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Determination of DNA Polymerase and Nuclease Activities of DNA-Dependent Polymerases Using Fluorescence Detection under Real-Time Conditions

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Abstract—A new method is proposed for estimation of polymerase activities using fluorescence detection during isothermal reaction. The method allows simultaneous determination of DNA-dependent DNA polymerase and 5′-3′-exonuclease activities using amplifiers supplied with an optical module for fluorescence detection under real-time conditions. Different primer–template combinations used as polymerase substrates were compared. Primer elongation (polymerase reaction) is detected by changes in SYBR Green I fluorescence upon binding to dsDNA during reaction; nuclease activities are detected by changes in fluorescence due to cleavage of the probe, containing the reporter fluorophore and fluorescence quencher, and hybridized in advance to the template single-stranded region. It was also shown that the method can be used for determination of relative activities of DNA polymerase preparations, estimation of temperature–time dissociation parameters of polymerase complexes with specific antibodies to its active center, and analysis of effects of inhibitors and activators of different nature on reaction rates of dsDNA polymerization and 5′-3′-exonuclease cleavage by polymerase. The method can be also used for estimation of endonuclease activities of DNA polymerases.

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Determination of DNA polymerase-specific activities is widely used in molecular biology and biotechnology, enabling solution of problems associated with optimization of the polymerase reaction conditions, revealing influencing factors, comparison of various enzyme processivity, standardization of polymerase batches, etc. Also, determination of specific activity makes possible investigation of parameters of formation and dissociation of polymerase complexes with antibodies or other synthesis-blocking proteins, estimation of alternative polymerase and nuclease activities of enzymes (like DNA- and RNA-dependent DNA polymerase activities of reverse transcriptases), and determination of conditions most favorable for these activities. Knowledge of exact specific activities of DNA- and RNA-dependent DNA polymerase preparations is important, in particular, for quantitative PCR and RT-PCR, because amounts of used enzymes and their ratios drastically influence the efficiency of amplification of the target sequence [1-4].

Classical methods of polymerase activity determination based on incorporation of radio-labeled nucleotides are characterized by rather high accuracy, but their laboriousness and danger make their regular usage difficult. A number of non-radioactive methods have been developed for measuring polymerase and nuclease activities of DNA polymerases. However, none of them is used in routine laboratory practice due to their laboriousness [5, 6], complicated data interpretation [7, 8], or necessity for special equipment or reagents not widely used in molecular biological laboratories [9-12]. A method was developed for

Abbreviations: Pfu) DNA polymerase of *Pyrococcus furiosus*; phi29) DNA polymerase of bacteriophage phi29; Taq polymerase) DNA polymerase of *Thermus aquaticus*; Vent) DNA polymerase of *Thermococcus litoralis*.

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the determination of DNA polymerase activity based on measurement of PCR efficiency in a series of the known concentration template dilutions. In this case, the amount of synthesized product, determined in one way or another, serves as a criterion of the efficiency of polymerase synthesis (as an example see [13]). However, in addition to polymerase activity, the dynamics of the DNA fragment amplification is influenced by dissociation conditions of the DNA template complementary strands, stability of primer–template complexes, buffer mixture composition, etc. [14, 15]. Thus, the use of different templates and primers can result in maximal efficiency of amplification under different conditions.

Optimization of PCR conditions is also hampered by the fact that it is often impossible to determine if a compound added to reaction mixture influences polymerase activity or it affects parameters of nucleic acid hybridization in solution. Measurement of DNA polymerase activity using PCR is suitable exclusively for determination of thermostable enzyme activities; in this case, thermal inactivation during reaction [16] is not considered. PCR is not isothermal because it is impossible to determine correctly the temperature dependence of enzyme activity, and required heating the reactive mixture (for template denaturation) makes impossible determination of the enzyme inactivation parameters at low temperatures and dissociation parameters of polymerase complexes with the polymerase activity inhibitors used in the "hot start" techniques [17, 18].

Since PCR product detection in a number of applications (like PCR using hydrolyzable probes [19, 20] and MALDI-TOF mass spectroscopy [21]) is based on the exhibition of the polymerase 5′-3′-exonuclease activity, it is necessary to have the possibility to determine both polymerase and exonuclease activities. It is impossible to adapt a great number of the nuclease activity determination methods to simultaneous determination of polymerase activity. Besides, these methods are expensive or laborious [11, 12, 22, 23]. So far there is no convenient method for simultaneous and rapid determination of specific polymerase and nuclease activities of polymerase preparations in routine practice.

A method for recording and estimation of DNA polymerase-specific activity using the real-time fluorescence detection during isothermal reaction is described below.

MATERIALS AND METHODS

Reagents and equipment. RNA-dependent DNA polymerases H^--MMLV and AMV (Fermentas, Lithuania), DNA-dependent DNA polymerases Vent and Pfu (SibEnzyme, Russia), phi29, recombinant Taq polymerase (Fermentas; Vector-Best, Russia), monoclonal antibodies to Taq polymerase (Clontech, USA), SYBR Green I dye (Molecular Probes, USA), BSA (Fermentas), and betaine (Sigma, USA) were used in this work. Fluorescence was detected under real-time conditions using amplifiers with an optical module for PCR in the real-time iCycler iQ4 (Bio-Rad, USA) and Rotor-Gene 3000 (Corbett Life Science, USA).

