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Supporting information

The 5-chlorouracil:7-deazaadenine as an alternative to dT:dA base pair

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CONTENTS

EXPERIMENTAL SECTION

Synthesis of phosphoramidites and oligonucleotides

***General*.** For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware under N_2. Reagents and solvents were provided by Acros (Fluka) or TCI. TLC: Pre-coated aluminum sheets (Fluka silica gel/TLC cards, 254 nm); the spots were visualized with UV light. Column chromatography (CC): ICN silica gel 63–200 60 P. ^{31}P-NMR spectra: a Bruker Avance 300-MHz, or a Bruker Avance 500-MHz spectrometer. Exact mass measurements were performed on a quadrupole time-offlight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard electrospray-ionization (ESI) interface; samples were infused in i-PrOH/H2O 1 : 1 at 3 cm^3/min.

The phosphoramidites were incorporated into the DNA sequences through solid-phase DNA synthesis[1] on an automated RNA synthesizer (for structures of chemically modified nucleotide monomers see Table S3). A_1, A_3, and T_1 containing modified oligonucleotides were obtained by using commercially available amidites (Glen Research). The chemical synthesis of A_2 modified phosphoramidite is previously described.[2,3] The T_2 and T_3 phosphoramidite were synthesized as O$^{5'}$-DMTr derivatives starting from 5-chloro-2' -deoxyuridine[4] and commercial 5-ethyl-2' -deoxyuridine, respectively.

For a standard DNA synthesis cycle (1 µmol scale), O$^{5'}$-DMTr protected DNA phosphoramidites for ultramild DNA synthesis (Glen Research) and common reagents were used and the stepwise coupling yield of all monomers was >99%. Following standard deprotection, purification and workup, the composition and purity (>90%) of the resulting oligonucleotides was confirmed by MALDI-MS analysis and ion exchange HPLC.

Melting temperatures (T_m) values were measured as the maximum of the first derivative of the melting curve (A_{260} and A_{270} *vs*. temperature 10 to 85 °C and 85 to 10 °C; increase 1 °C min^{-1}) recorded in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 4 µM concentrations with complementary DNA.

5'-Dimethoxytrityl-5-chloro-2'-deoxyuridine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite*.*

To a colorless solution of 5-chloro-2'-deoxyuridine[4] (600 mg, 2.28 mmol) in pyridine (20 mL) DMTrCl (930 mg, 2.75 mmol) was added in one portion at RT. The reaction mixture was stirred for 12 h and turn to yellow-orange color. When the starting material has disappeared the reaction mixture was cooled in an ice bath, methanol (1 mL) was added, and the reaction mixture was concentrated and co-evaporated twice with toluene. The residue was dissolved in DCM, washed with H_2O, dried over Na_2SO_4, and purified by column chromatography on silica gel to yield 5'-*O*-dimethoxytrityl-5-chloro-2'-deoxyuridine (1.08g, 84%). The separated compound (1.08 g, 1.91 mmol) was dissolved in DCM (10 mL) and cooled in an ice-bath. DIPEA (1.5 mL, 8.76 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.58 mL, 2.6 mmol) were added. The reaction solution was stirred for 30 min at RT. Upon completion the reaction mixture was concentrated and coevaporated twice with toluene. The crude material was purified by column chromatography on silica to yield 5'-dimethoxytrityl-5-chloro-2'-deoxyuridine, 3'-[(2-cyanoethyl)-(*N,N*-diisopropyl)]-phosphoramidite (1.0 g, 68%). ^{31}P NMR ($CDCl_3$, 25 °C): δ = 149.08, 148.7. HRMS calculated (calcd) for $C_{39}H_{46}ClN_4O_8P$, [MH+] 765.2794, found 765.2820.

5'-Dimethoxytrityl-5-ethyl-2'-deoxyuridine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite.

