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

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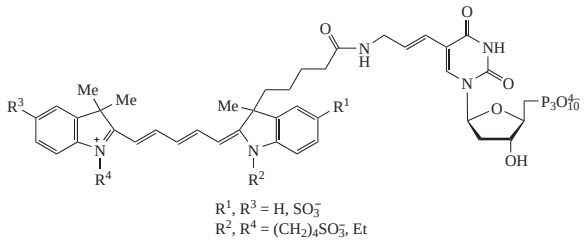
The novel C5-modified nucleotides containing indodicarbocyanine dye bearing a carboxyl group at the 3-position of indolenine have been synthesized and incorporated into DNA by commercially available thermostable Taq DNA polymerase. The efficiency of incorporation of labeled nucleotides during polymerase chain reaction was studied in a TB-biochip and THROMBO-Biochip test systems.

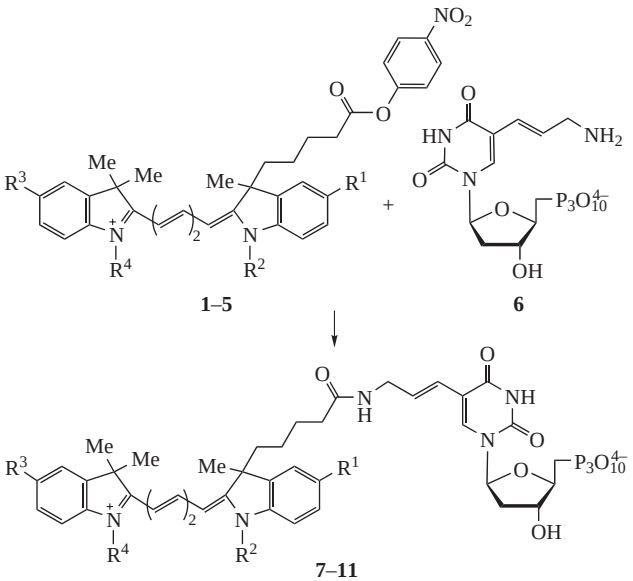


The incorporation of fluorescently labeled nucleotides into DNA by DNA polymerases has been extensively used for tagging genes and for labeling DNA.[1] Probes with high density of labels would increase detection sensitivity.[2] Polymerase chain reaction is a suitable method for simultaneous labeling and amplification of the DNA yielding high density of the fluorophore given a fluorescent nucleotide is a substrate for DNA polymerase during polymerase chain reaction (PCR).[3] However, the use of fluorescently labeled nucleotides has an inherent limitation, since most enzymes incorporate dye-labeled nucleotides poorly.[4–6] Labeling efficiency also varies significantly depending on the type of dye in modified nucleotides. Cyanine dyes are widely used for DNA labelling due to their attractive fluorescence properties.[7,8] However, one should keep in mind that cyanine dyes can interact with DNA through non-covalent interactions.[9] Interactions between the dye and the DNA may perturb the system being studied and can alter the fluorescent properties of the dye.[7,10]

It is known[1(a),11] that substituents at C5-position of pyrimidines provide the minimum interference with enzymatic polymerization and do not disturb significantly DNA structure. The purpose of this study was the synthesis of nucleotide derivatives containing cyanine dye at C5-position that minimize the interaction of the dye with the DNA and hence reduce the influence of the label on the DNA structure. Our approach is based on the synthesis of carbocyanine dyes with reactive group at the 3-position of indolenine heterocycle.

Previously, we reported the synthesis of cyanine dyes bearing a carboxyl group at the 3-position of indolenine, and their introduction into oligonucleotides.[12] Here we report the preparation of the fluorescent nucleotides (Scheme 1) and their application to the synthesis of fluorescently labeled DNA by PCR using Taq DNA polymerase. For conjugation of the nucleotide with dyes, highly reactive functional groups, such as *N*-hydroxysuccinimidyl and *p*-nitrophenyl (pNP) esters, are widely employed.[13] The pNP esters were found to be more stable than the *N*-hydroxysuccinimidyl esters under basic conditions of the reaction. The pNP esters have been prepared using bis(*p*-nitrophenyl) carbonate in DMF and corresponding dyes. Active esters of dyes were easily separated from reaction mixture by reversed-phase chromatography in ~90% yields. We did not observe any significant hydrolysis of pNP esters during chromatographic separation in a neutral water–acetonitrile mixture.

