

Supporting Information

for

Antibody-nucleotide conjugate as substrate for DNA polymerases

J. Balintová, M. Welter and A. Marx

*Department of Chemistry, University of Konstanz, Universitätsstrasse 10, 78457
Konstanz, Germany*

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1 Materials

5-(aminopentynyl)-2'-deoxycytidine triphosphate¹ was prepared according to the literature. 6-Maleimidohexanoic acid N-hydroxysuccinimide ester, DBCO-PEG4-NHS were purchased from Sigma Aldrich. Other chemicals were purchased from commercial suppliers and were used as received. Flash chromatography was performed using Merck silica gel G60 (230-400 mesh) and Merck precoated plates (silica gel 60 F254) were used for TLC. Reversed phase high pressure liquid chromatography (RP-HPLC) for the purification of compounds was performed using a Shimadzu system having LC8a pumps and a Dynamax UV-1 detector. A VP 250/16 NUCLEODUR C18 HTec, 5 μ m (Macherey-Nagel) column and a gradient of acetonitrile in 50 mM TEAA buffer were used. All nucleotides purified by RP-HPLC were obtained as their triethylammonium salts after repeated freeze-drying. NMR spectra were recorded on Bruker Avance III 400 (1 H: 400 MHz, 13 C: 101 MHz, 31 P: 162 MHz) or Bruker Avance III 600 (1 H: 600 MHz, 13 C: 150 MHz, 31 P: 243 MHz) spectrometer. The solvent signals were used as references and the chemical shifts converted to the TMS scale and are given in ppm (δ). HR-ESI-MS spectra were recorded on a Bruker Daltonics microTOF II.

DNA polymerases were expressed and purified as described before.² T4 polynucleotide kinase PNK was purchased from New England BioLabs. Oligonucleotides were purchased from Biomers.net. $[\gamma$ -³²P]ATP was purchased from Hartmann Analytics and natural dNTPs from Thermo Scientific. Streptavidin sepharose high performance was purchased from GE Healthcare (Matrix: highly cross-linked agarose, 6%; binding capacity/ml: >300 nmol biotin/ml medium; average particle size: 34 μ m). Primary and secondary antibodies were purchased from Thermo Scientific.

2 Synthesis of 16-azidohexadecanoic acid

To a stirred solution of 16-bromohexadecanoic acid (1 g, 2.98 mmol, 1 eq.) in DMF was added NaN₃ (0.39 g, 5.96 mmol, 2 eq.). Then the reaction mixture was warmed to 100 °C and refluxed for 16 h, then cooled to room temperature. The mixture was extracted with ethyl acetate (3x50 mL) and the combined organic phase was washed with water (3x50 mL) and brine (50 mL) and dried over MgSO₄ and then it was concentrated to get 16-azidohexadecanoic acid. The product was isolated as a white solid (0.7 g, 79 %). **¹H NMR (400 MHz, CDCl₃):** δ 3.25 (t, J =7.0 Hz, 2H, H-16), 2.35 (t, J =7.5 Hz, 2H, H-2), 1.69-1.54 (m, 4H, H-3, H-15), 1.42-1.21 (m, 22H, H-4-14). **¹³C NMR (101 MHz, CDCl₃):** δ 179.75, 51.66, 34.12, 29.76, 29.72, 29.68, 29.63, 29.57, 29.38, 29.30, 29.21, 28.99, 26.87, 24.84.

3 Synthesis of 5-[5-(16-azidohexadecanamido)pent-1-yn-1-yl]-2'-deoxycytidinetriphosphate

To a solution of 16-azidohexadecanoic acid (0.05 g, 0.18 mmol, 2 eq.) in DMF (2 ml) was added Et₃N (62 μ l, 0.45 mmol, 5 eq.) followed by HATU (0.07 g, 0.18 mmol, 2 eq.). The mixture was stirred for 15 min at rt and then 5-(aminopentynyl)-2'-deoxycytidine triphosphate (0.05 g, 0.09 mmol, 1 eq.) was added and mixture was stirred for 16 h at rt. The solvent was removed and the product was purified from the crude reaction mixture by RP-HPLC on a C18 column (95% 50 mM triethylammonium acetate (TEAA) buffer to 100% MeCN). The product was isolated as a white solid (26 mg, 35 %). **¹H NMR (400 MHz, Methanol-d₄):** δ 8.15 (s, 1H, H-6), 6.29 (t, J =6.4 Hz, 1H, H-1'), 4.69-4.59 (m, 1H, H-3'), 4.44-4.33 (m, 1H, H-5'a), 4.32-4.21 (m, 1H, H-5'b), 4.20-4.06 (m, 1H, H-4'), 3.37 (p, J =1.6 Hz, 22H, H-L-16), 3.25 (q, J =7.2 Hz, 17H, -CH₂CH₂CH₂NH-, Et₃N), 2.54 (t, J =6.8 Hz, 2H, -CH₂CH₂CH₂NH-), 2.45-2.36 (m, 1H, H-2'b), 2.30-2.18 (m, 3H, H-L-2, H-2'a), 1.84 (p, J =6.7 Hz, 2H, -CH₂CH₂CH₂NH-), 1.64 (p, J =7.3 Hz, 4H, H-L-3, H-L-15), 1.48-1.26 (m, 48H, H-L-4-14, Et₃N). **³¹P NMR (162 MHz, Methanol-d₄):** δ -10.48 (d, J =20.8 Hz, P), -11.43 (d, J =21.5 Hz, P), -23.83 (d, J =20.7 Hz, P). **HRMS (ESI):** calcd. for C₃₀H₅₁N₇O₁₄P₃: 826.2707; found 826.2729.