To a colorless solution of 5-ethyl-2'-deoxyuridine (0.50 g, 1.95 mmol) in pyridine (10 mL) DMTrCl (0.790 g, 2.34 mmol) was added in one portion at RT. The reaction mixture was stirred for 12 h and turn to yellow-orange color. When the starting material has disappeared the reaction mixture was cooled in an ice bath, methanol (1 mL) was added, and the reaction mixture was concentrated and coevaporated twice with toluene. The residue was dissolved in DCM, washed with H_2O, dried over Na_2SO_4, and purified by column chromatography on silica gel to yield 5'-*O*-dimethoxytrityl-5-ethyl-2'-deoxyuridine (0.95 g, 87%). The separated compound (0.95 g, 1.7 mmol) was dissolved in DCM (5 mL) and cooled in an ice-bath. DIPEA (1.45 mL, 8.4 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.62 mL, 2.8 mmol) were added. The reaction solution was stirred for 30 min at RT. Upon completion the reaction mixture was concentrated and coevaporated twice with toluene. The crude material was purified by column chromatography on silica to yield 5'-dimethoxytrityl-5-ethyl-2'-deoxyuridine , 3'-[(2-cyanoethyl)-(*N,N*-diisopropyl)]-phosphoramidite (1.2g, 93%). ^{31}P NMR ($CDCl_3$, 25 °C): δ = 148.9, 148.5. HRMS calcd for $C_{41}H_{51}N_4O_8P$, [MH+] 759.3523, found 759.3530.

Synthesis of deoxyribonucleoside 5'-triphosphate tetrabutylammonium salt.

General. A_1-A_3, T_2 2'-deoxyribonucosides were converted to their 5'-triphosphates in one-pot reaction by Ludwig method.[5–8] To an ice-cold solution or suspension of corresponding deoxyribonucleoside (0.1 mmol) in trimethyl phosphate (TMP) (1.0 mL) was added phosphoryl chloride (20µL, 0.22 mmol.) and the solution was stirred at 0°C for 5 hours. Tributylamine (300 uL, 1.6 mmol) and tetrabutylammonium pyrophosphate solution (0.5 M in DMF, 1.1 mmol) were added simultaneously, and the solution was stirred for a further 30 minutes. The reaction was then quenched by the addition of 0.5 M triethylammonium bicarbonate (TEAB) buffer (10 mL), and stored at 4°C overnight. The solvent was evaporated and the residue was treated with 25% ammonia (4 mL). The solution was evaporated to dryness and re-dissolved in water (5 mL) and applied to a Sephadex A25 column in 0.1 M TEAB buffer. The column was eluted with a linear gradient of 0.1-1.0 M TEAB. Appropriate fractions were pooled and evaporated to dryness to give desired product. HPLC (Alltima 5µ C-18 reverse phase column 10x250 mm, buffer A, 0.1 M TEAB; buffer B, 0.1 M TEAB, 25% MeCN. 0% to 100% buffer B over 60 minutes at 3 mL/min) showed the product to be pure.

8-Aza-2'-deoxyadenosine 5'-triphosphate tetrabutylammonium salt. ^{31}P NMR (D_2O): δ -7.62 (1P, br s), -11.00 (1P,d), -22.12 (1P, br s). ESIHRMS found: *m/z* 490.9884. calcd for $C_9H_{15}N_6O_{12}P_3$: $(M-H)^-$ 490.9888.

8-Aza-7-deaza-2'-deoxyadenosine 5'-triphosphate tetrabutylammonium salt. ^{31}P NMR (D_2O): δ -9.80 (1P, d), -10.85 (1P,d), -23.05 (1P, t). ESIHRMS found: *m/z* 489.9948. calcd for $C_{10}H_{16}N_5O_{12}P_3$: $(M-H)^-$ 489.9935.

5-Chloro-2'-deoxyuridine 5'-triphosphate tetrabutylammonium salt. ^{31}P NMR (D_2O): δ -7.38 (1P, d), -11.29 (1P, d), -22.18 (1P, t). ESIHRMS found: *m/z* 500.9279 calcd for $C_9H_{14}ClN_2O_{14}P_3$: $(M-H)^-$ 500.92735

SUPPORTING TABLES

Table S1. pKa and ΔpKa of investigated modified Tx and Ax nucleosides

Ax (pKa) / Tx (pKa)	A (3.5)	A_1 (5.2)	A_2 (2.4)	A_3 (4.2)
T (9.7)	6.2	4.5	7.3	5.5
T_1 (9.3)	5.8	4.1	6.9	5.1
T_2 (7.9)	4.4	2.7	5.5	3.7
T_3 (9.6)	6.1	4.4	7.2	5.4

Table S2. Full length product formation with modified adenosine triphosphates.[a]

