Significance of Methods for Purification of Oligodeoxyribonucleotide Probes for the Efficiency of Gene Diagnosis by Real-Time PCR

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 3, pp. 280-284, March, 2008
Original article submitted August 7, 2007

Analysis of the use of real-time PCR with fluorescent registration of results for gene diagnosis of infectious diseases showed that the sensitivity and reliability of quantitative evaluation of DNA targets directly depended on the method of purification of oligonucleotide probes. Chromatographic behavior of synthetic probes carrying various fluorophores and fluorescence quenchers was analyzed. Approaches to optimization of purification methods are proposed enabling elimination of previously undetectable admixtures. The importance of these studies is explained by the need in extending the armory of methods for the development and production of diagnosticums for detection of infectious and hereditary diseases, identification of genetically modified organisms, and for a wide spectrum of research in molecular biology and medicine.

Key Words: *oligodeoxyribonucleotide probes; polymerase chain reaction in the real-time mode; gene diagnosis; high performance liquid chromatography; mass spectrometry*

Real-time PCR is widely used in modern gene diagnosis [2-9]. The creation and use of diagnostic kits is often fraught with problems of insufficient sensitivity of diagnosticums and poor reproducibility of the results. The efficiency of the method largely depends on the work of the system of oligodeoxyribonucleotide primers and fluorescent probes. The quality of probes is the key factor for the development and production of diagnostic kits for the detection of infectious and hereditary diseases, meeting the reliability requirements, and for studies in molecular biology and medicine, including detection of genetically modified organisms. Improvement of reliable methods for obtaining labeled oligonucleotides is particularly important for the creation of complex diagnostic kits for simultaneous analysis of several DNA targets in a sample [3,4].

Real-time PCR probes are oligonucleotides partially or completely complementary to the sequence of the needed DNA target and carrying a fluorophore and a fluorescence quencher in the majority of modifications. The structure of the molecular "beacon" and "Perfect Probe" probes is characterized by short (5-10 nucleotides) terminal complementary sites [2, 4,6]. In solution, these oligomer molecules are usually present in the form of pins. The quencher and fluorophore in this conformation are closely approximated and the basal fluorescence is minimum.

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0007-4888/08/1453-0312

Real-time PCR probes are obtained under conditions of standard protocol of amidophosphite solid-phase synthesis of oligonucleotides in an automated mode and with postsynthetic introduction of non-nucleotide components [7]. In contrast to the many-stage approach, the synthesis with special amidophosphites and carriers allows obtaining the target oligomer at once. After cleavage of the solid phase bond and deblocking, the probe can be also purified in a single stage. Combination of both approaches is possible, that is, preliminary synthesis, for example, of the probe with the 5'-aminoalkyl—oligonucleotide-3'-quencher structure and subsequent attachment of 5'-fluorophore. We used both methods, with different combinations of labels and quenchers.

It is recommended to purify real-time PCR probes by PAAG electrophoresis and molecular sieve, ion exchange, and reverse phase chromatographies [6,7], and by using C18 cartridges (0.5-1.0 ml). These methods effectively separate derivatives of removed protective groups and short nucleotide sequences without 5'-terminal label (which are accumulated during elongation), as well as oligomer degradation products released during ammonolysis. However, in the majority of cases they fail to effectively separate products of incomplete deblocking of the oligomer and probes carrying instead of the target nucleotide labels, the products of their incomplete deblocking, destruction, or modification.

We think that the most promising approach for removal of these by-products is ion-pair or reverse-phase chromatography, a technology allowing single-step purification of the probe with subsequent removal of buffer salts easily sublimated under vacuum.

We studied chromatographic behavior of real-time PCR probes for finding approaches to rapid optimization of their HPLC purification and compared the sensitivities of real-time PCR diagnostic kits containing probes purified by the proposed and known methods [6-8].

