_N^{ER}** prepared by procedure described above in Section 1.4 using P15-II primer and *Bst* 3.0 DNA polymerase (Scheme S5). Cy5-labelled product **36ON^{II}_N^{ER}** was purified by G50 spin column and analyzed by PAGE (Figure S17A) and further digested at ribonucleotide position using RNase AT1 (5 U, 8 h) to provide **21ON_N^{ER}**. Optimized conditions were then applied for labelling of **20ON_N^{ER}** and are described as Method C.

Method C: Reaction mixture (20 µL) contained HPLC-purified **20ON_N^{ER}** (2 µM, 5 µL, prepared from **35NA^{III}_N^{ER}** according to Method B), ddA^{Cy5}TP (1 µL, 1 mM), $CoCl_2$ (2 µL, 2.5 mM), Terminal deoxynucleotidyl transferase (TdT) (10 U) and reaction buffer (10X, 2 µL) as supplied by the manufacturer. The reaction mixture was incubated for 12 h at 37 °C to achieve **21ON_N^{ER}**, stopped by cooling down to 4 °C and analyzed by PAGE (Figure S17B). Due to insufficient removal of free ddA^{Cy5}TP and its complexes by spin columns, Cy5-labelled **21ON_N^{ER}** product was purified by HPLC according to procedure described in section 1.5 (Figure S18) and confirmed by ESI (Figure S28).



Scheme S5. Non-templated labelling of **20ON_N^{ER}** (A) and **35ON^{II}_N^{ER}** (B) using TdT.

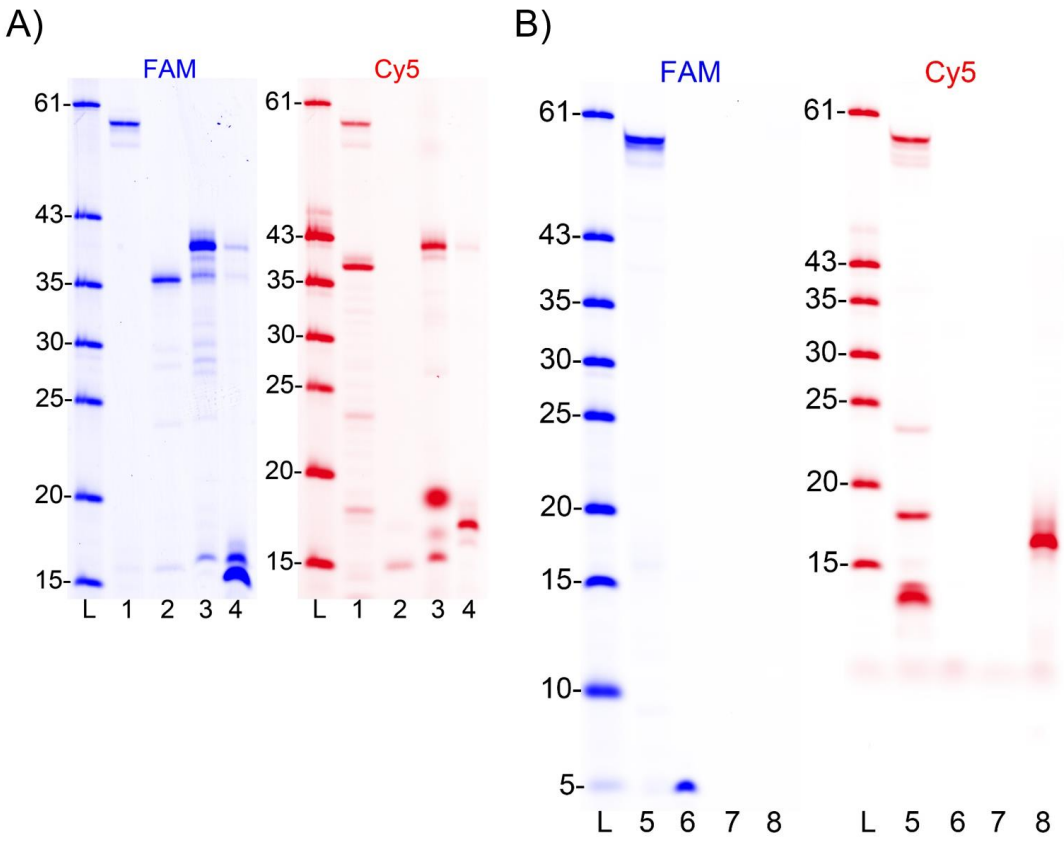


Figure S17. FAM (left) and Cy5 (right) scan of PAGE analyses for non-templated labelling of **35ON^{II}_N^{ER}** (A) and **20ON_N^{ER}** (B) using TdT: (L) single-stranded ladder; (lanes 1) **35NA^{II}_N^{ER}**; (lanes 2) **35ON^{II}_N^{ER}**; (lanes 3) **36ON^{II}_N^{ER}**; (lanes 4) **21ON_N^{ER}**, G50 spin column purified; (lanes 5) **35NA^{III}_N^{ER}**; (lanes 6) **20ON_N^{ER}**, not purified; (lanes 7) **20ON_N^{ER}**, HPLC purified; (lanes 8) **21ON_N^{ER}**, HPLC purified.