	Mn^{2+}	**A**		**A_1**		**A_2**		**A_3**	
		30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
Taq	-	79	81	79	85	5	15	12	24
	+	n.d.	n.d.	50	67	17	22	12	13
Vent exo-	-	90	94	77	89	80	90	18	47
	+	n.d.	n.d.	79	83	88	91	66	73
KF exo-	-	84	70	85	83	35	25	79	73
	+	n.d.	n.d.	97	86	79	66	84	57
PolIIIα	-	83	81	60	61	79	79	52	69
	+	n.d.	n.d.	77	75	89	83	78	81

a Yield of full length product is shown as a percentage in the line together with extended non-templated (>P+7) products. N.d. – non determined

Table S3. Calculated and found mass spectrometry data for the synthesized oligonucleotides containing A_1, A_2, A_3, T_1, T_2, and T_3 modified nucleotides.

A_1 *- 7-deaza-dA modified DNAs (5'→3')*	*MS*	*Calcd*	*Found*
CCG T***A_1***A ATG ACC		3611.7	3611.6
CCG T***A_1***A ATG ***A_1***CC		3610.7	3610.7
CCG T***A_1A_1 A_1***TG ACC		3609.7	3609.6
A_2 *- 8-aza-dA modified DNAs (5'→3')*	*MS*	*Calcd*	*Found*
CCG T***A_2***A ATG ACC		3613.7	3613.7
CCG T***A_2***A ATG ***A_2***CC		3614.6	3614.8
CCG T***$A_2A_2A_2$***TG ACC		3615.6	3615.8
A_3 *- 8-aza-7-deaza-dA modified DNAs (5'→3')*	*MS*	*Calcd*	*Found*
CCG T***A_3***A ATG ACC		3612.7	3613.4
CCG T***A_3***A ATG ***A_3***CC		3612.7	3612.7
CCG T***A_3A_3 A_3***TG ACC		3612.7	3612.6
T_1 *- dU modified DNAs (3'→5')*	*MS*	*Calcd*	*Found*
GGC A***T_1***T TAC TGG		3660.6	3660.7
GGC A***T_1***T TAC ***T_1***GG		3646.6	3646.7
GGC A***T_1T_1 T_1***AC TGG		3632.6	3632.8
T_2 *- 5-Cl-dU modified DNAs (3'→5')*	*MS*	*Calcd*	*Found*
GGC A***T_2***T TAC TGG		3694.6	3694.8
GGC A***T_2***T TAC ***T_2***GG		3714.5	3714.7
GGC A***$T_2T_2T_2$***AC TGG		3734.5	3734.7
T_3 *- 5-Et-dU modified DNAs (3'→5')*	*MS*	*Calcd*	*Found*
GGC A***T_3***T TAC TGG		3688.7	3688.8
GGC A***T_3***T TAC ***T_3***GG		3702.7	3702.8
GGC A***T_3T_3 T_3***AC TGG		3716.7	3716.9
Non-modified DNAs	*MS*	*Calcd*	*Found*
5'-CCG TAA ATG ACC-3'		3612.7	3612.5
3'-GGC ATT TAC TGG-5'		3674.6	3674.6
5'-CCG TGA ATG ACC-3'		3628.7	3628.6

Table S4. DNA sequences, templates and primers, used in the enzymatic studies

Name	Sequence, 5'→3'	Purpose
P1	^{33}P-CAGGAAACAGCTATGAC	dAxTP incorporation studies
T7T	TTTTTTTGTCATAGCTGTTTCCTG	
P2	^{33}P-CTTGTTGTGCTCCACGGTTA	dAxTP with dT_2TP
T37	CTCGCTATGACACTCGTCTCTTATATGCGTCTAATCATAACCGTGGAGCACAACAAG	incorporation studies
(Cy5) PrFW	(Cy5) CTCGCTATGACACTCGTCTCTT	Template and primers for PCR
(Cy3) PrRV	(Cy3) CTCGGTAGTTCTGTTGGTCT	amplification of 57bp fragment
T57	CTCGCTATGACACTCGTCTCTTATATGCGTCTAATCAAGACCAACAGAACTACCGAG	
(Cy3) M13RV	(Cy3) AGCGGATAACAATTTCACACAGGA	PCR primers for 149bp and
(Cy5) M13FW	(Cy5) CGCCAGGGTTTTCCCAGTCACGAC	360bp fragment synthesis
R67RV	TTAGTTGATGCGTTCAAGCG	PCR primers for 237bp
R67FW	ATGGAACGAAGTAGCAATGA	fragment synthesis
LongFW1	TAAGGAAGCTAAAATGGAGAAAAAAATCAC	Forward primer for synthesis of 523, 1023 and 1541bp fragments
LongFW2	ACAACTTCTTCGCCCCCGTTTTCACCATG	Forward primer for synthesis of 2074 and 2569bp fragments
LongRV1	AACGGGGGCGAAGAAGTTGTCCATATTGGCCAC	Reverse primer for synthesis of 523 and 2569bp fragments
LongRV2	GTCCGACCGCTGCGCCTTATCCGGTAACTATC	Reverse primer for synthesis of 1023bp fragment
LongRV3	TAGCGGAGTGTATACTGGCTTACTATGTTGGCACTGATG	Reverse primer for synthesis of 1541bp fragment
LongRV4	GATTTTTTTCTCCATTTTAGCTTCCTTAGC	Reverse primer for synthesis of 2074bp fragment