Amino group of 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate was reacted with 1.25 equiv. of pNP ester in 0.1 M $NaHCO_3$/Na_2CO_3 buffer (pH 8.5) containing DMF to furnish fluorescently labeled deoxyuridine in 35–49% yields depending on the com-



1, 7 $R^1 = R^3 = H$, $R^2 = R^4 = (CH_2)_4SO_3^-$
2, 8 $R^1 = R^3 = H$, $R^2 = Et$, $R^4 = (CH_2)_4SO_3^-$
3, 9 $R^1 = R^3 = H$, $R^2 = (CH_2)_4SO_3^-$, $R^4 = Et$
4, 10 $R^1 = H$, $R^2 = R^4 = Et$, $R^3 = SO_3^-$
5, 11 $R^1 = SO_3^-$, $R^2 = R^4 = Et$, $R^3 = H$

Scheme 1

pound structure. Fluorescently labeled nucleotides were further purified by a two-step procedure employing DEAE-anion exchange chromatography followed by a reverse-phase C18-RP chromatography. The structure and purity of compounds **7**–**11** were confirmed by ^{1}H and ^{31}P NMR spectroscopy and mass spectrometry (MALDI-TOF).†

Spectral properties of the fluorescently labeled nucleotides **7**–**11** are presented in Table 1.‡ Modified nucleotides containing cyanine dye are characterized by sharp intense absorption and emission bands and quantum yields of 0.14–0.18 in PBS buffer. The absorption, emission and the Stokes shifts show no significant variations for compounds **7**–**11**. The absorption and fluorescence emission spectra of modified nucleotide are shown in Figure 1. The strong NIR absorbance of the fluorophore (ε ~ 200000–245000 dm^3 mol^{-1} cm^{-1}) relative to the UV absorbance of the nucleotide (ε = 10200 dm^3 mol^{-1} cm^{-1})[14] can be observed. The high NIR molar absorptivity enhances the overall sensitivity. Modified nucleotides **7**–**11** have a NIR absorption maximum about 645 nm and fluorescence emission maximum about 663 nm allowing detection with IR imaging system.[15] The UV absorption maximum of the uridine-5′-triphosphate is shifted from 262 to ~300 nm by attachment of the linker arm plus fluorophore to the heterocyclic pyrimidine ring. The relative efficiency of fluorescence in PBS solutions at equal concentrations of fluorescently labeled nucleotides **7**–**11** was determined by the method described previously.[12] The results obtained were normalized to the highest

Table 1 Spectral-luminescent characteristics of the 10^{-6} M solution of probes in PBS buffer of the fluorescent labeled nucleotides.[a]

Nucleotide	λ_{max}^{abs}/nm	λ_{max}^{em}/nm	$\varepsilon/10^{-5}$ dm^3 mol^{-1} cm^{-1}	Q (%)	Relative efficiency of fluorescence ($\lambda_{max}^{abs}/\lambda_{max}^{em}$)
7	646	664	2.43±0.02	18	1.0
8	645	663	2.13±0.02	14	0.753
9	644	661	2.06±0.02	14	0.692
10	645	663	1.80±0.03	14	0.637
11	645	663	1.91±0.02	14	0.667

[a] ε is the molar extinction coefficient for the dyes;[12] Q is the quantum yield; PBS is the 10 mM potassium phosphate buffer solution, 0.9% NaCl, pH 7.4.

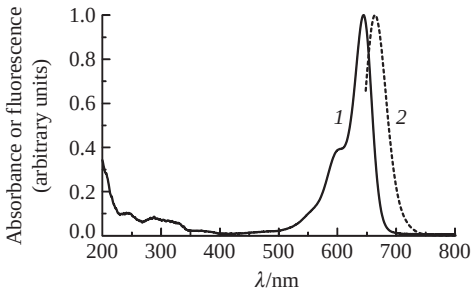


Figure 1 (*1*) Absorption and (*2*) fluorescence emission spectra of nucleotide **7** dissolved in PBS.