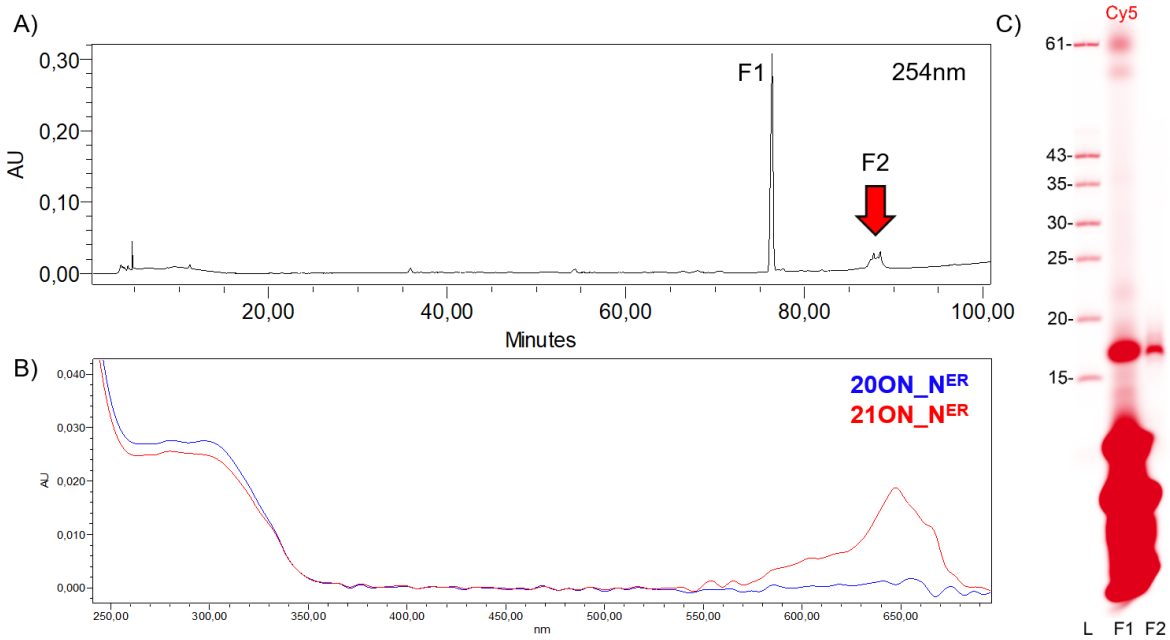


Figure S18. HPLC chromatogram of labelling reaction of **20ON_N^{ER}** (A) and absorption spectra (B) of fraction F2 containing both non-labelled **20ON_N^{ER}** (starting material) and Cy5-labelled **21ON_N^{ER}** (product); (C) Cy5 scan of PAGE analysis of HPLC fractions F1 and F2.

1.8 Reverse transcription – RNA template randomization

Products of reverse transcriptions synthesized from five random sequences of RNA template gave 30, 40, 50, 60 or 70% of GC content within synthesized part of extending P15-I primer (**Table S10**). Reactions were performed according to Method A described in Section 1.2 using *Bst* 3.0 DNA polymerase, digested with RNase AT1 and purified by MicroSpin G-50. PAGE analysis of these reactions is shown in **Figure S19**.

Table S10. Randomization of extended sequence within ssONs products of RT

RNA template	Product	Extended sequence	GC content of extended sequence	dNTPs distribution
35RNAα	**35ON^{I}α_N^{ER}**	AATACTACTACAGCTTTGTT	30%	T-rich
35RNAβ	**35ON^{I}β_N^{ER}**	ATAAGTGAACCGTCGAAACA	40%	A-rich
35RNA	**35ON^{I}_N^{ER}**	ACTGAGCTCATGCTAGACTG	50%	equal
35RNAγ	**35ON^{I}γ_N^{ER}**	TACCGACACGAACCCCAGCT	60%	C-rich
35RNAδ	**35ON^{I}δ_N^{ER}**	CGGAGCCCGCCGTAGGAGAA	70%	G-rich

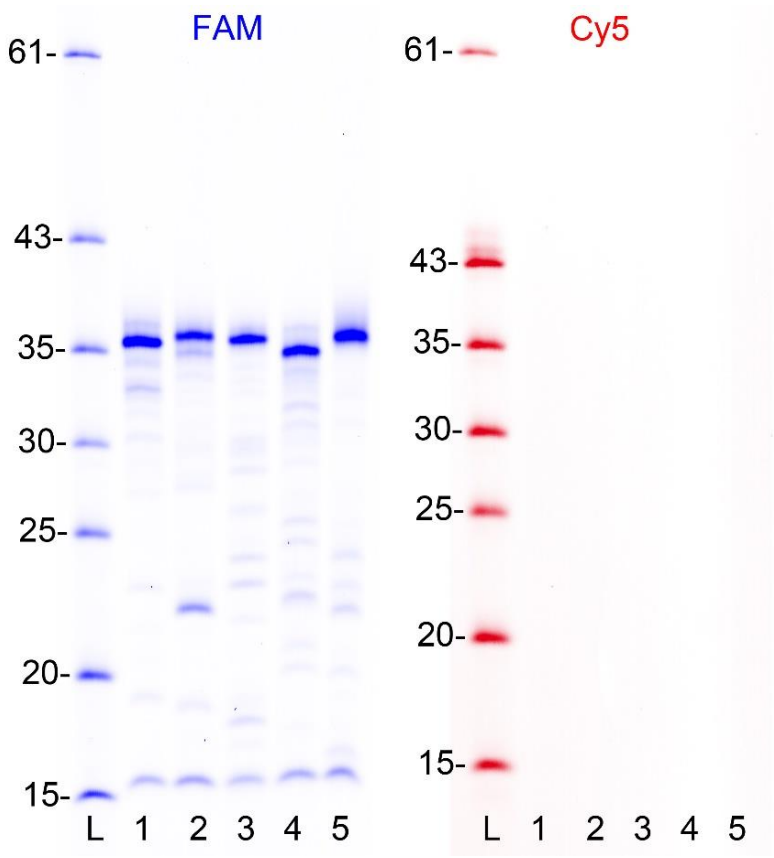


Figure S19. FAM (left) and Cy5 (right) scan of PAGE analysis of RT reactions performed on five random sequences of RNA template with P15-I primer and *Bst* 3.0 DNA polymerase, followed by RNA template digestion using RNase AT1 and purified by MicroSpin G-50: (L) single-stranded ladder; (lanes 1) **35ON^{I}α_N^{ER}**; (lanes 2) **35ON^{I}β_N^{ER}**; (lanes 3) **35ON^{I}_N^{ER}**; (lanes 4) **35ON^{I}γ_N^{ER}**; (lanes 5) **35ON^{I}δ_N^{ER}**.