Table S5. List of plasmids used in this work

Name	Company	Description	Purpose
pUC19	New England Biolabs	Cloning vector	PCR template for 149bp fragment
pXEN156	Laboratory collection	pSU18 derivative, P15A replicon, *cat*+[9,10]	PCR template for 237-2569bp fragmnets

SUPPORTING FIGURES

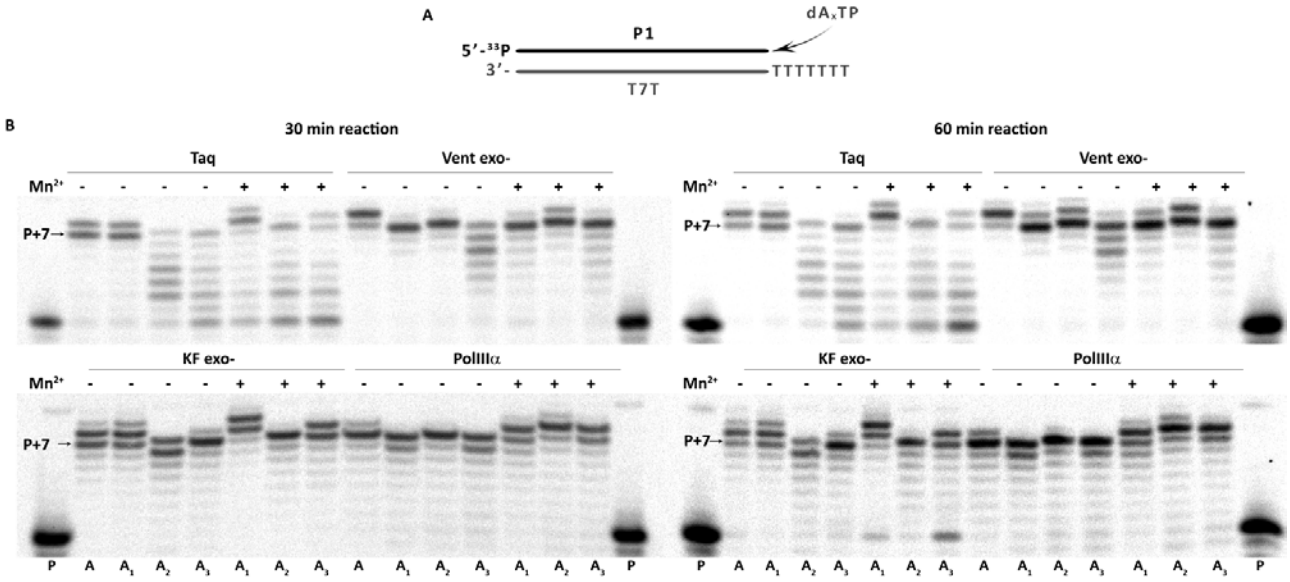

Fig. S1. Enzymatic incorporation of modified deoxyadenosine triphosphates (dAxTP) into a 50nM primer-template duplex (P1-T7T) in the presence of 25 U/ml Taq, Vent exo-, KF exo- or 1200 U/ml PolIIIα DNA polymerases. (A) Schematic representation of experiment. (B) Phosphorimages of polymerization reaction with 100μM modified dAxTPs after 30 min or 60 min of reaction in absence or presence of 1mM $MnCl_2$ P - primer only; P+7 – full length product.

