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dUTPs conjugated with zwitterionic Cy3 or Cy5 fluorophore analogues are effective substrates for DNA amplification and labelling by Taq polymerase

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ABSTRACT

To develop structural modifications of dNTPs that are compatible with Taq DNA polymerase activity, we synthesized eight dUTP derivatives conjugated with Cy3 or Cy5 dye analogues that differed in charge and charge distribution throughout the fluorophore. These dUTP derivatives and commercial Cy3– and Cy5-dUTP were studied in Taq polymerase-dependent polymerase chain reactions (PCRs) and in primer extension reactions using model templates containing one, two and three adjacent adenine nucleotides. The relative amounts of amplified DNA and the kinetic parameters K_m and V_{max} characterizing the incorporation of labelled dUMPs have been estimated using fluorescence measurements and analysed. The dUTPs labelled with electroneutral zwitterionic analogues of Cy3 or Cy5 fluorophores were used by Taq polymerase approximately one order of magnitude more effectively than the dUTPs labelled with negatively charged analogues of Cy3 or Cy5. The nucleotidyl transferase activity of Taq polymerase was also observed and resulted in the addition of dUMPs labelled with electroneutral or positively charged fluorophores to the 3′ ends of DNA. The introduction of mutually compensating charges into fluorophores or other functional groups conjugated to dNTPs can be considered a basis for the creation of PCR-compatible modified nucleoside triphosphates.

INTRODUCTION

Fluorescent labelling of DNA is extensively used in biology, biotechnology, medical diagnostics and nanotechnology (1–5). In particular, the enzymatic incorporation of fluorescently labelled dNMPs is a traditional method for generating fluorescently labelled DNA probes (2,3,6–15).

The development of polymerase-dependent synthesis of oligonucleotides using dNTPs modified with fluorophores or other functional groups is significant for future applications in medical diagnostics and for the creation of new modified aptamers with directed target-specificity. However, the corresponding methods are not sufficiently efficient and cheap to be routinely used.

Earlier studies showed that many DNA polymerases incorporate nucleotides conjugated with a variety of fluorophores (6–15). The efficiency of incorporating fluorescent nucleotides depends on the type of DNA polymerase and the structure of the fluorescent residue (13). Previous studies were mainly focused on increasing the efficiency of enzymatic labelling by examining the different types of DNA polymerases, their structures and modifications of their amino acid sequences (9–15). The absence of 3′→5′ exonuclease proofreading activity in polymerases was considered a positive factor for the effective incorporation of fluorescent nucleotides (9,13). Meanwhile, the structural properties of fluorophores that are necessary for the efficient incorporation of fluorescently labelled dNMPs have not been thoroughly analysed.

The introduction of ionized groups into fluorescent moieties used for dNTP labelling greatly improves their solubility. However, in 2003, Giller *et al*. and Tassara *et al*. compared the incorporation efficiencies of dUMPs conjugated with differently charged derivatives of the fluorophore Gnothis blue by Vent (exo-) during polymerase chain reactions (PCRs), but charge-dependent patterns were not observed (11,12). In our recently published short communication, we showed that the addition of 5% dUTP labelled with an electrically neutral Cy5 analogue leads to effective incorporation during PCR (16).

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In the present study, we investigated the influence of charge and charge distribution on the substrate compatibilities of Cy3 and Cy5 fluorophore analogues conjugated to dUTPs at the five position in relation to Taq polymerase activity. For this purpose, Taq polymerase-dependent PCR amplification in the presence of minor amounts (5%) of dUTPs labelled with differently charged Cy3 and Cy5 analogues was investigated. dUTPs labelled with electroneutral Cy3 and Cy5 analogues were used by Taq polymerase approximately one order of magnitude more effectively than dUTPs labelled with negatively charged Cy3 or Cy5 fluorophore analogues, including Amersham Cy3-dUTP and Cy5-dUTP, each of which carries negatively charged fluorophores. Additionally, primer extension by Taq polymerase after the complete replacement of dTTP with the differently labelled dUTPs mentioned above was studied. Gel electrophoresis of the primer extension products demonstrated that dUTPs labelled with electroneutral hydrophilic analogues of the Cy3 and Cy5 fluorophores are optimal for the preparation of full-length primer extension products containing highly fluorescent labelled nucleotides. The results of this study are the basis for a methodical approach to create Taq polymerase-compatible fluorescently labelled (or modified with other groups) nucleoside triphosphates to improve DNA labelling and hybridization for use in routine medical diagnostics and the design of the new modified aptamers.

MATERIALS AND METHODS

Deoxyuridine triphosphates labelled with various Cy3 and Cy5 fluorophore analogues

The chemical structures and optical properties of eight deoxyuridine triphosphates labelled with analogues of Cy3 and Cy5 fluorophores are summarized in Table 1. The methods used in the syntheses were partially described previously (17). Detailed synthetic procedures and spectroscopic characteristics, including data on the intermediates, are provided in the Supplementary Data (Part A: Reaction Schemes of Modified Nucleoside Triphosphates and Part B: Procedures and Spectroscopic Data). Amersham Cy3-dUTP and Amersham Cy5-dUTP were purchased from GE Healthcare (Little Chalfont, UK).

Oligodeoxyribonucleotides, PCR, primer extension and extra nucleotide addition to the 3′ ends of DNA fragments

The sequences of oligonucleotides listed in Supplementary Table S1 were selected to reduce the formation of hairpin structures. Possible hairpin structures were calculated using the DI-nucleic acid hybridization and melting prediction web server (http://unafold.rna.albany.edu). Detailed synthetic and analytical procedures are provided in the Supplementary data (Part C: Oligodeoxyribonucleotides, Supplementary Tables S1 and 2).

Detailed descriptions of PCRs, primer extension reactions and adding extra nucleotides to the 3′ ends of DNA fragments are in the Supplementary Data (Part D: PCR, primer extension and extra nucleotide addition to the 3′ ends of DNA fragments).

Electrophoresis and gel image acquisition

Before being loaded on denaturing 20% polyacrylamide gels for electrophoresis, the samples were desalted and purified from an excess of dNTPs and fluorescently labelled dUTPs on a Sephadex G25 Superfine gel filtration column (Pharmacia, Sweden; volume 1 ml, length 50 mm). The column was washed with Milli-Q water using a peristaltic pump at a speed of 1 mm^3 per second. Sample elution was monitored with a Pharmacia Dual Path Monitor UV2 double-beam sensor (Pharmacia, Sweden). The eluted samples were reprecipitated in 10 volumes of 2% $LiClO_4$ in acetone, diluted in 5 μl of 7 M urea and then loaded into gel wells.

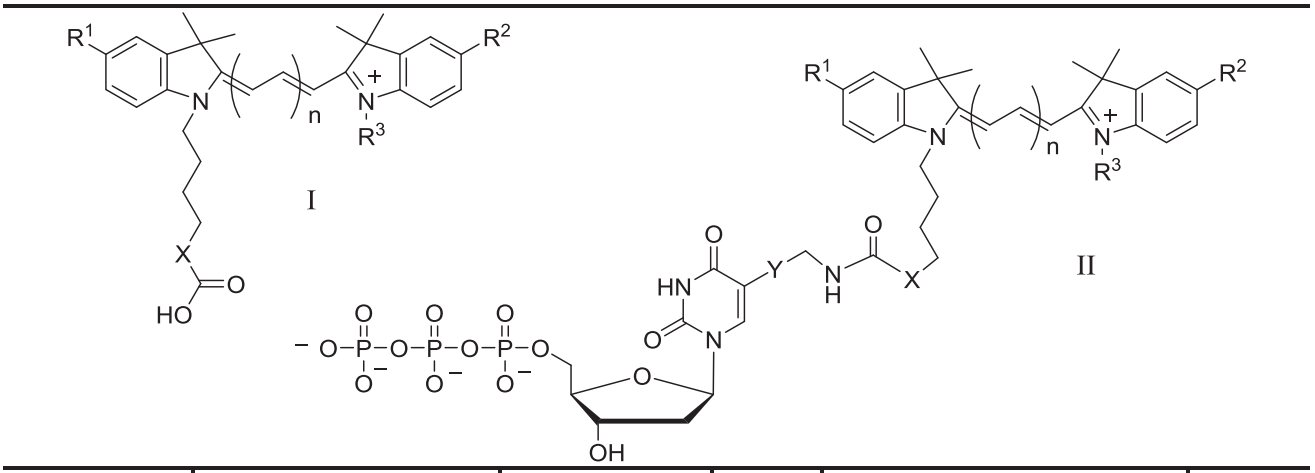
Electrophoresis was carried out in denaturing 20% polyacrylamide gels (19:1 (w/w) acrylamide/bis-acrylamide, 7 M urea; 600 V; thermostabilized 15 × 15 cm glass sandwich with 1-mm gel thickness; TBE buffer (89 mM Tris-borate and 2 mM ethylenediaminetetraacetic acid, pH 8.3)).

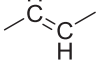
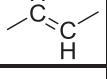
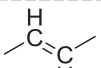
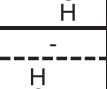
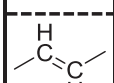
After electrophoresis, gel images were obtained using a research fluorescence Gel Imager with an image field of 15 × 15 cm equipped with an RTE/CCD-1536-K/1 CCD camera (Roper Scientific, Sarasota, FL, USA) and a mercury lamp. Fluorescence was recorded in the range of the Cy3 dye using a 535DF35 and 580DF27 filter pair, in the range of the Cy5 dye using a 630DF30 and 690DF40 filter pair, and in the range of the fluorescein isothiocyanate (FITC) dye using a 470DF40 and 535DF35 filter pair. In the broad spectral range of Cy3 and Cy5 dyes, fluorescence was recorded using a 535DF35 and 690DF40 filter pair. Additionally, the visualization of oligonucleotide bands in polyacrylamide gels was performed using an aluminium sheet coated with TLC Silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) as a gel support, a TCP-20 LC transilluminator with an excitation wavelength of 254 nm (Vilber Lourmat, Marne-la-Vallée, France) as a UV source and a 470DF40 filter for observation. All filters were purchased from Omega Optical, Brattleboro, VT, USA. The microscope was equipped with a computer running ImaGel Research software (18,19).

Quantitative analysis of electrophoretic bands containing PCR-amplified full-length DNA fragments

The relative quantities of PCR-amplified full-length DNA fragments containing dUMPs labelled with Cy5 dye analogues were considered proportional to the relative fluorescence intensities of the Cy3-labelled primers. These quantities were calculated separately for each of lanes 3–8 in Figure 1A (Results and Discussion section). For this purpose, the summed Cy3 fluorescence intensities of the upper bands in each of lanes 3–8 in Figure 1A (relative to the level of the doublet band in lane 2) that arose due to the incorporation of dUMPs labelled with the rather heavy Cy5 dye analogues were quantified using virtual rectangular frames that surrounded the upper bands. The fluorescence intensities of all the pixels surrounded by the frame were summed to obtain the fluorescence intensity within the frame. The fluorescence intensities of the blank gel regions within the same frames were then subtracted from the obtained values. The obtained quantities were normalized to the fluorescence intensity of the full-length DNA in lane 2 in Figure 1A, which contained only natural nucleotides.

