Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING INFORMATION

Generation of long, fully modified, and serum-resistant oligonucleotides by rolling circle amplification

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1. General experimental section:

The base modified nucleoside triphosphates $dA^{Hs}TP$ (**1**), $dU^{POH}TP$ (**2**), $dC^{Val}TP$ (**3**), and the modified dUTP analogs **S1**-**S4** were synthesized according to literature protocols.[1, 2] 7-deaza-dGTP (**4**), 2'-fluoro-rNTPs (**5**), and α-thio-dNTPs (**6**) were purchased from TriLink. The purity of all triphosphates was verified by RP-HPLC using an Äkta™ basic 10/100 system (Amersham Pharmacia Biotech) equipped with a Phenomenex Jupiter analytical RP-HPLC column (10µ C4 300Å).

Vent (*exo*$^-$) DNA polymerase, DNA polymerase I, Large (Klenow) Fragment, 9°N_m DNA polymerase, ϕ29 DNA polymerase, exonuclease I, and the low molecular weight DNA ladder were all purchased from New England Biolabs. The *Pwo* DNA polymerase was purchased from Peqlab. T4 Polynucleotide kinase, *Pfu* DNA polymerase, and T4 DNA Ligase were purchased from Promega. Sequenase Version 2.0 was purchased from Affymetryx. S1 nuclease and T4 PNK were bought from Thermo Scientific. CircLigase™ II ssDNA Ligase was obtained from Epicentre. Heat-deactivated Fetal Bovine Serum was purchased from Gibco. γ-^{32}P-ATP was purchased from Hartmann Analytic and the natural dNTPs from Promega. Oligonucleotides were purchased from Microsynth and gel purified (PAGE 15-20%) prior to use. Acrylamide/bisacrylamide (19:1, 40%) was obtained from Serva. Radioactivity was detected using a Storm 820 phosphorimager with the ImageQuant software (both from GE Healthcare).

Oligonucleotides used (shown 5' to 3'):

P1: CTAACCCTAACCCTAACC

P2: TAGACCAAGGCAATCCGTA

P3: GTTAGGGTTAGGGTTAGG

T1: 5'-phosphate-TAGGGTTAG$(GGTTAG)_4$GGTTAGGGT

T2: 5'-phosphate-TAGGGTTAG$(GGTTAG)_5$GGTTAGGGT

T3: 5'-phosphate-TAGGGTTAG$(GGTTAG)_6$GGTTAGGGT

T4: 5'-phosphate-CTTGGTCTACTGGAGN_{20}CTACGGATTGC

T5: 5'-phosphate-CTTGGTCTACTGGAGAGGTGCAGGTCGTTGTAGCTAGCCTGGAG AGCACTACGGATTGC

2. Additional procedures

General procedure for ligation with T4 DNA ligase:

Primer **P2** (7 nmoles) and template **T4** or **T5** (5 nmoles) were annealed by heating to 95°C and then gradually cooling to room temperature. 100 μL T4 DNA ligase buffer 10X (300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), 2 μL BSA, and 40 U of T4 DNA ligase were then added to the annealed oligonucleotides for a total reaction volume of 1 mL. The reaction mixture was then incubated at 16°C for 12 h. Following heat deactivation (80°C, 10 min), the mixture was evaporated to dryness. The ligated oligonucleotide was precipitated (EtOH) and gel purified (PAGE 20%). The bands corresponding to products were then eluted (crush and soak method using a buffer consisting of $LiClO_4$ 1%, NEt_3 1 mM, pH 8), precipitated (EtOH), and desalted (G10 spin columns). 300-350 pmol of the circular templates **cT4** and **cT5** were regularly obtained (A_{260} measurement). Circularity was confirmed by exonuclease I treatment (data not shown).

Sequencing of the RCA product:

First, the general protocol for rolling circle amplification with natural dNTPs was applied. The resulting product was diluted to 40 μL and extracted with 40 μL phenol/chloroform. The reaction mixture was then precipitated with EtOH (400 μL) and passed through a spin column (30k cutoff, from Millipore) and stored in 15 μL H_2O. The 5'-[^{32}P]-labeled primer **P3** was annealed in Sequenase buffer 5x to the RCA product. A mixture of natural dNTPs (175 μM final concentration) and DTT (4.5 mM final concentration) was then added to the annealed oligonucleotides. The resulting solutions were then split into 6 eppendorfs for the different sequencing reactions. 4 of these vials contained each of the dideoxynucleoside triphosphates (ddNTPs, 20 μM final concentration), while one eppendorf only contained water (negative control), and the last was used for the positive control. A 1:5:1 mixture of thermostable inorganic pyrophosphatase (1.2 U/reaction), Sequenase diluent, and Sequenase Version 2.0 (5.2 U/reaction) was added to the vials as a cocktail at 4°C, for a final volume of 10 μL. The reaction mixtures were then incubated at 37°C for 15 min and quenched by adding of 10 μL of stop solution. The reaction mixtures were subjected to gel electrophoresis in 7% denaturing polyacrylamide gel containing TBE 1x buffer (pH 8) and 7 M urea (Figure S14). Visualization was performed by phosphorimaging.

3. Additional gel images and quantifying autoradiography:

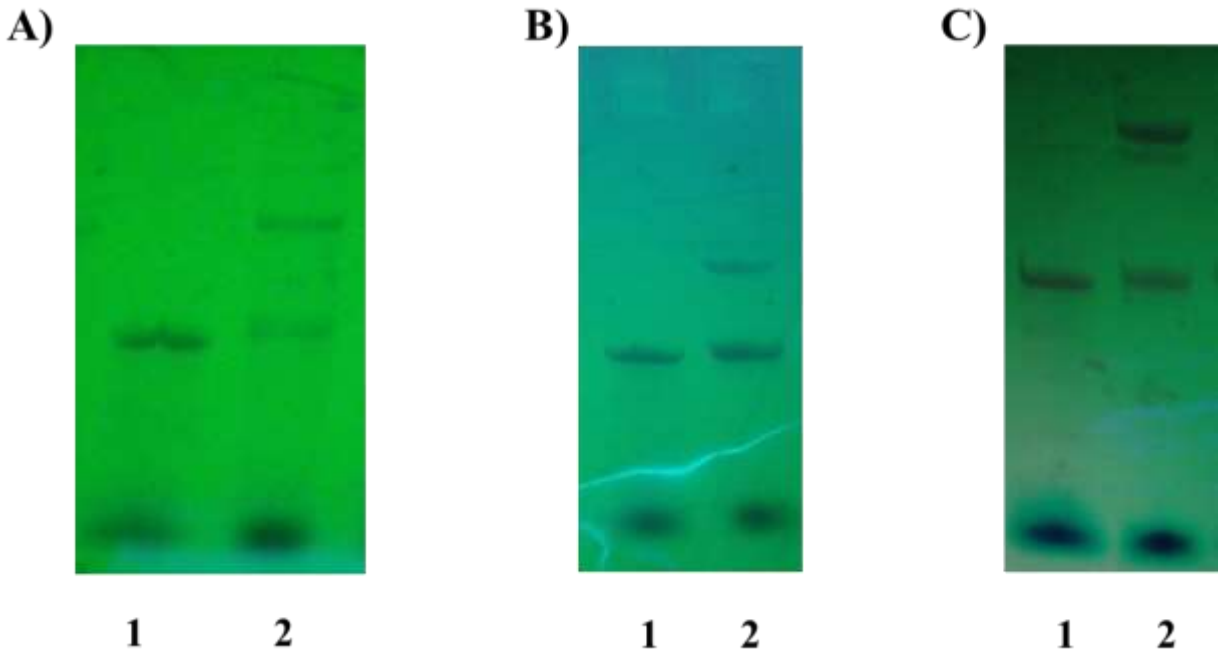

Figure S1. Gel images (PAGE 20%) of the circularization reactions with Circligase™ (UV shadowing) and linear precursors **T1** (Gel A)), **T2** (Gel B)) and **T3** (Gel C)). Lane 1: linear precursor; lane 2: reaction mixture. The top bands correspond to the circular DNA templates and present a substantial reduction of mobility. The bands corresponding to the linear templates in lanes 2 present a slightly reduced gel mobility compared to the starting oligonucleotides since these are the 5'-adenylylated intermediates of the linear precursors.