Nucleotide material. DNA of plasmid pGEM-T Easy Vector (Promega, USA) with cloned fragments of the *orf-B* and *atpA* genes from sugar beet mtDNA [24] was isolated from an overnight culture of *E. coli* strain MRF-XL-Blue by the thermal lysis technique [25]. Sonicated DNA from salmon testes (500-6000 bp, on the average 3000 bp; Medigen Laboratory, Russia), poly(dA)$_{80\text{-}120}$, oligo(dA)$_{16}$, poly(dT)$_{80\text{-}120}$, hexanucleotides with random N_6 sequence (Vector-Best), specific oligonucleotide primers, oligonucleotides containing at 3′ end a hairpin structure, and probes labeled by a reporter fluorophore (Syntol, Russia) were used. The rhodamine dye Rhodamine 6G (R6G, 6-carboxyrhodamine) and an azo-dye BHQ-1 (4′-(2-nitro-4-toluyldiasa)-2′-methoxy-5′-methyldiazobenzyl-4′-(N-ethyl)-N-2-ethanol) were used as reporter fluorophore and quencher. R6G was chosen for its high quantum yield, photostability, and the ability of detection using standard filters from the set of amplifiers for real-time PCR. BHQ-1 was chosen because it forms with R6G a FRET pair with high efficiency energy transfer and is characterized by practically complete absence of its own fluorescence.

Used oligonucleotide sequences. Specific oligonucleotide primers:

orf-Bfor 5′-TGCCTGTCCCATGCGTTGTT-3′,

orf-Brev 5′-AGGCATGACCAGAAGAATTGTGTGA-3′,

atpAfor 5′-GGACCATGCCTGACCTTGTTCG-3′,

atpArev 5′-GAATGCCGCTGCTGATAGATCCC-3′.

Oligonucleotides with a hairpin structure at the 3′ end:

TaqC1 5′-TAC T<u>CG GTG TAC TCA CCG GTT CCG CAG A</u>TA GAC ATA CTT ATT AAC TTA TAT TCA CTC TTA CTT ATA CTC **ATC GAT AC**T TTT **GTA TCG AT**-3′,

TaqC1.2 5′-ATC T<u>CG GTG TAC TCA CCG GTT CCG CAG A</u>TA GAC ATA CTT ATT AAC TTA TCT ATC CTC TTA CTT CAT CTC **ATC GAT AA**T TTT **TTA TCG AT**-3′.

Probes labeled by the reporter fluorophore:

24d 5′R6G-<u>TCTGCGGAACCGGTGAGTACACCG</u>-3′BHQ-1,

endo24d(10/24) 5′R6G-CAATCTGAATTCTGCGGAACCGGT(BHQ-1)GAGTACACCG-3′,

endo24d(10/15) 5′R6G-CAATCTGAATTCTGC(BHQ-1)GGAACCGGTGAGTACACCG-3′,

endo24d(4/9) 5′R6G-GAATTCTGC(BHQ-1)GGAACCGGTGAGTACACCG-3′,

Cendo24d(10/4) 5′R6G-CAAT(BHQ-1)CTGAATTCTGCGGAACCGGTGAGTACACCG-3′

(probe fragments forming duplexes with oligonucleotides TaqC1, TaqC1.2, and corresponding regions within the abovementioned oligonucleotides are underlined; sequences forming the hairpin stem under the reaction conditions are shown in bold).

Determination of polymerase activity. The reaction mixture (50 μl) contained 67 mM Tris-HCl (pH 8.9), 1.6 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 0.01% Tween 20, 200 μM dNTP, 0.25 μM of the fluorophore-labeled oligonucleotide probe, SYBR Green I (1 : 10,000 of the commercial stock solution), different types of DNA templates at concentration 5-20 ng/μl, and different amounts of DNA polymerase. The amount of Taq polymerase that catalyzes incorporation of 10 nmol deoxynucleotides into the polynucleotide fraction in 30 min at 70°C in reaction mixture containing 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 mM NaCl, 0.1 mg/ml BSA, 0.75 mM activated calf thymus DNA, 200 μM each dNTP, and 0.4 MBq/ml [^{3}H]dTTP was taken as activity unit (U). Activities of other polymerases were determined relative to the activity of Taq polymerase. Before addition of polymerase, the reaction mixture was incubated for 2 min at 94°C followed by cooling to 4°C for 120-160 sec to allow primer–template complex formation. Polymerase was added to the cooled reaction mixture and mixed for 10 sec. Then the tubes were placed into an amplifier (well temperature 4°C). Thus, before fluorescence detection, the temperature of reaction mixtures was kept at the level at which polymerase and nuclease activities of the used DNA polymerases are negligible.