Table 1. Chemical structures and optical characteristics of Cy3 and Cy5 analogues, I (Cy3a+, Cy5a+, Cy3a1±, Cy5a1±, Cy5a2±, Cy5a3±, Cy3a−, Cy5a−, Cy3, Cy5), and their corresponding fluorescently labelled deoxyuridine triphosphates, II (dU(Cy3a+)TP, dU(Cy5a+)TP, dU(Cy3a1±)TP, dU(Cy5a1±)TP, dU(Cy5a2±)TP, dU(Cy5a3±)TP, dU(Cy3a−)TP, dU(Cy5a−)TP, Cy3-dUTP, Cy5-dUTP), measured in PBS buffer (0.15 M NaCl, 10 mM potassium phosphate, pH 7.4)



Abbreviations for the Cy3 and Cy5 analogues and corresponding dUTPs	Substituents of fluorophore				Linker fragment	Linker fragment	ε (x10^{-5}) [a] ($M^{-1}cm^{-1}$)	λ^{abs}_{max} (nm)	λ^{em}_{max} (nm)	Quantum yield [a, b] (%)	Total electrical charge of fluorophore
	R^1	R^2	R^3	n	X	Y					
Cy3a+	-H	-H	$-C_2H_5$	1	$-CH_2-$	-	1.3 [c]	543	558	6	+1
Cy5a+				2			1.9 [c]	639	655	17	
dU(Cy3a+)TP				1			-	545	561	14	
dU(Cy5a+)TP				2			-	642	660	17	
Cy3a1±	-H	$-SO_3^-$	$-C_2H_5$	1	$-CH_2-$	-	1.3	546	559	7	0
Cy5a1±				2			2.1	643	661	12	
dU(Cy3a1±)TP				1			-	547	561	13	
dU(Cy5a1±)TP				2			-	644	662	14	
Cy5a2±	-H	$-SO_3^-$	$-C_2H_5$	2	$-(CH_2)_5NHCOCH_2-$	-	2.2	642	660	14	0
dU(Cy5a2±)TP							-	644	661	17	
Cy5a3±	-H	-H	$-(CH_2)_4SO_3^-$	2	$-CH_2-$	-	2.1	644	660	14	0
dU(Cy5a3±)TP							-	646	663	16	
Cy3a-	$-SO_3^-$	$-SO_3^-$	$-C_2H_5$	1	$-CH_2-$	-	1.5	549	564	15	-1
Cy5a-				2			2.5	647	663	27	
dU(Cy3a-)TP				1			-	550	564	29	
dU(Cy5a-)TP				2			-	648	665	31	
Cy3	$-SO_3^-$	$-SO_3^-$	$-C_2H_5$	1	$-CH_2-$	-	1.5 [d]	550 [d]	570 [d]	>15 [d]	-1
Cy5				2			2.5 [d]	648 [d]	670 [d]	27 [d]	
Cy3-dUTP [e]				1		—C≡C—	1.5 [f]	550 [f]	570 [f]	>15 [f]	
Cy5-dUTP [e]				2			2.5 [f]	649 [f]	670 [f]	>28 [f]	

[a]Standard deviations for the values of molar extinction (ε) and quantum yield were 5 and 10%, respectively.
[b]The fluorescence quantum yields were determined as described previously (25) using 15% as the reference value for the quantum yield for Cy3 (see Amersham CyDye mono-reactive NHS Esters product booklet) and 27% as the reference value for the quantum yield for Cy5 (26).
[c]The molar extinctions (ε) of Cy3a+ and Cy5a+ were measured in methanol.
[d]Optical characteristics of Cy3 are from Amersham CyDye mono-reactive NHS Esters product booklet. Optical characteristics of Cy5 are from Mader *etal.* (26).
[e]Amersham Cy3-dUTP and Amersham Cy5-dUTP were purchased from GE Healthcare.
[f]Optical characteristics of Amersham Cy3-dUTP and Amersham Cy5-dUTP were provided by the producer (GE Healthcare).

The same quantitative analysis was performed for lanes 9–13 in the Cy5 fluorescence range (Figure 1B) to separately estimate the relative quantities of synthesized full-length DNA fragments containing dUMPs labelled with Cy3 dye analogues. The corresponding values characterizing the efficiency of the enzymatic incorporation of dUMPs labelled with Cy3 or Cy5 dye analogues are shown in Supplementary Table S3 and are also plotted in the histogram in Figure 1C (grey columns).

Additionally, the normalized fluorescence intensities characterizing the brightness of incorporated dUMPs labelled with Cy3 and Cy5 dye analogues were calculated in the Cy3 (lanes 10–13, Figure 1A) and Cy5 (lanes 3–8, Figure 1B) fluorescence ranges, respectively. For this purpose, the Cy3 fluorescence intensities of the bands containing full-length DNA products in each of lanes 10–13 (Figure 1A) were summarized using the virtual rectangular frames surrounding the bands (the fluorescence intensities of the blank gel regions within the same frames were subtracted from the obtained values). In a similar manner, the summed Cy5 fluorescence intensities of the bands containing full-length DNA products in each of lanes 3–8 (Figure 1B) were calculated. The obtained two sets of fluorescence intensities were normalized to their maximum values (separately for the Cy3 and Cy5 fluorescence ranges) and plotted in the histogram shown in Figure 1C (blue and orange columns, respectively).

Quantitative analysis of electrophoretic bands containing full-length DNA fragments obtained during primer extension

To estimate the relative quantities of the full-length products of primer extensions in Figures 2 and 3 and Supplementary Figure S1, we calculated the fluorescence intensity of each analysed band in the fluorescence range of the labelled primer using a virtual rectangular frame surrounding the band (Results and Discussion section). Fluorescence intensities of all the pixels surrounded by the frame were summed to obtain the fluorescence intensity within the frame. The fluorescence intensity of a blank gel region within the same frame was then subtracted from the obtained value. The obtained fluorescence signals of the analysed bands within each gel image were normalized to the fluorescence intensity of the band containing the full-length natural product of primer extension. Within each gel image in Supplementary Figure S14, the results were normalized to the full-length natural product of primer extension obtained in the presence of dATP, dGTP and dCTP.

Within each gel image in each of the remaining quantitatively analysed figures (Figure 6 and Supplementary Figures S2, 6, 8, 9 and 11), the fluorescence signals were obtained in the same way and normalized to the maximum value.

The fluorescence intensities characterizing the brightness of incorporated dUMPs labelled with Cy3 and Cy5 dye analogues were also similarly calculated in the Cy3 and Cy5 fluorescence ranges, respectively. Values were normalized to the maximum value.

Mass spectrometry

The details of mass spectroscopic analysis are provided in the Supplementary data (Part E: Mass spectrometry).

Michaelis–Menten kinetics measurements

When measuring the kinetic parameters, including the Michaelis–Menten constant (K_m), maximum substrate incorporation rate (V_{max}), and substrate incorporation rate at [S] = 5×10^{-4} M (V([S] = 5×10^{-4} M)), 1.5 units of Taq polymerase were added to the primer extension reaction mixtures. The dTTP, dU(Cy5a1±)TP, dU(Cy5a2±)TP, dU(Cy5a3±)TP, dU(Cy5a+)TP and dU(Cy5a−)TP concentrations were varied from 5×10^{-8} M to 5×10^{-4} M, and the reactions proceeded for 40 min (Figures 6 and 7; Supplementary Figures S9–12). The other conditions were the same as described in the Primer extension and extra nucleotide addition to the 3′ ends of DNA fragments subsection of the Supplementary data (Part D: PCR, primer extension and extra nucleotide addition to the 3′ ends of DNA fragments).

Each electrophoretic gel included a single lane containing the product of a control reaction. The control reaction mixture contained 15 units of Taq polymerase and 5×10^{-4} M dTTP. In this case, the primer extension reaction proceeded for 2 h, until the entire primer was extended. The fluorescence signals from other full-length reaction products on the gel image were normalized to the fluorescence signal from abovementioned control reaction product.

Plots of the product synthesis rate, d[P]/dt, against substrate concentration, [S], were approximated by curves according to the Michaelis–Menten equation (20):

$$d[P]/dt = V_{max} \cdot [S] / (K_m + [S])$$

where V_{max} is the maximum substrate incorporation rate and K_m is the Michaelis–Menten constant. The approximation was performed using OriginPro 8.6 software (OriginLab Corp., Northampton, MA, USA), which provided estimations of the parameters V_{max} and K_m and the coefficient of determination, R^2.

For dU(Cy5a1±)TP, dU(Cy5a2±)TP, dU(Cy5a3±)TP, dU(Cy5a+)TP and dU(Cy5a−)TP, the d[P]/dt values at a substrate concentration of 5×10^{-4} M were five to ten times smaller than those obtained at substrate concentrations of $5 \times 10^{-6} - 5 \times 10^{-5}$ M. Therefore, 5×10^{-4} M fluorescently labelled dUTPs greatly inhibited the reactions and the corresponding points were not considered in the approximations.

RESULTS AND DISCUSSION

Effect of the charge of dUTP-conjugated fluorophores on PCR by Taq polymerase

We first compared the effect of the charge of the Cy3 and Cy5 dye analogues on PCRs performed by Taq polymerase in the presence of the M0 DNA template (Supplementary Table S1), natural nucleoside triphosphates, and minor amounts (5%) of the dUTPs labelled with differently charged analogues of the Cy3 and Cy5 fluorophores (Table 1).

Figure 1A and B shows the results of the electrophoretic separation of PCR products recorded in both the Cy3 and Cy5 fluorescence ranges. The full-length complementary DNA strands amplified using natural nucleoside triphosphates are represented by two closely spaced bands. In the