Reverse transcriptions using five random sequences of RNA template and P15-III primer were performed according to Method B described in Section 1.5 using *Bst* 3.0 DNA polymerase and digested with RNase H or AT1 (**Table S11**). Non-labelled fully-modified ssONs formed after template digestion and primer cleavage with RNase AT1 were purified by HPLC according to HPLC method described in Section 1.5 and applied for ESI measurements (**Figures S29-S32**). PAGE analysis of these reactions is shown in **Figure S20**.

Table S11. Products of RT (using five random sequences of RNA template) and digestion with RNase H or AT1

RNA template	After RNase H	Figure S20 lane	After RNase AT1	ESI spectrum
35RNAα	**35ON^{III}α_N^{ER}**	1	**20ONα_N^{ER}**	Figure S29
35RNAβ	**35ON^{III}β_N^{ER}**	2	**20ONβ_N^{ER}**	Figure S30
35RNA	**35ON^{III}_N^{ER}**	3	**20ON_N^{ER}**	Figure S26
35RNAγ	**35ON^{III}γ_N^{ER}**	4	**20ONγ_N^{ER}**	Figure S31
35RNAδ	**35ON^{III}δ_N^{ER}**	5	**20ONδ_N^{ER}**	Figure S32

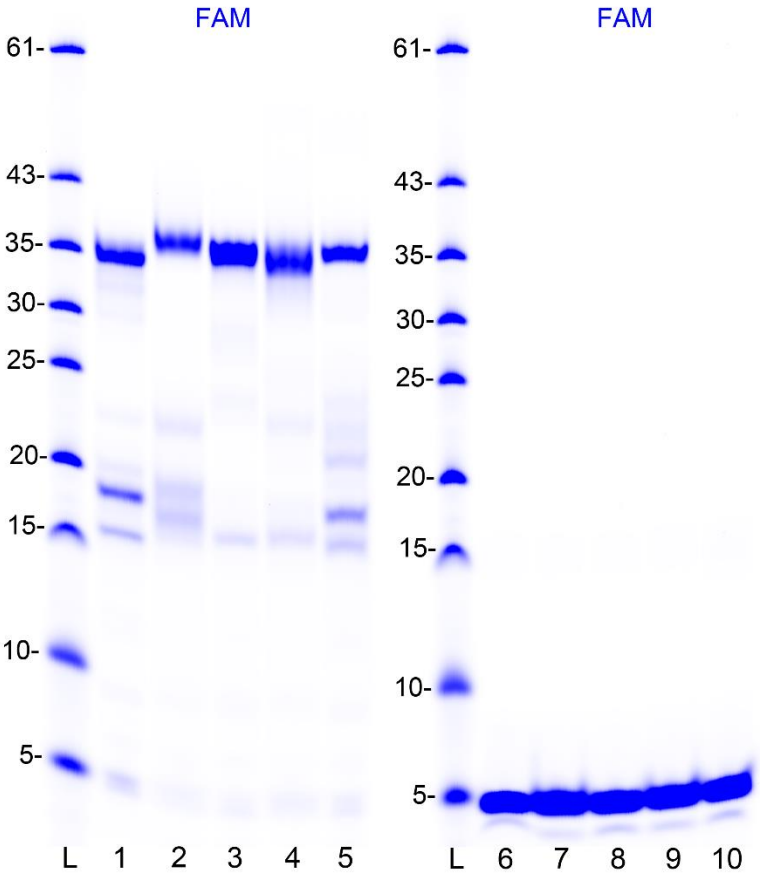


Figure S20. FAM scan of PAGE analysis of RT reactions performed on five random sequences of RNA templates with P15-III primer, *Bst* 3.0 DNA polymerase, followed by template digestion using RNase H (lanes 1-5) or AT1 for additional primer cleavage (lanes 6-10): (L) single-stranded ladder; (lane 1) **35ON^{III}α_N^{ER}**; (lane 2) **35ON^{III}β_N^{ER}**; (lane 3) **35ON^{III}_N^{ER}**; (lane 4) **35ON^{III}γ_N^{ER}**; (lane 5) **35ON^{III}δ_N^{ER}**; (lane 6) **20ONα_N^{ER}**; (lane 7) **20ONβ_N^{ER}**; (lane 8) **20ON_N^{ER}**; (lane 9) **20ONγ_N^{ER}**; (lane 10) **20ONδ_N^{ER}**.

2) MALDI-TOF and ESI

Table S12. Overview of synthesized (hypermodified) ssONs and their masses

ON	Mass calculated [Da]	Mass found [Da]	Mass spectrum
35ON^{I}_N^{ER}	13166.4	13167.0 and 13616.9	Figure S21
35ON^{I}_N^{ER}	13166.4	13168.0 and 13619.0	Figure S22
60ON^{I}_N^{ER}	21987.8	21990.0	Figure S23
20ON_N^{ER}	7892.8	7894.0 and 8346.0	Figure S24
28ON_N^{ER}	10461.5	10464.0 and 10915.0	Figure S25
20ON_N^{ER}	7892.8	8345.0	Figure S26
20ON_N^{ER}	7892.8	8346.5	Figure S27
21ON_N^{ER}	9335.4	9334.0	Figure S28
20ONα_N^{ER}	7976.7	7978.0	Figure S29
20ONβ_N^{ER}	8168.0	8621.0	Figure S30
20ONγ_N^{ER}	7805.8	7806.0	Figure S31
20ONδ_N^{ER}	7870.0	7870.0	Figure S32