Protocol of polymerase activity detection. Polymerase activity was followed in real time on amplifiers with an optical block using a standard set of filters (filters 490/20 and 530/30 for excitation/detection of the SYBR Green I fluorescence and filters 530/30 and 575/20 for excitation/detection of the R6G fluorescence). Reactions were carried out at constant temperature of 65°C (except reactions for determination of stability of the polymerase–antibody complexes, in which temperature was varied from 50 to 65°C, as well as reactions for determination of dissociation temperature of the polymerase–antibody complexes, in which temperature was increased 0.5°C at each step), and the level of fluorescence was detected every 10 sec in the case of polymerase reaction and every 20 sec in the case of simultaneous determination of polymerase and nuclease activities.

Polymerase chain reaction. The reaction mixture of 50 μl volume contained 67 mM Tris-HCl (pH 8.9), 1.6 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 0.01% Tween 20, 200 μM dNTP, 0.25 μM the fluorophore-labeled oligonucleotide probe, 0.5 μM forward and reverse primers (*orf-Bfor*, *orf-Brev*), DNA template at concentration 5 ng/μl, and 1 U Taq polymerase. The following temperature profile was used: 1 min at 94°C, 65 cycles consisting of steps including 10 sec at 94°C and 20 sec at 65°C.

RESULTS AND DISCUSSION

Choosing primer–template complex optimal for recording polymerase activity. The following requirements for primers and templates were formulated:

– primer elongation by polymerase should be seen as increased fluorescence; the maximal ratio of fluorescence of a mixture in which all primer–template complexes are completed to fluorescence of the mixture without added polymerase provides for the highest accuracy of measurements and reliability of results;

– the fluorescence of polymerase-free reaction mixture should undergo minimal changes in the course of reaction;

– the fluorescence increase should be rather rapid (to reduce the time necessary for the experiment), but in this case it should allow highly precise determination of the slope of the curve of fluorescence time dependence at the first (linear) part;

– templates characterized by fixed number of priming complexes per mass unit and equal increase in fluorescence upon completion of each priming complex should have an additional advantage because this will enhance the reproducibility of the technique.

The following requirements were imposed on oligonucleotide sequences containing a hairpin structure at the 3′ end: the duplex part is short (9 nucleotide pairs) and maximally high-melting, and the unpaired part is no longer than 60 nucleotides; an extensible hairpin should be a unique stable secondary structure formed by an oligonucleotide under standard conditions of polymerase reaction (potential secondary structures were analyzed using a program package available online at www.idtdna.com). Comparison of several variants meeting these criteria did not reveal any principal distinctions between them (typical sequence variants are given in "Materials and Methods"). Reactions were performed with such primer–template combinations as plasmid DNA with specific primers (plasmid pGEM-T Easy with the insert containing sites for primers *orf-Bfor/rev* and *atpAfor/rev*) and plasmid or sonicated genomic DNA with random primers (N_6), poly(dA)$_{80\text{-}120}$/poly(dT)$_{80\text{-}120}$, oligo(dA)$_{16}$/poly(dT)$_{80\text{-}120}$, a synthetic oligonucleotide containing a

hairpin (acting as a primer) at the 3′ end, and a site for oligonucleotide probe (TaqC oligonucleotides). The use of different primer–template complexes resulted in different results, from reliable detection of fluorescence increase due to formation of double-stranded DNA or hybrid DNA/RNA duplexes to the impossibility of detection of complex elongation due to insufficient amplitude of increasing signal. Detection of primer–template complex elongation became possible using oligo$(dA)_{16}$/poly$(dT)_{80\text{-}120}$ and synthetic TaqC oligonucleotides with a hairpin at the 3′ end and a site for the probe containing reporter fluorophore R6G. The advantages, shortcomings, and fields of application of these templates are shown in the table; use of other templates did not allow estimation of the fluorescence increase due to the extremely low signal/noise ratio. The use of both oligo$(dA)_{16}$/poly$(dT)_{80\text{-}120}$ and TaqC makes possible the estimation of the activity of the polymerase preparation at the qualitative level (yes/no) which can be used, for example, for estimation of temperature–time dissociation parameters of polymerase complexes with antibodies. However, reliable estimation of polymerase activity using oligo$(dA)_{16}$/poly$(dT)_{80\text{-}120}$ is possible only at relatively high (10-30 mU/μl) enzyme concentrations in the reaction mixture. Besides, owing to low melting temperatures of dA_n/dT_n duplexes, the reaction is possible only at temperatures below 60°C (in this case the use of oligo(dG)/poly(dC) and oligo$(dC)_{16}$/poly(dG) is difficult due to complicated synthesis of GC-rich oligonucleotides and high efficiency of secondary structure formation in the case of poly(dG)). Even at 60°C melting of primer–template complexes in early cycles contributes significantly to the results of the measurements. An additional feature of the reaction with poly(dT)/oligo(dA) as template is a lower rate of polymerization. At poly(dT) concentration 10 ng/μl, oligo(dA) 2 ng/μl, and polymerase 15 mU/μl, the reaction reaches a plateau in 20 min, whereas with 10 ng/μl hairpin structure and 15 mU/μl polymerase the increase in fluorescence ceases in 10-20 sec from the beginning of the reaction, which shows that the majority of substrate was transformed into the double-stranded form. Stability of the primer–template complex is probably the factor limiting reaction rate in the case of poly(dT)/oligo(dA). The low rate of primer–template complex elongation and uncertain increase in fluorescence (accompanied by changes in the character of the increase) restricts the range of detectable polymerase amounts to concentrations of 10-30 mU/μl.