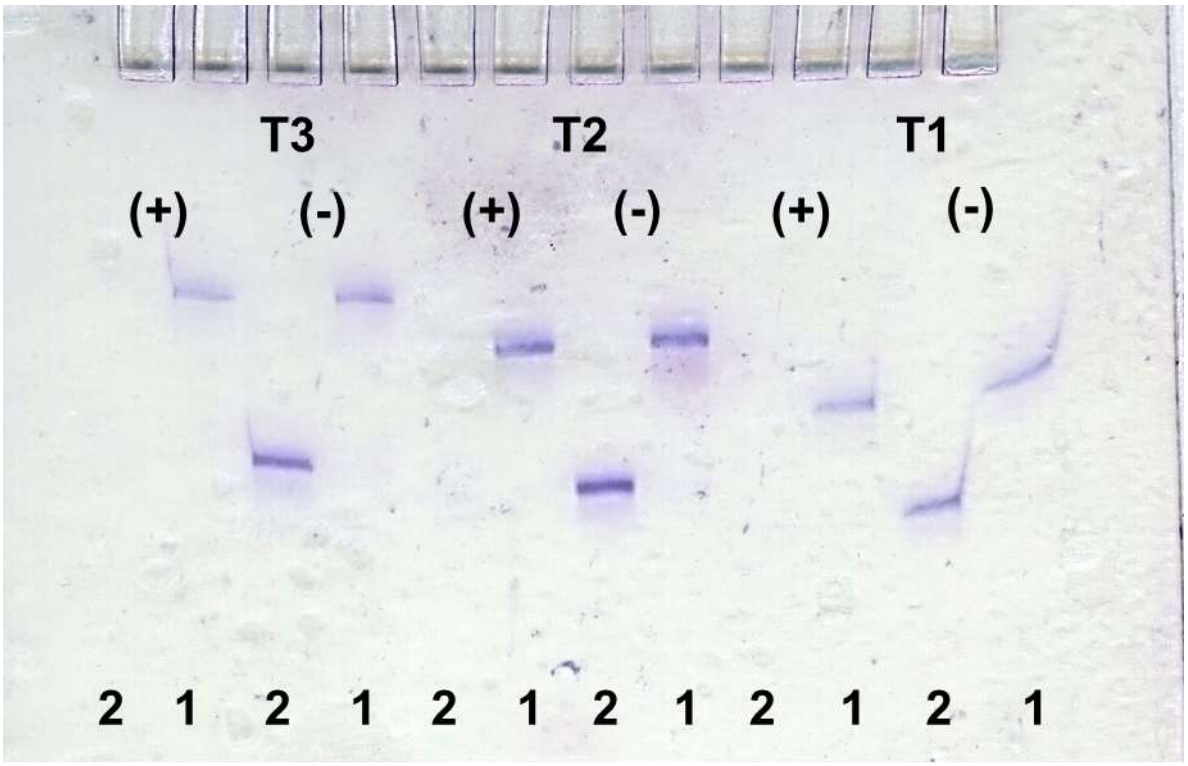

Figure S2. Gel image (PAGE 20%; visualized with stains-all) of the circular templates **cT1-3** and linear oligonucleotides **T1-3** with (+) and without (-) exonuclease I treatment. Lanes 1: gel purified circular templates; lanes 2: linear templates.

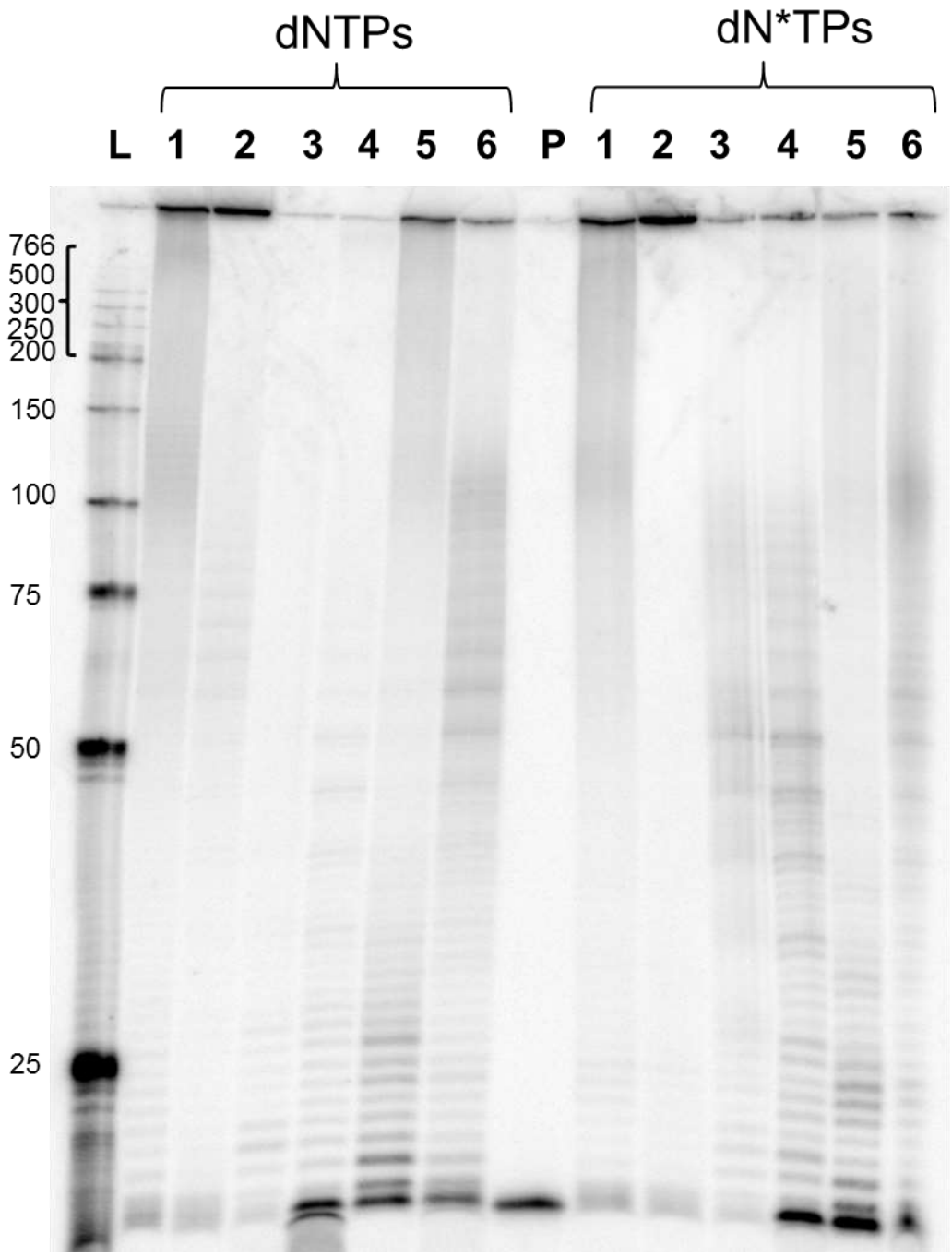

Figure S3. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT3** and α-thio dNTPs **6** (the close-up view of this gel is shown in Fig. 2A of the manuscript). DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L: low molecular weight DNA ladder.

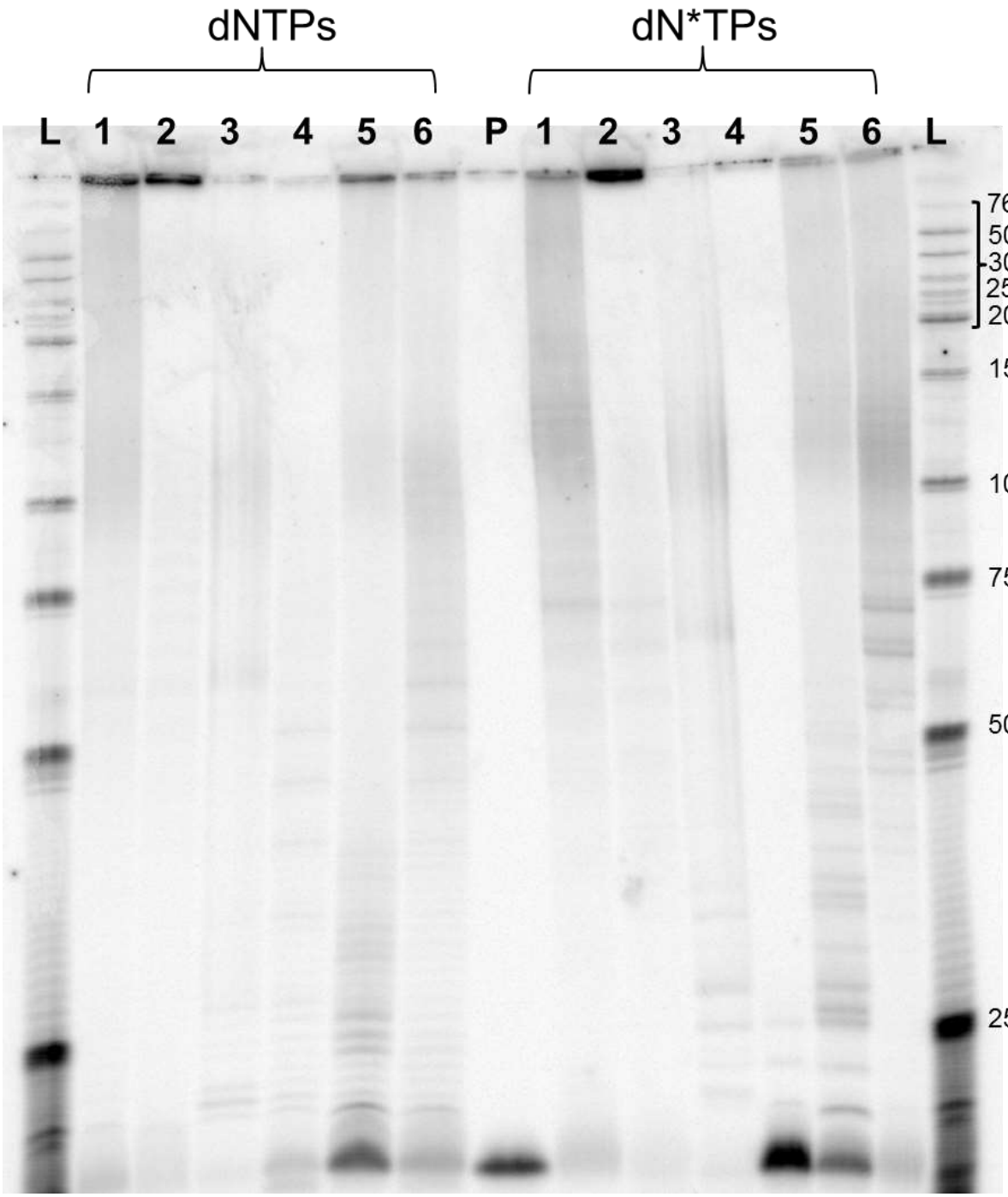

Figure S4. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT3** and base-modified dNTPs **1-3** (the close-up view of this gel is shown in Fig. 2B of the manuscript). DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L: low molecular weight DNA ladder.