MATERIALS AND METHODS

Oligonucleotides were obtained by solid-phase phosphoramide method on an ASM 800 automated DNA synthesizer (Biosset) using standard commercial phosphoramidite nucleosides, carriers with aminoalkyl substitute, 3'-Amino-Modifier C7 CPG and quencher-containing, 3'-BHQ-1 CPG and 3'-BHQ-2 CPG, amidophosphite fluorophores 5'-(6-FAM) Amidite and 5'-HEX Amidite, Cy3 Amidite, Cy5 Amidite (all reagents from Glen Research). Removal of the synthesized oligomers from carriers and deblocking were carried out using 28% ammonia (5 h, 50°C for 5'-(6-FAM)-derivatives and 24 h at ambient temperature for 5'-HEX-, Cy3-, Cy5-derivatives). Postsynthetic attachment of substitutes to oligonucleotide 3'-aminoalkyl derivatives was carried out using commercial activated derivatives, for example NHS-Tamra (Glen Research).

High-performance liquid chromatography was carried out on an Agilent Chemstation 1100 chromatograph in acetonitrile gradient in 0.1 M ammonium acetate using C16, C18, and C4 reverse phase columns (Fig. 1, 2).

Oligonucleotides were separated by electrophoresis in 20% PAAG under denaturing conditions [9], amplicons in 2% agarose gel.

Purification of oligonucleotides on C18 Nucleosil (1 ml) or Poly-PakTM II Cartridges (Glen Research) was carried out on an OPS-201 device (Biosset).

The fluorescence spectra were obtained by scanning function of G1321A FLD matrix chromatographic detector (Agilent), UV spectra were recorded on an ND-1000 spectrophotometer (NanoDrop Technologies Inc.). Mass spectra were obtained on UltraFlex and MicroFlex Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI TOF) mass spectrometers (Bruker) using a standard MSP target polished steel (Bruker). The matrix was prepared using 3-hydroxypicolinic acid (Fluka).

Real-time PCR of DNA samples isolated from clinical material (*e.g.* scraping samples of urogenital epithelium) was carried out using commercial DNA express kit (Litech Firm) on a MiniOpticon amplifier (Bio-Rad). Amplification mixtures (50 μl) were prepared on the basis of 10-fold buffer (0.5 M Tris, 0.5 M KCl, 30 mM $MgCl_2$); they contained 10 μl solution of isolated DNA, 1000 μM of each deoxynucleoside triphosphate, 200 nm of each PCR primer, 100 nM of the probe, and 10 U Taq polymerase (Litekh Company). PCR started by preliminary denaturation at 94°C (90 sec), followed by 40 cycles: 94°C (10 sec), 64°C (10 sec), 72°C (40 sec), and 50°C (reading of fluorescent signal).

RESULTS

Chromatographic behavior of real-time PCR probes is determined by the structure of oligomers (high G/C content, presence of mutually complementary terminal sites) and their liability to intra- and intermolecular aggregation. The proportion of oligomer conformations depends on temperature protocol of chromatography (Fig. 1, *a*, *b*) and often differs for diluted and concentrated solutions. Therefore, the profiles of analytical and preparative chromatograms can contain different peaks, while the chromatography conditions found for the analytical variant can be ineffective for preparative purifica-