4 Synthesis of antibody-labeled nucleotide (dC^{pAbTP})

The antibody was first reacted with DBCO-PEG₄-NHS following a standard procedure for antibody conjugation through the NH₂ group. In brief, primary (mouse monoclonal IgG1-Apolipoprotein A1 monoclonal antibody, 100 µg in PBS buffer) was incubated with DBCO-PEG₄-NHS (5 eq.) in PBS buffer (pH 7.4) for 2 h at rt. After reaction, the free DBCO-PEG₄-NHS was removed using the Amicon Ultra Centrifugal Filter (50 kDa). To determine the number of DBCO molecules on the antibody, the absorbance at 309 and 280 nm was measured. The molar concentrations of DBCO and antibody were determined using their respective molar extinction coefficient (12 000 M⁻¹cm⁻¹ for DBCO at 309 nm and 204 000 M⁻¹cm⁻¹ for antibody at 280 nm). The number of DBCO molecules per antibody was calculated by dividing the molar concentration of DBCO by the molar concentration of antibody. We obtained 3.1 DBCO molecules per pAb after reaction. The DBCO-functionalized antibody reacted with azide-modified nucleotide ($\text{dC}^{\text{C16N3TP}}$, 10 eq.). The reaction was conducted at 4 °C for 16 h. The solution was purified either by anion exchange FPLC (HiTrap Q HP, GE Life Science) with a gradient from Buffer A (20 mM TRIS, pH 9) to Buffer B (20 mM TRIS, 1 M NaCl, pH 9) or by the Amicon Ultra Centrifugal Filter Amicon Ultra Centrifugal Filter (10 kDa). The final concentration of the obtained conjugate was determined using their respective molar extinction coefficient (204 000 M⁻¹cm⁻¹ at 280 nm).

5 Primer extension experiments

The reaction mixture (10 µL) contained DNA polymerase (KTq 100 nM, KOD 150 nM, 9°N 150 nM), template (Template^{BRAF}, 0.2 µM), primer (Primer^{BRAF}, 0.15 µM), modified dNTPs (1 µM) and unmodified dATP, dTTP, dGTP and dC^{pAbTP} (for experiment in Figure 2 lane BRAF-I, each 1 µM) in 1x polymerase buffer (DNA sequences see table S1, buffer composition see table S2). Primer was labeled by use of [³²P]ATP according to standard techniques. Reaction mixtures were incubated for time points at 55 °C and analysed by 9% PAGE electrophoresis.

6 Primer extension experiment on streptavidin-coated sepharose beads (dC^{pAbTP})

10 µL of a streptavidin-coated sepharose bead slurry (GE Life Science) were spun down (2400 x g) and the supernatant was discharged carefully. The beads were then washed three times with 100 µL 1 x binding buffer and 9.5 µL binding buffer and 0.5 µL 5'-biotinylated primer (Primer^{BIOTIN-BRAF}, 100 µM) were added. Following 30 min of incubation with gentle mixing, 5.5 µL of 1 mM (D)-+-biotin in binding buffer were added to block the remaining streptavidin moieties. After further 10 min, the beads were spun down again and washed three times with 200 µL binding buffer and three times with 200 µL 1x polymerase buffer. For the primer extension reaction, 22.4 µL milliQ, 3 µL 10x polymerase buffer and 1 µL template (Template^{BRAF-MATCH}) were added to the beads and the mixture was mixed every minute for 10 min to prevent the beads from settling down in the tube. Subsequently, DNA polymerase and 1 µL of 5 µM dC^{pAbTP} were added. The reaction mixture was incubated at 55°C for 5 min, then the beads were spun down and transferred to an empty spin column cartridge. After removing the reaction mixture by short spin centrifugation in a table top centrifuge, the beads were washed three times with 200 µL 1x polymerase buffer and five times with 200 µL PBS buffer (pH 7.4) and 29 µL PBS buffer and 1 µL of 0.1 mg/ml secondary antibody (goat anti-mouse IgG H+L Superclonal, HRP) was added to the reaction mixture and incubated for 4h at rt and then beads were washed 5 times with PBS buffer (pH 7.4) for 5 minutes.

Visualization with o-dianisidine: The reaction mixture was washed 5 times with 200 µL

detection buffer I. Subsequently, the supernatant was discharged and 20 μ L of the developing solution was applied (1 mM *o*-dianisidine/H₂O₂ in detection buffer I). The reaction was quenched by the addition of 5 μ L of 10 N sulfuric acid. Absorptions were measured with a Nanodrop ND-1000 spectrometer at 450 nm and 520 nm. Pictures were taken with a digital camera.

