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RESEARCH ARTICLE

Investigation of the substrate properties of fluorescently labeled pyrimidine triphosphates in recombinase polymerase amplification

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Abstract

Objectives. To study the substrate properties of Cy5-labeled deoxynucleoside triphosphates of various natures (dU and dC) in the process of incorporation in the DNA chain during recombinase polymerase amplification (RPA).

Methods. The work used the real-time RPA method. The method of horizontal electrophoresis was used to control the quality of the amplification products obtained.

Results. The influence of the fluorophore structure and linker lengths on the substrate properties for deoxynucleoside triphosphates Cy5-dUTP and Cy5-dCTP was studied. The following values of the substrate efficiency parameters were determined: amplification efficiency (kinetic indicator), normalized product yield, and embedding coefficient.

Conclusions. Modified deoxynucleoside triphosphates (dNTP) with long linkers between the fluorophore and the nitrogenous base, as well as between the quaternary ammonium group and the second heterocycle of the fluorophore, showed greater substrate efficiency than fluorescently labeled dNTP with short linkers. The modified dU in each pair demonstrated greater substrate efficiency compared to the modified dC.

Keywords

recombinase polymerase amplification, fluorescently labeled deoxynucleoside triphosphates

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НАУЧНАЯ СТАТЬЯ

Исследование субстратных свойств флуоресцентно-меченых пиримидинтрифосфатов в рекомбиназной полимеразной амплификации

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Аннотация

Цели. Изучить субстратные свойства трифосфатов дезоксинуклеозидов различной природы (dU и dC), флуоресцентно-меченых красителями цианинового ряда Cy5, при их встраивании в цепь ДНК в процессе рекомбиназной полимеразной амплификации (RPA).

Методы. В работе использовали метод RPA в режиме реального времени. Для контроля качества получаемых продуктов амплификации использовали метод горизонтального электрофореза.

Результаты. Исследовано влияние строения флуорофора и длин линкеров между красителем и азотистым основанием нуклеотида, а также вторым гетероциклом флуорофора и четвертичной аммониевой группой, на субстратные свойства для дезоксинуклеозидтрифосфатов Cy5-dUTP и Cy5-dCTP. Определены значения параметров субстратной эффективности: эффективности амплификации (кинетического показателя), а также нормированного выхода продукта и коэффициента встраивания.

Выводы. Модифицированные дезоксинуклеозидтрифосфаты (dNTP) с длинными линкерами между флуорофором и азотистым основанием нуклеотида, а также между четвертичной аммониевой группой и вторым гетероциклом флуорофора, показывали большую субстратную эффективность, в отличие от флуоресцентно-меченых dNTP с короткими линкерами. Модифицированные dU в каждой паре демонстрировали большую субстратную эффективность по сравнению с модифицированными dC.

Ключевые слова

рекомбиназная полимеразная амплификация, флуоресцентно-меченые дезоксинуклеозидтрифосфаты

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INTRODUCTION

Fluorescent labeling of nucleic acids is currently widely used in molecular biology and medical diagnostics. Deoxynucleoside triphosphates (dNTPs) modified by fluorophore introduction [1–4], more often of pyrimidine nature, are used to introduce DNA directly in the process of amplification, which greatly simplifies the procedure of analysis of amplification products using the immobilized phase (on biological microarrays, various strip systems, etc.) [5]. The fluorophores used to obtain fluorescently labeled DNA need the following properties: chemical stability, low background fluorescence, and resistance to multiple irradiation (fluorescence). Such tags include deoxyribonucleoside triphosphate derivatives modified with Cy5 cyanine dyes (Cy5-dNTP) [6], where the fluorophore is attached via a linker to the nitrogenous base of the nucleotide [7–9]. The wide application of fluorescently labeled nucleoside triphosphates in molecular biological diagnostic systems makes it extremely important for their compatibility with enzymatic systems [2, 3, 10] to be studied.

The aim of the study is to investigate the substrate properties of fluorescently labeled dNTPs of pyrimidine nature (dU and dC) in the enzymatic system of recombinase polymerase amplification (RPA) [11–13]. A fragment of the *ebpS* gene of the human pneumonia pathogen *Staphylococcus aureus* was selected as a bacterial genetic target to study the substrate efficiency of Cy5-dUTP and Cy5-dCTP.