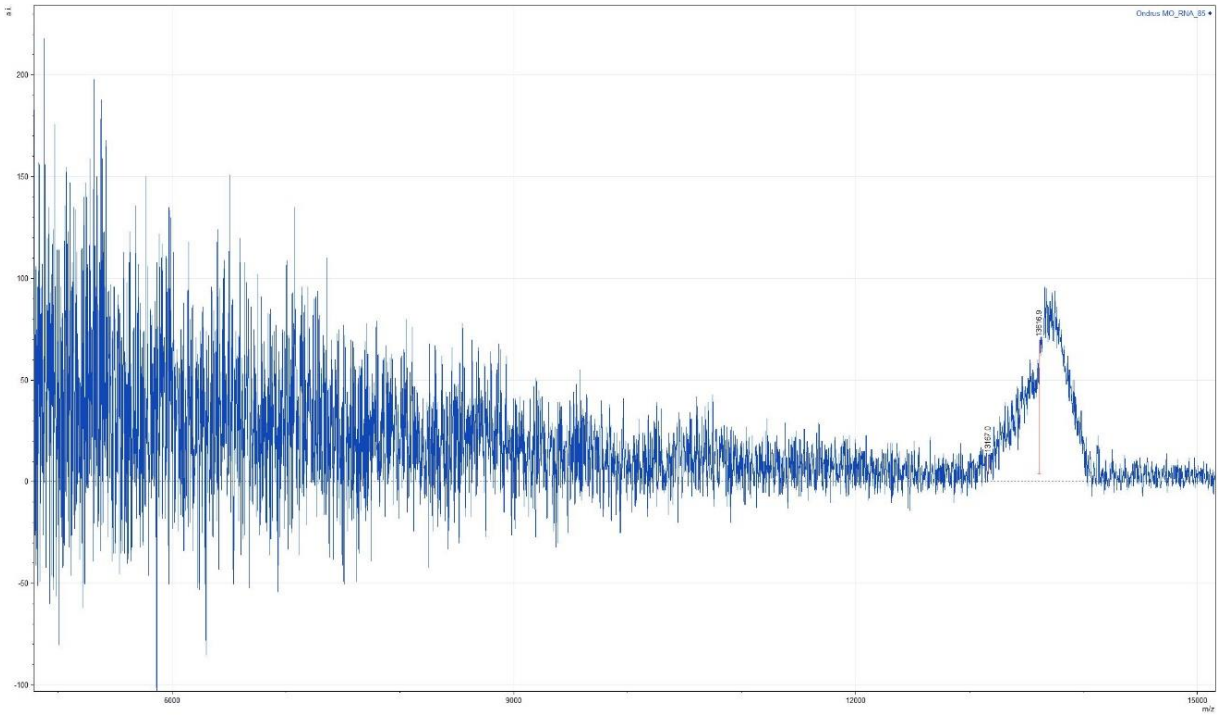


Figure S21. MALDI-TOF spectrum of **35ON^{I}_N^{ER}**: calculated: 13166.4 Da, found: 13167.0 Da, Δ = 0.6 Da. The peak at 13616.9 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 13617.7 Da, Δ = 0.8 Da).

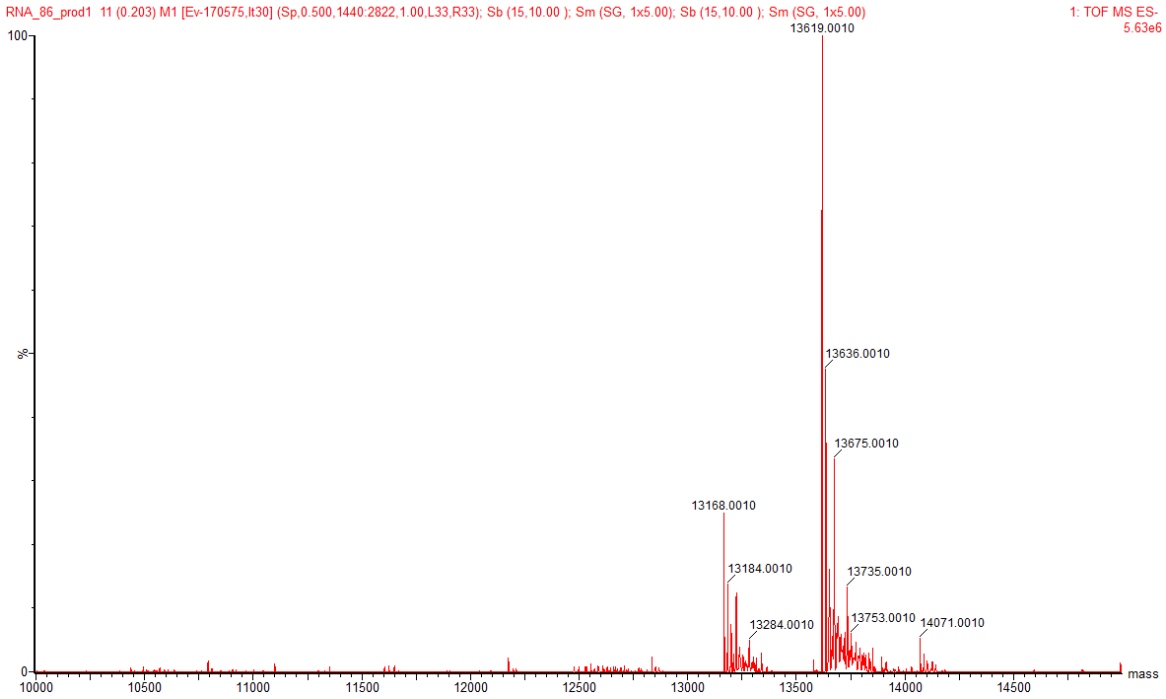


Figure S22. ESI spectrum of **35ON^{I}_N^{ER}**: calculated: 13166.4 Da, found: 13168.0 Da, Δ = 1.6 Da. The peak at 13619.0 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 13617.7 Da, Δ = 1.3 Da).