Visualization with Amplex red: The reaction mixture was washed 5 times with 200 μ L detection buffer II. Subsequently, the supernatant was discharged and 20 μ L of the developing solution was applied (250 μ M Amplex red/H₂O₂ in detection buffer II). Absorption was measured with a Nanodrop ND-1000 spectrometer at 570 nm. Pictures were taken with a digital camera.

The antibody-modified DNA was incubated with the secondary antibody (goat anti-mouse IgG H+L Superclonal, HRP, final concentration is 3.3 μ g/ml) for 2 hours at rt. We found that at higher concentration of the secondary antibody the background signal increased. The reaction condition and the purification process (to remove any unbound antibody-labeled nucleotide and the secondary antibody) were optimized in order to determine the detection limit and exclude the possibility of non-specific binding.

7 Sequences of primers and templates

Table S1. Primers and templates used for PEX experiments.^[a]

Sequences	
Primer ^{BRAF}	5'-d(GACCCACTCCATCGAGATTT)-3'
Template ^{BRAF-G}	5'-d(TGCCTGGTGTTTGGGA <u>G</u> AAATCTCGATGGAGTGGGTC)-3'
Template ^{BRAF-A}	5'-d(TGCCTGGTGTTTGGGA <u>A</u> AAATCTCGATGGAGTGGGTC)-3'
Template ^{BRAF-C}	5'-d(TGCCTGGTGTTTGGGA <u>C</u> AAATCTCGATGGAGTGGGTC)-3'
Template ^{BRAF-T}	5'-d(TGCCTGGTGTTTGGGA <u>T</u> AAATCTCGATGGAGTGGGTC)-3'
Template ^{BRAF-M}	5'-d(TGCCTGGTGTTTGGGG <u>G</u> AAATCTCGATGGAGTGGGTC)-3'
Template ^{BRAF-I}	5'-d(TGCCTGGTGTTT <u>GATC</u> GAAATCTCGATGGAGTGGGTC)-3'
Primer ^{BIOTIN-BRAF}	5'-biotin-d(TTTTTTTTTTTTTTTTTTTTGACCCACTCCATCGAGATTT)-3'
Template ^{MATCH-BRAF}	5'-d(GGTCTAGCTACAGAGAAATCTCGATGGAGTGGGTC)-3'

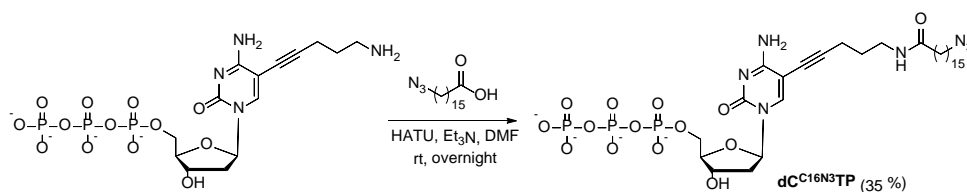
^[a] In the template, segments that form a duplex with the primer are in **bold**. First incorporation site is underlined, expected incorporation sites are marked in italic.

8 Buffers and solutions

Table S2. Buffers and solutions.