† *General procedure for the synthesis of fluorescently labeled 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate*. pNP ester **1**–**5** (2 μmol) in DMF (0.2 ml) was added to a solution of AAdUTP **6** (1 mg, 1.6 μmol) in $NaHCO_3/Na_2CO_3$ buffer (0.2 ml, 0.1 M, pH 8.5) and the reaction mixture was stirred at 5 °C for 12 h. Conjugates **7**–**11** were separated from excess free dye by precipitation with 1.6 ml of a 2% solution of $NaClO_4$ in acetone. The precipitates were collected by centrifugation at 13000 rpm for 1 min, washed with acetone (0.2 ml), and dissolved in 1 ml of 30% acetonitrile in water.

The solution of modified nucleotide in 1 ml of 30% MeCN in water was loaded onto DEAE-cellulose (DE-52, Whatman) column (2×8 cm) equilibrated with 30% MeCN in 0.025 M TEAHC buffer (pH 8.5). Elution was carried out with step salt gradient concentration running from 30% MeCN in 0.025 M TEAHC buffer (pH 8.5) to 30% MeCN in 0.3 M TEAHC buffer (pH 8.5). The fraction containing a modified nucleotide was diluted to 50 ml with 0.1 M TEAHC buffer (pH 8.5) and loaded onto reverse-phase C18-RP column (2×8 cm) (Analtech, Newark, DE), washed with 0.1 M $NaClO_4$, 0.1 M EDTA (pH 8.0) and Milli-Q water, and eluted with an acetonitrile–Milli-Q water mixture.

The purified fractions containing the target compound were combined and diluted to 50 ml with MQ-water. The concentration of modified nucleotide was determined using an extinction coefficient of the cyanine dye.[12] The absorbance was measured in three various dilutions of the stock solution in MQ-water so that optical density of the samples did not exceed 0.1. The solutions were evaporated to dryness *in vacuo* and dissolved in TE-buffer (pH 8.0) to yield a fluorescently labeled nucleotides at concentration of 1 μmol ml^{-1} and stored at –20 °C.

Sodium salt 5-{9-[3,3′,3′-trimethyl-1,1′-di(4-sulfonatobutyl)indodicarbocyanin-3-yl]-5-oxo-4-azanon-1-en-1-yl}-2′-deoxyuridine-5′-triphosphate **7**: 0.72 μmol, yield 46%, navy blue powder. ^{1}H NMR (D_2O) δ: 0.45, 0.77 (2m, 2H, dye C^{8}H$_2$), 1.35 (m, 2H, dye C^{9}H$_2$), 1.62 (s, 9H, dye C^{3}H$_3$, C$^{3',3''}$H$_3$), 1.75 [m, 4H, CH$_2$(C*H*$_2$)$_2$CH$_2$SO$_3$], 1.9 (m, 2H, dye C^{7}H$_2$), 2.25 [m, 6H, C$^{2'}$H$_2$, dye C^{6}H$_2$, (CH$_2$)$_3$C*H*$_2$SO$_3$], 3.85 (m, 2H, dye C^{3}H$_2$), 4.12 [br.m, 8H, C$^{3'}$H, C$^{4'}$H, C$^{5'}$H$_2$, C*H*$_2$(CH$_2$)$_3$SO$_3$], 6.15 (m, 3H, C$^{1'}$H, dye α,α′-CH), 6.31 (m, 2H, dye C^{1}H, C^{2}H), 6.39 (m, 1H, dye γ-CH), 7.11, 7.25, 7.41, 7.59, 7.64 (5m, 9H, dye H$_{Ar}$, C^{6}H), 8.08 (m, 2H, dye β,β′-CH). ^{31}P NMR (D_2O) δ: –22.86 (t, $^{\beta}$P), –11.94 (d, $^{\alpha}$P), –7.05 (d, $^{\gamma}$P). MS (MALDI), *m*/*z*: 1219.3 (M$^+$).