EXPERIMENTAL

DNA samples. Decontaminated DNA of *Staphylococcus aureus* obtained from the collection of the State Scientific Center of Applied Microbiology and Biotechnology (Obolensk, Moscow oblast) was used in the work.

PCR. The polymerase chain reaction (PCR) method was used to produce the target fragment of the *ebpS* gene. The reaction mixture (30 μL) contained a mixture of natural dNTPs at a concentration of 0.2 mM; species-specific primers: forward (5'-TTAGAAGCGTCTTTAGATGTGTC-3') and reverse (5'-GGAACAGCGGGGGTGTTGTTGCAGGTGC-3'); 5U Taq-DNA polymerase (*Thermo Scientific*, USA). Its reaction buffer was in the amount recommended by the manufacturer. Amplification was performed on a Gentier 96E (*Tianlong*, China) using the following program: preheating at 95°C for 3 min, then 32 cycles: 95°C for 20 s, 60°C for 30 s, 72°C for 30 s. This was followed by final incubation at 72°C for 3 min. The resulting PCR product of 497 base pairs (bp) in length was purified and isolated according to the method described in [14] and then used in RPA to study the kinetic characteristics and substrate properties.

Real-time RPA. The reaction mixture (50 μL) contained the components of the TwistAmp Basic kit (*TwistDX*, United Kingdom) at the manufacturer's recommended concentrations; the kit reagents with the addition of a pair of primers: 5'-CTCCAAATATATCGCTAATGCACCGATAATTAGTACAGTACAGCTGC-3') and reverse (5'-ACTCGACTGAGGAGGAGGATAAAGCGTCTCTCTCAAGATAAGATAAGTCTAAGAAGA-3'). EvaGreen intercalating dye (*Biotium*, USA) and purified PCR product in the amount of 1 μL were included in the total mixture. After careful stirring it was poured into 200 μL reaction tubes with the lyophilizate from the kit. Fluorescently labeled triphostaphates were introduced into the reaction volume at a concentration of 8 μM. The reaction was carried out on a Gentier 96E DNA amplifier (*Tianlong*, China) in real time according to the program: 50 min at 40°C and fluorescence signal acquisition once per minute. The accumulation of reaction product was visualized using intercalating dye. The RPA product with a length of 282 bp obtained was purified and isolated according to the method described in [14].

Horizontal electrophoresis for RPA product control. RPA products were separated in 4% agarose gel Agarose LE (*Helicon*, Russia) for 5 min at 5 V/cm, then 50 min at 10 V/cm, SYBR Green I (*Molecular Probes*, USA) was used for staining. For DNA detection by SYBR Green I staining, visualization was performed on a ChemiScope 6200 Touch gel-documentation system (*Clinx Science Instruments*, China) using built-in LEDs, and "Green light excitation/emission" light filters with excitation spectrum corresponding to Cy3 dye ($\lambda_{max.excitation}$ = 550 nm, $\lambda_{max.emission}$ = 570 nm). For selective detection of the tag embedded in DNA, "Red light excitation/emission" light filters were used. Their excitation spectrum is similar to the Cy5 dye ($\lambda_{max.excitation}$ = 650 nm, $\lambda_{max.emission}$ = 670 nm).

RESULTS AND DISCUSSION

For a comparative analysis of the substrate efficiency of fluorescently labeled nucleotides, we used dNTPs (Fig. 1) containing zwitter-ionic indodicarbocyanine dyes in their structure. They differ in terms of the spatial structure of the fluorophore [7], the length of the linker between the dye and the nitrogenous base of the nucleotide, the second heterocycle of the fluorophore and the quaternary ammonium group.

Designations were introduced depending on the length of the two linkers for the fluorescently labeled derivatives in each pair (dU and dC). For the linker connecting the aromatic group of the dye to the nitrogenous base of the nucleotide, the following numbering was adopted: (1) for short and (2) for long. The linker connecting the second heterocycle of the fluorophore to the quaternary ammonium group was designated as (a) for short and (b) for long.