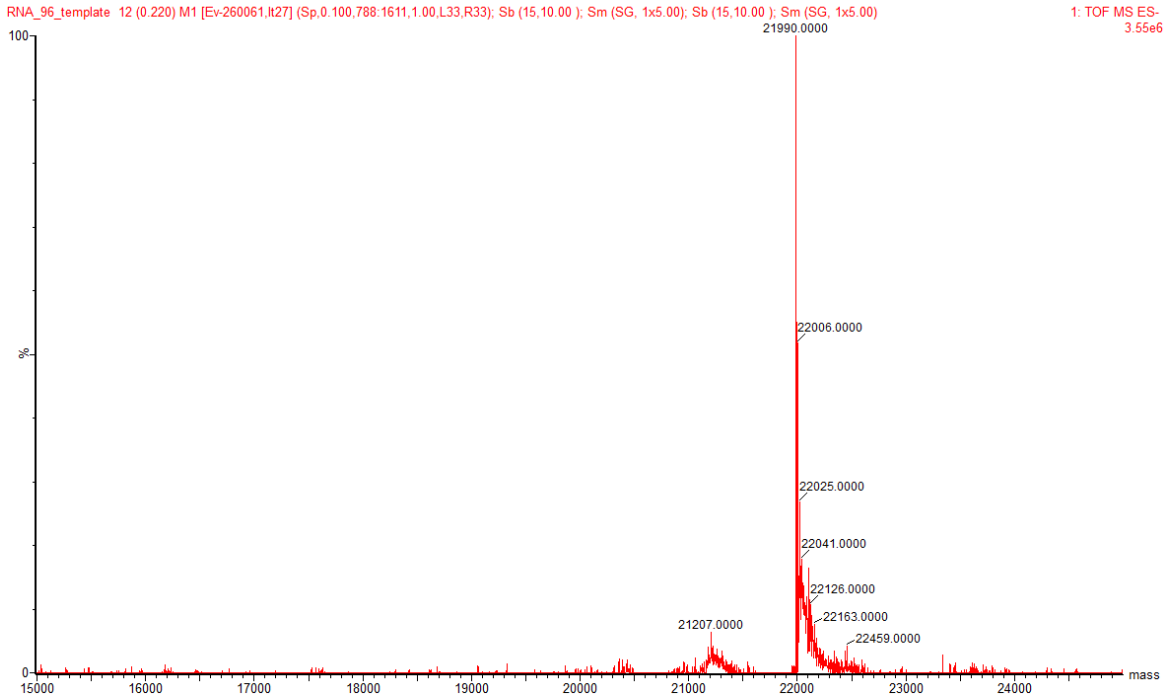


Figure S23. ESI spectrum of **60ON^{I}_N^{ER}**: calculated: 21987.8 Da, found: 21990.0 Da, Δ = 2.2 Da.

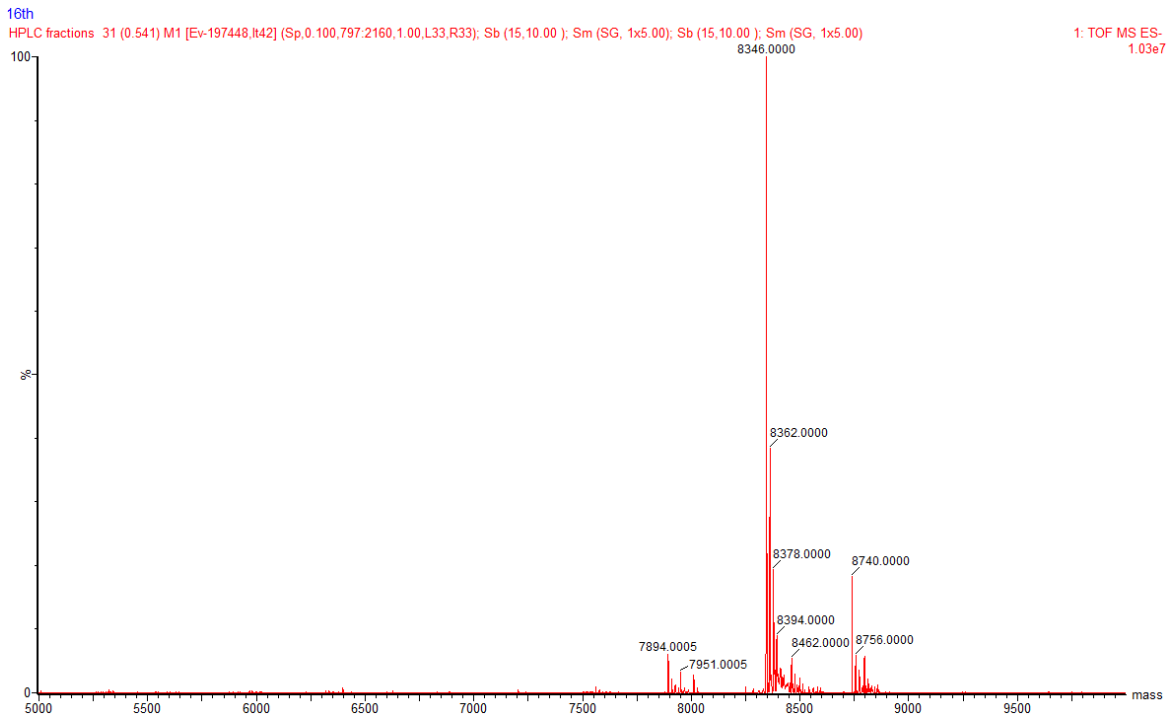


Figure S24. ESI spectrum of **20ON_N^{ER}**: calculated: 7892.8 Da, found: 7894.0 Da, Δ = 1.2 Da. The peak at 8346.0 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 8344.1 Da, Δ = 1.9 Da).