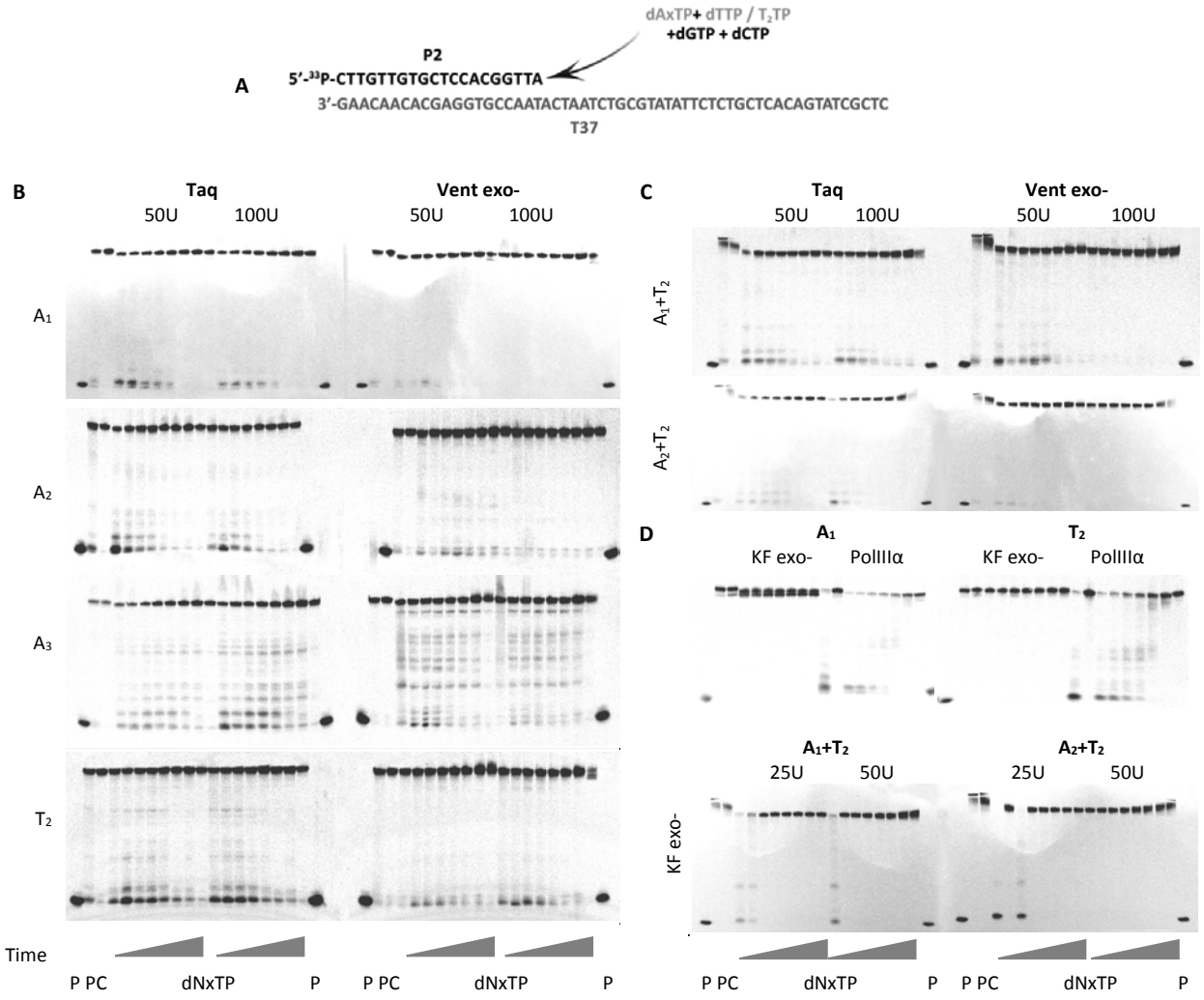

Fig. S2 Examples of primer extension reactions with dAxTP analogues together with or without 5-Cl-dUTP on a 57mer template. (A) The sequences of primer-template duplex (50nM) used in studies. (B) Product formation catalyzed by thermophilic DNA polymerases, Taq and Vent exo-, in concentration of 50U/ml or 100U/ml and different reaction time, 3-60 min. From top to bottom are listed PAGE images of the incorporation reaction with A_1, A_2, A_3 and T_2 triphosphates (200μM) with other non-modified dNTPs. (C) Primer extension catalyzed by thermophilic DNA polymerases, Taq and Vent exo-, in concentration 50U/ml or 100U/ml, with A_1 or A_2 compounds together with T_2 triphosphate, dGTP and dCTP (200μM each). Reactions are stopped at different periods of time (3-60 min). (D) Examples of primer extension catalyzed by mesophilic DNA polymerases, 25 or 50U/ml KF exo- and 2400U/ml PolIIIα, with modified A_1 or A_2 triphosphates, dTTP or 5-Cl-dUTP, together with dGTP and dCTP (200μM each). First shown denaturing PAGE image of the products containing A_1 or T_2, within reaction time 3-120 min; next products containing A_1 with T_2 or A_2 with T_2 in different periods of time (1-20 min) catalyzed by KF exo-. P – ^{33}P-labeled primer only, PC-positive control with natural dNTP after minimum and maximum reaction time. dNxTP- modified nucleoside triphosphates (A_1-A_3 and T_2 triphosphates).