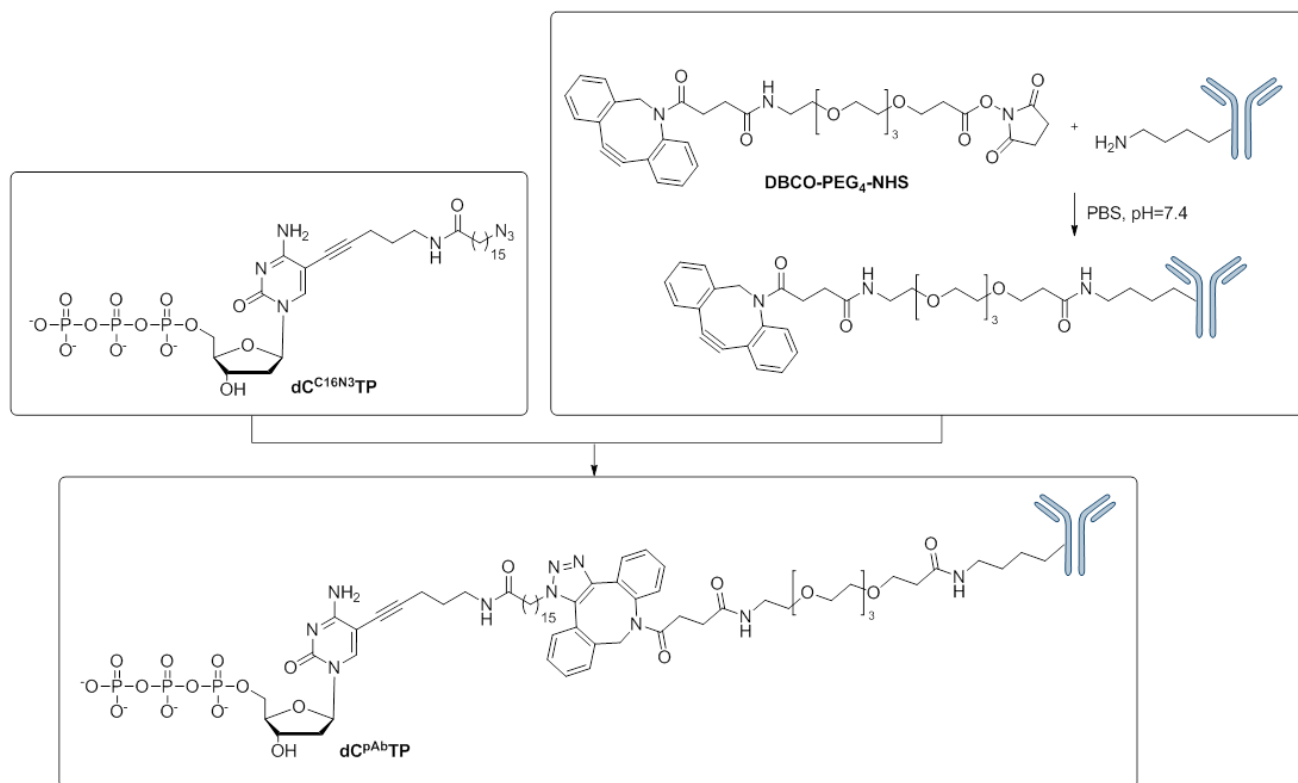
Buffers and solutions	
TEAA buffer	50 mM triethylammonium acetate, pH 7
10x <i>KTq</i> reaction buffer	160 mM Tris, 500 mM (NH ₄) ₂ SO ₄ , 25 mM MgCl ₂ , 1% Tween 20, pH 9.2
10x <i>KOD</i> reaction buffer	160 mM Tris, 500 mM (NH ₄) ₂ SO ₄ , 25 mM MgCl ₂ , 1% Tween 20, pH 8
10x 9°N reaction buffer	200 mM Tris, 100 mM (NH ₄) ₂ SO ₄ , 100 mM KCl, 20 mM MgCl ₂ , 1% Triton, pH 8.8
Stopping solution	80% v/v formamide, 20 mM EDTA, 0.025% w/v bromphenol blue, 0.025% w/v xylene cyanol
Binding buffer	200 mM Na ₃ PO ₄ , 1.5 M NaCl, pH 7.5
PBS buffer	20 mM Na ₃ PO ₄ , 150 mM NaCl, pH 7.4
Detection buffer I	100 mM sodium citrate, pH 5
Detection buffer II	50 mM Tris, pH 7.4

9 Synthesis of azido-modified nucleotide (**dC^{C16N3}TP**)



Scheme 1. Synthesis of the azide-modified nucleotide (**dC^{C16N3}TP**).

10 Synthesis of antibody-labeled dNTP (dC^{pAbTP})



Scheme 2. Synthesis of the antibody-labeled nucleotide (dC^{pAbTP}).