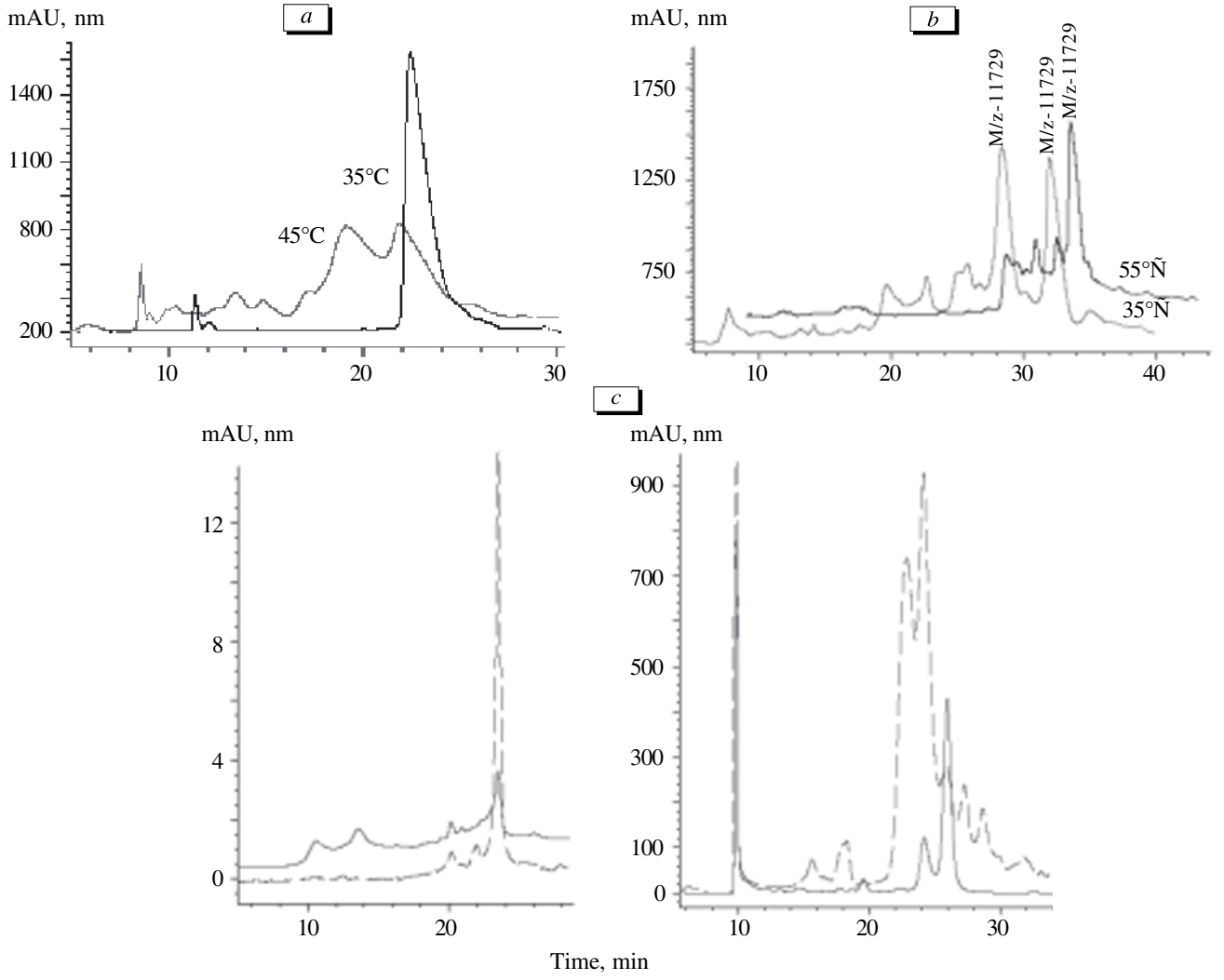

Fig. 1. Chromatographic profiles of separation (acetonitrile gradient in 0.1 M ammonium acetate) of 5'-(6-FAM)-37-mer-3'-BQH1 (*a*), 5'-HEX-46-mer-3'-Dabsyl (*b*), 5'-(6-FAM)-35-mer-3'-BQH2 (*c*) oligonucleotide synthesis reaction mixtures (for 0.1-0.2 μmol); *c*, left: chromatogram of $^{1}/_{150}$ fraction of reaction mixture. *a*, *c*) C16T 4×250 mm column (Diasorb); *b*) Nucleosil 10×250 mm column (Teknokroma).