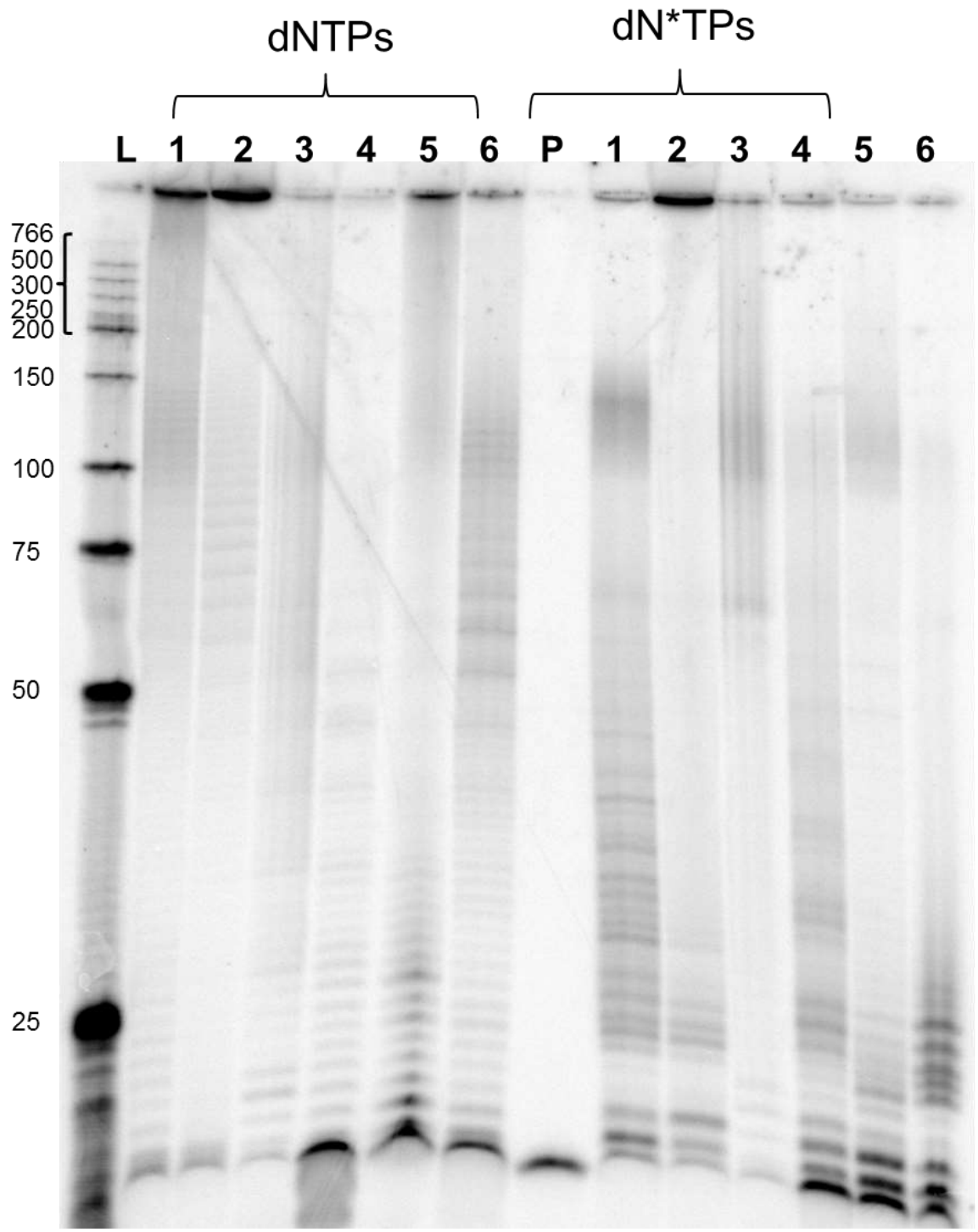

Figure S5. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT3** and 2'-fluoro-rNTPs **5** (the close-up view of this gel is shown in Fig. 2C of the manuscript). DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L: low molecular weight DNA ladder.

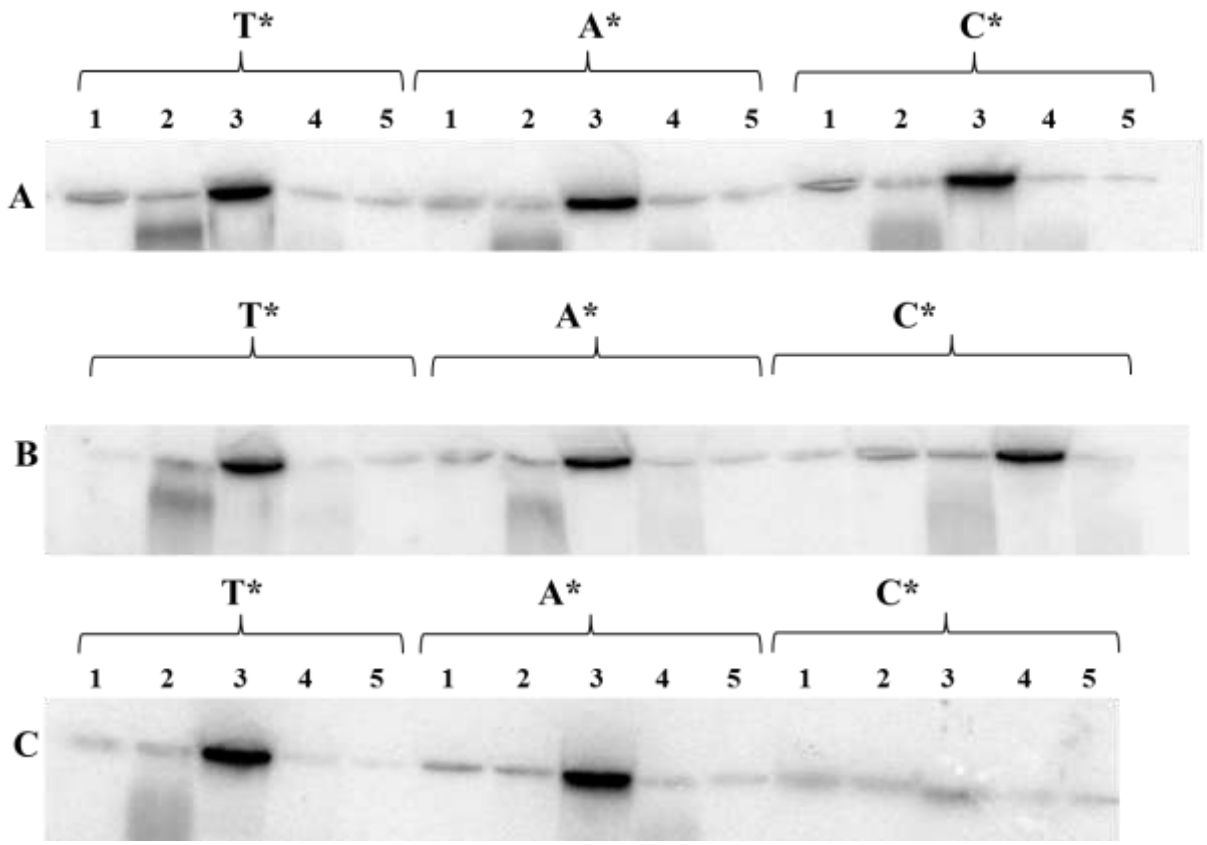

Figure S6. Gel images (close-up view; PAGE 10%) of RCA products with circular template **cT1**, primer **P1**, and single modified α-thio dNTPs **6** (A), base-modified dNTPs **1**-**3** (B) and 2'-fluoro-rNTPs **5** (C). DNA polymerases used: lane 1: Klenow fragment; lane 2: Vent (*exo*$^-$); lane 3: 9°N_m; lane 4: *Pfu*; lane 5: *Pwo*;

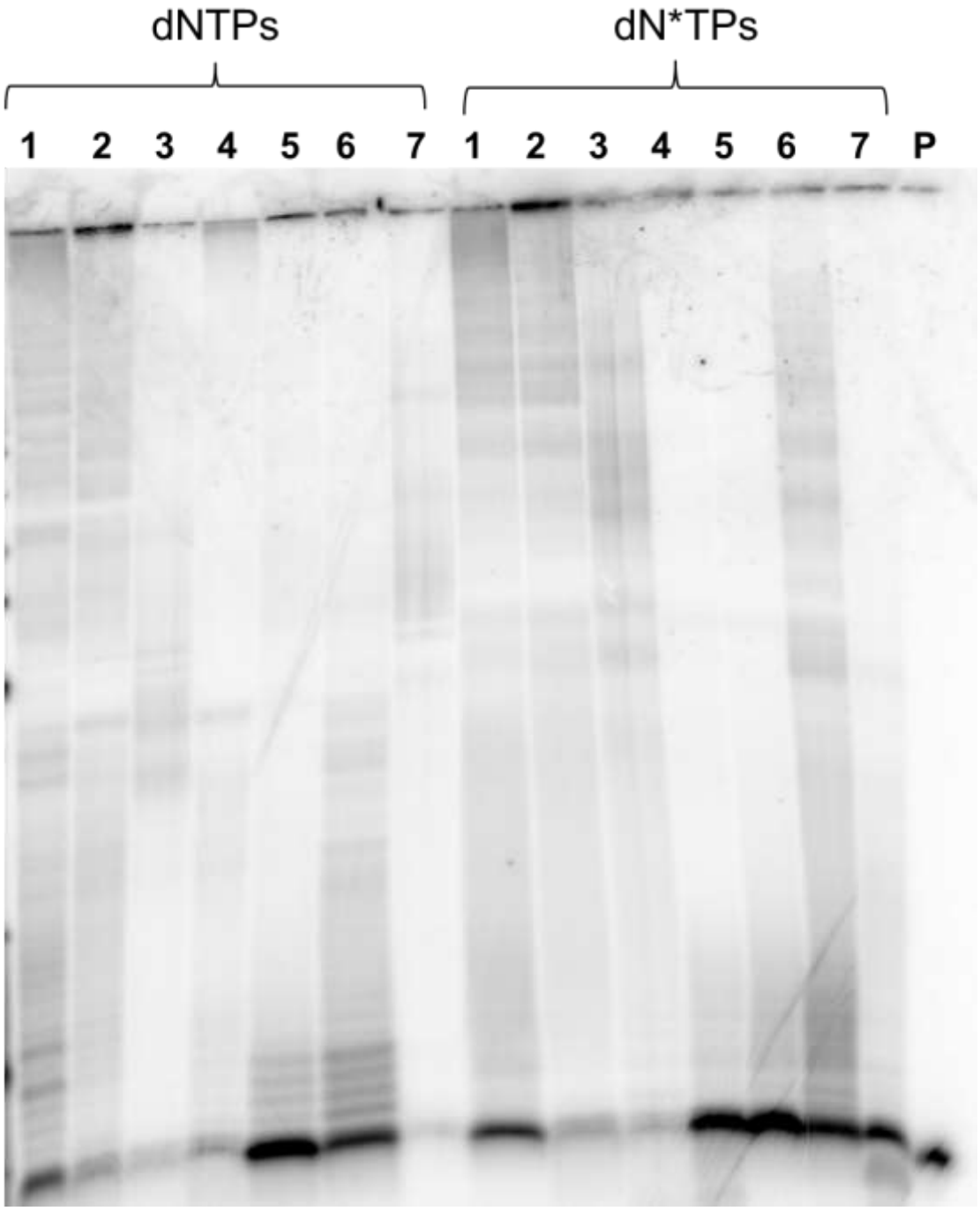

Figure S7. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT1** and base-modified dNTPs **1-3**. DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; lane 7: Sequenase; P = control without polymerase.