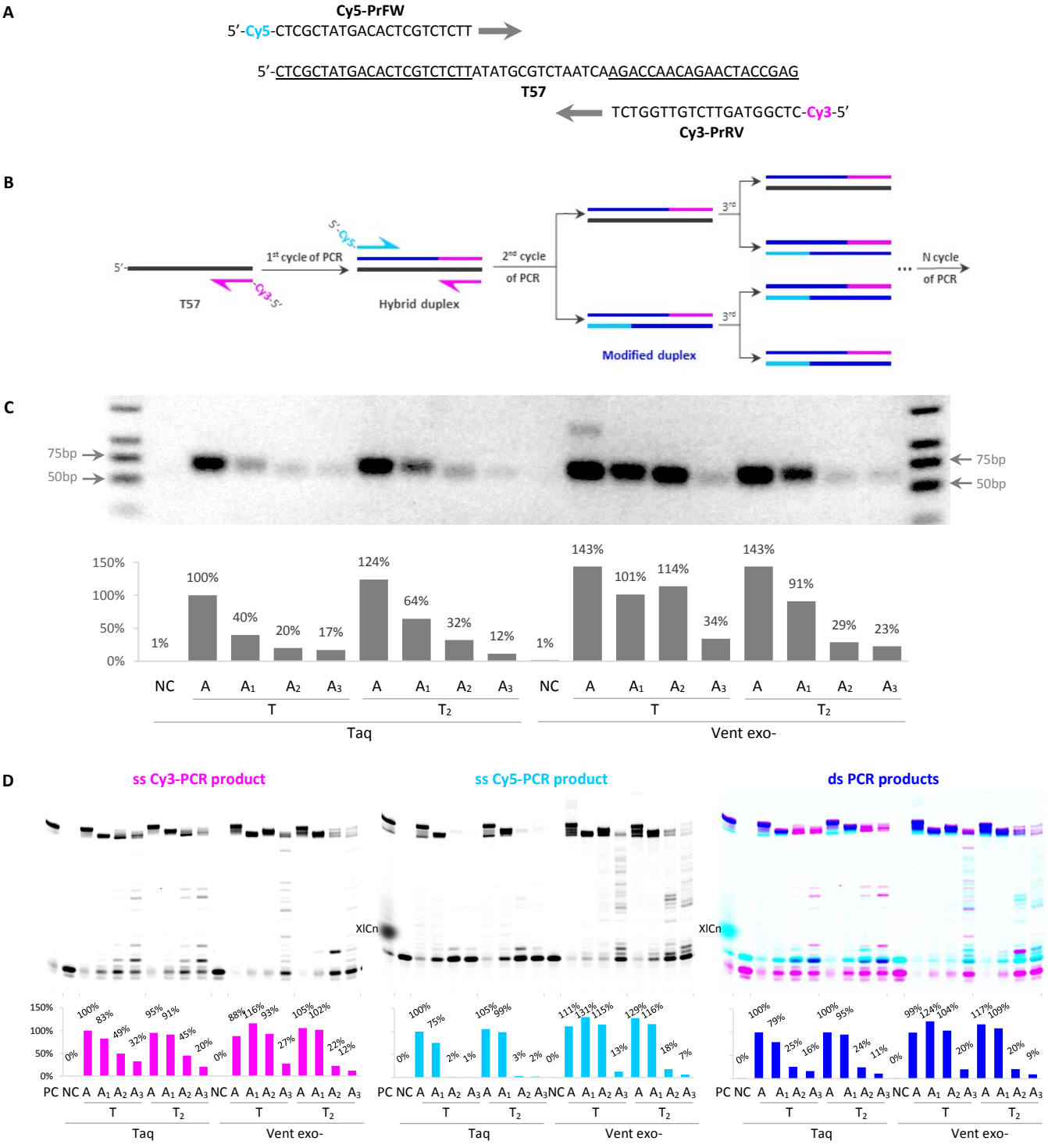

Fig. S3 PCR amplification of 57mer DNA template in the presence of natural dNTP or dAxTP, dT_2TP together with dCTP and dGTP triphosphates. (A) Sequences of primers and template used in the PCR assay. (B) Schematic representation of the PCR experiment. (C) Image of 3% agarose gel with relative yields of PCR product formation to the reaction yield of natural PCR product formed by Taq DNA polymerase (below). (D) Image of 15% denaturing PAGE with relative yields below. Cy3- or Cy5-labeled single-stranded (ss) PCR products are shown in pink or in light blue, respectively. Double-stranded (ds) PCR product is shown as dark blue and represents the average yields from Cy3 and Cy5 labeled DNA strands. The yield of natural PCR product formed by Taq polymerase was taken