The amplification kinetics in the presence of Cy5-dNTP were analyzed by means of real-time RPA.

The sample labeled as dU-K was taken as a comparison control. The choice was dictated by its wide application in gel biological microarray technology due to its good substrate efficiency [5]. Similar to the samples under study, it is characterized by the electroneutral structure of the fluorophore.

In order to analyze the kinetics of DNA fragment amplification, 8 μM was chosen as the working concentration of the tested labeled dNTPs. This was to ensure partial substitution of natural triphosphates during the formation of the growing DNA chain. Amplification was performed in the presence of all four natural dNTPs at a concentration of 200 μM in three repeats for each of the tested labeled dU and dC (Fig. 2). A reaction mixture containing only unmodified (natural) dNTPs at a concentration of 200 μM was used as a control sample.

The amplification efficiency in the RPA process (E_t) was determined using the slope of the straight line of the S-shaped signal accumulation curve on a logarithmic scale according to the methodology used for PCR [15]. The *t* letter implies plotting by reaction time due to the absence of cycles as in PCR (*E*).

The effect of linkers of different lengths on the inhibition of RPA enzyme system polymerases was studied, and the experimental data summarized in Table.

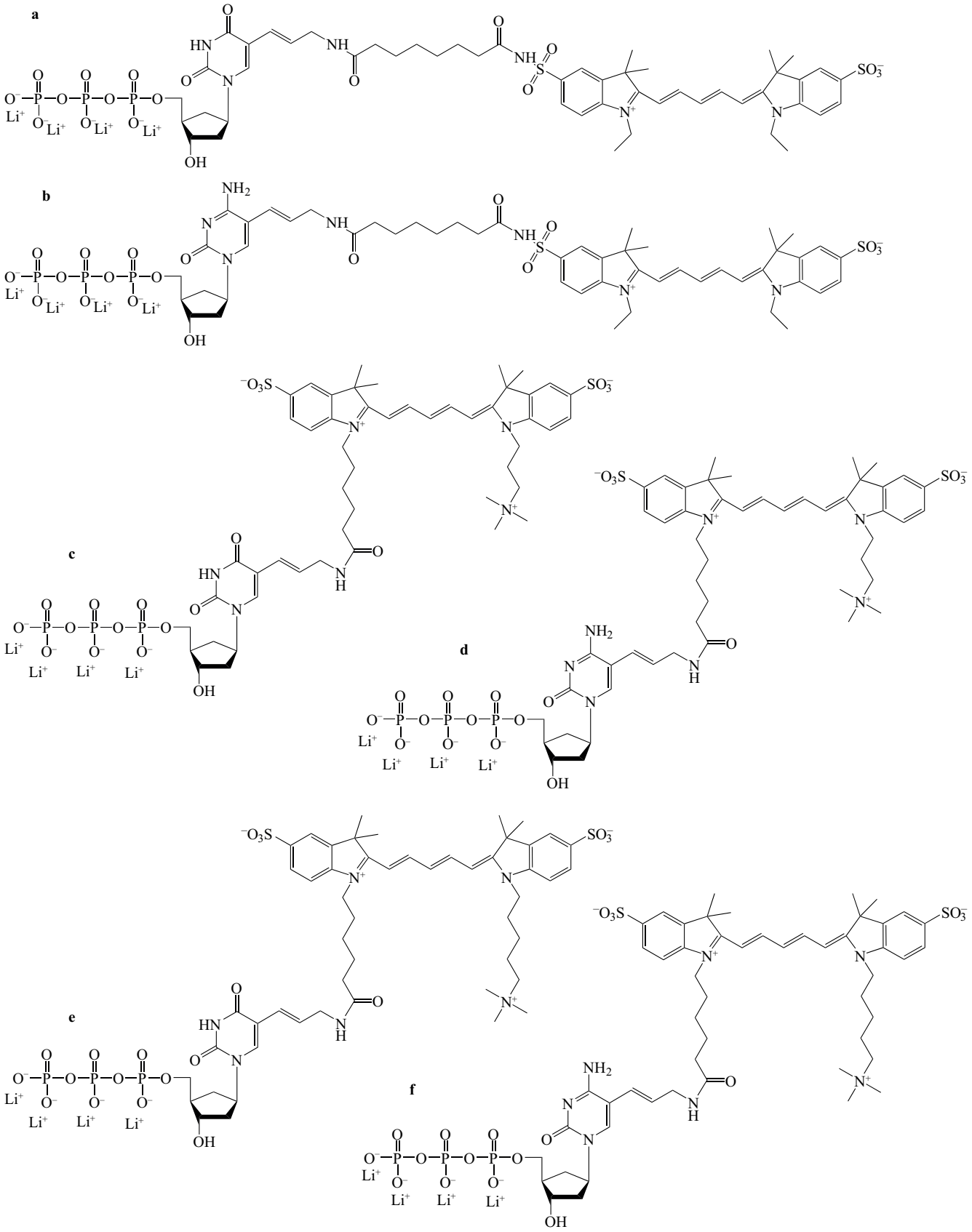

Fig. 1. Structure of fluorescently labeled deoxyuridine and deoxycytidine triphosphates:
(**a**) dU-K, (**b**) dC-K, (**c**) U1a, (**d**) C1a, (**e**) U1b, (**f**) C1b