11 Primer extension experiments (9°N, *KlenTaq*)

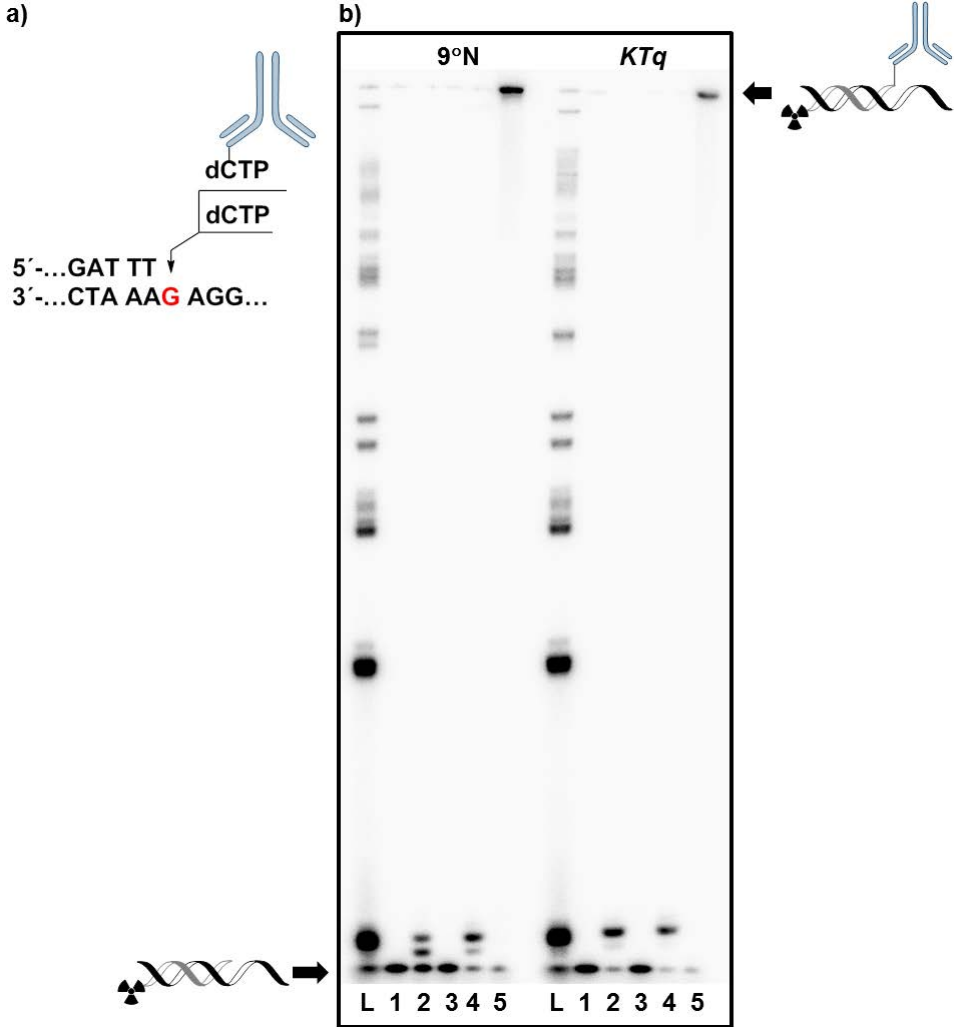


Figure 1. PAGE of PEX single incorporations of employing the modified nucleotides ($dC^{C16N3}TP$, $dC^{pAb}TP$); a) Partial sequence of the primer and the template at the incorporation site; b) Autoradiography of a primer extension reaction employing natural dCTP (Lane 2), no dCTP (Lane 3), $dC^{C16N3}TP$ (Lane 4) and conjugate ($dC^{pAb}TP$, lane 5) with 9°N and *Ktq* DNA polymerase on a template containing the BRAF T1796A point mutation site; Samples were collected after 5 min. Lane 1: Primer, L: Marker.

12 Primer extension experiments with mismatch and multiple incorporation templates – SDS-PAGE

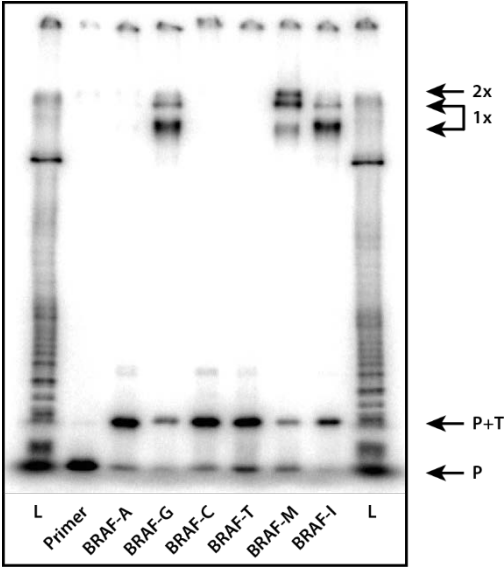


Figure 2. SDS-PAGE of PEX single and multiple incorporation of the modified nucleotide ($\text{dC}^{\text{pAb}}\text{TP}$). Autoradiography of a primer extension reaction employing the conjugate ($\text{dC}^{\text{pAb}}\text{TP}$) with KOD DNA polymerase on templates containing the BRAF T1796A point mutation site with mismatch pairings and multiple incorporation sites (see sequence table); Samples were collected after 5 min and 30 min and resolved on a 7.5-12.5% SDS-PAGE gradient gel. L: Marker.

13 Competition experiments

A typical competition experiment (10 μ L) employing KOD DNA polymerase contained 1x polymerase reaction buffer, 32 P-labelled primer (Primer^{BRAF}, 0.15 μ M), template (Template^{BRAF}, 0.2 μ M), ratio dCTP: dC^{pAb}TP mixture, and KOD DNA polymerase (150 nM). First primer and template were annealed. Afterwards the primer-template complex, nucleotides and DNA polymerase were incubated (55 $^{\circ}$ C; 5 min). The reactions were quenched by addition of PAGE gel loading buffer/stop solution and the product mixtures were analysed by 12% denaturing polyacrylamide gel and subjected to autoradiography. Quantification was done by using the Bio-Rad Quantity One software. The conversion in percent was plotted versus the concentration using the program ImageLab.

