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Novel fluorescently labeled nucleotides: synthesis, spectral properties and application in polymerase chain reaction

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Experimental

^{1}H NMR spectra were recorded on a Bruker AMX-400 spectrometer (400 MHz) (Bruker, USA) in D_2O and DMSO-d_6 solutions. Chemical shifts δ are given in ppm. Coupling constants (J) are given in Hz. Mass spectra analysis was carried out with a MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) mass spectrometer KOMPACT MALDI 4 (Kratos Analytical, Manchester, UK). Mass spectra were obtained in the linear mode and positive ions were registered using 2,5-dihydroxybenzoic acid and 3-hydroxypyridine-2-carboxylic acid as a matrix. Fluorescence spectra were recorded on a Cary Eclipse spectrofluorimeter (Agilent Technologies, USA). Absorption spectra were measured using a Jasco V-550 spectrophotometer (JASCO International Co., Japan). The pH values were determined using a Thermo Orion 330 pH-meter (Thermo Scientific, USA).

Analytical thin layer chromatography was performed using C18 reversed-phase plates (C18-RP) (Merck). Purification of fluorescently labeled nucleotides was performed using a reversed-phase C18-RP column (Analtech, Newark, DE) and DEAE column (Analtech, Newark, DE).

All the solvents were purified before use. DMF was distilled over phthalic anhydride and ninhydrin. All reagents were obtained from Sigma-Aldrich or Fluka unless otherwise stated. Indodicarbocyanine dyes were synthesized according to the procedure reported in our previous

work.[12] 5-(3-Aminoallyl)-2′-deoxyuridine 5′-triphosphate trilithium salt was purchased from Biosan (Novosibirsk, Russia).

TB-Biochip and THROMBO-Biochip test systems were purchased from the manufacturer (Biochip-EIMB, Moscow, Russia). PCR amplification was carried out with a T100 thermal cycler (Bio-Rad, USA). Hybridization on biochips was performed using incubator with mechanical control (B 28, Binder, Germany). The results of hybridization were analyzed using a Chipdetector portable fluorescence analyzer equipped with Imageware software (Biochip-EIMB, Moscow, Russia). The horizontal gel electrophoresis was performed in electrophoretic chamber (SE-1, Helicon, Russia) connected with PowerPac Basic Power Supply (Bio-Rad Laboratories Inc., Hercules, CA, USA). The electrophoresis images were taken with ChemiDoc MP Imaging System using Image Lab Software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The abbreviations used throughout the paper are as follows: AA-dUTP [5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate trilithium salt], DMF (*N*,*N*-dimethylformamide), EDTA (ethylenediaminetetraacetic acid), EIMB (Engelhardt Institute of Molecular Biology), PBS (potassium phosphate buffer solution), PCR (polymerase chain reaction), pNP (*p*-nitrophenol), TE-buffer (Tris-EDTA buffer solution), TEAHC (triethylammonium hydrogen carbonate buffer), Tris (tris(hydroxymethyl)aminomethane).

General procedure for the synthesis of p-*nitrophenyl esters of cyanine dyes.* The mixture of dye (0.044 mmol) and bis(*p*-nitrophenyl) carbonate (26 mg, 0.087 mmol) was dissolved in DMF (1 ml) and stirred at room temperature for 2 h. The mixture was then diluted with water (10 ml) and precipitate obtained was removed by filtration. The filtrate was purified by reverse-phase (C18-RP) chromatography using a water-acetonitrile mixture as eluent. The quantitative yields (about 90%) of cyanine active esters were obtained.

TB-Biochip analysis. DNA samples were analyzed according to the protocol provided by the manufacturer (Biochip-EIMB, Moscow, Russia).[14b] DNA sample was amplified by a two-round PCR. The presence of PCR products was confirmed by agarose gel electrophoresis after each reaction. In the first round PCR 3 μl of genomic DNA was amplified in a total reaction volume of 30 μl. The first round PCR amplification was carried out in a 30 μl reaction mixture containing a 3 μl dilution of Taq buffer; a 2.5 U Taq polymerase; 0.2 μM each deoxynucleoside triphosphate (dNTP); primers; and a 3 μl of the DNA sample. PCR was performed according to the following protocol: 4 min at 95^{o}C, (36 cycles): 30 s at 95^{o}C, 30 s at 67^{o}C, and 30 s at 72^{o}C, and then 5 min at 72^{o}C. The volume of 1 μl after the first round of PCR was used for the second PCR round. The reaction mixture for the second round PCR contained a fluorescently labeled

nucleotide 8 μmol and was carried out with an excess of one primer in each primer pair to obtain predominantly single-stranded fluorescently labeled product for hybridization. Amplification was performed according to the following protocol: 5 min at 95°C, (37 cycles): 20 s at 95°C, 30 s at 65°C, and 30 s at 72°C, and then 5 min at 72°C. Single-stranded DNA was then hybridized with a microarray chip by dispensing 12 μl of the labeled PCR product in hybridization buffer into the hybridization chamber and incubating the chip for 18 h at 37°C. The chip was washed with deionized water and air dried. The presence and nature of mutation were determined by analysis of the fluorescence intensity pattern on the chip, using a Chipdetector portable fluorescence analyzer equipped with Imageware software (Biochip-EIMB, Moscow, Russia) of which 1 μl in a total reaction volume of 60 μl was used for the second round PCR.