as 100%. PCR reactions were performed with 25 U/ml Taq or Vent exo- DNA polymerases. NC – negative control, PCR without dNTP, PC - Cy3/Cy5 labeled ds T57 template. XlCn - xylene cyanol, loading buffer compound (tracking dye).

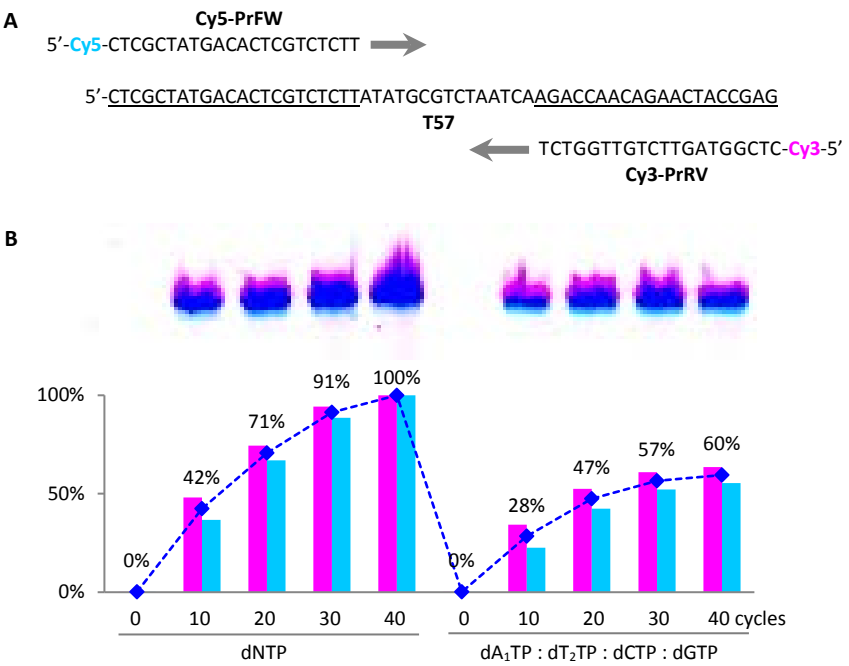

Fig. S4 PCR amplification of 57mer template in presence of natural or 7-deaza-dA, 5-Cl-dU together with dGTP and dCTP triphosphates. (A)The sequences of primers and template for PCR. (B) Image of 15% denaturing PAGE with relative yields below. Cy3- or Cy5-labeled PCR products are shown in pink or in light blue respectively, total product yield are shown in dark blue. PCR reactions were performed with 25U/ml Taq DNA polymerase. The yield of natural PCR product after 40 PCR cycles was taken as 100%. 3 min of extension time was used.