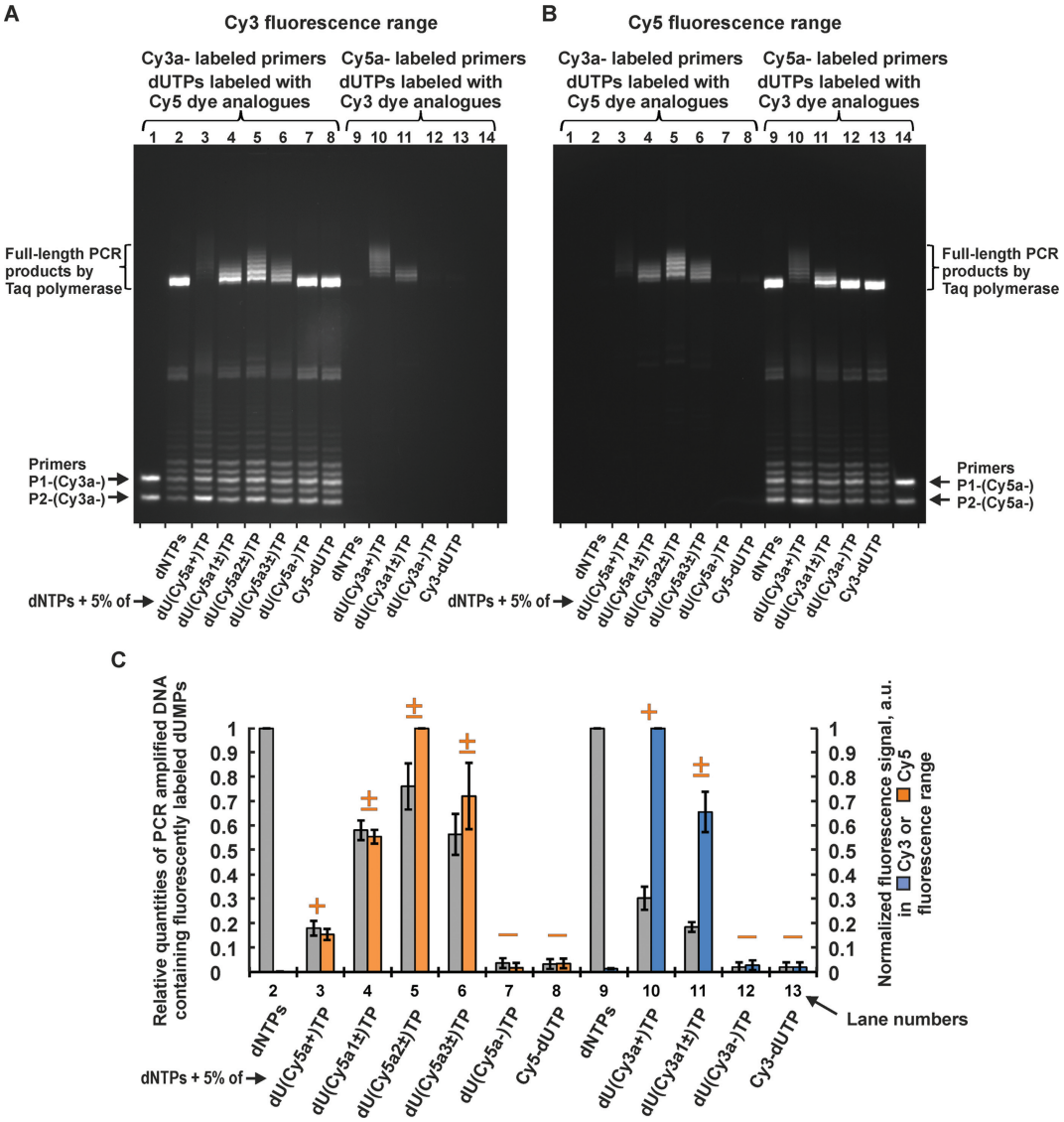


Figure 1. Effect of the charge of dUTP-conjugated Cy3 and Cy5 analogues on PCR performed by Taq polymerase using a 68-nt M0 template that contained varying numbers of consecutive adenine nucleotides: a single A, an AA doublet and an AAA triplet (Supplementary Table S1). (**A** and **B**) Electrophoretic separation of PCR products obtained in the presence of dNTPs and 5% fluorescently labelled dUTPs. (Table 1, Supplementary Schemes S1–3 and Table S2). The experiment shown in A and B was performed two times**.** Lane 1: P1-(Cy3a−) and P2-(Cy3a−) primers; lane 2: PCR products prepared using natural nucleoside triphosphates and P1-(Cy3a−) and P2-(Cy3a−) primers; lanes 3–8: PCR products obtained after the addition of 5% dU(Cy5a+)TP, dU(Cy5a1±)TP, dU(Cy5a2±)TP, dU(Cy5a3±)TP, dU(Cy5a−)TP or Amersham Cy5-dUTP, respectively. Lane 9: PCR products prepared using natural nucleoside triphosphates and P1-(Cy5a−) and P2-(Cy5a−) primers; lanes 10–13: PCR products obtained after the addition of 5% dU(Cy3a+)TP, dU(Cy3a1±)TP, dU(Cy3a−)TP or Amersham Cy3-dUTP, respectively. Lane 14: P1-(Cy5a−) and P2-(Cy5a−) primers. (**C**) Histogram of the relative quantities (grey columns) of PCR-amplified full-length DNA fragments containing fluorescently labelled dUMPs and normalized fluorescence signals characterizing the brightness of these incorporated dUMPs (see also Supplementary Table S3). The brightness of incorporated dUMPs in the Cy3 and Cy5 ranges are shown in blue and orange, respectively. The values averaged over the two experiments are shown. The bars indicate the absolute deviations.

Cy3 fluorescence range (Figure 1A), a closely spaced doublet is observed in lane 2 for the products amplified using P1-(Cy3a−) and P2-(Cy3a−) primers. A doublet is similarly observed in lane 9 in the Cy5 fluorescence range (Figure 1B) for the products amplified using the P1-(Cy5a−) and P2-(Cy5a−) primers.

The full-length PCR products prepared with 5% dUTPs that were labelled with Cy3 and Cy5 analogues carrying positive or electroneutral charges are observed in the corresponding fluorescence ranges as multiple discrete bands that are shifted upward (lanes 10 and 11 in Figure 1A and lanes 3–6 in Figure 1B) from the abovementioned doublets seen in lanes 2 and 9. These up-shifted bands have arisen due to the incorporation of one or more fluorescently labelled dUMPs carrying the rather heavy fluorescent labels (500–650 Da) into the DNA strands.

In the case of the positively charged fluorophores, full-length PCR products containing from one to four dU(Cy3a+)MPs are represented by seven or eight bands in lane 10 of Figure 1A. Products containing from one to three

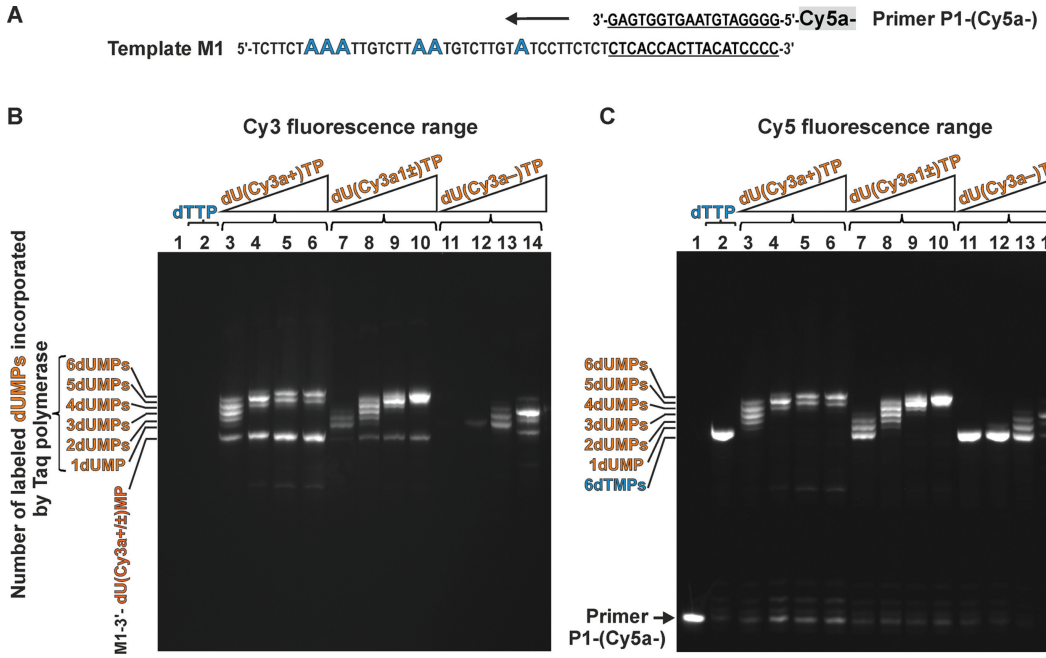


Figure 2. Incorporation of deoxyuridines labelled with Cy3 fluorophore analogues carrying positive, neutral or negative charges by Taq polymerase in a P1-(Cy5a−) primer extension reaction using the M1 template. (**A**) Primer extension reaction scheme. (**B** and **C**) Electrophoretic separation of primer extension products obtained in the presence of dTTP, dU(Cy3a+)TP, dU(Cy3a1±)TP and dU(Cy3a−)TP. The experiment shown in B and C was performed two times. Lane 1: P1-(Cy5a−) primer; lane 2: reaction products prepared using natural nucleoside triphosphates. Lanes 3–6, 7–10 and 11–14: reaction products prepared using dATP, dCTP, dGTP, increasing amounts of modified dUTPs (10, 50, 90 and 100%) and the corresponding decreasing amounts of dTTP (90, 50, 10 and 0%). Lanes 3–6: dU(Cy3a+)TP, lanes 7–10: dU(Cy3a1±)TP and lanes 11–14: dU(Cy3a−)TP.

dU(Cy5a+)MPs are represented by four or five barely visible bands in lane 3 of Figure 1B.

Figure 1A (lane 11) accordingly shows that products containing one or two dU(Cy3a1±)MPs are represented by three or four visible bands of different brightness. Similarly, Figure 1B shows that the products containing one or two dU(Cy5a1±)MPs (lanes 4) or dU(Cy5a3±)MPs (lane 6) are represented by four bands, while the products containing one, two or three dU(Cy5a2±)MPs (lane 5) are represented by five bands.

The bands that are visible in the specific fluorescence ranges of fluorescently labelled dUTPs are also visible in the same lanes when observed in the fluorescence ranges of the fluorescently labelled primers.

In contrast, the incorporation of dUMP labelled with a negatively charged fluorophore, i.e. dU(Cy3a−)MP (lane 12, Figure 1A), Amersham Cy3-dUMP (lane 13, Figure 1A), dU(Cy5a−)MP (lane 7, Figure 1B) and Amersham Cy5-dUMP (lane 8, Figure 1B), was poorly observed in the corresponding Cy3 and Cy5 fluorescence ranges.

Quantitative analysis of the efficiency of PCR amplification using dUTPs labelled with differently charged fluorophores

The quantitative analysis of the gel images presented in Figure 1A and B was performed according to the procedure described in the Quantitative analysis of electrophoretic bands containing PCR-amplified full-length DNA fragments section ('Materials and Methods' section). In accordance with this procedure, the relative quantities of PCR-amplified full-length DNA fragments containing incorporated dUMPs labelled with Cy3 dye analogues were estimated based on the fluorescence intensity of the primers that were labelled with the Cy5a− analogue. In turn, the relative quantities of amplified DNA fragments containing incorporated dUMPs labelled with Cy5 dye analogues were estimated based on the fluorescence intensity of the primers that were labelled with the Cy3a− analogue. The obtained data are summarized in Supplementary Table S3 and plotted in the histograms shown in Figure 1C (grey columns).

The data in Supplementary Table S3 and the histogram in Figure 1C show that the efficiency of Taq polymerase using minor amounts (5%) of dUTPs labelled with electroneutral, positively or negatively charged Cy3 and Cy5 fluorophore analogues (including Cy3− and Cy5-dUTP (Amersham)) in PCRs was 20–75, 20–30 and 2–4%, respectively, of the values when only natural dNTPs were used. The addition of 5% dUTPs labelled with electroneutral or positively charged Cy3 or Cy5 analogues thus leads to approximately one order of magnitude more effective DNA labelling than the addition of the same amount of dUTPs labelled with negatively charged Cy3 or Cy5 dye analogues.

The blue and orange columns in the histogram in Figure 1C show the relative fluorescence intensities of fluorescently labelled dUMPs incorporated into the full-length PCR products measured in the Cy3 and Cy5 fluorescence ranges, respectively. The incorporated dUMPs labelled with electroneutral Cy3 (lane 11, Figure 1A) and Cy5 (lanes 4–6, Figure 1B) analogues and dU(Cy3a+)MP (lane 10, Figure 1A) emitted bright fluorescence in the corresponding fluorescence ranges. In contrast, the brightness of the product containing dU(Cy5a+)MP (lane 3, Figure 1B) is rather

weaker than these emissions. Additionally, the brightness of full-length DNA fragments amplified using dUTPs labelled with negatively charged fluorophores was at least one order of magnitude lower than that for fragments amplified using dUTPs labelled with electroneutral fluorophores (lanes 7, 8, 12 and 13, Figure 1C).

Primer extension by Taq polymerase using dUTPs labelled with differently charged Cy3 or Cy5 dye analogues

To assess the effects of fluorophore charge on the incorporation efficiency of fluorescently labelled dUMPs, we studied primer extension by Taq polymerase in the presence of the synthesized dUTPs. For this purpose, the P1-(Cy3a−) and P1-(Cy5a−) primers, the M1 template (Supplementary Table S1) and dUTPs labelled with positively charged, neutral, or negatively charged analogues of Cy3 and Cy5 dyes were used. The M1 template (Figure 2A) contained varying numbers of consecutive adenine nucleotides (a single A, then an AA doublet and an AAA triplet) to test the both the single and sequential insertion of modified deoxyuridines.

Figure 2B and C shows the results of the electrophoretic separation of P1-(Cy5a−) primer extension products recorded in the Cy3 and Cy5 fluorescence ranges. An increase in the amount of dUTP labelled with Cy3a+, Cy3a1± or Cy3a− and the corresponding decrease in the amount of dTTP in the reaction mixture resulted in increased amounts of fluorescently labelled deoxyuridines incorporated into full-length DNA fragments. The levels of the bands corresponding to the positions of full-length products containing one to six incorporated fluorescently labelled deoxyuridines are shown schematically to the left of the gel images.

When dU(Cy3a+)TP or dU(Cy3a1±)TP completely replaced dTTP in the reaction mixtures, full-length DNA fragments containing all six modified deoxyuridines were obtained (the upper bands in lanes 6 and 10 of Figure 2B and C). The intensities of these upper bands on the gel images in the Cy5 fluorescence range were used to calculate the relative quantities of full-length DNA fragments containing six incorporated dU(Cy3a+)MPs or six incorporated dU(Cy3a1±)MPs (the 'Materials and Methods' section).