THROMBO-Biochip analysis. DNA samples were analyzed according to the protocol provided by the manufacturer (Biochip-EIMB, Moscow, Russia). Briefly, the genomic DNA sequence was amplified by a two-round PCR. The reaction mixture (25 μl) for the first round contained 10× buffer for PCR, 0.2 mM each deoxynucleoside triphosphate, 0.4 pmol of each primers, 2.5 U Taq polymerase, and 10 ng of the genomic DNA sample. At the first round, PCR was performed according to the following protocol: 4 min at 94°C, 35 cycles: 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, and then 5 min at 72°C. PCR product obtained at first round (2 μl) was used as the template at the second round. The reaction mixture, which was the same as in the first round, was supplemented with fluorescently labeled nucleotide (8 μmol) in order to obtain an excess of the labeled single-stranded PCR product. Amplification was performed according to the following protocol: 4 min at 94°C, 35 cycles: 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, and then 5 min at 72°C. Hybridization on biochips was performed according to the protocol provided by the manufacturer (Biochip-IMB, Moscow, Russia).[18d]

Gel electrophoresis. The DNA samples with equal volumes were mixed with 6× DNA loading dye and analyzed on 2% agarose gel according to the protocol provided by the manufacturer (Biochip-EIMB, Moscow, Russia). The running buffer contained 40 mM Tris acetate and 1 mM EDTA in water. All solutions were freshly prepared prior to use.

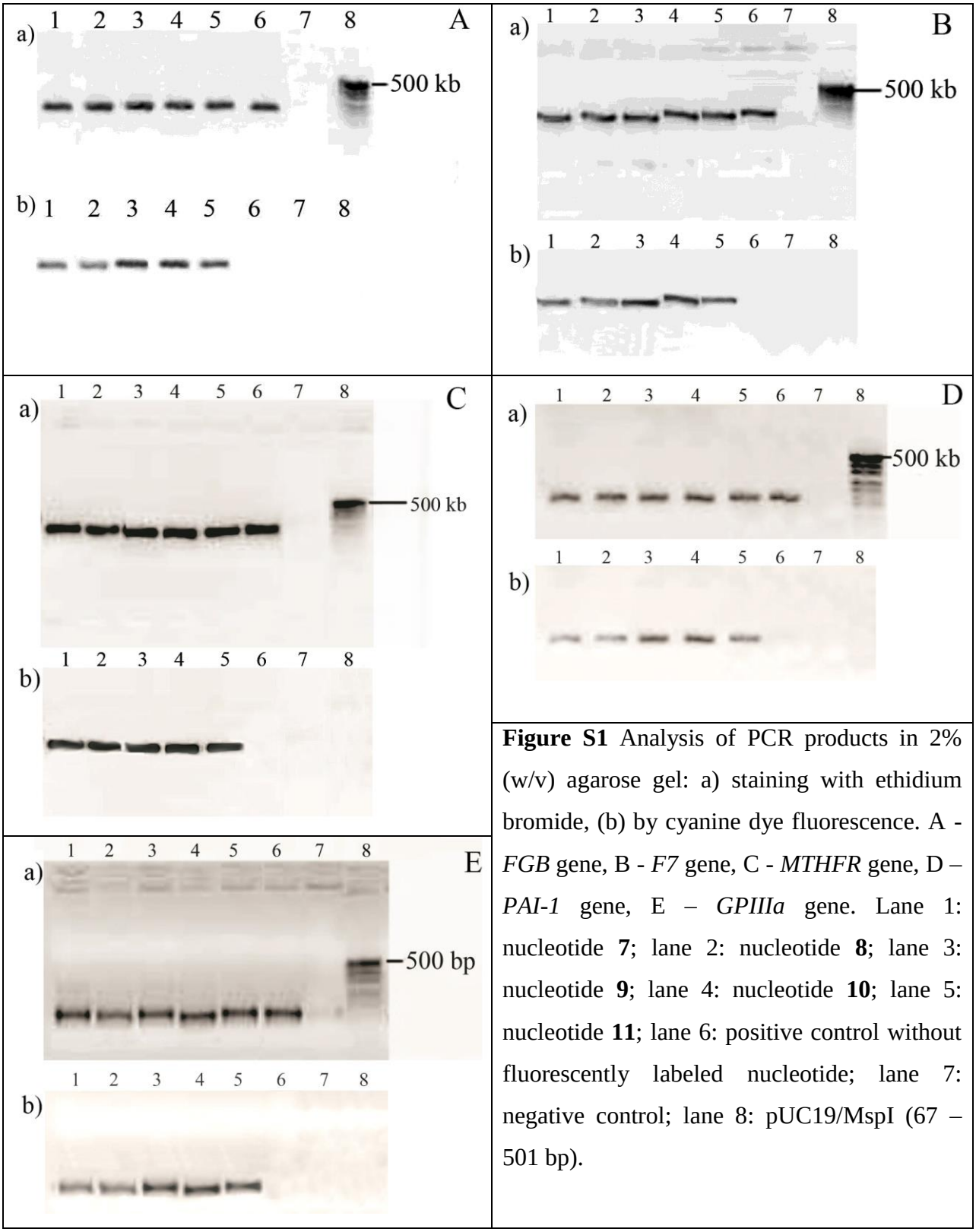

Figure S1 Analysis of PCR products in 2% (w/v) agarose gel: a) staining with ethidium bromide, (b) by cyanine dye fluorescence. A - *FGB* gene, B - *F7* gene, C - *MTHFR* gene, D – *PAI-1* gene, E – *GPIIIa* gene. Lane 1: nucleotide **7**; lane 2: nucleotide **8**; lane 3: nucleotide **9**; lane 4: nucleotide **10**; lane 5: nucleotide **11**; lane 6: positive control without fluorescently labeled nucleotide; lane 7: negative control; lane 8: pUC19/MspI (67 – 501 bp).