Sodium salt 5-{9-[1-ethyl-3,3′,3′-trimethyl-1′-(4-sulfonatobutyl)indodicarbocyanin-3-yl]-5-oxo-4-azanon-1-en-1-yl}-2′-deoxyuridine-5′-triphosphate **8**: 0.62 μmol, yield 39%, navy blue powder. ^{1}H NMR (D_2O) δ: 0.45, 0.78 (2m, 2H, dye C^{8}H$_2$), 1.21 (t, 3H, CH$_2$*Me*, *J* 7 Hz), 1.34 (m, 2H, dye C^{9}H$_2$), 1.62 (s, 9H, dye C^{3}H$_3$, C$^{3',3''}$H$_3$), 1.72 [m, 4H, CH$_2$(C*H*$_2$)$_2$CH$_2$SO$_3$], 1.91 (m, 2H, dye C^{7}H$_2$), 2.24 [m, 6H, C$^{2'}$H$_2$, dye C^{6}H$_2$, (CH$_2$)$_3$C*H*$_2$SO$_3$], 3.81 (m, 2H, dye C^{3}H$_2$), 4.12 [br.m, 8H, C$^{3'}$H, C$^{4'}$H, C$^{5'}$H$_2$, C*H*$_2$Me, C*H*$_2$(CH$_2$)$_3$SO$_3$], 6.12 (m, 3H, C$^{1'}$H, dye α,α′-CH), 6.32 (m, 2H, dye C^{1}H, C^{2}H), 6.45 (m, 1H, dye γ-CH), 7.12, 7.25, 7.38, 7.65, 7.75 (5m, 9H, dye H$_{Ar}$, C^{6}H), 7.99 (m, 2H, dye β,β′-CH). ^{31}P NMR (D_2O) δ: –21.56 (t, $^{\beta}$P), –11.26 (d, $^{\alpha}$P), –7.13 (d, $^{\gamma}$P). MS (MALDI), *m*/*z*: 1111.2 (M$^+$).

Sodium salt 5-{9-[1′-ethyl-3,3′,3′-trimethyl-1-(4-sulfonatobutyl)indodicarbocyanin-3-yl]-5-oxo-4-azanon-1-en-1-yl}-2′-deoxyuridine-5′-triphosphate **9**: 0.56 μmol, yield 35%, navy blue powder. ^{1}H NMR (D_2O) δ: 0.47, 0.76 (2m, 2H, dye C^{8}H$_2$), 1.22 (t, 3H, CH$_2$*Me*, *J* 7 Hz), 1.31 (m, 2H, dye C^{9}H$_2$), 1.64 (s, 9H, dye C^{3}H$_3$, C$^{3',3''}$H$_3$), 1.77 [m, 4H, CH$_2$(C*H*$_2$)$_2$CH$_2$SO$_3$], 1.92 (m, 2H, dye C^{7}H$_2$), 2.21 [m, 6H, C$^{2'}$H$_2$, dye C^{6}H$_2$, (CH$_2$)$_3$C*H*$_2$SO$_3$], 3.80 (m, 2H, dye C^{3}H$_2$), 4.08 [br.m, 8H, C$^{3'}$H, C$^{4'}$H, C$^{5'}$H$_2$, C*H*$_2$Me, C*H*$_2$(CH$_2$)$_3$SO$_3$], 6.19 (m, 3H, C$^{1'}$H, dye α,α′-CH), 6.34 (m, 2H, dye C^{1}H, C^{2}H), 6.41 (m, 1H, dye γ-CH), 7.15, 7.21, 7.38, 7.61, 7.71 (5m, 9H, dye H$_{Ar}$, C^{6}H), 7.95 (m, 2H, dye β,β′-CH). ^{31}P NMR (D_2O) δ: –21.16 (t, $^{\beta}$P), –11.06 (d, $^{\alpha}$P), –7.98 (d, $^{\gamma}$P). MS (MALDI), *m*/*z*: 1111.6 (M$^+$).