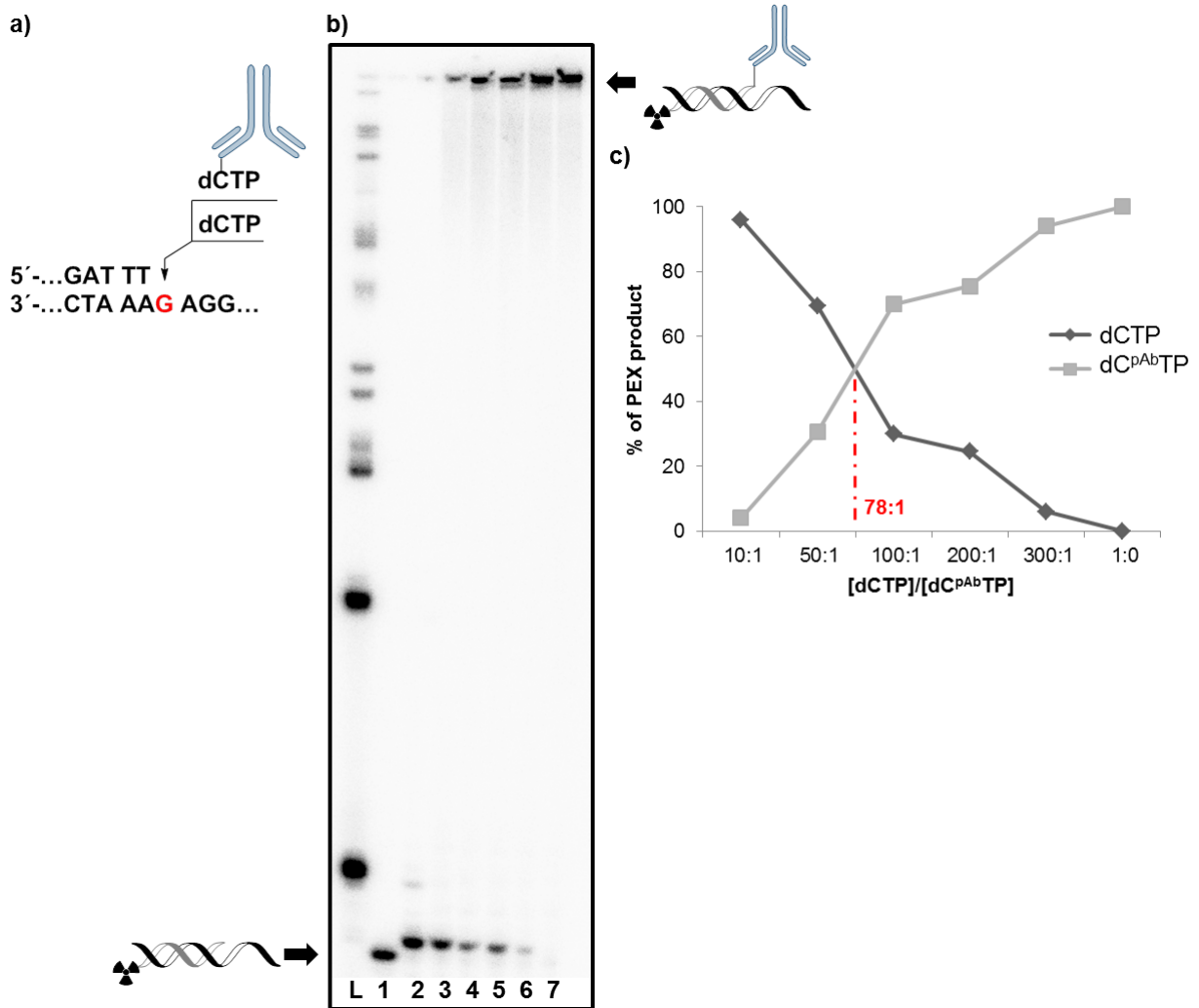
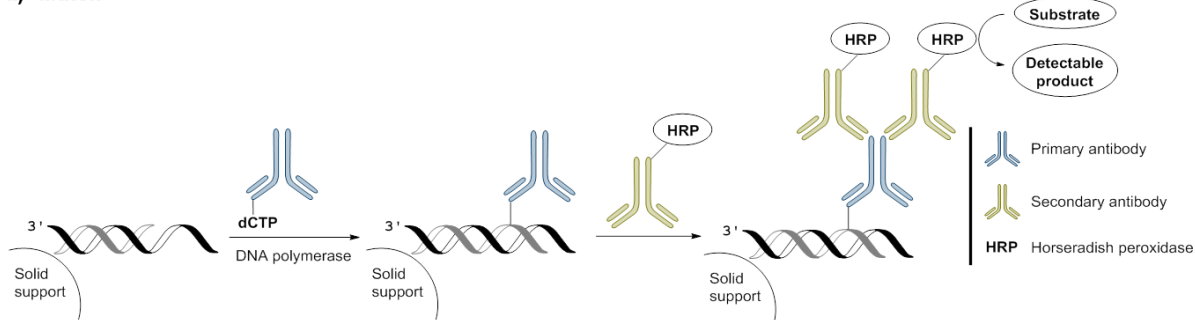


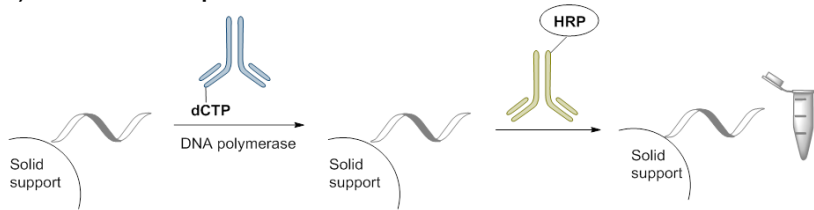
Figure 3. Incorporation competition experiments between the dC^{pAb}TP and dCTP with KOD DNA polymerase. a) Partial sequence of the primer and the template at the incorporation site; b) PAGE analysis of the competition experiments with various ratios of dC^{pAb}TP versus dCTP: Ratios applied for dC^{pAb}TP:dCTP were 2) 10:1, 3) 50:1, 4) 100:1, 5) 200:1, 6) 300:1; 7) 1:0; c) Quantification of the PAGE analysis. The point of equal incorporation is indicated by a red dashed line. Samples were collected after 5 min. Lane 1: Primer, L: Marker.