tion (Fig. 1, *c*). Fluorescent chromatographic profiles are in most cases little informative because of the presence of fluorescent label and quencher in the same molecule; moreover, the data on the time of elution of the target substance can be wrong for this reason (Fig. 1, *c*; 2, *b*). Therefore, in order to optimize chromatographic purification of oligonucleotides, the oligomer synthesis reaction mixtures (0.1-0.2 μmol) were used after removal of ammonia under vacuum; both parameters of solvent gradient and temperatures were varied. The fractions corresponding to the chromatogram peaks were collected and studied by MALDI TOF mass spectroscopy, spectrophotometry in visible and UV spectra, and electrophoretic analysis in PAAG; the efficiencies of their work under respective conditions of real-time PCR were compared (Figs. 1, 2). Comparative analysis of the profiles showed that, for example, 5'-(6-FAM)-37-mer-3'-BHQ1 was more effectively separated from admixtures at 35°C, while 5'-HEX-35-mer-3'-BHQ2 at 55°C (Fig. 1, *a*, *b*).

In most cases, temperature and gradient regimens were effective for C16 and C18 reverse phase columns from 0.4×15 to 1×25 cm with at least 130-nm porosity. At the same time, for some oligomers, such as G-rich 5'-FAM-35-mer-BHQ1, we failed to select adequate conditions for HPLC purification by just modifying the temperature and elution gradient slope. The use of columns with C4 phase was effective in these cases (Fig. 2, *a*). The use of less hydrophobic phases, for example, C8 or C4 instead of columns with C16 or C18 adsorbent for some probes led to improvement of the purification parameters.

Another problem in the real-time PCR probe purification consisted in the possibility of structural

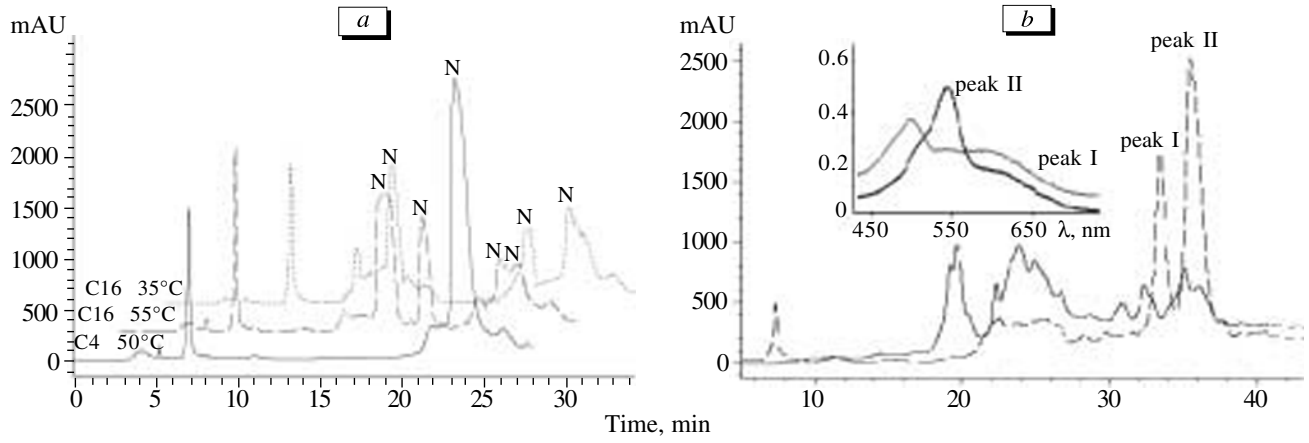

Fig. 2. Examples of chromatographic detection of probes with the black hole labels. *a*) effects of HPLC thermal protocol and adsorbent type on chromatographic separation of oligonucleotide (N) 5'-(6-FAM)-35-mer-3'-BQH1; *b*) chromatographic profile of reaction mixture separation for synthesis of oligonucleotide 5'-HEX-47-mer-3'-BQH2 (flow 0.8 ml/min, 50°C). Insert: UV spectra (in water) of oligonucleotides, corresponding to peaks I and II.

degradation of some labels during deblocking. As a result, oligonucleotides coinciding by sequences with the target probe but having other fluorescent characteristics accumulate in the reaction mixture. Under conditions of PCR they compete with the probe for the target and distort the results of the analysis. The process of ammonolysis of oligonucleotide containing fluoresceinisothiocyanate-based label was previously studied. It was shown that NH-C(=X)-NH-(X=S) thiourea fragment in the label is partially transformed into urea and guanidine residues (X=O and NH) [1]. In high resolution HPLC this phenomenon in our study caused the appearance of additional peaks near the target peak on the chromatogram, which prompted in further studies replacing the so-called fluorescein-phosphoramidite by another fluorescein derivative (6)-FAM phosphoramidite, which did not contain the above fragment.