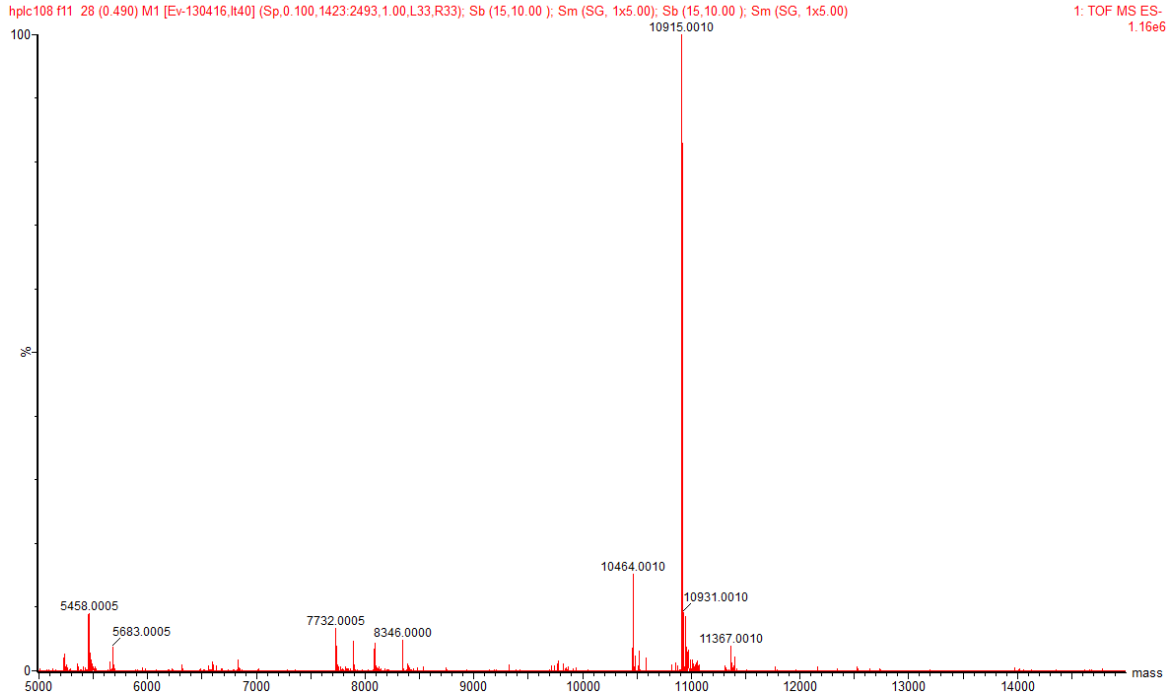


Figure S25. ESI spectrum of **28ON_N^{ER}**: calculated: 10461.5 Da, found: 10464.0 Da, Δ = 2.5 Da. The peak at 10915.0 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 10912.8 Da, Δ = 2.2 Da).

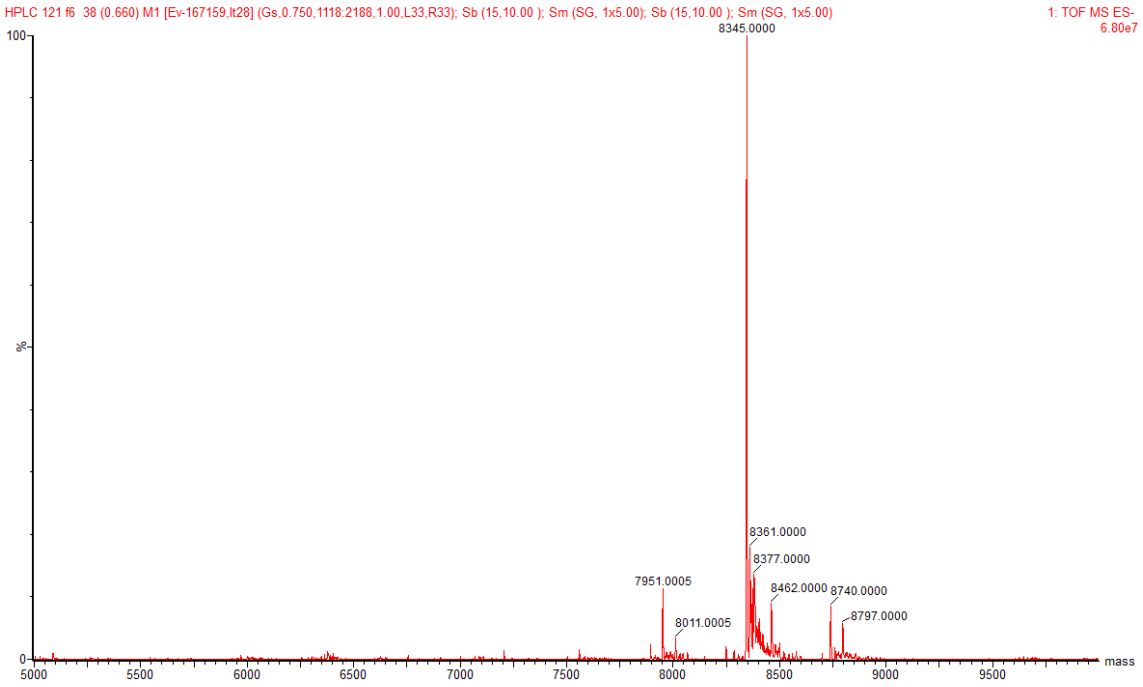


Figure S26. ESI spectrum of **20ON_N^{ER}**: calculated: 7892.8 Da, found: 8345.0 Da. The peak at 8345.0 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 8344.1 Da, Δ = 0.9 Da).