Determination of polymerase and nuclease activities of DNA polymerases using hairpin-forming oligonucleotides. The use of synthetic oligonucleotides containing a hairpin structure at the 3′ end and a site for the

Characteristics and fields of application of studied templates

Primer–template complex	Advantages	Shortcomings	Fields of application
oligo$(dA)_{16}$ /poly$(dT)_{80\text{–}120}$	high signal/noise ratio, low cost	absence of fixed hybridization site (additional complications in calculation of the number of priming complexes); primer annealing on the template is cooperative; low melting temperature (upper limit of the temperature of activity determination about 60°C)	determination of DNA polymerase activity* within the interval between 10 and 30 mU/μl, studying the effect of the chemical composition of the reaction mixture on DNA polymerase activity, determination of parameters of DNA polymerase activity inhibition by specific antibodies within temperature interval 25-60°C
Synthetic oligonucleotides containing a hairpin and a site for the hydrolyzable probe (TaqC)	high signal/noise ratio, high sensitivity, possibility of simultaneous determination of polymerase and exonuclease activities, measuring enzyme activities in a broader temperature interval due to a higher melting temperature of the primer–template complex, fixed number of primer complexes per nucleic acid mass unit, fixed fluorescence increase per primer complex	not shown	determination of DNA polymerase and 5′-3′-exonuclease activities* within intervals from 0.5 to 80 mU/μl and from 2.5 mU to 2.5 U/μl, respectively; studying the effect of reaction mixture chemical composition and temperature on DNA polymerase and nuclease activities; determination of parameters of DNA polymerase and nuclease activity inhibition by specific antibodies within temperature interval 25-90°C; determination of endonuclease activity of DNA polymerases

* Estimated using Taq polymerase.

hydrolyzable probe (hairpin extension assay) has the following advantages over the use of complexes of homopolymeric templates and primers:

– it makes possible determinations of polymerase and exonuclease activities of the preparations over a broad range of concentrations (from 0.5 to 80 mU/μl for polymerase and from 2.5 mU/μl to 2.5 U/μl for 5′-3′-exonuclease activities of Taq polymerase);

– it allows measurement of enzyme activities over a broader temperature range due to a higher melting temperature of the primer–template complex;

– it produces higher signal/noise ratio that enables the reliable detection of fluorescence increase (the level of fluorescence upon template completion at least threefold exceeds that of the original templates);

– it provides for formation of a fixed number of priming complexes per nucleic acid mass unit (one priming complex per oligonucleotide molecule);

– it produces fixed fluorescence increase per priming complex (uniformity of reaction products);

– it allows independent detection of polymerase (the increase in the SYBR Green I fluorescence) and exonuclease activities of polymerase (the oligonucleotide contains a site for the probe with R6G as fluorophore) (Fig. 1a).

A series of reactions with different Taq polymerase concentrations in reaction mixture was carried out to determine the dependence of the rate of fluorescence increase on enzyme concentration. Activity was determined according to the following scheme:

– the fluorescence of reaction mixtures with different amounts of polymerase at different moments of time was determined (Fig. 1b);

– plots of the fluorescence time dependence were leveled by the initial fluorescence (normalization (Fig. 1c)) and the arithmetic means of fluorescence at each time were found for repeated samples (Fig. 1d);

– initial parts of the plots of the fluorescence time dependence with linear fluorescence increase were chosen. Linear approximation to these parts was plotted and tangent of the slope was determined (Fig. 1e);

– the dependence of the slope on polymerase concentration in reaction mixture was approximated by a function (determination of the exact kind of function was not an aim of this work; we used second degree polynomial approximation, to which there corresponded high values (>0.95) of the approximation reliability coefficient (R^2) for all studied polymerase samples). To determine activities of experimental samples, the inverse functions for appropriate slopes were calculated.

Thus, the activity of the preparation was normalized relative that of a standard preparation. Figure 2 shows an example of the application of the above-mentioned algorithm in determination of polymerase activities of two Taq polymerase preparations. The activities of preparations normalized to that of standard Taq polymerase, estimated by the radiolabel incorporation, were 25 and 6.25 mU/μl for samples I and II, respectively.

The scheme can be used in determination of both polymerase and nuclease activities. In this case, a good correlation is observed between the rates of increase in fluorescence. Figure 3 shows dependences of the increase in fluorescence in response to increased amounts of dsDNA in the reaction mixture (fluorescence of SYBR Green I) and fluorescence caused by the uncoupling of the fluorophore and quencher upon hydrolysis of the oligonucleotide probe (detection of exonuclease activity). The mean value of the ratio of the rates of fluorescence increase in the SYBR Green I and R6G channels for Taq polymerase preparations of different specific activity varied insignificantly and was 2.32 ± 0.35 ($p = 0.999$). Thus, this method can be used to compare the effect of different factors (temperature, chemical composition of the mixture, etc.) on polymerase and endonuclease activities of polymerase, which makes it possible to differentiate the influence of various factors on optimization of protocols using both activities.