As for other labels (HEX, TAMRA, and Cy3), manufacturers warn about their vulnerability at the oligonucleotide ammonolysis stages, but side reactions are poorly studied and no methods for purification from their products are described. We most often observed unstable results in real-time PCR with probes carrying HEX label. Chromatographic analysis under conditions optimized for each oligomer showed that in all cases the postammonolysis mixtures contained admixture similar to peak I in Fig. 2, *b*, coinciding by electrophoretic mobility in PAAG with the target compound (peak II), virtually not differing from it by mass spectrometry values, but with a different long-wave maximum in UV spectrum and other fluorescent characteristics (Fig. 2, *b*).

Comparison of changes in the kinetics of amplification product accumulation under conditions of PCR analysis showed the obvious advantages of the suggested methods for obtaining oligonucleotide probes in comparison with the traditional methods (Fig. 3). Replacement of an electrophoretically purified probe by a highly purified oligomer in the diagnostic kit increased the summary sensitivity of the analysis by at least 20% (Fig. 3).

Hence, the sensitivity and reliability of diagnosticums for evaluation of DNA targets of infectious diseases by real-time PCR directly depend on the method for purification of oligonucleotide probes. The proposed approaches to optimization of the

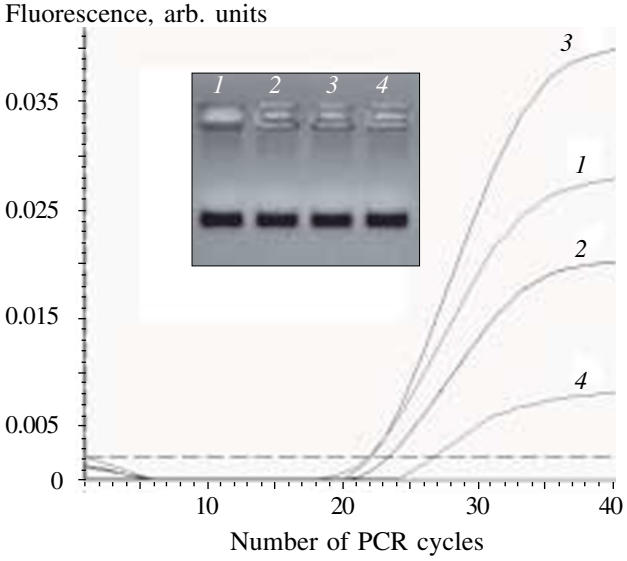

Fig. 3. Curves of accumulation kinetics for DNA amplification products (DNA from a clinical sample, urogenital epithelium scrape) in real-time PCR and electrophoretic analysis (2% agarose, ethidium bromide; insert) of their resultant content in analysis using Fluoropol-Chl.tr EP (Litech Firm) with a probe purified by PAAG electrophoresis (*1*), by Poly-PakTM II Cartridge (*2*), and HPLC (*3*, corresponds to peak II and *4* to peak I in Fig. 2).

methods for probe obtaining were successfully used (Litech Company) for the creation and fabrication of new kits for detection of numerous pathogens (*Bacteroides spp.*, *Chlamydia trachomatis*, *Cytomegalovirus hominis*, *Epstein-Barr virus*, *Gardnerella vaginalis*, *Herpes simplex virus* 1 and 2, *Lactobacillus spp.*, *Mobiluncus curtissi*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Ureaplasma urealyticum*). These studies are particularly important because of constant need in extending the armory of diagnostic method meeting to the reliability criteria.

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