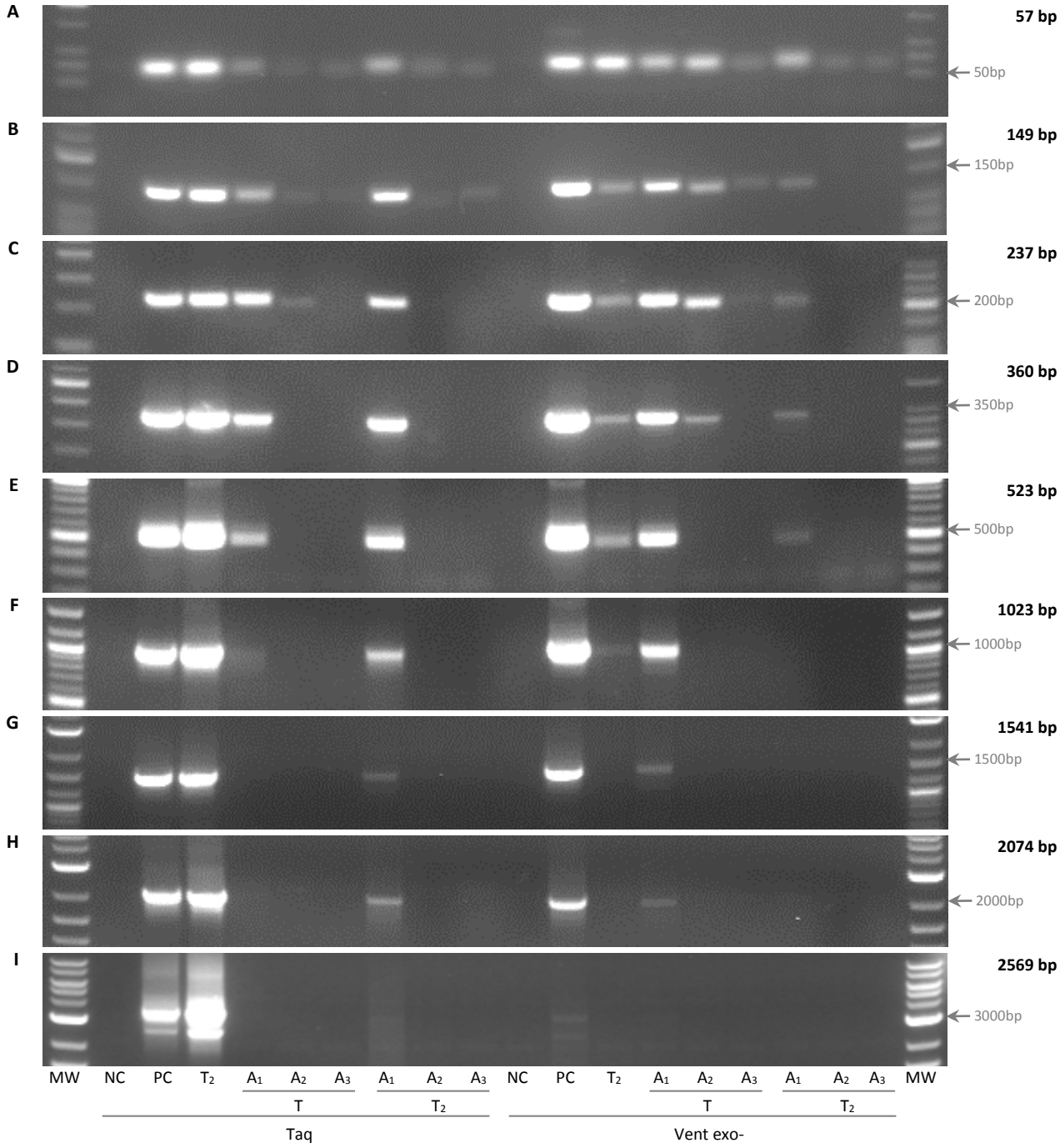

Fig. S5 PCR amplification of templates with different lengths in presence of natural or dAxTP, 5-Cl-dUTP together with dGTP and dCTP triphosphates. (A-I) Images of agarose gel electrophoresis: (A) 3% gel with 57 bp product, (B) 3% gel with 149 bp product, (C) 2% gel with 237 bp product, (D) 2% gel with 360 bp product, (E) 1% gel with 523 bp product, (F) 1% gel with 1023 bp product, (G) 1% gel with 1541 bp product, (H) 1% gel with 2074 bp product, (I) 1% gel with 2569 bp product. PCR reactions were performed with 25U/ml Taq or Vent exo- DNA polymerases. MW – molecular weights, NC- negative controls, PCR without dNTP, PC – positive controls, PCR with all four natural dNTP.

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