Sodium salt 5-[9-(1,1′-diethyl-3,3′,3′-trimethyl-5′-sulfoindodicarbocyanin-3-yl)-5-oxo-4-azanon-1-en-1-yl]-2′-deoxyuridine-5′-triphosphate **10**: 0.78 μmol, yield 49%, navy blue powder. ^{1}H NMR (D_2O) δ: 0.43, 0.72 (2m, 2H, dye C^{8}H$_2$), 1.28 (m, 6H, CH$_2$*Me*), 1.35 (m, 2H, dye C^{9}H$_2$), 1.58 (s, 9H, dye C^{3}H$_3$, C$^{3',3''}$H$_3$), 1.88 (m, 2H, dye C^{7}H$_2$), 2.24 (m, 4H, C$^{2'}$H$_2$, dye C^{6}H$_2$), 3.85 (m, 2H, dye C^{3}H$_2$), 4.13 (br.m, 8H, C$^{3'}$H, C$^{4'}$H, C$^{5'}$H$_2$, C*H*$_2$Me), 6.21 (m, 3H, C$^{1'}$H, dye α,α′-CH), 6.31 (m, 2H, dye C^{1}H, C^{2}H), 6.45 (m, 1H, dye γ-CH), 7.14, 7.19, 7.31, 7.58, 7.68 (5m, 8H, dye H$_{Ar}$, C^{6}H), 7.98 (m, 2H, dye β,β′-CH). ^{31}P NMR (D_2O) δ: –22.35 (t, $^{\beta}$P), –12.64 (d, $^{\alpha}$P), –8.68 (d, $^{\gamma}$P). MS (MALDI), *m*/*z*: 1083.1 (M$^+$).

Sodium salt 5-[9-(1,1′-diethyl-3,3′,3′-trimethyl-5-sulfoindodicarbocyanin-3-yl)-5-oxo-4-azanon-1-en-1-yl]-2′-deoxyuridine-5′-triphosphate **11**: 0.67 μmol, yield 42%, navy blue powder. ^{1}H NMR (D_2O) δ: 0.44, 0.71 (2m, 2H, dye C^{8}H$_2$), 1.25 (m, 6H, CH$_2$*Me*), 1.37 (m, 2H, dye C^{9}H$_2$), 1.59 (s, 9H, dye C^{3}H$_3$, C$^{3',3''}$H$_3$), 1.89 (m, 2H, dye C^{7}H$_2$), 2.26 (m, 4H, C$^{2'}$H$_2$, dye C^{6}H$_2$), 3.83 (m, 2H, dye C^{3}H$_2$), 4.08 (br.m, 8H, C$^{3'}$H, C$^{4'}$H, C$^{5'}$H$_2$, C*H*$_2$Me), 6.23 (m, 3H, C$^{1'}$H, dye α,α′-CH), 6.33 (m, 2H, dye C^{1}H, C^{2}H), 6.43 (m, 1H, dye γ-CH), 7.15, 7.17, 7.30, 7.56, 7.66 (5m, 8H, dye H$_{Ar}$, C^{6}H), 7.99 (m, 2H, dye β,β′-CH). ^{31}P NMR (D_2O) δ: –22.15 (t, $^{\beta}$P), –12.54 (d, $^{\alpha}$P), –8.24 (d, $^{\gamma}$P). MS-MALDI, *m*/*z*: 1083.4 (M$^+$).

‡ The fluorescence quantum yields of the modified nucleotides were determined by the method described previously[19] using a reference standard Cy5-dUTP (Amersham PA55022, QY = 0.28 in PBS at 25 °C).

value detected for nucleotide **7**. The aqueous solution of conjugate **7** possesses the peak value of relative efficiency of fluorescence.

Enzymatic incorporation of fluorescent nucleotides was studied in a diagnostic TB-Biochip system for the detection of mutations associated with drug resistance in *M. tuberculosis*[15] (see Online Supplementary Materials). The procedure of the molecular identification of mutations consisted of three consecutive steps: (1) multiplex PCR amplification of five DNA fragments; (2) asymmetric multiplex PCR to yield five fluorescently-labelled single-stranded DNA fragments; and (3) hybridization of the labelled fragments with oligonucleotide biochip. Fluorescently labeled deoxyuridine triphosphates were added to the mixture of natural dNTPs during PCR. It was shown that complete replacement of dTTP with fluorescently labeled dUTP does not yield full length labeled DNA.[15(*b*)]

The formation of the full-length PCR products was analyzed by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide (Figure 2). Also, the formation of the fluorescence-labeled DNA was detected by cyanine dye fluorescence using custom-made imaging system.[16] Electrophoretic bands of PCR products (lines 3–7) obtained by either methods are identical. During PCR amplification nucleotides **7**–**11** were stable and did not inhibit the polymerase activity.