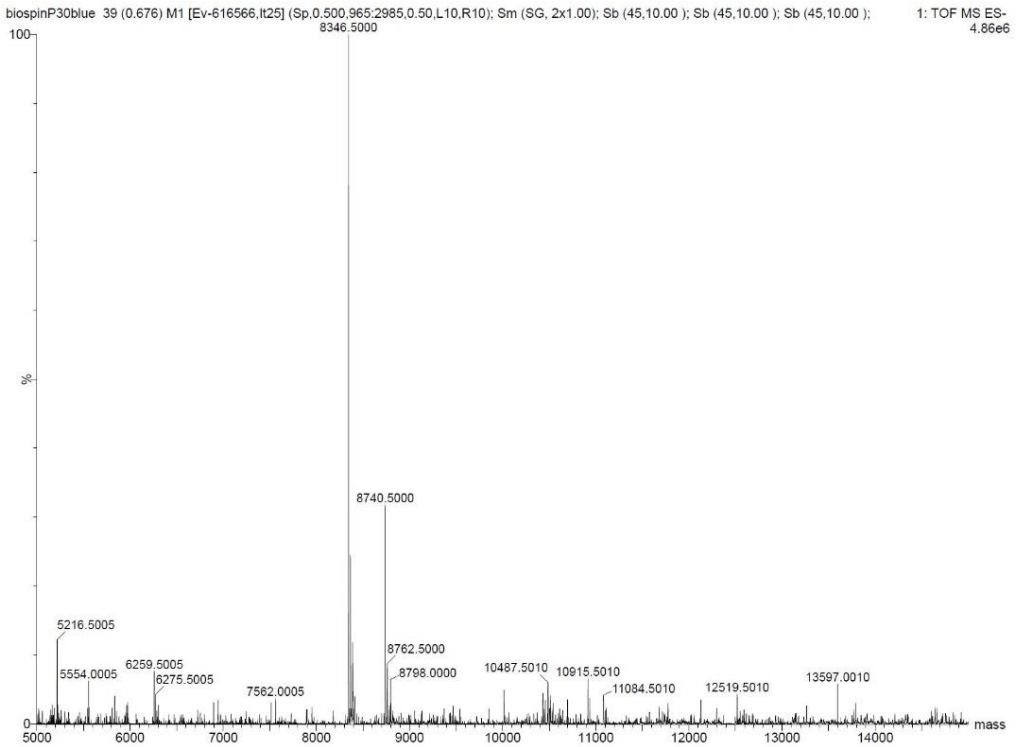


Figure S27. ESI spectrum of **20ON_N^{ER}**: calculated: 7892.8 Da, found: 8346.5 Da. The peak at 8346.5 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 8344.1 Da, Δ = 2.4 Da).

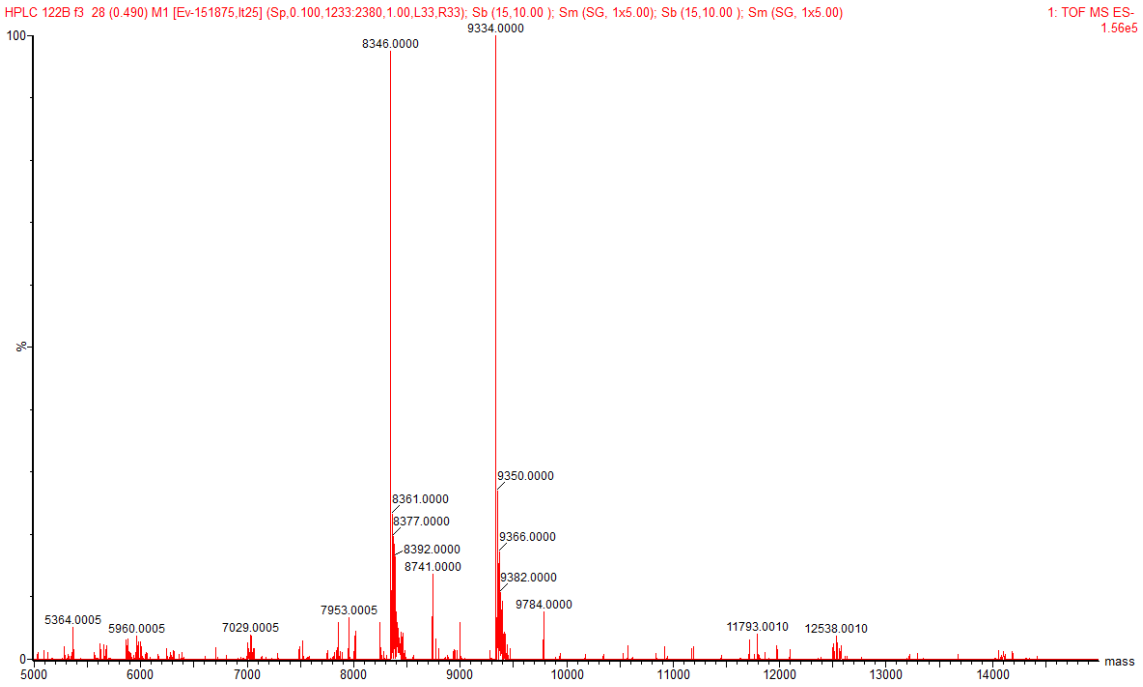


Figure S28. ESI spectrum of **21ON_N^{ER}**: calculated: 9335.4 Da, found: 9334.0 Da, Δ = 1.4 Da. The peak at 8346.0 Da is assigned to **20ON_N^{ER}** (calculated: 8344.1 Da, Δ = 1.9 Da).

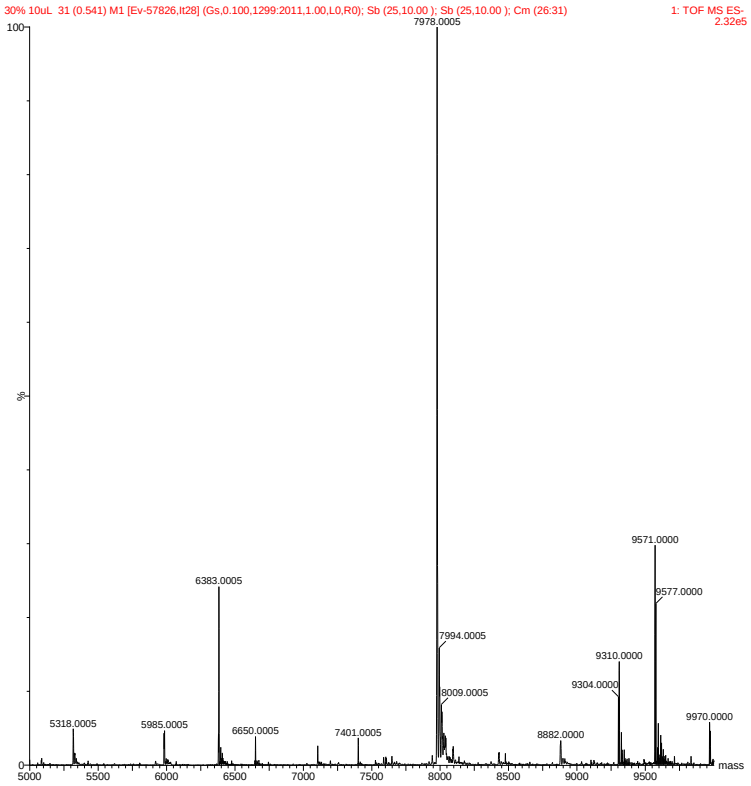


Figure S29. ESI spectrum of **20ONα_N^{ER}**: calculated: 7976.7 Da, found: 7978.0 Da, Δ = 1.3 Da.