14 Scheme of naked eye detection assay

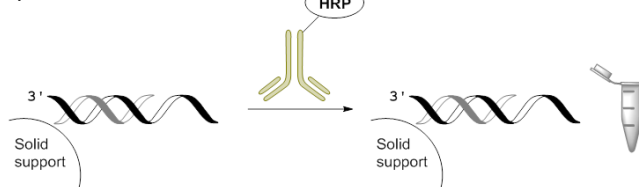
a) Match



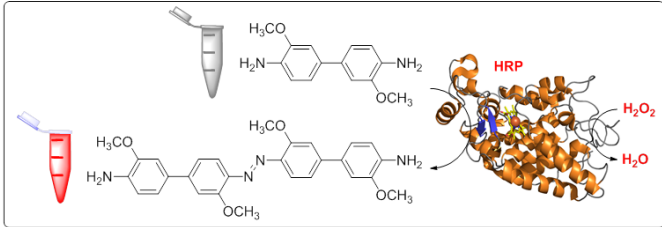
b) Absence of template



c) Absence of dC^{pAb}TP



d) Peroxidase oxidation of *o*-dianisidine



e) Peroxidase oxidation of Amplex Red

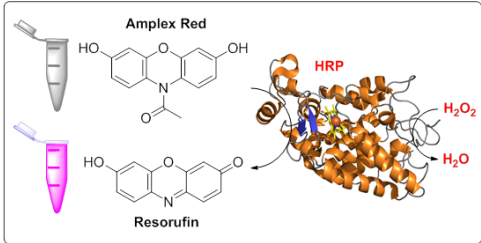
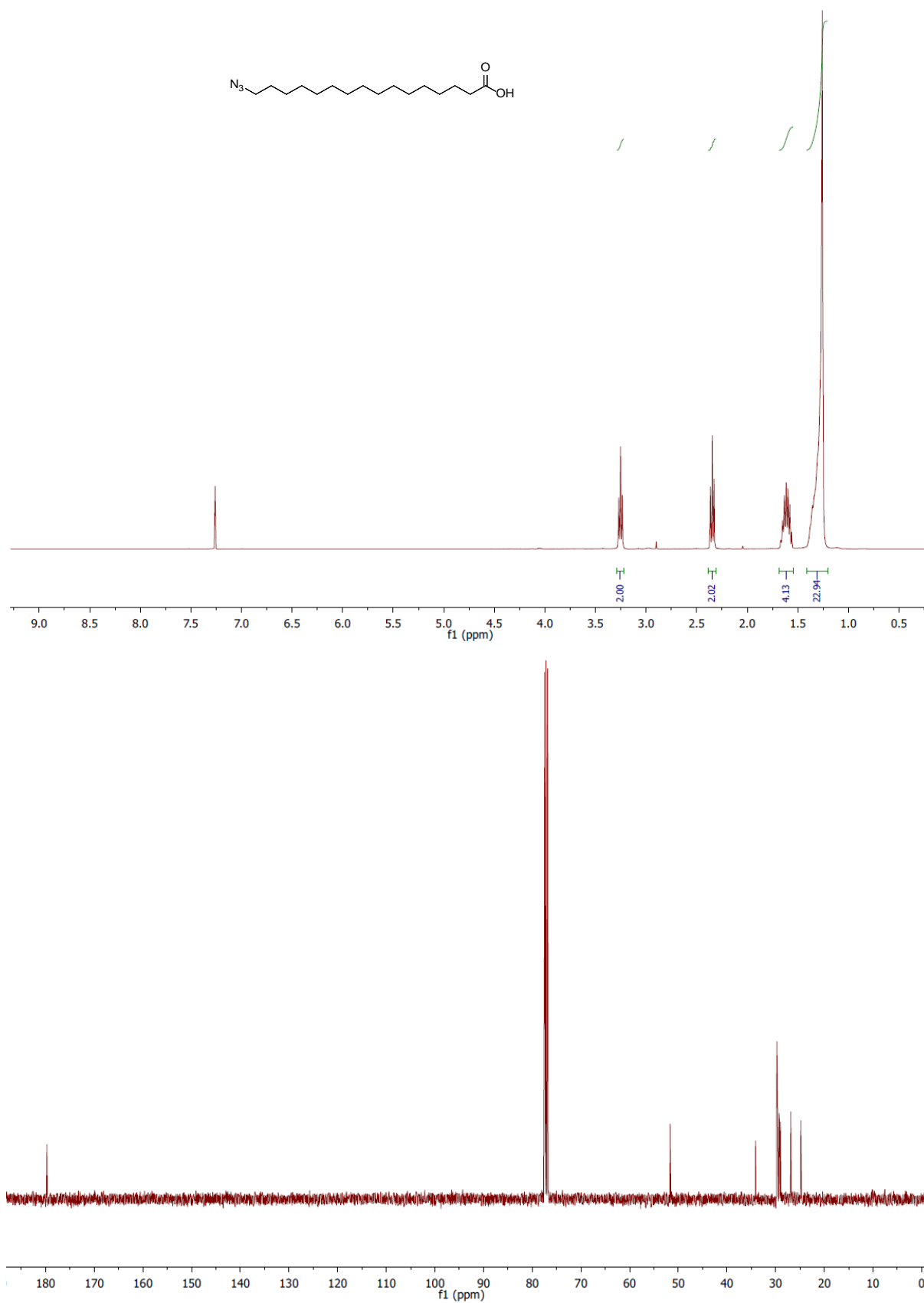