The hybridization of wild-type DNA of *M. tuberculosis* sample with the microarray is illustrated by Figure 3. Gel pads with immobilized oligonucleotides were clustered in 23 groups.[15(*b*)] Following hybridization, the fluorescent signals were analyzed within each group separately to determine whether mutation were present in DNA sample.

The fluorescence in biochip gel pads was found to vary depending on the structure of modified nucleotides used as substrates in PCR. To compare the incorporation of modified nucleotides, we calculated an average fluorescence signal from the match probe and then normalized it to the same value from DNA labeled with the commercial nucleotide Cy5-dUTP.[17] It

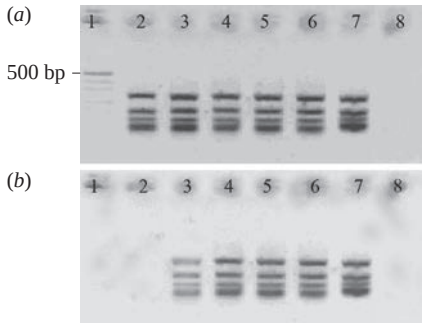


Figure 2 Analysis of PCR products in 2% (w/v) agarose gel: (*a*) staining with ethidium bromide, (*b*) by cyanine dye fluorescence. Lane 1: pUC19/MspI (67–501 bp), lane 2: positive control (without fluorescently labeled nucleotide); lane 3: nucleotide **7**; lane 4: nucleotide **8**; lane 5: nucleotide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

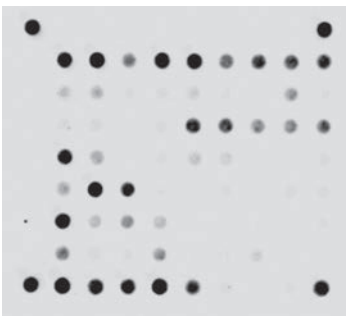


Figure 3 Hybridization of a wild-type DNA sample on the TB-biochip.

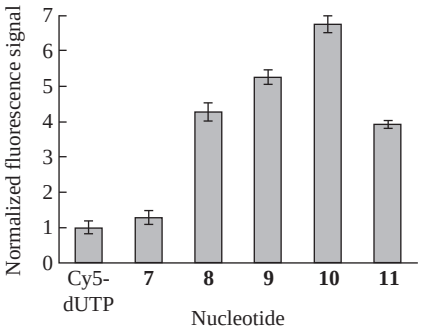


Figure 4 The fluorescence signal from a TB-biochip for different modified nucleotides **7**–**11**.

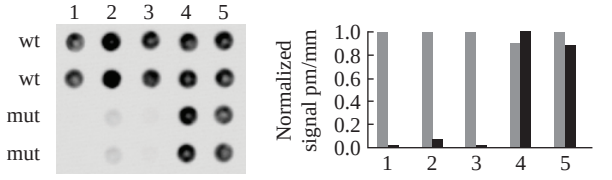
appeared, that formation of the full-length PCR product depends not only on the chemical structures of the substitution but also on the position of the substituents in dye residue. Fluorescent signals are higher for nucleotides **8**–**11** containing cyanine dye with one sulfo group (Figure 4). The fluorescence signals for nucleotide **7** and Cy5-dUTP bearing two sulfo groups were the lowest and approximately equal. The location of the sulfo group in the cyanine dye has been found to influence incorporation efficiency of modified nucleotide in the PCR product. The nucleotide **10** containing sulfo group directly attached to indolenine moiety and positioned remotely from pyrimidine heterocycle revealed a higher incorporation efficiency than nucleotides **8** and **9** containing sulfobutyl group. It is important to note that in contrast to Cy5-dUTP nucleotides **7**–**11** did not show non-specific binding with acrylamide gel pads of biochip.