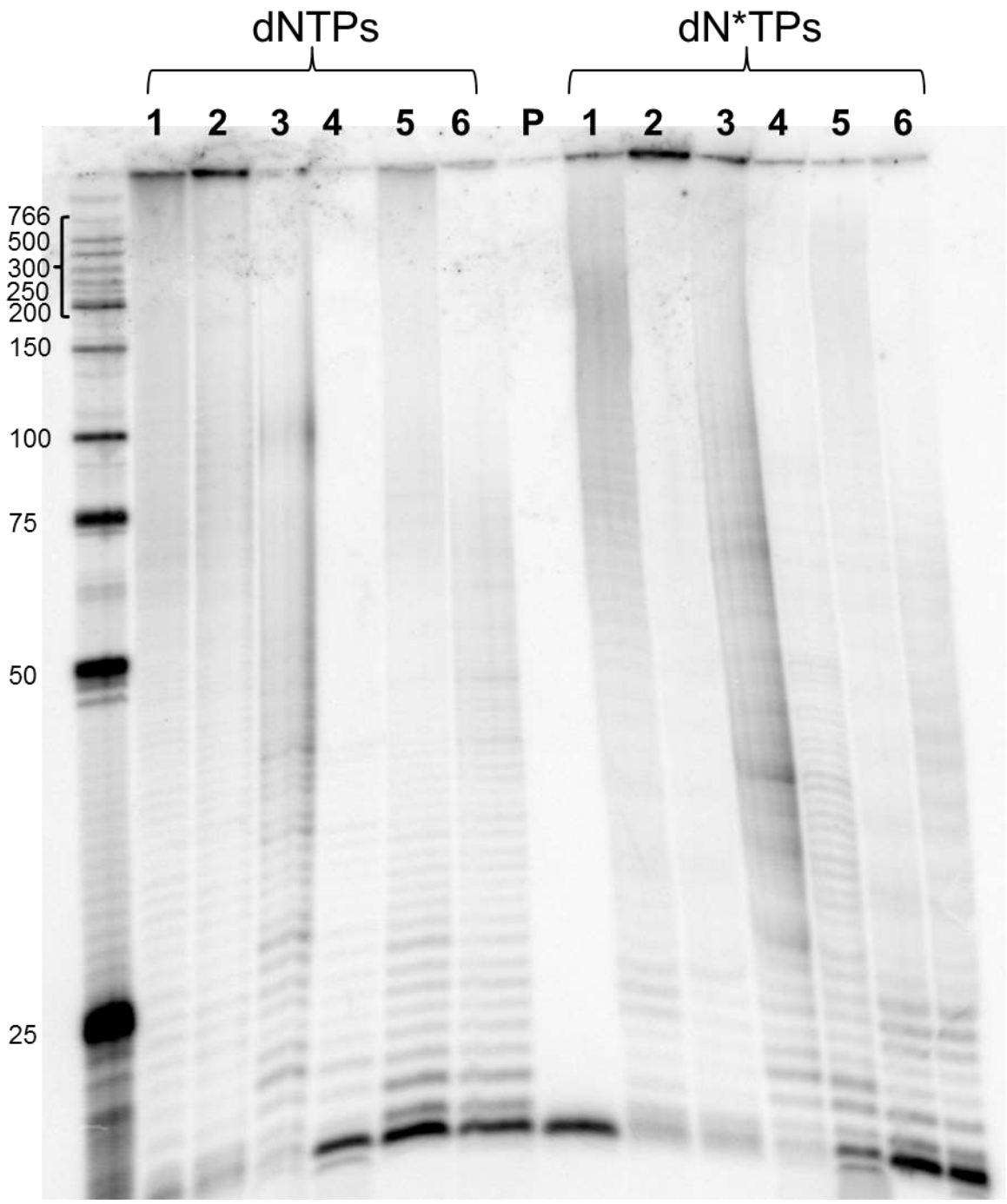

Figure S8. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT1** and α-thio dNTPs **6**. DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L = low molecular weight DNA ladder.

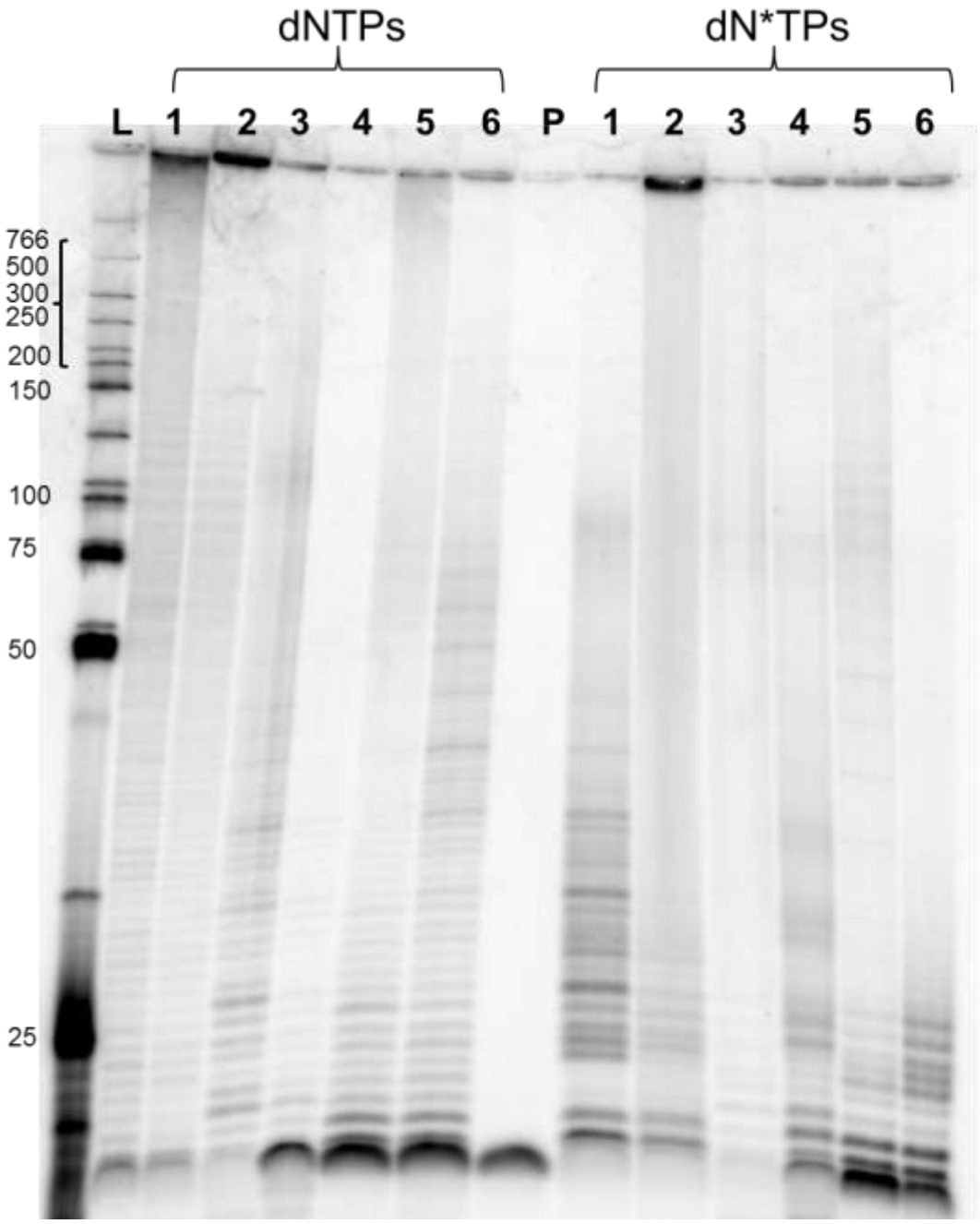

Figure S9. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT1** and 2'-fluoro-rNTPs **5**. DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L = low molecular weight DNA ladder.