In contrast, when dU(Cy3a−)TP completely replaced dTTP in the reaction mixtures, the band at the level corresponding to six included dU(Cy3a−)MPs, was absent; the fluorescence intensity at the corresponding position was therefore estimated to be close to zero. In this case, a brightly visible band occupied the position corresponding to only three included dU(Cy3a−)MPs (lane 14 in Figure 2B and C). Because the full-length product was nevertheless completed, one can conclude that Taq polymerase probably failed to incorporate adjacent dU(Cy3a−)MPs into the sequences complementary to the AA and AAA blocks of the M1 template that resulted in nucleotide deletions (21) or the mistaken incorporation of other dNMPs.

The full-length product in lane 6 (Figure 2B and C) has an additional band representing a product with a smaller mass that corresponds to the incorporation of only five fluorescently labelled dUMPs. The decreased incorporation of dU(Cy3a+)MPs was most likely due to a deletion that occurred when Taq polymerase failed to incorporate some adjacent dU(Cy3a+)MPs (21).

The relative quantities of full-length DNA fragments containing six dU(Cy3a+)MPs, dU(Cy3a1±)MPs or dU(Cy3a−)MPs are shown in Supplementary Table S3. Supplementary Table S3 shows that the relative quantities of DNA fragments containing only natural dNMPs (lane 2), six incorporated dU(Cy3a+)MPs (lane 6), six incorporated dU(Cy3a1±)MPs (lane 10) or six incorporated dU(Cy3a−)MPs (lane 14) can be written in the following ratios: 1:(0.35 ± 0.05):(1.0 ± 0.1):(0 + 0.02). Thus, the ability of Taq DNA polymerase to use dU(Cy3a+)TP, dU(Cy3a1±)TP and dU(Cy3a−)TP was estimated at 35, 100 and 0%, respectively, of its ability to use dTTP (Supplementary Table S3).

Similar to previous experiments, quantitative analysis of the incorporation of dUMPs labelled with Cy5 dye analogues was performed by extending the P1-(Cy3a−) primer (Figure 3A) and determining the fluorescence intensities of the bands in the Cy3 fluorescence range. Images of electrophoretically separated primer extension products are shown in Figure 3B–E and Supplementary Figure S1B and C.

Analysis of the data presented in Figure 3B shows that the relative quantities of full-length DNA fragments containing only natural dNMPs (lane 2), six incorporated dU(Cy5a+)MPs (lane 6), six incorporated dU(Cy5a1±)MPs (lane 10) or six incorporated dU(Cy5a−)MPs (lane 14) can be written in the following ratios: 1:(0.3 ± 0.2):(0.7 ± 0.2):(0 + 0.02). The same analysis of the data presented in Figure 3D showed that the relative quantities of full-length DNA fragments containing only natural dNMPs (lane 2), six incorporated dU(Cy5a1±)MPs (lane 6) or six incorporated Cy5-dUMPs (lane 10) can be written in the following ratios: 1:(0.6 ± 0.05):(0.06 ± 0.02). Combining these ratios estimates the ability of Taq DNA polymerase to use dU(Cy5a+)TP, dU(Cy5a1±)TP, Cy5-dUTP and dU(Cy5a−)TP to be 30, 66, 6 and 0%, respectively, of its ability to use dTTP (Supplementary Table S3).

The same approach showed that Taq DNA polymerase used dU(Cy5a2±)TP and dU(Cy5a3±)TP during primer extension with efficiencies of ∼70 and 50%, respectively, of its efficiency at using dTTP (Supplementary Figure S1B and Table S3).

Incorporation features of dUMPs labelled with negatively charged fluorophores

The image obtained when dU(Cy5a−)TP completely replaced dTTP in the reaction mixture (lane 14 in Figure 3B and C) was quite similar to that obtained for dU(Cy3a−)TP. The band at the position corresponding to six incorporated dU(Cy5a−)MPs was absent, i.e. the fluorescence intensity at the corresponding position was estimated as zero. In this case, a brightly visible band occupied the position corresponding to only three incorporated dU(Cy5a−)MPs (lane 14 in Figure 3B and C). Taq polymerase thus has difficulty incorporating adjacent dUMPs labelled with negatively charged Cy3 or Cy5 dye analogues. These results are consistent with data previously reported by Zhu and Wag-

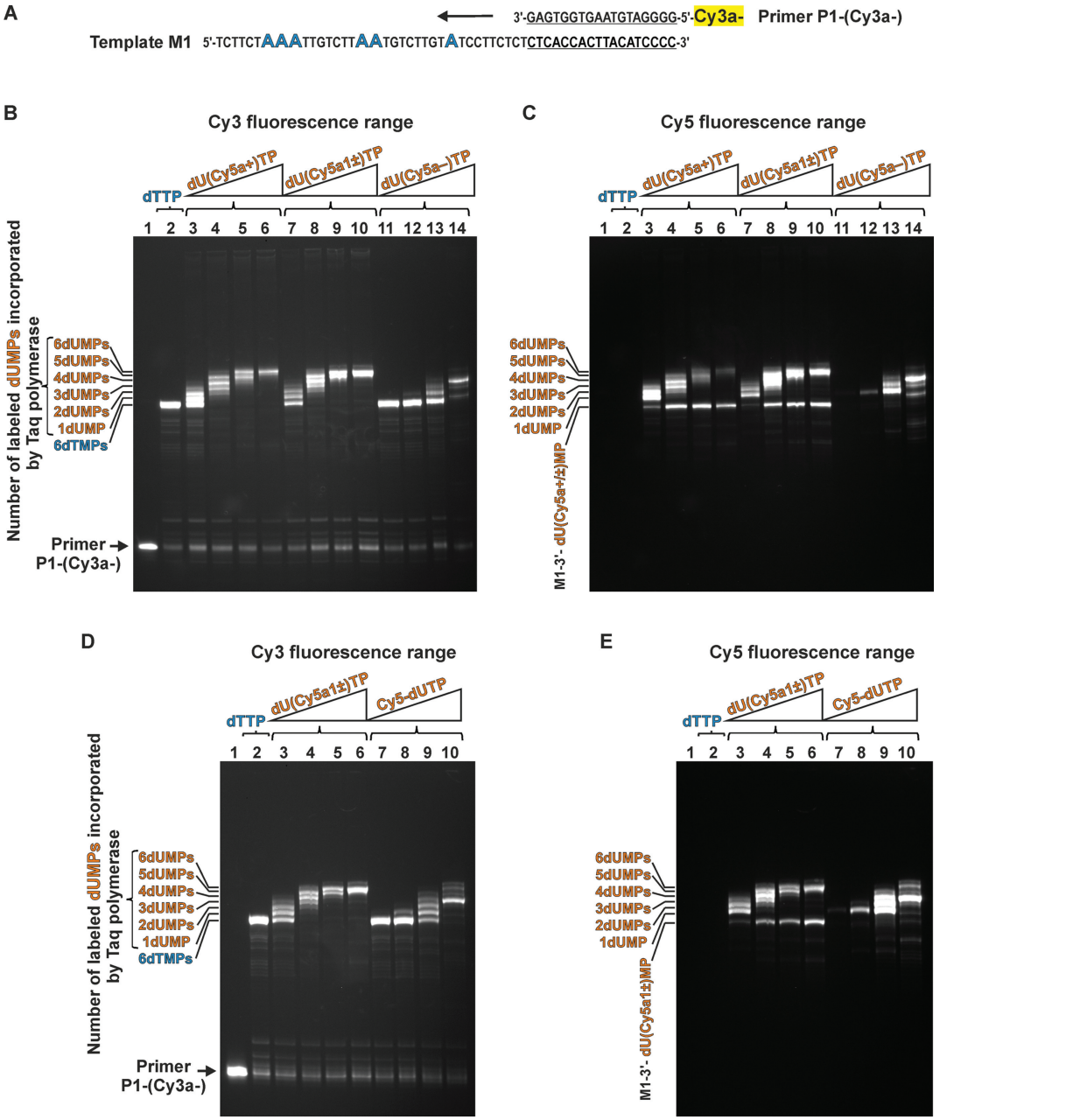


Figure 3. Incorporation of deoxyuridines labelled with Cy5 fluorophore analogues carrying positive, neutral or negative charges by Taq polymerase in a P1-(Cy3a−) primer extension reaction using the M1 template. (**A**) Primer extension reaction scheme. (**B** and **C**) Electrophoretic separation of primer extension products obtained in the presence of dTTP, dU(Cy5a+)MP, dU(Cy5a1±)MP and dU(Cy5a−)MP. The experiment shown in B and C was performed two times. Lane 1: P1-(Cy3a−) primer; lane 2: reaction products prepared using natural nucleoside triphosphates. Lanes 3–6, 7–10 and 11–14: reaction products prepared using dATP, dCTP, dGTP, increasing amounts of modified dUTPs (10, 50, 90 and 100%) and the corresponding decreasing amounts of dTTP (90, 50, 10 and 0%). Lanes 3–6: dU(Cy5a+)TP, lanes 7–10: dU(Cy5a1±)TP and lanes 11–14: dU(Cy5a−)TP. (**D** and **E**) Electrophoretic separation of primer extension products obtained in the presence of dTTP, dU(Cy5a1±)TP and Amersham Cy5-dUMP. The experiment shown in D and E was performed two times. Lane 1: P1-(Cy3a−) primer; lane 2: reaction products prepared using natural nucleoside triphosphates. Lanes 3–6 and 7–10: reaction products prepared using dATP, dCTP, dGTP, increasing amounts of modified dUTPs (10, 50, 90 and 100%) and the corresponding decreasing amounts of dTTP (90, 50, 10 and 0%). Lanes 3–6: dU(Cy5a1±)TP and lanes 7–10: Amersham Cy5-dUTP.

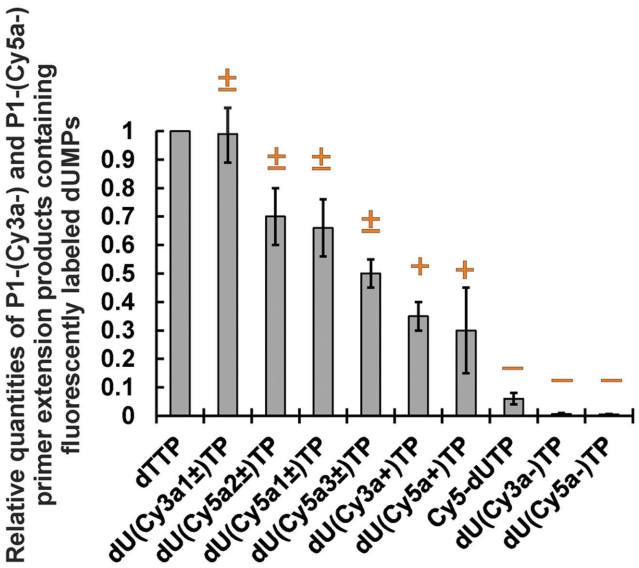


Figure 4. Histogram of the relative quantities of full-length DNA fragments containing up to three adjacent fluorescently labelled dUMPs incorporated during primer extension in the presence of dUTPs labelled with Cy3 or Cy5 dye analogues instead of dTTP. These values were obtained in the experiments shown in Figures 2 , 3 and Supplementary Figure S1 (see also Supplementary Table S3). For dU(Cy5a1±)MP and dU(Cy5a+)MP, the values were averaged over five and three experiments, respectively, and the bars indicate the mean absolute deviations. For the other fluorescently labelled dUTPs, the values were averaged over two experiments, and the bars indicate the absolute deviations.

goner (8) regarding dUTPs labelled with negatively charged Cy3 dye analogues.

Use of Amersham Cy5-dUTP (GE Healthcare), which also contains a negatively charged fluorophore, yielded a result similar to that obtained using dU(Cy3a−)TP and dU(Cy5a−)TP (Figure 3D and E). After complete replacement of dTTP with Cy5-dUTP, the main product resulting from Taq polymerase activity contained only three incorporated Cy5-dUMPs (lane 10 in Figure 3D and E). However, unlike the cases of dU(Cy3a−)TP and dU(Cy5a−)TP, visible bands that correspond to the inclusion of four, five and even six Cy5-dUMPs are observed in this lane. In the cases of dU(Cy3a−)TP and dU(Cy5a−)TP, which were synthesized in our laboratory, the bands corresponding to the inclusion of six dU(Cy3a−)MPs or six dU(Cy5a−)TPs were not detected (lanes 14 in Figures 2B and C and 3B and C). This difference can be explained by the difference in the chemical structures of the linkers connecting the fluorophores to dUTP (Table 1, linker fragment Y).