To estimate the suitability of the proposed method for determination of effects of different substances on activity of the DNA polymerase preparations, we studied the effects of two widely used in PCR compounds, BSA and betaine, on polymerase activity of three samples of Taq polymerase. The effects of these compounds at different concentrations were estimated similarly to comparison of activities of different enzyme samples. Addition of 10-50 μg/ml BSA to the reaction mixture stimulated the DNA-dependent DNA polymerase activity of Taq polymerase. Measurement of BSA fluorescence at this concentration under the same conditions showed that the fluorescence does not exceed the threshold level and does not increase with time. The positive effect of betaine described in [26] is not associated with increased polymerase activity (addition of 0.1-0.5 M betaine does not influence the rate of synthesis). This is probably due to the effect on nucleic acids present in the reaction mixture (for example, in the presence of betaine enhanced specificity of the primer–template complex formation is observed).

Determination of temperature–time parameters of dissociation for Taq-polymerase–antibody complexes (with Taq-polymerase-specific sites for antigen binding). Different "hot-start" techniques are often used in PCR-based protocols for detection of specific nucleotide sequences (blocking of the polymerization reaction is relieved only at the required rather high temperature). Hot start decreases the probability of undesirable synthesis of unspecific products (as a result of primer hybridization with the partially complementary template regions, formation of primer dimers, etc.) during mixing of the reaction components and at initial low-temperature reaction stages due to polymerase activity that is significant for many polymerases already at 25-28°C.

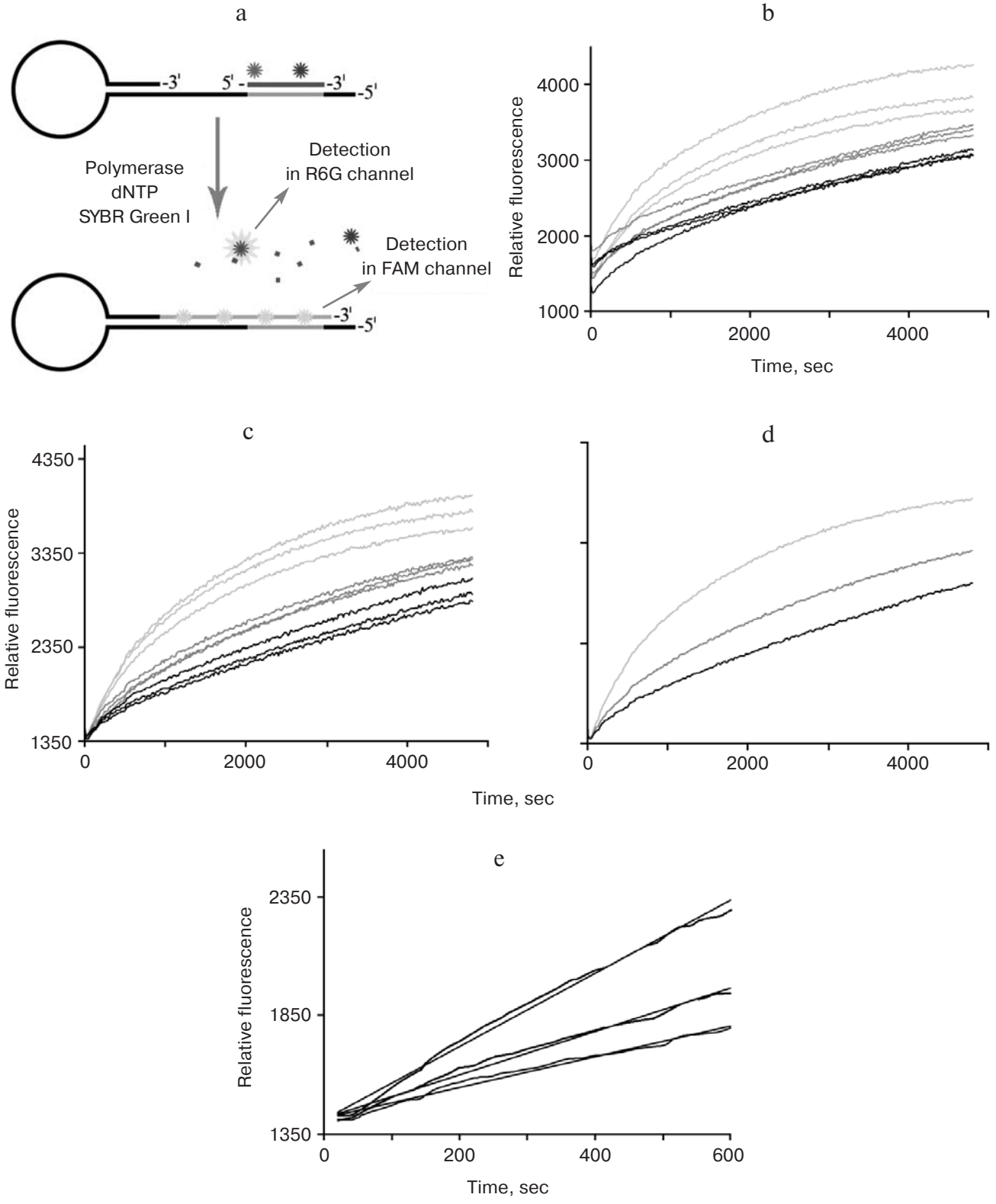

Fig. 1. Principle of simultaneous detection of polymerase and 5′-3′-exonuclease activities of polymerases using the intercalating dye SYBR Green I and the hybridizable fluorescence probe with uncoupled fluorescence spectra (F and Q, fluorophore and quencher, respectively) (a). Scheme for determination of activity of DNA-dependent DNA polymerase preparation (b-e). Plots of fluorescence increase (b) are normalized by the initial fluorescence level (c) and averaged out (d). Linear approximations to the initial parts of the curve are plotted (e). Slope correlates with the polymerase content in the mixture.