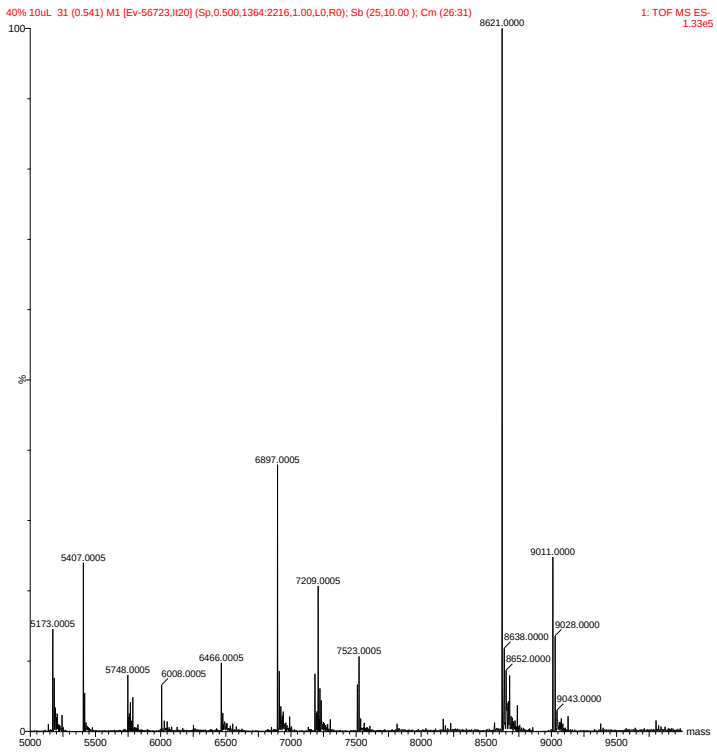


Figure S30. ESI spectrum of **20ONβ_N^{ER}**: calculated: 8168.0 Da, found: 8621.0 Da. The peak at 8621.0 Da is assigned to product of non-templated addition of **dA^{EIn}MP** (calculated: 8619.2 Da, Δ = 1.8 Da).

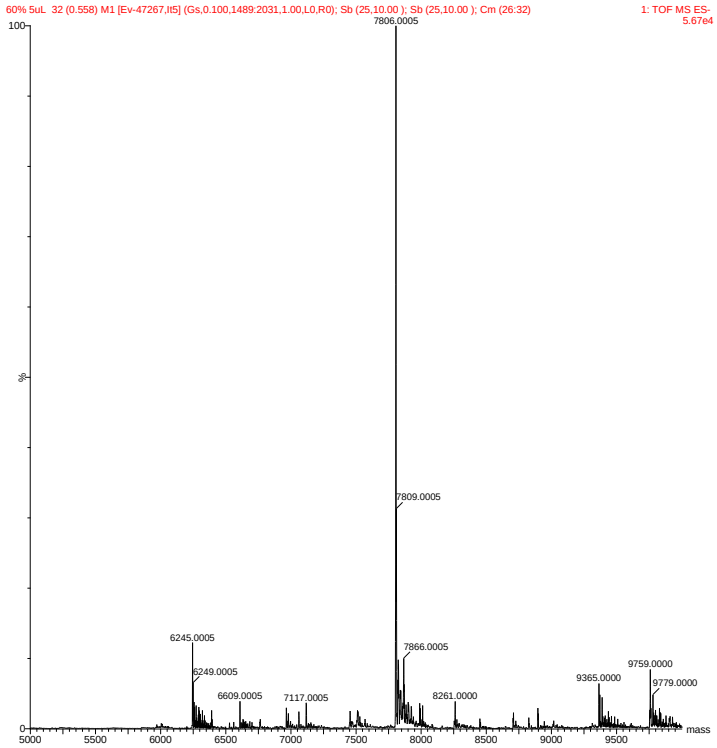


Figure S31. ESI spectrum of **20ONγ_N^{ER}**: calculated: 7805.8 Da, found: 7806.0 Da, Δ = 0.2 Da.

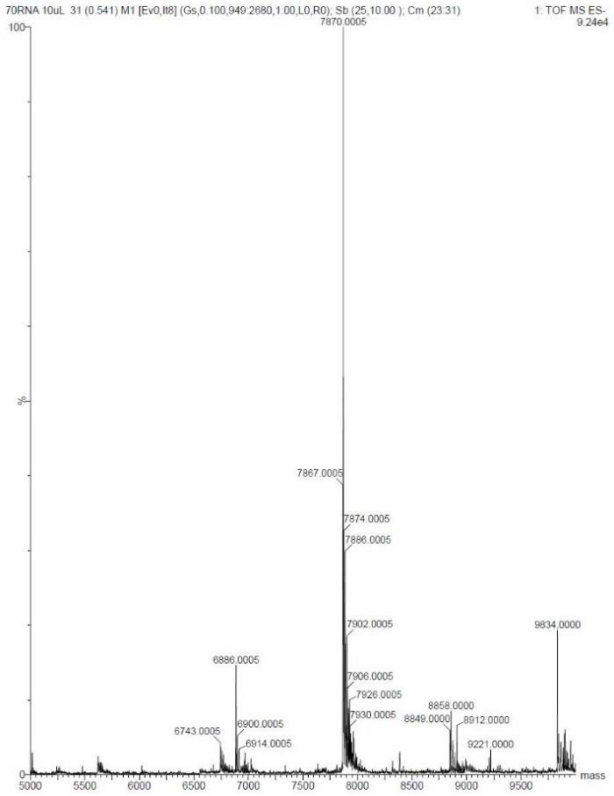


Figure S32. ESI spectrum of **20ONδ_N^{ER}**: calculated: 7870.0 Da, found: 7870.0 Da, Δ = 0 Da.

3) References

[1] M. Ondruš, V. Sýkorová, L. Bednárová, R. Pohl and M. Hocek, *Nucleic Acids Res.*, 2020, **48**, 11982–11993.