Comparison of the incorporation efficiencies of dUMPs labelled with Cy3 and Cy5 analogues carrying different charges. Effect of linker length and charge distribution of an electroneutral fluorophore

The data presented in Supplementary Table S3 and illustrated in the histogram in Figure 4 show that the efficiency of Taq polymerase using the different fluorescently labelled dUTPs as substrates in DNA synthesis decreases in the following order: dTTP ≥ {dU(Cy3a1±)TP, dU(Cy5a1±)TP, dU(Cy5a2±)TP, dU(Cy5a3±)TP} > {dU(Cy3a+)TP, dU(Cy5a+)TP} > Cy5-dUTP > {dU(Cy3a−)TP, dU(Cy5a−)TP}.

The efficiency with which Taq polymerase extends primers using dUTPs labelled with electroneutral or positively or negatively charged Cy3 and Cy5 fluorophore analogues was 50–100, 30–40 or 0–6%, respectively, of the efficiency using dTTP.

The data described above (Supplementary Table S3) indicate that the efficiency of Taq polymerase using dUTPs labelled with electroneutral Cy3 and Cy5 dye analogues, i.e. dU(Cy3a1±)TP, dU(Cy5a2±)TP, dU(Cy5a1±)TP and dU(Cy5a3±)TP, for primer extension decreases in the following order: 100, 70, 66 and 50%, respectively. Thus, the difference in charge distribution in electroneutral Cy3 and Cy5 fluorophore analogues and the longer linker conjugating the fluorophore to the base (which is two times longer for dU(Cy5a2±)TP than for dU(Cy5a1±)TP) did not significantly influence the ability of Taq polymerase to use these substrates.

Incorporation of natural and fluorescently labelled nucleotides using DNA templates modified with fluorescently labelled deoxyuridines

It was also demonstrated that modified templates containing incorporated dUMPs labelled with electroneutral analogues of the Cy5 dye can be used by Taq polymerase for the synthesis of unmodified full-length DNA fragments (Supplementary data, Part H: Incorporation of natural nucleotides during primer extension by Taq polymerase using DNA templates modified with deoxyuridines labelled with electrically neutral and positively charged Cy5 analogues, Supplementary Figures S2 and 3). Meanwhile, when dU(Cy5a1±)MP had been efficiently incorporated into the template instead of dTMP, Taq polymerase failed to synthesize full-length copies modified with the same dU(Cy5a1±)MPs that had been used in the template (Supplementary Data, Part I: Incorporation of dU(Cy5a1±)MP during primer extension from DNA template modified with dU(Cy5a1±)MPs, Figure S4). Taq polymerase cannot therefore perform PCRs if dTTP is completely replaced with fluorescently labelled dUTP in the reaction mixture.

Nucleotidyl transferase activity of Taq polymerase in the presence of fluorescently labelled dUTPs: electrophoretic analysis of primer extension products

The appearance of the lower bands in lanes 3–10 in Figure 2B that are visible in the Cy3 fluorescence range and not visible in the Cy5 fluorescence range (Figure 2C) can be explained by nucleotidyl transferase activity of Taq polymerase (22). Similarly, the extension of the 3' end of the M1 template by one dU(Cy5a+)MP or dU(Cy5a1±)MP can explain the origin of the lower visible bands in lanes 3–10 in Figure 3C that are not visible in Figure 3B. Moreover, the addition of one dU(Cy5a2±)MP or dU(Cy5a3±)MP to the 3' end of the M1 template can also explain the origin of the lower bands in lanes 3–10 in Supplementary Figure S1C that are not visible in Supplementary Figure S1B. Assuming that these bands consist of the original M1 template extended at the 3′ end

by a single labelled dUMP, we termed the corresponding products M1–3′-dU(Cy3a+)MP, M1–3′-dU(Cy3a1±)MP, M1–3′-dU(Cy5a+)MP, M1–3′-dU(Cy5a1±)MP, M1–3′-dU(Cy5a2±)MP and M1–3′-dU(Cy5a3±)MP.

To examine the incorporation of dU(Cy5a1±)MP by Taq polymerase during P1-(Cy3a−) primer extension in more detail, we synthesized an M5 template containing one adenine to enable the enzymatic incorporation of the complementary dU(Cy5a1±)MP. The 3′ end of this template was either unmodified and contained an OH group or was modified to contain an FITC fluorophore or NH_2 group (the reaction scheme presented in Figure 5A).

Figure 5B–D shows an electrophoretic separation of P1-(Cy3a−) primer extension products obtained using the M5 template; the images were recorded in the Cy3, Cy5 and FITC fluorescence ranges. The reaction products obtained using natural dNTPs fluoresced as single bands in the Cy3 fluorescence range (lanes 2, 4 and 6; Figure 5B). Naturally, these bands were not visible in the Cy5 fluorescence range.

The reaction products of a P1-(Cy3a−) primer extension using the M5 template (both unmodified and modified at the 3′ end with either FITC or an NH_2 group) in the presence of dU(Cy5a1±)TP instead of dTTP fluoresced as doublet bands in the Cy3 fluorescence range (lanes 3, 5 and 7; Figure 5B), demonstrating the presence of two primer extension products. We termed these products 1dU(Cy5a1±)MP and 2dU(Cy5a1±)MPs to correspond to containing one and two dU(Cy5a1±)MP molecules.

When the unmodified M5 template was used, the third lowest band, M1–3′-dU(Cy5a1±)MP, was observed in the Cy5 fluorescence range (lane 3; Figure 5C) in addition to the doublet bands containing the products mentioned above. Modification of the M5 template with either an FITC or NH_2 group at the 3′ end prevented Taq polymerase from adding dU(Cy5a1±)MP, and the third band was thus absent (lanes 5 and 7; Figure 5C). In this case, the M5 template modified with FITC dye on the 3′ end fluoresced in the FITC range, as expected (lanes 4 and 5 in Figure 5D).

Based on these findings, we conclude that the appearance of the lowest bands on the gel images in the Cy3 (Figure 2B) and Cy5 fluorescence ranges (Figure 3C and Supplementary Figure S1C) is due to the addition of dUMP labelled with positively charged or electroneutral Cy3 or Cy5 dye analogues to the 3′ end of the original DNA template by Taq polymerase. This activity was not observed for Taq polymerase when dUTPs labelled with negatively charged Cy3 and Cy5 dye analogues, dU(Cy3a−)TP, dU(Cy5a−)TP and Amersham Cy5-dUTP, were used (Figures 2B and 3C and E).

Nucleotidyl transferase activity of Taq polymerase in the presence of fluorescently labelled dUTPs: mass spectral analysis of primer extension products

Figure 5E illustrates the results of a mass spectral analysis of P1-(Cy3a−) primer extension products obtained using the M5 template and natural dNTPs or dNTPs in which dTTP was completely replaced by dU(Cy5a1±)TP (mass spectra 1 and 2, respectively). As shown in mass spectrum 1 (Figure 5E), the reaction product from natural dNTPs contained the original M5 template with either a dAMP or a dGMP added to the 3′ end (M5–3′-dAMP/dGMP; main peak, m.w. = 13 750.8 Da) and two types of enzymatically extended P1-(Cy3a−) primer: unmodified (peak at m.w. = 15 023.4 Da) and containing either a dAMP or dGMP added to the 3′ end (peak at m.w. = 15 335.4 Da). These two DNA fragments differed in molecular weight by only 312 Da, and they migrated as a single band that fluoresced in the Cy3 fluorescence range (labelled as dTMP on the gel image; lane 2 in Figure 5B). The mass of 312 Da corresponds well to the masses of dAMP and dGMP added to the 3′ ends of DNA fragments (313 Da and 329 Da, respectively).

Mass spectrum 2 (Figure 5E) allows one to conclude that the products of the reaction in the presence of dU(Cy5a1±)TP (instead of dTTP) included the original template in several forms: an unmodified original M5 template (peak at m.w. = 13 437.7 Da), the original template with a dAMP or a dGMP added to its 3′ end (M5–3′-dAMP/dGMP; main peak, m.w. = 13 750.8 Da), and the original template with dU(Cy5a1±)MP added to its 3′ end (the peak at m.w. = 14 341.6 Da), which fluoresced in the Cy5 fluorescence range (also the lowest weak band in the triplet in lane 3 in Figure 5C).

The products of the reaction that occurred with dU(Cy5a1±)TP instead of dTTP included an enzymatically extended P1-(Cy3a−) primer with incorporated dU(Cy5a1±)MP (peak at m.w. = 15 624.7 Da) and a similar enzymatically extended P1-(Cy3a−) primer that contained not only one incorporated dU(Cy5a1±)MP but an additional dU(Cy5a1±)MP at the 3′ end (peak at m.w. = 16 527.7 Da). The difference between the molecular weights of these DNA fragments is 903 Da, which is exactly equal to the mass of dU(Cy5a1±)MP, which had been added to the 3′ end. Both of the last two P1-(Cy3a−) primer products are visible in the Cy5 fluorescence range as the upper two (bright) bands of the triplet (lane 3 in Figure 5C). The addition of natural deoxyribonucleotides (dAMP, dGMP or dCMP) to the 3′ end of the enzymatically extended P1-(Cy3a−) primer was not detected.

When the primer extension reaction was performed in the presence of dU(Cy5a1±)TP instead of dTTP, Taq polymerase synthesized the extended P1-(Cy3a−) primer with incorporated dU(Cy5a1±)MP. Part of this product contained an additional dU(Cy5a1±)MP at the 3′ end, and the original template was extended at its 3′ end by one dU(Cy5a1±)MP. A doublet in the Cy3 fluorescence range (lane 3 in Figure 5B) and a triplet in the Cy5 fluorescence range (lane 3 in Figure 5C) consequently appeared on the gel images.

Electrophoretic analysis of the nucleotidyl transferase activity of Taq polymerase with blunt-ended DNA fragments

A mass spectral analysis of P1-(Cy3a−) primer extension products obtained using the M5 template was performed, but the nucleotidyl transferase activity of Taq polymerase was also examined using blunt-ended DNA fragments (Supplementary Data, Part J: Electrophoretic analysis of the nucleotidyl transferase activity of Taq polymerase with blunt-ended DNA fragments, Supplementary Figure S5).