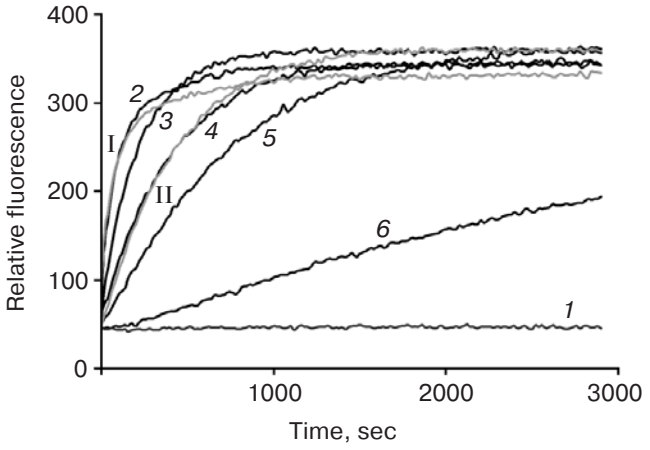

Fig. 2. Example of polymerase activity determination in two Taq polymerase preparations (gray plots I and II). *1*) Sample without polymerase ("negative control"). Taq polymerase from Fermentas company was used as standard (activities of samples *2-6* were 25.0, 12.5, 6.25, 3.13, and 1.56 mU/μl, respectively).

The approach based on blocking of the polymerase reactive center by specific antibodies irreversibly dissociating upon increase in temperature is widely used. Determination of parameters of polymerase–antibody complex dissociation is important for optimization of the reaction protocol and for comparison of stability of enzyme complexes with different antibodies.

To estimate the efficiency of inhibition of polymerase activity by antibodies at different temperatures and determination of activation parameters, polymerase activities of native enzyme and that complexed with specific antibodies were evaluated. Taq polymerase activity in the absence of antibodies was revealed at temperatures above 28°C. At 50°C, the enzyme activity already reached 50% of that at 70°C. At the same time, the polymerase complex with specific antibodies exhibited only the background activity (the rate of fluorescence increase was no greater than 3% of that for native enzyme). Incubation of reaction mixture at 55, 60, and 65°C resulted in the maximal efficiency of the reaction in 4000, 1300, and 100 sec, respectively (Fig. 4).

To determine the dependence of the efficiency of inhibition of the polymerase activity and temperature of complex dissociation on the Taq polymerase ratio to specific antibodies, we used a protocol with gradual increase in temperature (0.5°C per 10 sec). At the antibody concentrations in the reaction mixture below 1 μg/ml, their inhibitory effect was insignificant (samples *2* and *3* in Figs. 4e and 4f), while at concentrations above 15 μg/ml the efficiency of inhibition remained constant (samples *7* and *8* in Figs. 4e and 4f). On the whole, disintegration of the polymerase–antibody complexes depended both on the temperature and time of incubation, but at temperatures below 50°C the complex remained stable for practically unlimited time.

Recording endonuclease activity of Taq polymerase. It has been shown in a number of publications that Taq polymerase is characterized both by 5′-3′-exonuclease and endonuclease activities [27, 28]. The existence of endonuclease activity in Taq polymerase makes possible cleavage of branched structures, in which the 5′ part of oligonucleotide probe is not complementary to the template, and as a result it is able to decrease the specificity of concrete sequence detection by PCR under real time conditions using hydrolyzable probes. To estimate endonuclease activity, we used oligonucleotide probes in which fluorophore is joined to the 5′end, unpaired with the template hairpin oligonucleotide (Fig. 5a), and the quencher is bound to the nucleotide remote from the branching site for five (endo24d(4/9) and endo24d(10/15)) or 14 (endo24d(10/24)) nucleotides (the first number in the name shows the length of unpaired single-stranded region