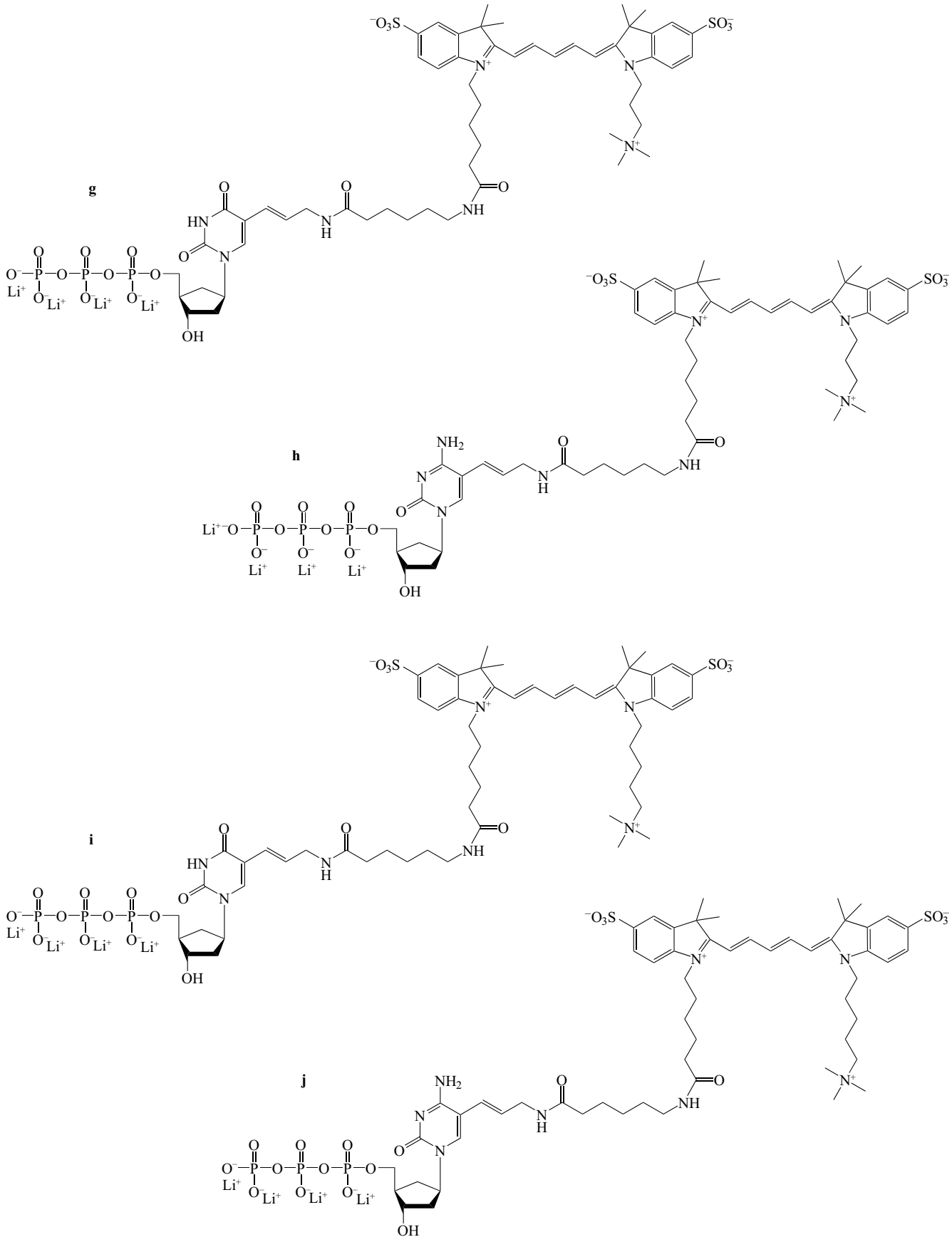

Fig. 1. Structure of fluorescently labeled deoxyuridine and deoxycytidine triphosphates:
(**g**) U2a, (**h**) C2a, (**i**) U2b, (**j**) C2b

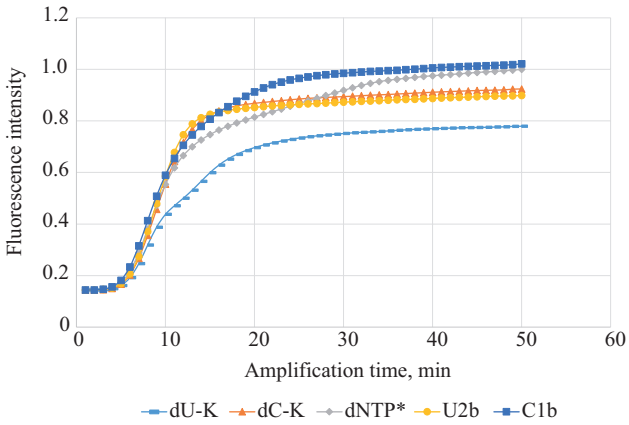

Fig. 2. Kinetics of fluorescence signal accumulation in the presence of modified dU and dC (case study of U2b and C1b). *Unmodified deoxynucleoside triphosphates.

The data shows that the amplification efficiency for each of the Cy5-dNTPs was lower than when compared to natural dNTPs (except U1b, C2a), indicating inhibition. There was no significant effect of the length of the linker connecting the aromatic group of the dye to the nitrogenous base of the nucleotide on amplification efficiency. Increasing the length of the linker connecting the second heterocycle of the fluorophore to the quaternary amine resulted in a more pronounced inhibitory effect modified dC when compared to paired dU.

In order to estimate the yield of the amplification product, a normalized conditional product yield (η) was introduced according to the pixel brightness (pixel signal intensity of the analyzed image section) of the corresponding band in the electrophoregram. Normalization was performed, in order to visualize the effect of Cy5-(dU, dC) on the amplification process when compared to the control sample (natural dNTPs). The normalized conditional product yield was determined by means of the pixel brightness of the amplification product bands on an agarose gel in terms of the control band. The fluorescently labeled substrate under study had no significant effect on product yield (except for U1b).

Pixel brightness in conventional units (c.u.) was measured using ImageJ software (*NIH*, USA) (Table) and used to determine the normalized conditional product yield and label incorporation rate.

In order to evaluate the incorporation of fluorescently labeled dNTPs into the growing DNA strand, we introduced the incorporation coefficient (K_{in}). This is the ratio of the total pixel brightness of the area of the phoregram corresponding to the band of the labeled amplification product as captured on the Cy5 channel to the brightness of the same band as captured on the Cy3 channel (Table). Three RPA series were performed for each pair of fluorescently labeled triphosphates.

It is evident from the data obtained that all modified dU incorporate into DNA better compared to their Cy5-dC analogs. It was found that increasing the linker connecting the second heterocycle of the fluorophore to the quaternary ammonium group significantly increases the incorporation rate of fluorescently labeled dNTPs. Increasing the length of the linker connecting the aromatic group of the dye to the nitrogenous base of the nucleotide had a less significant effect on substrate characteristics.