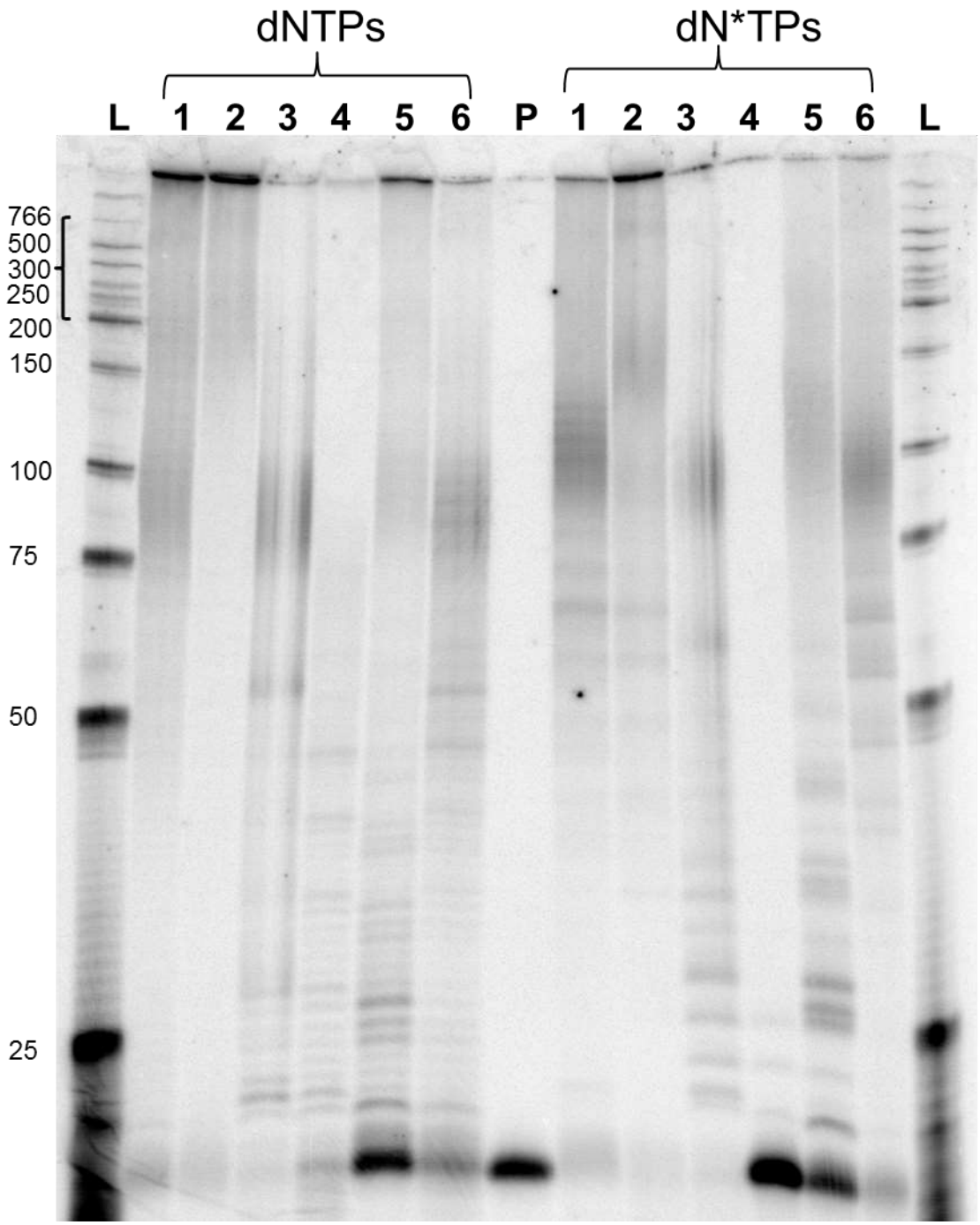

Figure S10. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT2** and base-modified dNTPs **1-3**. DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; lane 7: Sequenase; P = control without polymerase.

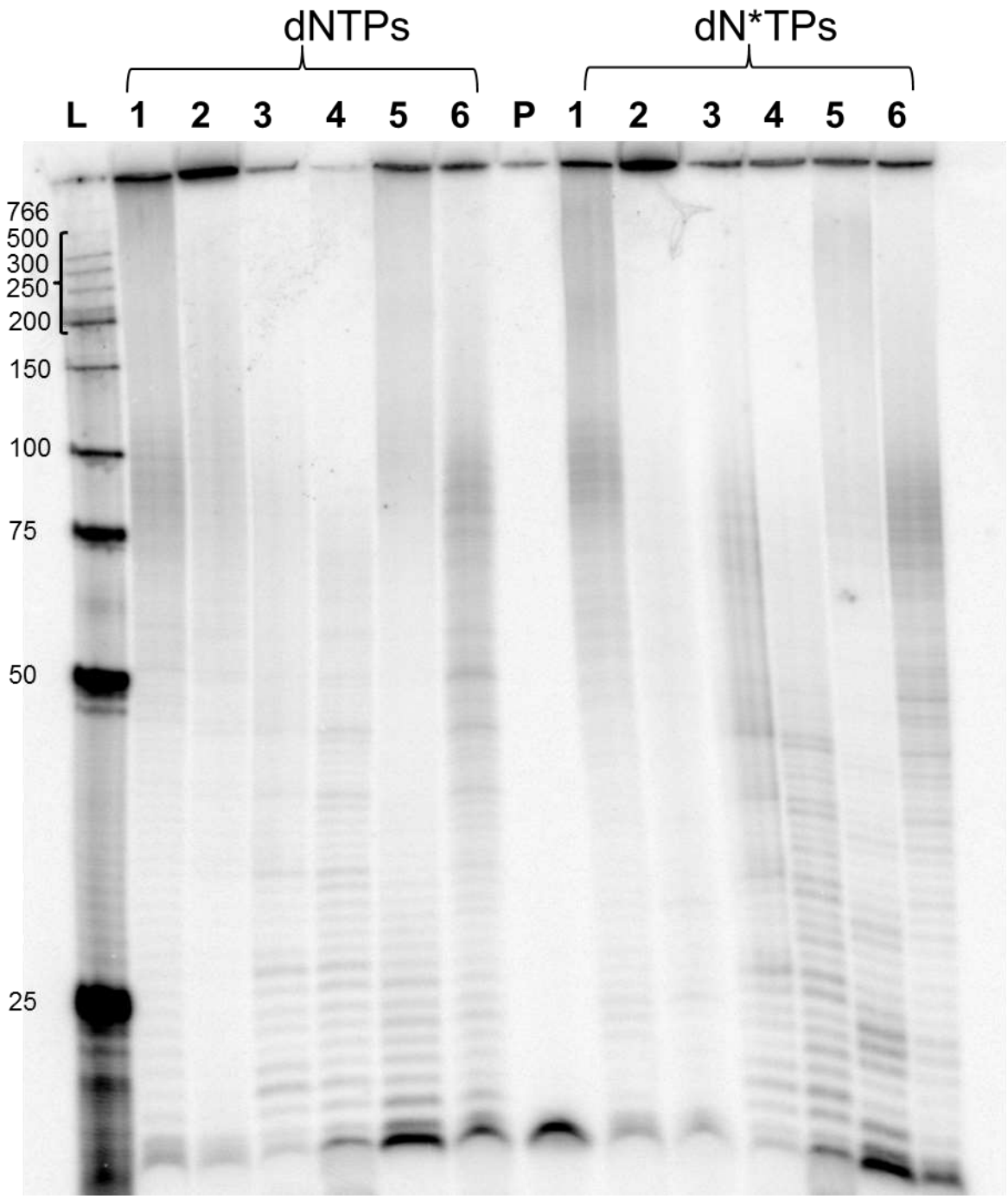

Figure S11. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT2** and α-thio dNTPs **6**. DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L = low molecular weight DNA ladder.

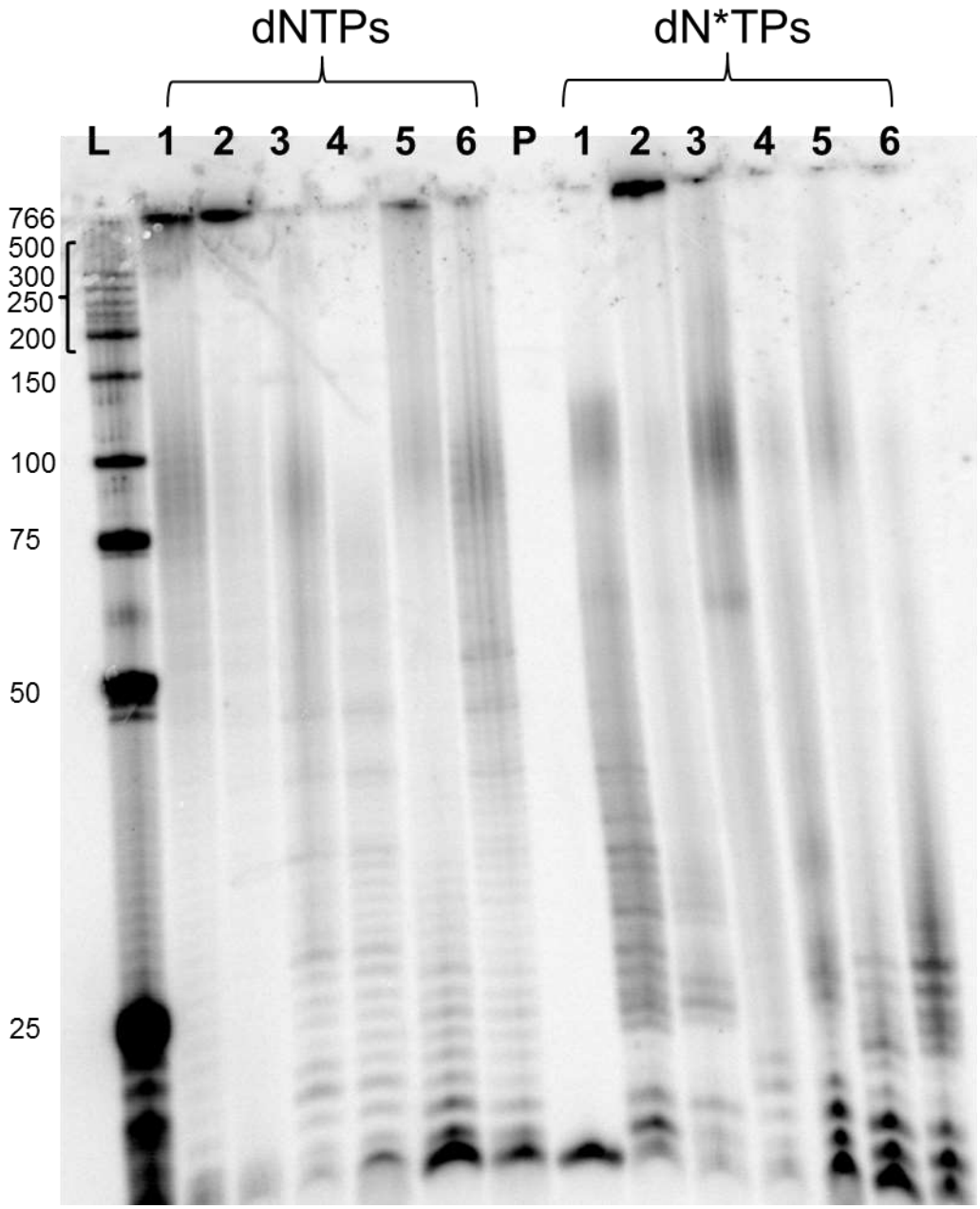

Figure S12. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT2** and 2'-fluoro-rNTPs **5**. DNA polymerases used: lane 1: Vent (*exo*$^-$); lane 2: 9°N_m; lane 3: Klenow fragment; lane 4: Φ29; lane 5: *Pfu*; lane 6: *Pwo*; P = control without polymerase; L = low molecular weight DNA ladder.