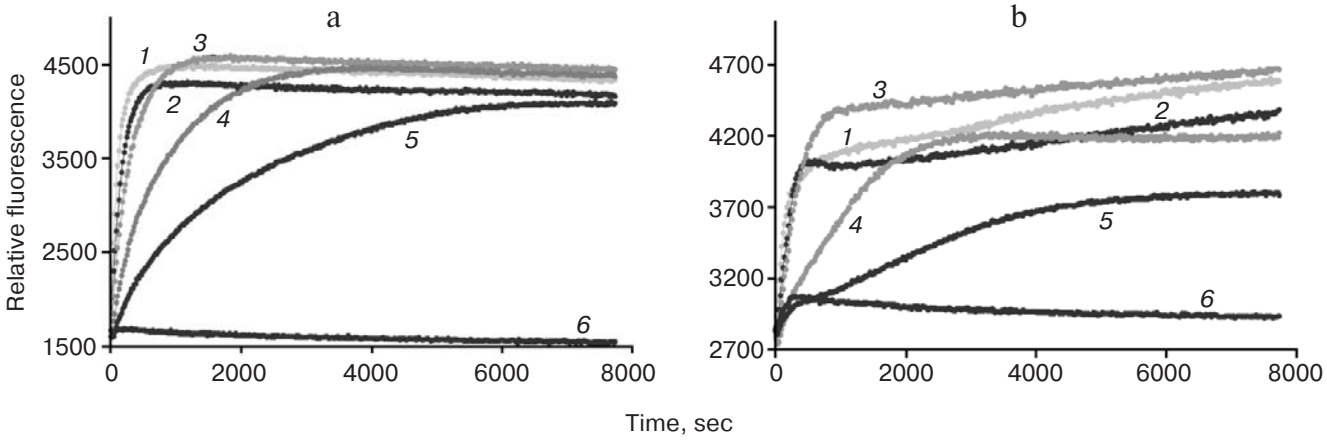

Fig. 3. Titration of Taq polymerase preparation. *1-6*) Enzyme concentrations in reaction mixture are 10.0, 4.0, 2.0, 1.0, 0.4, and 0 mU/μl, respectively. a) Fluorescence in the SYBR Green I channel indicating the amount of synthesized dsDNA (polymerase activity); b) fluorescence in the R6G channel showing the amount of hydrolyzed probe (exonuclease activity).