Table. Amplification efficiency (E_t), normalized product yield (η), coefficient of incorporation (K_{in}) obtained for RPA with Cy5-dU and Cy5-dC

dNTP No.	Amplification efficiency $E_t \pm \sigma$**	Normalized product yield $\eta \pm \sigma$**	Coefficient of incorporation $K_{in} \pm \sigma$**
dNTP*	1.35 ± 0.02	1.00 ± 0.00	–
dU-K	1.27 ± 0.06	0.76 ± 0.25	1.74 ± 0.07
dC-K	1.29 ± 0.02	0.97 ± 0.17	0.50 ± 0.07
U1a	1.28 ± 0.01	0.96 ± 0.06	0.27 ± 0.05
C1a	1.28 ± 0.03	0.98 ± 0.11	0.17 ± 0.04

Table. Continued

dNTP No.	Amplification efficiency $E_t \pm \sigma$**	Normalized product yield $\eta \pm \sigma$**	Coefficient of incorporation $K_{in} \pm \sigma$**
U1b	1.34 ± 0.06	0.77 ± 0.29	0.94 ± 0.13
C1b	1.33 ± 0.03	1.18 ± 0.58	0.35 ± 0.04
U2a	1.33 ± 0.04	0.99 ± 0.34	0.29 ± 0.10
C2a	1.35 ± 0.07	0.88 ± 0.14	0.23 ± 0.03
U2b	1.33 ± 0.03	0.84 ± 0.34	0.87 ± 0.21
C2b	1.28 ± 0.02	1.10 ± 0.51	0.68 ± 0.47

* Unmodified deoxynucleoside triphosphates.

** The mean square deviation.

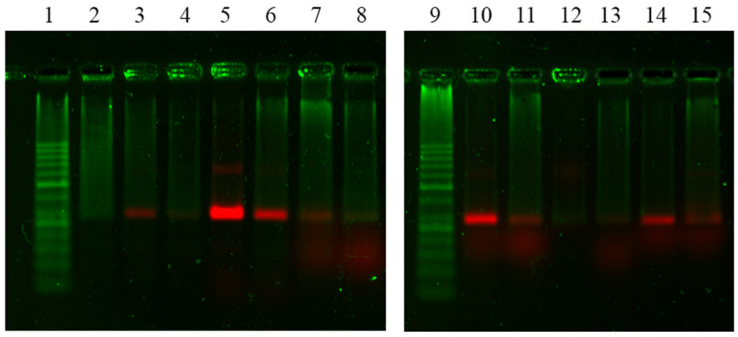

Fig. 3. Electrophoregram obtained in digital TIFF 24 format with saving pixel-by-pixel signal intensity data for measuring the embeddability coefficient (K_{in}) of the RPA product: (*1*) marker of the lengths of double-stranded DNA GeneRuler 50 bp (*Thermo Scientific*, USA); (*2*) dNTP; (*3*) laboratory control mod-U1, not considered in this study; (*4*) laboratory control mod-C1, not considered in this study; (*5*) dU-K; (*6*) dC-K; (*7*) U1a; (*8*) C1a; (*9*) marker of the lengths of double-stranded DNA GeneRuler 50 bp (*Thermo Scientific*, USA); (*10*) U2a; (*11*) C2a; (*12*) U1b; (*13*) C1b; (*14*) U2b; (*15*) C2b

CONCLUSIONS

The study showed that the spatial structure of the fluorophore and differences in linker lengths affect the substrate characteristics of the modified dNTPs. It was found that fluorescently labeled triphosphates with a long linker connecting the nitrogenous base of the nucleotide to the aromatic group of the dye, and a long linker connecting the second heterocycle of the fluorophore to the quaternary ammonium group were significantly better incorporated into the growing DNA strand and were characterized by high product yields.

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Authors' contributions

A.S. Epifanov—conducting research, writing the text of the manuscript.

V.E. Shershov—synthesis of fluorescently labeled dNTPs.

S.A. Surzhikov—synthesis of primers.

A.V. Chudinov—academic advising.

S.A. Lapa—construction of primers for PCR and RPA, planning experiments, and editing the manuscript.

The authors declare no conflicts of interest.

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