Table S1 Evaluation of the efficiency of RCA with different circular templates and nucleoside triphosphates.[a]

Triphosphate used	Template **cT1**	Template **cT2**	Template **cT3**
dN*TPs **1**-**3**	17.8	38.7	23.1
2'-fluoro-rNTPs **5**	4.6	4.6	2.4
α-thio dNTPs **6**	26.9	32.4	28.2
Natural dNTPs	14.1	11.4	12.2

[a] Determined as the fraction of long RCA product formed over the sum of unused primer and shorter products.

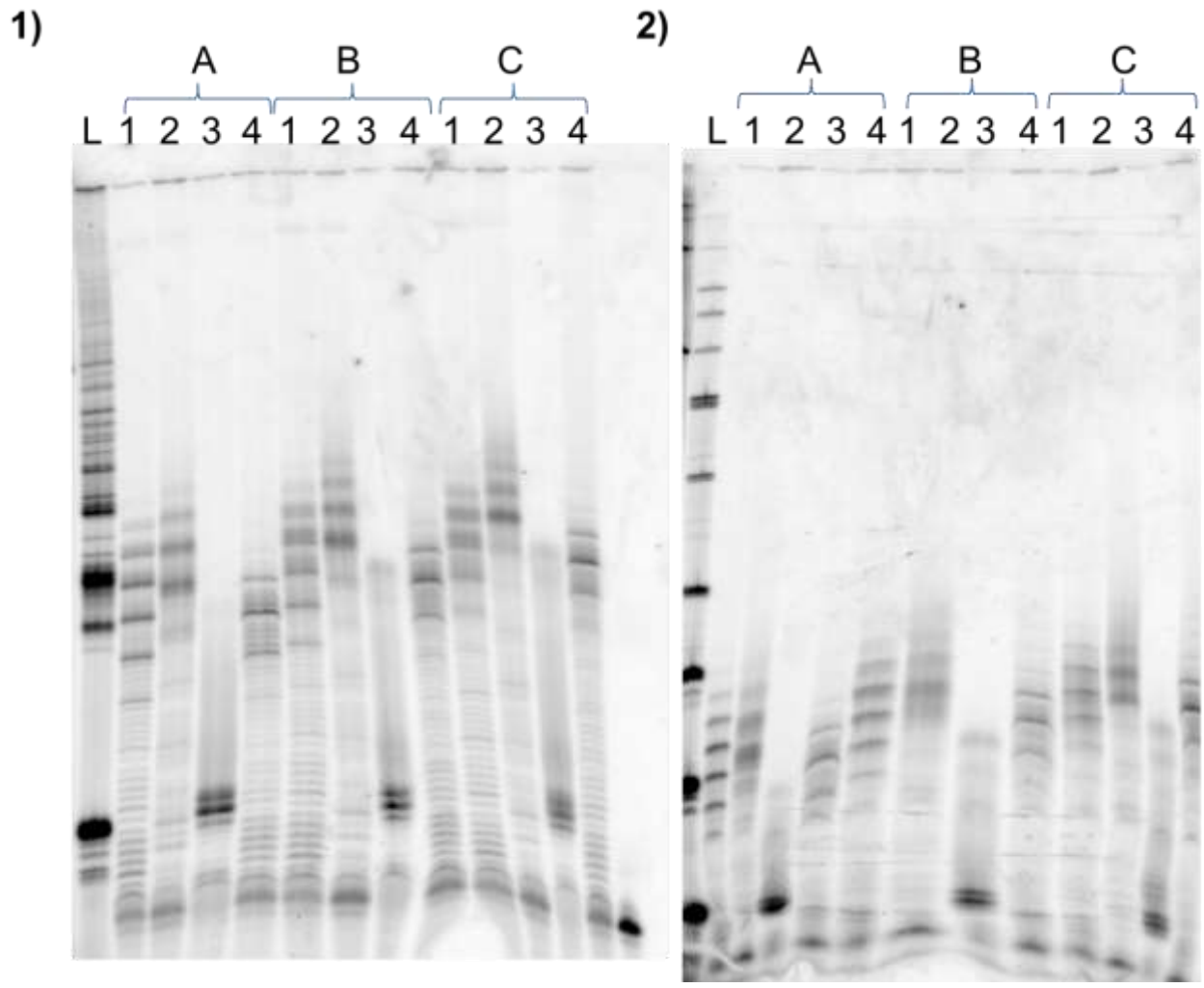

Figure S13. Primer extension reactions using the linear templates **T1** (A), **T2** (B) and **T3** (C) with primer **P1** and the 9°N_m DNA polymerase; the reactions were analyzed by 1) 10% PAGE and 2) 5% PAGE. Lane 1: natural dNTPs; lane 2: base-modified dN*TPs **1**-**3**; lane 3: 2'-fluoro-rNTPs **5**; lane 4: α-thio-dNTPs **6**; L: low molecular weight DNA ladder.

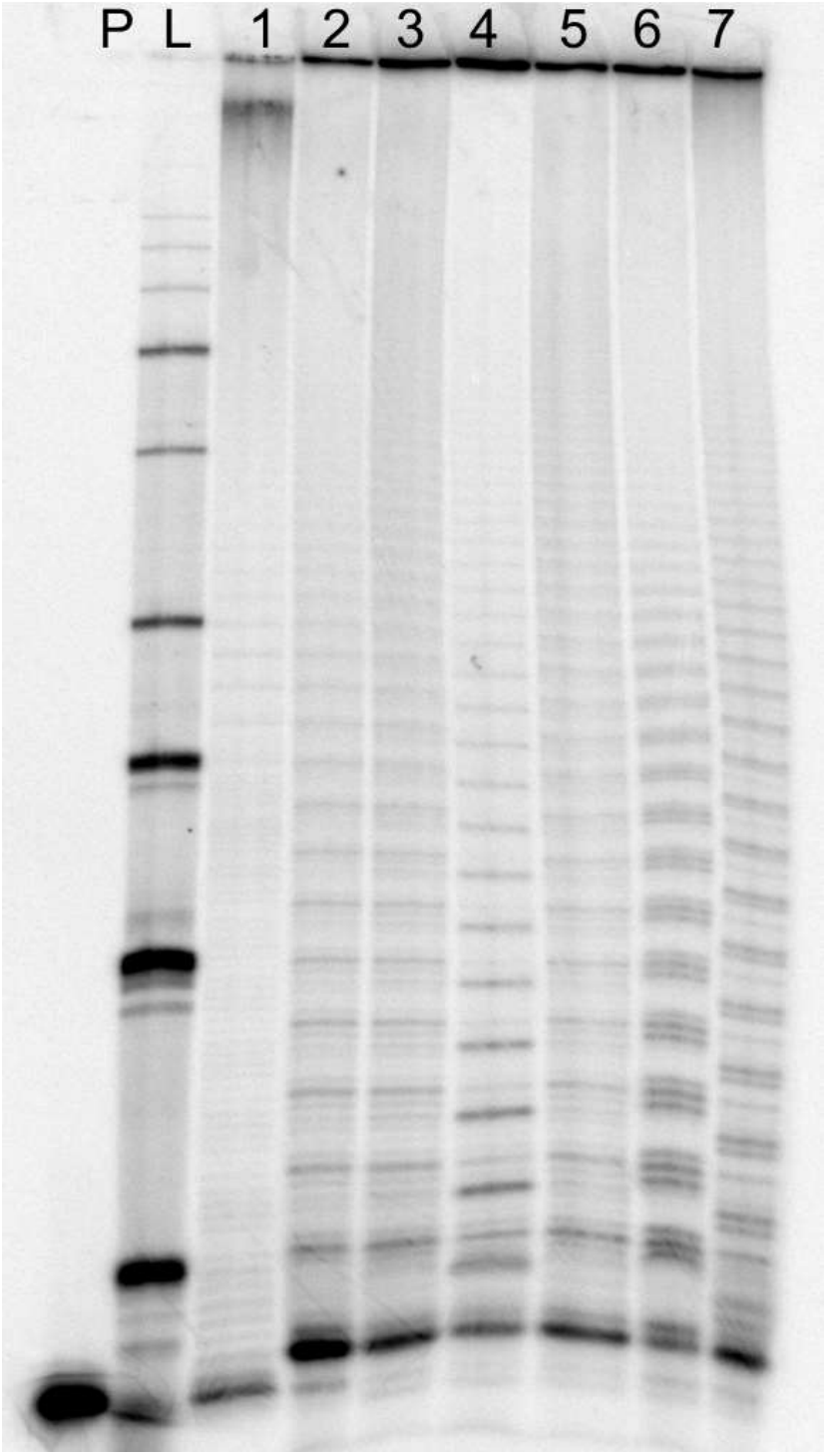

Figure S14. Gel image (PAGE 7%) of the sequencing reaction of the product stemming from the rolling circle amplification using template **cT1** and primer **P1** and natural dNTPs. P: primer **P3** (used for the sequencing reaction); L: low molecular weight DNA ladder; lane 1: untreated RCA product; lane 2: sequencing reaction without dNTPs and ddNTPs; lane 3: sequencing reaction only with dNTPs (i.e. no ddNTPs); Lane 4: with ddATP; Lane 5: with ddCTP; Lane 6: with ddGTP; Lane 7: with ddTTP.