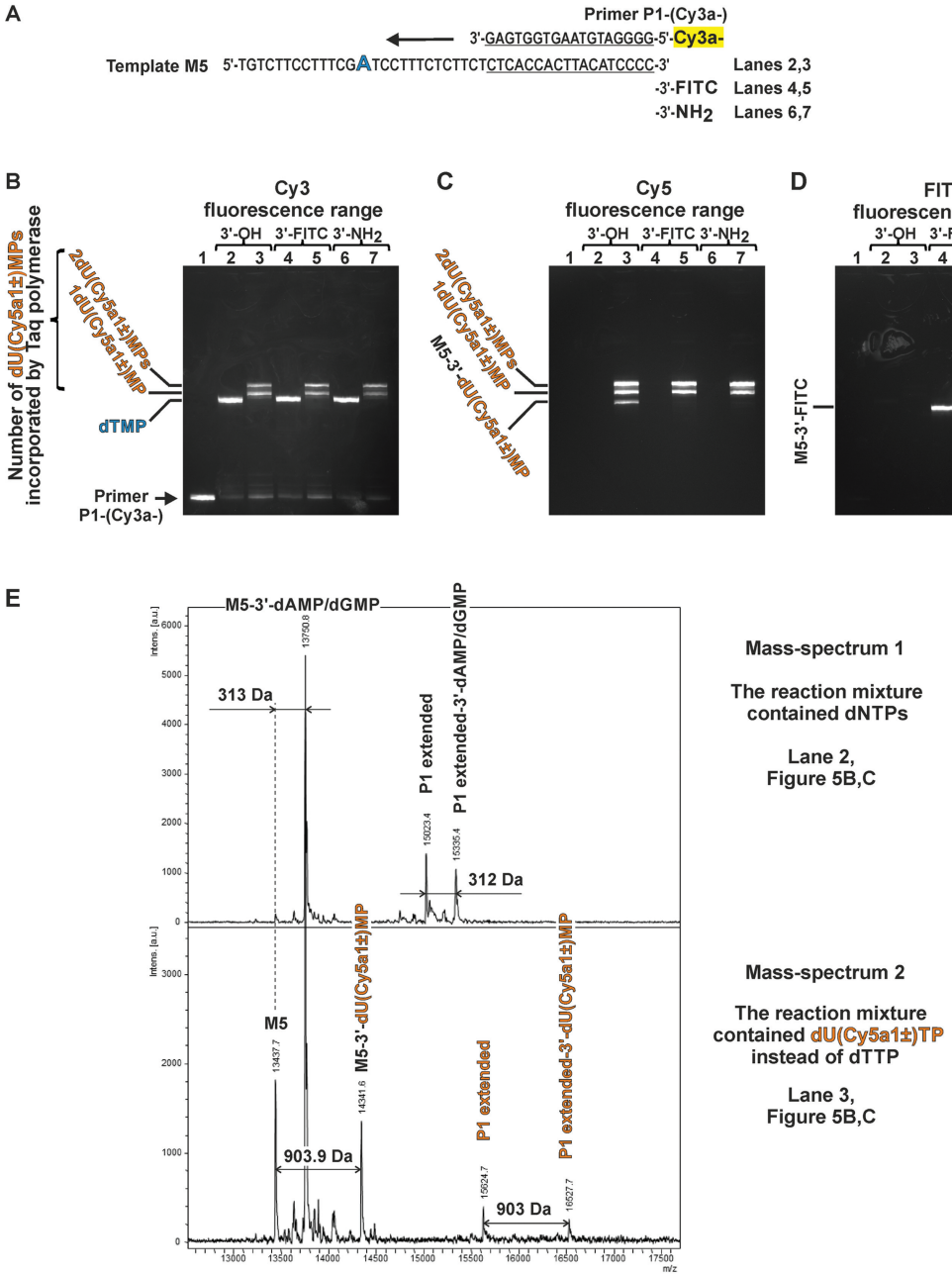


Figure 5. Analysis of Taq polymerase-dependent extension of the 3′ ends of DNA fragments by one nucleotide. (**A**) Primer extension reaction scheme. (**B**–**D**) Effect of modification of the 3′ end of the M5 template with an FITC fluorophore or NH_2 group on the incorporation of dU(Cy5a1±)MP by Taq polymerase during P1-(Cy3a−) primer extension. Lane 1: P1-(Cy3a−) primer; lane 2: reaction products obtained in the presence of unmodified M5 template and natural dNTPs, lane 3: reaction products obtained using the same template in the presence of dU(Cy5a1±)TP instead of dTTP. Lane 4: reaction products obtained in the presence of natural dNTPs using the M5 template with the FITC fluorophore covalently bound to the 3′ end, lane 5: reaction products obtained using the same template as for lane 4 with complete replacement of dTTP by dU(Cy5a1±)TP. Lane 6: reaction products obtained when the 3′ end of the M5 template was modified with an NH_2 group and in the presence of natural dNTPs, lane 7: reaction products obtained from the same template as for lane 6 in the presence of dU(Cy5a1±)TP instead of dTTP. The gel images were obtained in the Cy3 and Cy5 fluorescence ranges (B and C) and in the FITC fluorescence range ($\lambda^{ex}_{max} = 492$ nm, $\lambda^{em}_{max} = 517$ nm, D) (27). (**E**) Mass spectrometric analysis of the reaction mixtures after P1-(Cy3a−) primer extension using the M5 template and natural dNTPs or completely replacing dTTP with dU(Cy5a1±)TP. Mass spectrum 1: the reaction mixture contained natural dNTPs (lane 2 in B and C); mass spectrum 2: the reaction mixture contained dU(Cy5a1±)TP instead of dTTP (lane 3 in B and C). The reaction products are indicated above the spectral maxima.

The nucleotidyl transferase activity of Taq polymerase was observed by having both natural dNTPs and dU(Cy5a1±)TP in the reaction mixture, allowing both natural dNMPs and dU(Cy5a1±)MP to be added to the 3′ ends of double-stranded DNA fragments.

Effect of T, C and G nucleotides adjacent to A on the incorporation of dU(Cy5a1±)MP, dU(Cy5a2±)MP and dU(Cy5a3±)MP by Taq polymerase during primer extension

The incorporation of dUMPs labelled with electroneutral Cy5 analogues by Taq polymerase was studied in the P1-(Cy3a−) primer extension reaction using 45-nt M2-M10 templates whose sequences contained one A nucleotide surrounded by three other nucleotides (T, C and G) in all possible variants, i.e. TAT, TAG, TAC, GAT, GAG, GAC, CAT, CAG and CAC. The remaining nucleotide sequences of these templates were identical. The data analysis is described in Supplementary Data (Part K: Effect of T, C and G nucleotides adjacent to A on the incorporation of dU(Cy5a1±)MP, dU(Cy5a2±)MP and dU(Cy5a3±)MP by Taq polymerase during primer extension, Supplementary Figures S6 and 7).

The incorporation of one dU(Cy5a1±)MP, dU(Cy5a2±)MP or dU(Cy5a3±)MP by Taq polymerase does not depend on the surrounding T, C and G nucleotides in the template sequence to within 15–20% of the measurement accuracy.

Experimental approach to studying the kinetics of fluorescently labelled dUMP incorporation by Taq polymerase

To characterize the Taq polymerase-dependent incorporation efficiency of fluorescently labelled dUMPs with kinetics parameters, the Michaelis–Menten constant, K_m and the maximum substrate incorporation rate, V_{max}, were determined. These parameters for dTTP and for the fluorescently labelled substrates dU(Cy5a1±)TP, dU(Cy5a2±)TP, dU(Cy5a3±)TP, dU(Cy5a+)TP and dU(Cy5a−)TP were determined using templates containing one (template M2), two (template M11) or three (template 12) adjacent adenines (Figure 6, Supplementary Table S1 and the Michaelis–Menten kinetics measurements section in the 'Materials and Methods' section).

The experimental conditions used for the kinetic measurements were chosen in accordance with the data presented in Supplementary Figure S8. Supplementary Figure S8B shows the electrophoretic separation of time-dependent primer extension products obtained in the presence of natural dNTPs using the M2 template, the P1-(Cy3a−) primer and low (1.5 units, lanes 7–11) and large (15 units, lanes 2–6) amounts of Taq polymerase. The fluorescence intensities of the bands corresponding to full-length products increased with primer extension time. The dependences for the normalized fluorescence signals shown in Supplementary Figure S8C therefore made determining the linear interval of time for measuring the extension reaction rates possible, and a time of 40 min was chosen for subsequent experiments.

Figure 6 shows an electrophoretic separation of products obtained after 40 min of primer extension by Taq polymerase using the M2, M11 and M12 templates, which contained a single A, an AA doublet and an AAA triplet, respectively (Figure 6A–C), in the presence of the P1-(Cy3a−) primer, dATP, dCTP, dGTP and increasing concentrations of dTTP or dU(Cy5a1±)TP. The upper row of bands in each gel image shown in Figure 6D–F in the Cy3 fluorescence range belongs to the products containing one (lanes 4, 6, 8, 10, 12, 14 and 16 in Figure 6D), two (lanes 4, 6, 8, 10, 12 and 14 in Figure 6E) and three (lanes 4, 6, 8, 10, 12 and 14 in Figure 6F) incorporated dU(Cy5a1±)MP(s). The slightly lower row in each of the images are the products containing one (lanes 3, 5, 7, 9, 11, 13 and 15 in Figure 6D), two (lanes 3, 5, 7, 9, 11 and 13 in Figure 6E) and three (lanes 3, 5, 7, 9, 11 and 13 in Figure 6F) incorporated dTMP(s).

The middle, bright row of bands in each image represents an incomplete chain, the synthesis of which was terminated because of low substrate concentrations (dTTP or dU(Cy5a1±)TP) and the corresponding long time that the polymerase waited for substrate. These middle rows of bands were not observed in the Cy5 fluorescence range because dU(Cy5a1±)MP was not incorporated.

The bright bands in lane 17 of Figure 6D and lane 15 of Figure 6E and F are the products obtained after 2 h of primer extension using the corresponding templates, which was performed to ensure that all of the primer molecules were extended. Since the primer concentration was known (5×10^{-6} M), the proportionality coefficients between the fluorescence intensity of each of these bright bands and the concentration of the product containing the P1-(Cy3a−) primer could be calculated. The concentrations of all the products synthesized by Taq polymerase per 40 min were estimated using the fluorescence intensities of the bands, and the corresponding plots of the extended product synthesis rates (d[P]/dt) versus the substrate concentration [S] were obtained.

Determination and analysis of the K_m and V_{max} values characterizing the incorporation of dUMPs labelled with differently charged Cy5 analogues

The plots of incorporation rates, dP/dt, against dTTP and dU(Cy5a1±)TP concentrations in primer extension reactions along the M2, M11 and M12 templates (the experiments shown in Figure 6) are presented in Figure 7. Approximations of the experimental points by curves according to the Michaelis–Menten equation ('Materials and Methods' section) provided estimated K_m and V_{max} values (Table 2) characterizing the incorporation of dTMP or dU(Cy5a1±)MP by Taq polymerase along the templates containing a single A, an AA doublet or an AAA triplet. The K_m value for dTTP when the template contained a single A was $(1.0 \pm 0.4) \times 10^{-6}$ M (Table 2), which is within experimental error of the result (0.49×10^{-6} M) obtained previously by the steady-state method (23). The K_m and V_{max} values were similarly estimated for the incorporation of dU(Cy5a2±)MP, dU(Cy5a3±)MP, dU(Cy5a+)MP and dU(Cy5a−)MP using the same templates (Supplementary Figures S9–12).

Plots of K_m and V_{max} values against the number of adjacent nucleotides incorporated by Taq polymerase are shown in Supplementary Figure S13, while the values themselves