Further, we examined the incorporation of the fluorescently labeled nucleotides **7**–**11** during PCR using THROMBO-Biochip for detection of genetic predisposition to thrombosis. Using fluorescently labeled nucleotides we carried out the analysis of polymorphism in hemostatic system genes (FGB, F7, GPIIIa, PAI-1, MTHFR).[18] The procedure included the amplification of selected gene sequences followed by hybridization of fluorescently labeled fragments with gel pads carrying immobilized oligonucleotides.

To ensure the nucleotides **7**–**11** did not inhibit the polymerase activity a symmetric PCR amplification was performed using standard protocol, in which the primers are used in equal amounts. Agarose gel electrophoresis was used to determine the presence and size of PCR products of FGB, F7, GPIIIa, PAI-1, MTHFR. As can be seen from Figure S1 (Online Supplementary Materials), the PCR products of genes migrated between the bands of 242 bp and 100 bp of the pUC19/MspI (67–501 bp). Amplification both with only the natural nucleotides (lane 6) and in the presence of modified substrates **7**–**11** (lanes 1–5) demonstrated the formation of the full length product as expected. Electrophoretic patterns of PCR products detected by ethidium staining and by cyanine dye fluorescence were identical for FGB, F7, GPIIIa, PAI-1 and MTHFR.

Hybridization on biochips was carried out after two-round multiplex PCR yielding single stranded fluorescently labeled DNA fragments. The results of hybridization are demonstrated in Figure 5. The two upper gel pads perfect-match (pm), as well as the two lower gel pads – mismatch (mm), are identical (*i.e.*, the probes are used in duplicate). The fluorescent signals from duplicate gel pads were averaged and normalized.[18(*d*)] Signals from the pm of the first, second and third columns are more than two times higher than those from the corresponding mm. Signals from the pm and mm cells of the fourth and fifth columns are comparable with each other, suggesting heterozygosity.

The efficiency of incorporation of the modified nucleotides **7**–**11** in the PCR was estimated by measuring the fluorescence



	Genotype				
	1	2	3	4	5
wt/mut	FGB_G/G	F7_G/G	GPIIIa_T/T	PAI-1_4G/5G	MTHFR_C/T

Figure 5 Hybridization, normalized signals and genotype of labelled DNA sample fragments on the THROMBO-Biochip using fluorescently labeled nucleotides **7**–**11** (wt – wild type, mut – mutation).

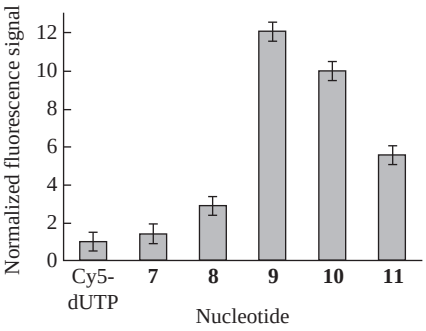


Figure 6 The fluorescence signal in a THROMBO-Biochip gel pads for fluorescently labeled nucleotides.

from the perfect duplexes for each oligonucleotide probe analyzed. The data obtained were corrected to a background signal and then normalized to the value for the Cy5-dUTP (Figure 6). The efficiency of incorporation of labeled nucleotides during PCR was found to be similar in both systems (TB-biochip and THROMBO-Biochip). Fluorescence signals are higher for the nucleotide **9** containing cyanine dye with one sulfo group positioned remotely from pyrimidine.

In conclusion, we have described the synthesis and properties of fluorescently labeled nucleotides that contain cyanine dye with one or two negatively charged sulfo groups and linked to pyrimidine heterocycle at the 3-position of indolenine. The use of new fluorescently labeled nucleotides in PCR was found to provide 8 and 12 times higher fluorescence signals as compared with commercial fluorescent probe Cy5-dUTP in detection of *M. tuberculosis* strains mutations and for the detection of genetic predisposition to thrombosis using TB-Biochip and THROMBO-Biochip oligonucleotide microarray system, respectively.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2016.03.002.

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