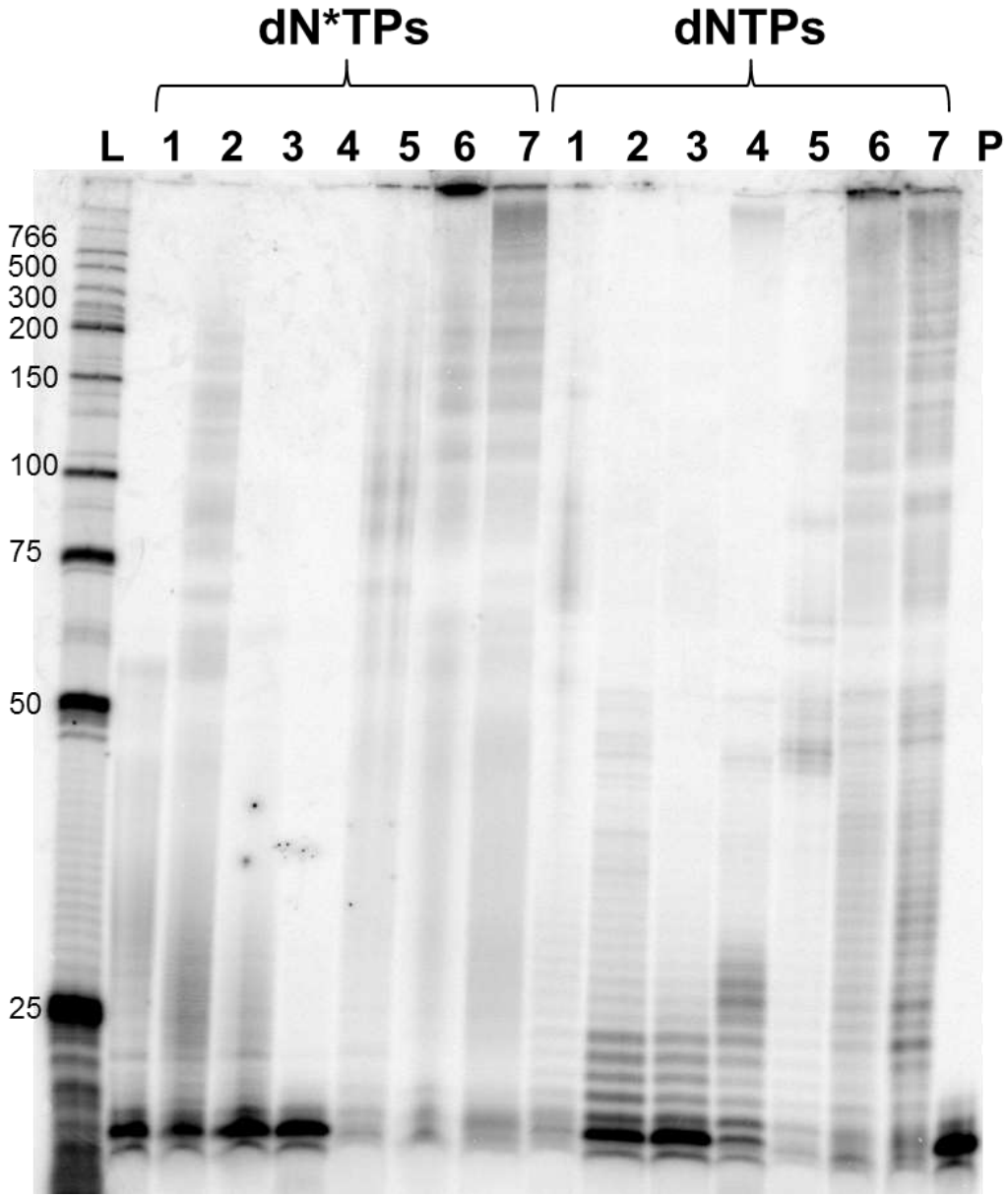

Figure S15. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT4**, primer **P2**, and base-modified dNTPs **1**-**4**. DNA polymerases used: lane 1: Sequenase; lane 2: *Pwo*; lane 3: *Pfu*; lane 4: Φ29; lane 5: Klenow fragment; lane 6: 9°N_m; lane 7: Vent (*exo*$^-$); P = control without polymerase; L = low molecular weight DNA ladder.

Table S2 Evaluation of the efficiency of RCA with template **cT4**, primer **P2**, and various polymerase and natural dNTPs

	Natural dNTPs (10 mM)	
Enzyme	Fraction of primer extended	Long RCA product[a]
Sequenase	64.5 %	15.3 %
Pwo	25.9 %	1.5 %
Pfu	13.0 %	0.2 %
Φ29	60.0 %	3.3 %
Klenow fragment	77.1 %	18.8 %
9°N_m	85.6 %	57.4 %
Vent (*exo*$^-$)	84.6 %	32.0 %

[a] Determined as the fraction of long RCA product formed over the sum of unused primer and shorter products

Table S3 Evaluation of the efficiency of RCA with template **cT4**, primer **P2**, and various polymerase and four base-modified dNTPs

	Base-modified dNTPs (10 mM)	
Enzyme	Fraction of primer extended	Long RCA product[a]
Sequenase	34.9 %	1.5 %
Pwo	59.3 %	1.1 %
Pfu	13.8 %	0.2 %
Φ29	7.7 %	0.7 %
Klenow fragment	82.8 %	35.5 %
9°N_m	92.8 %	87.8 %
Vent (*exo*$^-$)	85.8 %	37.1 %

[a] Determined as the fraction of long RCA product formed over the sum of unused primer and shorter products

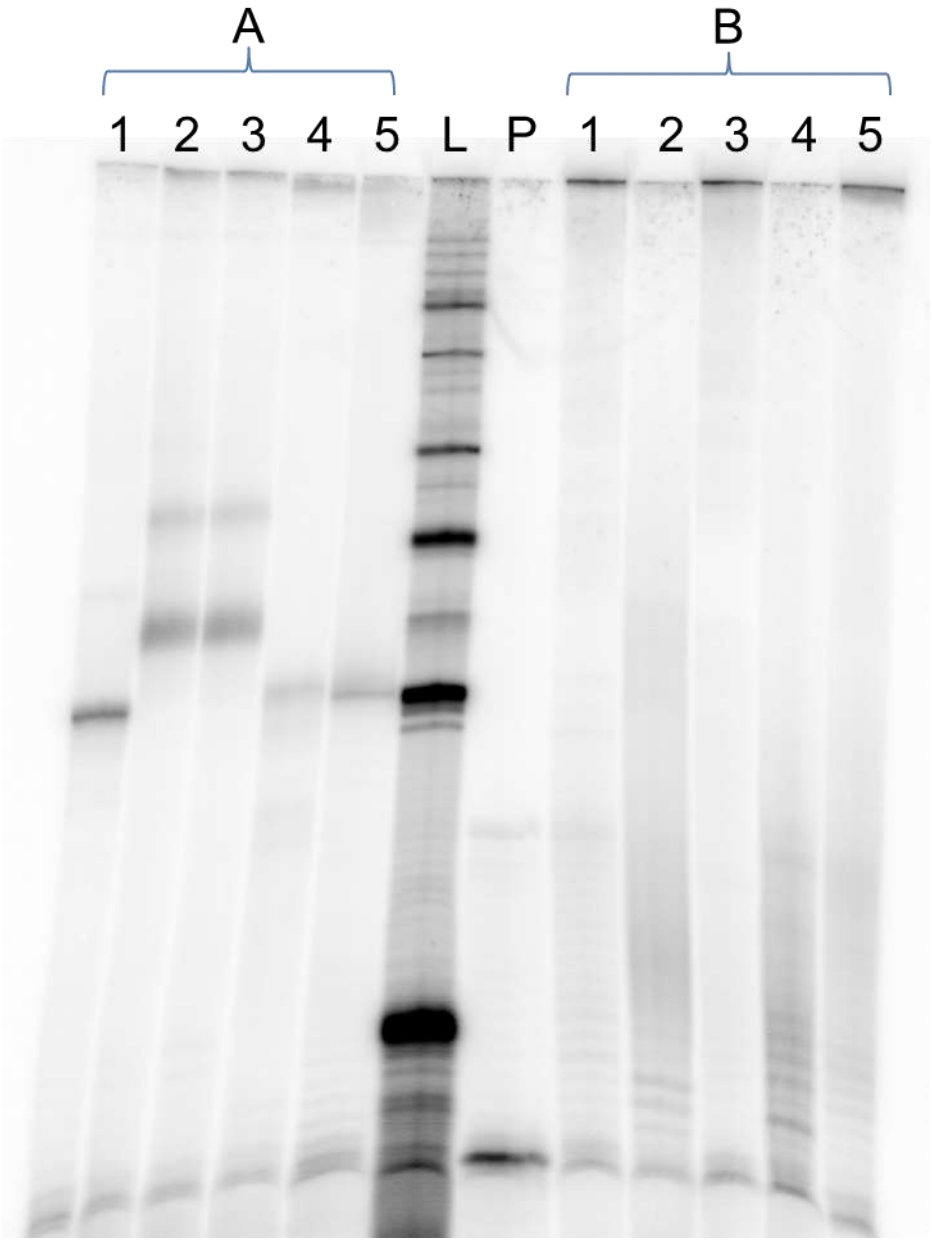

Figure S16. Gel image (PAGE 10%) of A) primer extension using the linear template **T4**, primer **P2** and the 9°N_m DNA polymerase, and B) RCA with template **cT4**, primer **P2**, and the 9°N_m DNA polymerase. Lane 1: natural dNTPs; lane 2: base-modified dN*TPs **1**-**3**; lane 3: base-modified dNTPs **1**-**4**; lane 4: 2'-fluoro-rNTPs ; lane 5: α-thio-dNTPs; L: low molecular weight DNA ladder; P: primer.