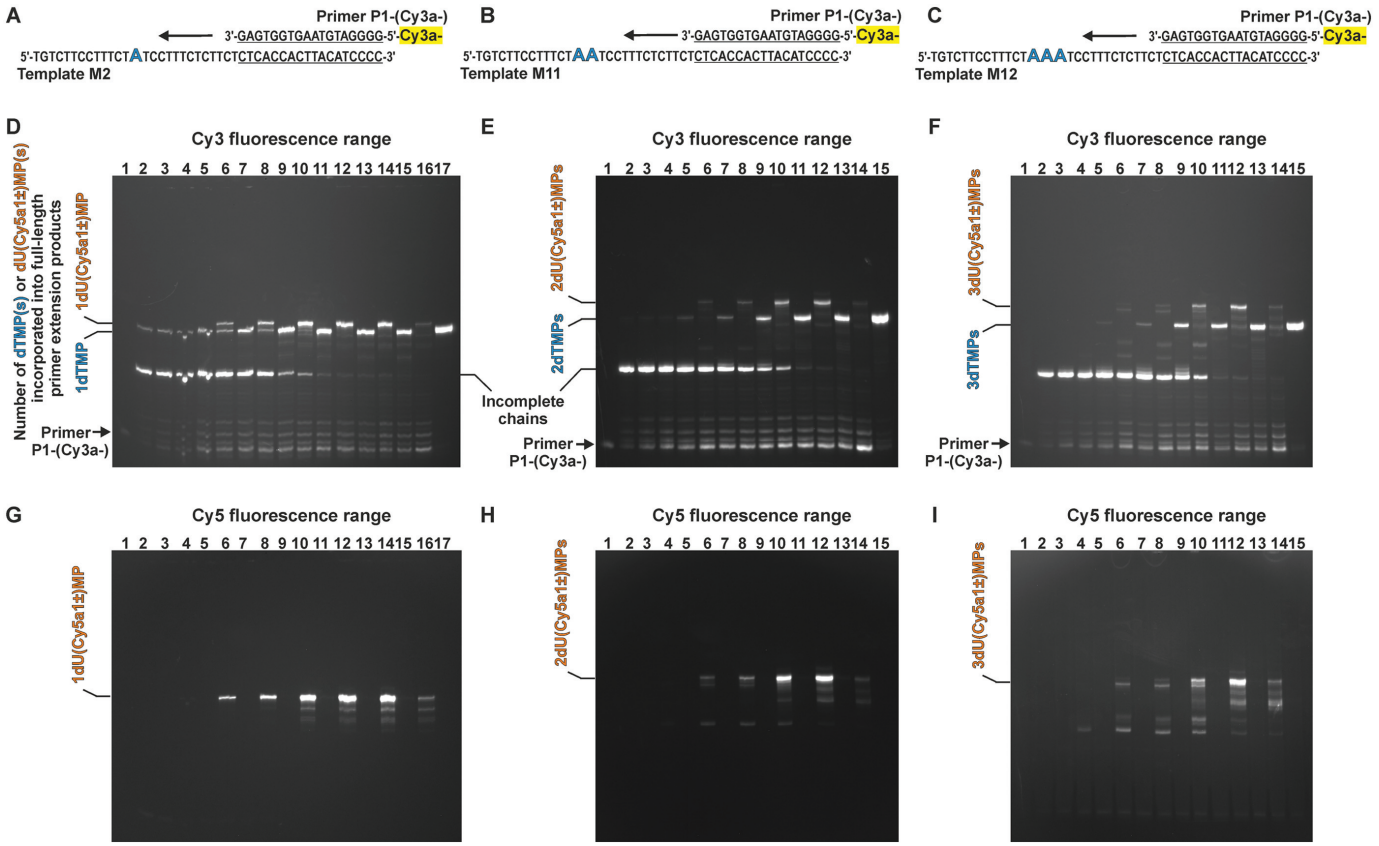


Figure 6. Determining the Michaelis–Menten constants (K_m) and the maximum substrate incorporation rates (V_{max}) for dTMP and dU(Cy5a1±)MP incorporation by Taq polymerase. (**A**–**C**) Primer extension reaction schemes. (**D**–**I**) Electrophoretic separation of the reaction products. Lane 1: P1-(Cy3a−) primer. Lane 2: products of a 40-min reaction in the presence of dATP, dCTP and dGTP (5×10^{-4} M each); 5×10^{-6} M M2 (D and G), M11 (E and H) or M12 (F and I) template; 5×10^{-6} M P1-(Cy3a−) primer and 1.5 units of Taq polymerase. D and G: lanes 3, 5, 7, 9, 11, 13 and 15—the same mixture as for lane 2 with an increasing concentration of dTTP: 5×10^{-8}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 2.5×10^{-5}, 5×10^{-5} and 5×10^{-4} M, respectively. E, F, H and I: lanes 3, 5, 7, 9, 11 and 13—the same mixture as for lane 2 with an increasing concentration of dTTP: 5×10^{-8}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 5×10^{-5} and 5×10^{-4} M, respectively. D and G: lanes 4, 6, 8, 10, 12, 14 and 16—the same mixture as for lane 2 with an increasing concentration of dU(Cy5a1±)TP: 5×10^{-8}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 2.5×10^{-5}, 5×10^{-5} and 5×10^{-4} M, respectively. E, F, H and I: lanes 4, 6, 8, 10, 12 and 14—the same mixture as for lane 2 with an increasing concentration of dU(Cy5a1±)TP: 5×10^{-8}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 5×10^{-5} and 5×10^{-4} M, respectively. Lane 17 in D and lane 15 in E and F contained the reaction products obtained in 2-h primer extension reactions in the presence of dATP, dCTP, dGTP and dTTP (5×10^{-4} M each); 5×10^{-6} M M2 (D), M11 (E) or M12 (F) template; 5×10^{-6} M P1-(Cy3a−) primer and 15 units of Taq polymerase. The experiments shown in the figure were performed two times. A quantitative analysis of the gel images shown in the figure is presented in Figure 7.

are presented in Table 2. The dUTPs labelled with electroneutral or positively charged Cy5 analogues are characterized by the values of K_m and V_{max}, which are closer to the corresponding parameters for dTTP as opposed to K_m and V_{max} values characterizing dU(Cy5a−)TP labelled with the negatively charged fluorophore.

The V_{max}/K_m ratios (the relative efficiencies of converting fluorescently labelled dUTPs into products by Taq polymerase) are also presented in Table 2 and plotted in Figure 8A versus the number of adjacent nucleotides incorporated into the positions complementary to A, AA or AAA sequences by Taq polymerase. Figure 8A shows that the relative efficiency of converting dUTPs labelled with positively charged or electroneutral Cy5 analogues is close to the efficiency of converting natural dTTP and is approximately one order of magnitude greater than the efficiency of converting dU(Cy5a−)TP.

Inhibition of Taq polymerase at high concentrations of fluorescently labelled dUTPs

The kinetic curves that were used for the estimation of K_m and V_{max} (Figure 7, Supplementary Figures S10 and 12) show that the incorporation rates decreased significantly (excluding that for dTMP incorporation) when the concentration of the fluorescently labelled dUTPs was much more than 5×10^{-5} M. To learn more about the inhibition of Taq polymerase by dUTPs labelled with Cy3 or Cy5 analogues carrying a positive, a negative or no total charge, we introduced the parameter $\alpha = V([S] = 5 \times 10^{-4}\ M)/V_{max}$ (the relative factor of inhibition). This parameter characterizes the decrease in the incorporation rate when the concentration of a dNTP is 5×10^{-4} M. The values of α were estimated for the cases when A, AA or AAA were presented in the corresponding M2, M11 and M12 templates; these values are shown in Table 2.

The template M13 was also synthesized and is identical to the previously mentioned templates M2, M11 and M12

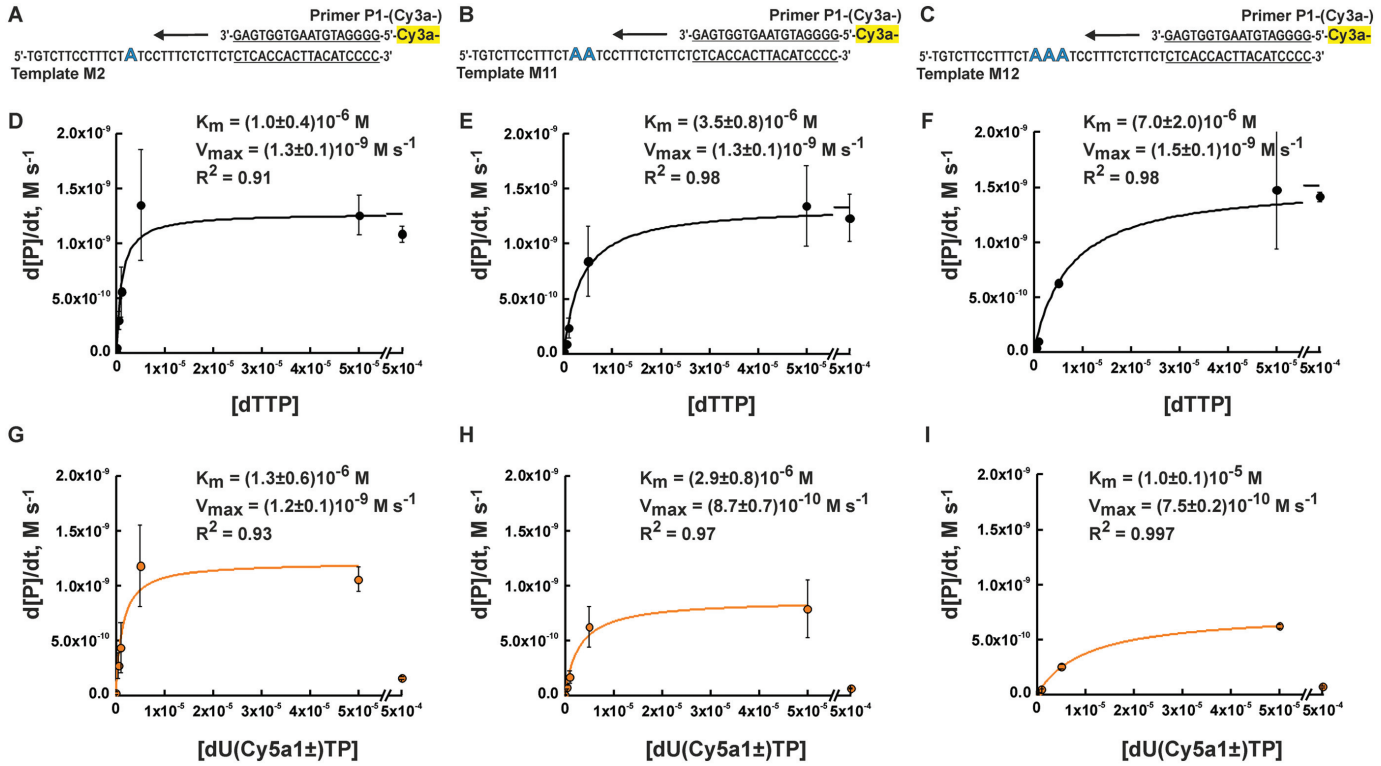


Figure 7. Determination of K_m and V_{max} values characterizing the incorporation of dTMP and dU(Cy5a1±)MP by Taq polymerase. (**A**–**C**) P1-(Cy3a−) primer extension reaction schemes. (**D**–**F**) Plots of the incorporation rates, d[P]/dt, for one (D), two (E) and three (F) consecutively incorporated dTMP(s) (black circles and curves) as a function of dTTP concentration. (**G**–**I**) Plots of d[P]/dt for one (G), two (H) and three (I) consecutively incorporated dU(Cy5a1±)MP(s) (orange circles and curves) as a function of dU(Cy5a1±)TP concentration. The values averaged over the two experiments are shown. The bars indicate absolute deviations.

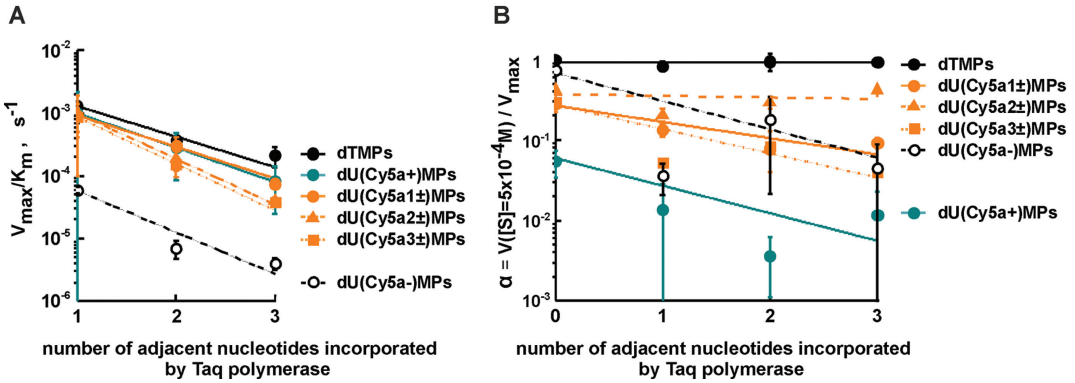


Figure 8. Plots of kinetic parameter ratios, V_{max}/K_m (**A**) and $\alpha = V([S] = 5 \times 10^{-4}\ M)/V_{max}$ (**B**), characterizing the incorporation of dTMP and fluorescently labelled dUMPs during primer extension by Taq polymerase against the number of incorporated nucleotides, x. V_{max} is the maximum substrate incorporation rate, K_m is the Michaelis–Menten constant and $V([S] = 5 \times 10^{-4}\ M)$ is the substrate incorporation rate when the substrate concentration is 5×10^{-4} M (Table 2, Figures 6 and 7, and Supplementary Figures S9–12). The bars indicate standard deviations. The plots of V_{max}/K_m (A) were approximated using the equation $V_{max}/K_m = 10^{Ax+B}$ as previously performed by Kuwahara *et al.* (28), where A and B are fitting parameters. The plots of $\alpha = V([S] = 5 \times 10^{-4}M)/V_{max}$ (B) were approximated using the equation $\alpha = 10^{Ax+B}$.