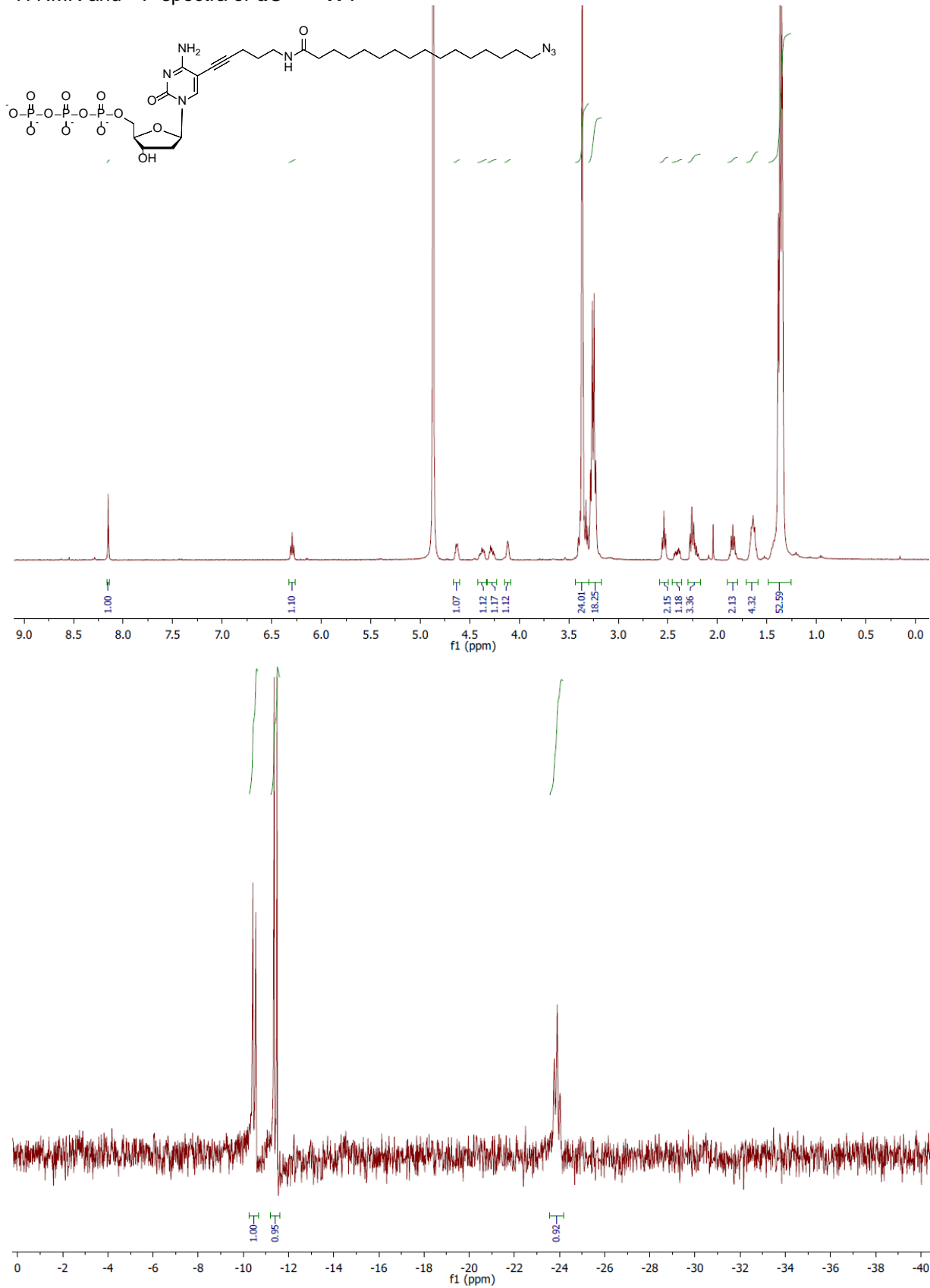
Figure 4. a-c) The naked-eye detection assay employing dC^{pAb}TP; d-e) Peroxidase oxidation of the substrate: d) chromogenic substrate: *o*-dianisidine; e) fluorogenic substrate: Amplex red.

15 NMR spectra

^1H and ^{13}C NMR of 16-azidehexadecanoic acid



^1H NMR and ^{31}P spectra of **dC¹⁶N³TP**.



16 References

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² a) D. Summerer, N. Z. Rudinger, I. Detmer, A. Marx, *Angew. Chem. Int. Ed.*, 2005, **44**, 4712; b) K. Betz, F. Streckenbach, A. Schnur, T. Exner, W. Welte, K. Diederichs, A. Marx, *Angew. Chem. Int. Ed.*, 2010, **49**, 5181; c) H.M. Kropp, K. Betz, J. Wirth, K. Diederichs, A. Marx *Plos ONE* **2017**, **12**, e0188005.