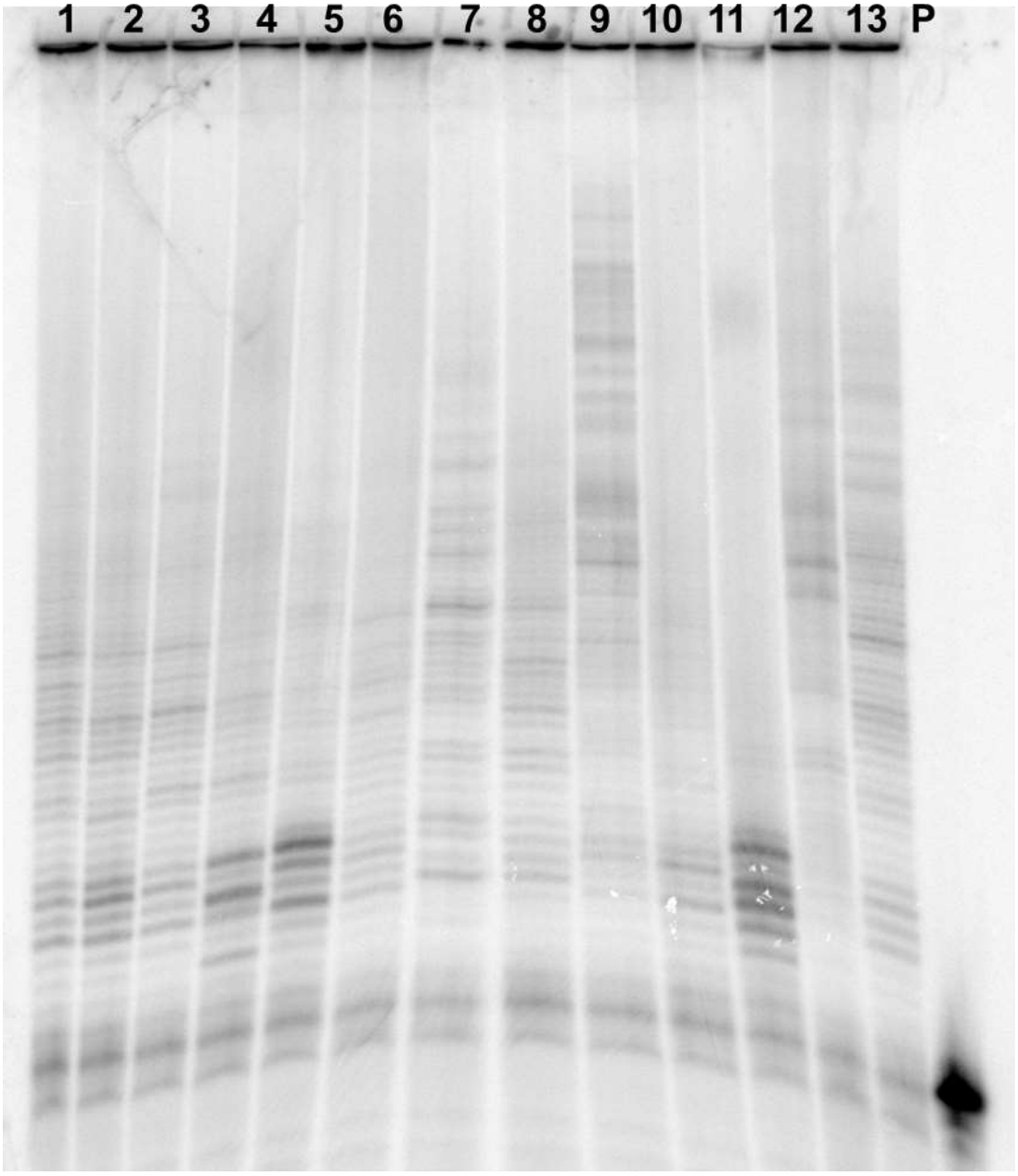

Figure S17. Gel image (PAGE 10%) of the products from the rolling circle amplification with circular template **cT5** and primer **P2**. Lanes **1**-**9** result from reaction mixtures containing one modified dN*TP along with three natural dNTPs, while products in lanes **10**-**12** were generated using three modified dNTPs. Lane 1: α-thio-dTTP; lane 2: α-thio-dCTP; lane 3: α-thio-dATP; lane 4: 2'-fluoro-TTP; lane 5: 2'-fluoro-CTP; lane 6: 2'-fluoro-ATP; lane 7: base-modified dUTP **2**; lane 8: base-modified dCTP **3**; lane 9: base-modified dATP **1**; lane 10: three α-thio-dNTPs and dGTP; lane 11: three 2'-fluoro-rNTPs and dGTP; lane 12: three base-modified dNTPs and dGTP; lane 13: natural dNTPs; P: primer.

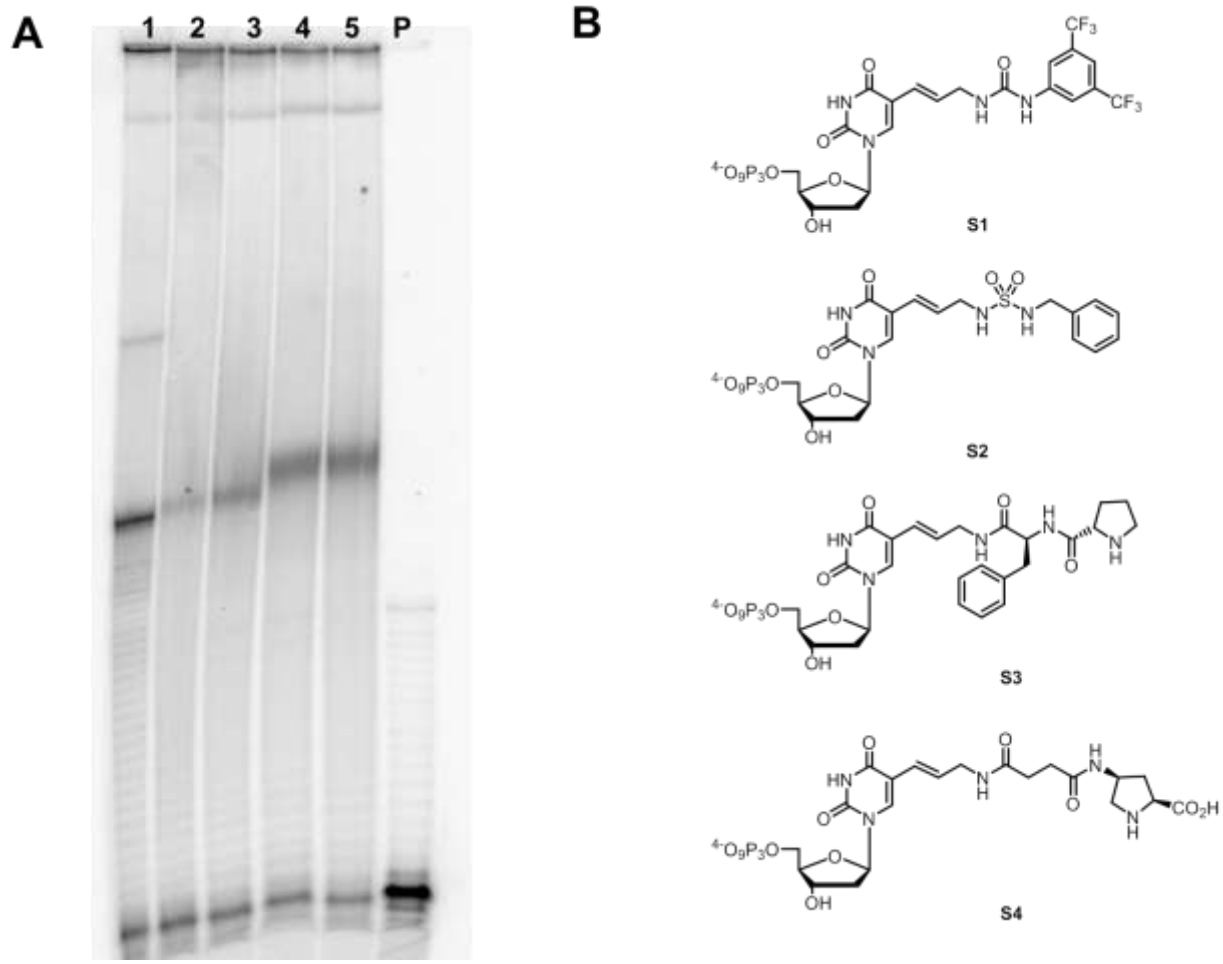

Figure S18. A) Gel image (PAGE 10%) of the fully modified products stemming from the rolling circle amplification with circular template **cT4**, primer **P2**, and the 9°N_m DNA polymerase. The dN*TPs used were α-thio-dATP, α-thio-dCTP, 7-deaza-dGTP **4**, and the dU*TPs shown in B). Lane 1: natural dNTPs; lane 2: dU*TP = **S1**; lane 3: dU*TP = **S2**; lane 4: dU*TP = **S3**; lane 5: dU*TP = **S4**; P : primer.

4. References:

[1] M. Hollenstein, *Chem. Eur. J.* **2012**, *18*, 13320-13330.
[2] M. Hollenstein, *Org. Biomol. Chem.* **2013**, *11*, 5162-5172.