except that it does not contain adenine for complementary nucleotide incorporation (Supplementary Figure S14A). As shown in Supplementary Figure S14, primer extension by Taq polymerase along template M13 was inhibited to different extents in the presence of different fluorescently labelled dUTPs at a concentration of 5×10^{-4} M. This inhibition was characterized by the fluorescent intensities of the bands containing the full-length primer extension products (visible in lanes 3–8 in Supplementary Figure S14B and lanes 10–13 in Supplementary Figure S14C), which were normalized to the fluorescence intensities of the bands containing the products obtained using only dATP, dGTP and dCTP (lanes 2 and 9). The normalized fluorescence intensities are presented in Supplementary Figure S14D and are considered the values of the relative factor of inhibition, α, extended to the case in which the template does not con-

Table 2. The kinetic parameters for Taq polymerase synthesis of oligonucleotides complementary to M2, M11 or M12 templates containing one, two or three adjacent adenines, correspondingly, in the presence of dTTP or Cy5a-labelled dUTP (dU(Cy5a1±)TP, or dU(Cy5a2±)TP, or dU(Cy5a3±)TP, or dU(Cy5a+)TP or dU(Cy5a−)TP) and dGTP, dCTP, dATP in reaction mixture

Nucleoside triphosphates	K_m, M			V_{max}, M s^{-1}		
	Number of incorporated nucleotides			Number of incorporated nucleotides		
	One nucleotide[a]	Two adjacent nucleotides[b]	Three adjacent nucleotides[c]	One nucleotide[a]	Two adjacent nucleotides[b]	Three adjacent nucleotides[c]
dTTP	$(1.0 \pm 0.4)10^{-6}$	$(3.5 \pm 0.8)10^{-6}$	$(7.0 \pm 2.0)10^{-6}$	$(1.3 \pm 0.1)10^{-9}$	$(1.3 \pm 0.1)10^{-9}$	$(1.5 \pm 0.1)10^{-9}$
dU(Cy5a+)TP	$(1.0 \pm 1.0)10^{-6}$	$(2.5 \pm 1.4)10^{-6}$	$(6.0 \pm 3.0)10^{-6}$	$(1.0 \pm 0.2)10^{-9}$	$(7.0 \pm 1.0)10^{-10}$	$(5.0 \pm 1.0)10^{-10}$
dU(Cy5a1±)TP	$(1.3 \pm 0.6)10^{-6}$	$(2.9 \pm 0.8)10^{-6}$	$(1.0 \pm 0.1)10^{-5}$	$(1.2 \pm 0.1)10^{-9}$	$(8.7 \pm 0.7)10^{-10}$	$(7.5 \pm 0.2)10^{-10}$
dU(Cy5a2±)TP	$(1.0 \pm 0.7)10^{-6}$	$(2.6 \pm 1.0)10^{-6}$	$(1.0 \pm 0.1)10^{-5}$	$(1.0 \pm 0.2)10^{-9}$	$(4.8 \pm 0.5)10^{-10}$	$(4.0 \pm 0.1)10^{-10}$
dU(Cy5a3±)TP	$(1.5 \pm 0.5)10^{-6}$	$(4.1 \pm 0.6)10^{-6}$	$(5.7 \pm 0.6)10^{-6}$	$(1.3 \pm 0.1)10^{-9}$	$(6.2 \pm 0.3)10^{-10}$	$(2.2 \pm 0.1)10^{-10}$
dU(Cy5a−)TP	$(1.4 \pm 0.1)10^{-5}$	$(8.0 \pm 2.0)10^{-6}$	$(9.0 \pm 1.4)10^{-6}$	$(8.3 \pm 0.3)10^{-10}$	$(5.6 \pm 0.4)10^{-11}$	$(3.6 \pm 0.2)10^{-11}$
Nucleoside triphosphates	**V_{max}/K_m, s^{-1}**			**$\alpha = V([S] = 5 \times 10^{-4}\ M)/V_{max}$**		
	Number of incorporated nucleotides			**Number of incorporated nucleotides**		
	One nucleotide[a]	**Two adjacent nucleotides[b]**	**Three adjacent nucleotides[c]**	**No nucleotide[d]**	**One nucleotide[a]**	**Two adjacent nucleotides[b]**	**Three adjacent nucleotides[c]**
dTTP	$(1.3 \pm 0.6)10^{-3}$	$(3.7 \pm 1.1)10^{-4}$	$(2.1 \pm 0.8)10^{-4}$	$(9.9 \pm 0.9)10^{-1}$	$(8.3 \pm 1.2)10^{-1}$	$(9.5 \pm 2.4)10^{-1}$	$(9.4 \pm 0.9)10^{-1}$
dU(Cy5a+)TP	$(1.0 \pm 1.0)10^{-3}$	$(2.8 \pm 2.0)10^{-4}$	$(8.3 \pm 5.8)10^{-5}$	$(5.4 \pm 2.0)10^{-2}$	$(1.4 \pm 1.4)10^{-2}$	$(3.6 \pm 2.6)10^{-3}$	$(1.2 \pm 1.1)10^{-2}$
dU(Cy5a1±)TP	$(9.0 \pm 5.0)10^{-4}$	$(3.0 \pm 1.1)10^{-4}$	$(7.5 \pm 0.3)10^{-5}$	$(2.8 \pm 0.4)10^{-1}$	$(1.3 \pm 0.2)10^{-1}$	$(7.8 \pm 1.0)10^{-2}$	$(9.3 \pm 1.5)10^{-2}$
dU(Cy5a2±)TP	$(1.0 \pm 0.9)10^{-3}$	$(1.9 \pm 0.9)10^{-4}$	$(4.0 \pm 0.1)10^{-5}$	$(4.1 \pm 0.5)10^{-1}$	$(2.0 \pm 0.4)10^{-1}$	$(2.9 \pm 0.5)10^{-1}$	$(4.2 \pm 0.1)10^{-1}$
dU(Cy5a3±)TP	$(8.7 \pm 3.6)10^{-4}$	$(1.5 \pm 0.3)10^{-4}$	$(3.9 \pm 0.6)10^{-5}$	$(2.9 \pm 0.7)10^{-1}$	$(5.2 \pm 0.4)10^{-2}$	$(8.3 \pm 4.4)10^{-2}$	$(4.0 \pm 0.2)10^{-2}$
dU(Cy5a−)TP	$(5.9 \pm 0.6)10^{-5}$	$(7.0 \pm 2.0)10^{-6}$	$(4.0 \pm 0.8)10^{-6}$	$(7.4 \pm 1.1)10^{-1}$	$(3.6 \pm 1.5)10^{-2}$	$(1.8 \pm 1.6)10^{-1}$	$(4.5 \pm 4.4)10^{-2}$

[a] K_m, V_{max} and $V([S] = 5 \times 10^{-4}\ M)$ for one nucleotide incorporation followed by chain elongation were measured using M2 template (see Supplementary Table S1 and Figures 6A, D, G and 7A, D, G, Supplementary Figures S9A, D, G, S10A, D, G, S11A, D, G and S12A, D, G).
[b] K_m, V_{max} and $V([S] = 5 \times 10^{-4}\ M)$ for two adjacent nucleotides incorporation followed by chain elongation were measured using M11 template (see Supplementary Table S1 and Figures 6B, E, H and 7B, E, H, Supplementary Figures S9B, E, H, S10B, E, H, S11B, E, H and S12B, E, H).
[c] K_m, V_{max} and $V([S] = 5 \times 10^{-4}\ M)$ for three adjacent nucleotides incorporation followed by chain elongation were measured using M12 template (see Supplementary Table S1 and Figures 6C, F, I and 7C, F, I, Supplementary Figures S9C, F, I, S10C, F, I, S11C, F, I and S12C, F, I).
[d] For the case when M13 template which did not contain any adenine (Supplementary Table S1 and Figure S14) was used, dTMP or dUMPs labelled with Cy5 dye analogues (dU(Cy5a+)MP, dU(Cy5a1±)MP, dU(Cy5a2±)MP, dU(Cy5a3±)MP or dU(Cy5a−)MP) were not incorporated. Here, the values of relative factor of inhibition $\alpha = V([S] = 5 \times 10^{-4}\ M)/V_{max}$ were estimated from the data presented in Supplementary Figure S14.
K_m is the Michaelis–Menten constant, V_{max} is the maximum substrate incorporation rate, V_{max}/K_m is the relative efficiency of substrate conversion into product by Taq polymerase, $\alpha = V([S] = 5 \times 10^{-4}\ M)/V_{max}$ is the relative factor of inhibition at 5×10^{-4} M of substrate.

tain adenine for complementary nucleotide incorporation. The histogram in Supplementary Figure S14D allows the α values for the primer extension reaction catalysed by Taq polymerase in the presence of 5×10^{-4} M dUTPs labelled with positively charged, neutral or negatively charged Cy3 or Cy5 analogues can be estimated as 0.06, 0.25–0.40 and 0.65–0.75, respectively.

A similar conclusion can be made from an analysis of α values (the relative factor of inhibition) plotted against the number of adjacent adenine nucleotides in the template (zero, one, two or three adjacent adenine nucleotides). The data shown in Figure 8B (also Table 2) indicate that the relative factor of inhibition, α, generally decreases as the number of adenine nucleotides in the templates is increased. In the case of dUTPs labelled with electroneutral or negatively charged Cy5 analogues, the parameter α decreased to $\sim 10^{-1}$, while in the case of positively charged Cy5 analogue, the estimated α value was $\sim 10^{-2}$, indicating one order of magnitude greater inhibition of synthesis by Taq polymerase. This observation also supports the choice of electroneutral fluorophores for labelling the dNTPs that can be used efficiently by Taq polymerase.

Possible enzymatic activities of other DNA polymerases in DNA amplification and labelling with dNTPs carrying differently charged fluorophores

As was reported by Cline et al. (24), Taq polymerase is characterized by a low error rate and stands in a series of high fidelity polymerases: Pfu (1.3×10^{-6}) < Deep Vent (2.7×10^{-6}) < Vent (2.8×10^{-6}) < Taq (8.0×10^{-6}) << Pfu (exo-) (5×10^{-5}). Polymerases Pfu, Deep Vent and Vent exhibit somewhat higher fidelity due to their 3′→5′ exonuclease proofreading activity. These polymerases are inefficient at incorporating fluorescently labelled nucleotides (9,13). Turning off the proofreading function decreases fidelity but significantly improves the efficiency of incorporating fluorescently labelled nucleotides (9). Anderson et al. (13), who tested the ability of Taq and Vent (exo-) to use Amersham Cy5-dCTP, have found that Vent (exo-) incorporated Cy5-dCMP approximately two times better than Taq, which lacks 3′→5′ exonuclease proofreading activity. Polymerases with a switched out proofreading activity such as Pfu (exo-), Vent (exo-) and Deep Vent (exo-) are expected to be as or more efficient as Taq polymerase at incorporating dUMPs labelled with electroneutral Cy dye analogues.

Investigating the efficiencies of Pfu (exo-), Vent (exo-) and Deep Vent (exo-) polymerases to incorporate fluorescently labelled nucleotides as a function of fluorophore charge is therefore undoubtedly of interest. Moreover, the application of the original versions of the mentioned polymerases (Pfu, Vent and Deep Vent) can lead to new interesting results.

The results obtained in this study clearly demonstrate that the presence of mutually compensating charges on a nucleotide-conjugated molecule is a positive factor in the design of modified nucleoside triphosphates bearing fluorophores or other functional structures to be used in Taq polymerase-dependent DNA synthesis. This finding can be significant for increasing the effectiveness of DNA detection methods used in medical diagnostics and for the development of technology associated with the use of modified DNA aptamers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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