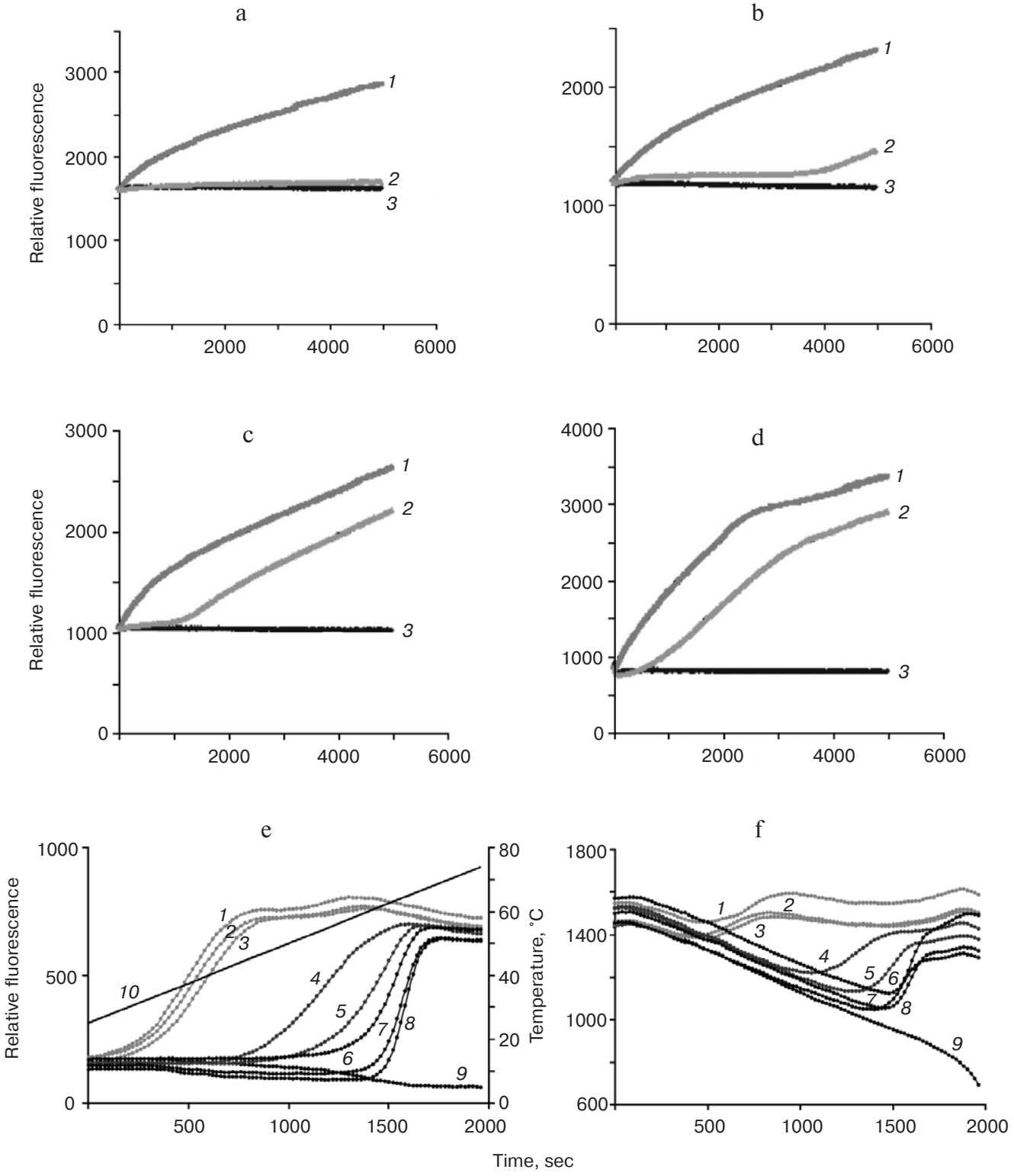

Fig. 4. Accumulation of product at different temperatures in reaction mixtures containing 62.5 mU/μl Taq polymerase in free form (*1*), Taq polymerase complexed with antibodies in a concentration 31.25 μg/ml (*2*) and containing no polymerase (*3*) at 50, 55, 60, and 65°C (a-d, respectively). Dissociation of polymerase complexes with antibodies in a protocol with gradual increase in temperature: recording polymerase (e) and 5′-3′-exonuclease (f) activities. Taq polymerase concentration was 62.5 mU/μl, concentration of antibodies was 0, 0.49, 0.97, 1.95, 3.9, 7.8, 15.6, and 31.3 μg/ml (*1-8*, respectively); sample *9* did not contain polymerase. Curve *10* on the diagram (e) shows the change in temperature (on diagram (f) temperature changed in the same way).

at the 5′ end, the other corresponds to the distance between the fluorophore and quencher in nucleotides). Cleavage of the probe is possible if endonuclease activity is present in Taq polymerase or if the polymerase is able to bind an unpaired 5′ end and cleave it using exonuclease activity. To check the second hypothesis, the control probe Cendo24d(10/4) was used in which the fluorophore and quencher, separated by four bases, were located in the template unpaired region. Since cleavage of control probe was not accompanied by increase in fluorescence (data not shown), it was concluded that polymerase is not able to efficiently hydrolyze the oligonucleotide from the unpaired 5′ end.

With endo24d probes, in all considered cases fluorescence increase, depending on the polymerase concentration, was observed in the R6G channel, which suggests that polymerase introduces a nick in the branching site, i.e. it exhibits endonuclease activity. In this case exonuclease (probe 24d, Fig. 5b) and endonuclease (probes endo24d(4/9), endo24d(10/15), and endo24d(10/24) in Figs. 5c-5e, respectively) cleavages of probes occur at very similar rates.

The existence of endonuclease activity in Taq polymerase was confirmed by real-time PCR (Fig. 5f). A synthetic DNA fragment having a site for the 24d probe was used as template; in this case, the flanking sequence was not complementary to the 5′ ends of endo24d probes. The fluorescence associated with the cleavage of the probe increased both with 24d (Fig. 5f, sample *1*) and in all cases with probes having protruding 5′ ends (endo24d(4/9), endo24d(10/15), endo24d(10/24) in Fig. 5f, samples *2-4*, respectively). The fact that the activity cleaving endonuclease of the probe is inherent in Taq polymerase rather than being due to contaminants present in the mixture is confirmed by the similarity of kinetic curves of endo- and exonuclease cleavage of the probe and by the absence of probe cleavage in the unhybridized state (when template was not present in the solution) both in PCR and in isothermal reaction of the probe cleavage on the hairpin (data not shown).

These data show that the use of systems based on oligonucleotide probe degradation by nuclease for discrimination between similar sequences makes impossible the use of the specificity of the 5′-terminal region hybridization of the probe as the discriminating factor.

Thus a new method is proposed for simultaneous determination of DNA-dependent DNA polymerase and nuclease activities of polymerase enzymes using PCR amplifiers with an optical module. The variant using oligo$(dA)_{16}$/poly$(dT)_{80\text{-}120}$ allows one to register polymerase activity at the qualitative level, which enables the application of this method for determination of dissociation parameters of polymerase complexes with specific antibodies (antibody screening for choosing the polymerase complex with antibodies, having specified dissociation parameters, and optimization of the "hot start activation" step in the PCR protocol). The variant using the hairpin-containing synthetic oligonucleotide in combination with hydrolyzable probes makes possible simultaneous quantitative determination of the polymerase and exonuclease activities of the enzyme. This allows one to compare different polymerases or different batches of the same polymerase, as well as to estimate the DNA-dependent DNA polymerase activity of reverse transcriptases. Determination of the relative activity of Taq polymerase allows one to normalize amounts of each new enzyme batch (or preparations obtained from different manufacturers) comparing to the chosen standard, which might enhance the reproducibility of techniques in which the accuracy of the determination of the enzyme amount can be important (like RT-PCR). Besides, estimation of the activity of a polymerase preparation under different conditions can be used for determination of the enzyme half-life time at a certain temperature under particular conditions and for evaluation of the effect of the chemical composition of the reaction mixture on enzyme activity. The method is suitable for evaluation of activities of a broad circle of polymerases on the basis of any contemporary amplifier with sufficiently sensitive optical module. The possibility of determination under above-mentioned conditions of relative activity of DNA-dependent DNA polymerases Vent, Pfu, phi29, and DNA-dependent DNA polymerase activity of reverse transcriptase H^--MMLV and AMV (data not shown) was demonstrated. In all these cases, quantitative activity estimation was possible at relatively low polymerase concentrations (from 10 mU to 1 U per reaction), which makes preferable the use of the proposed technique if the enzyme preparation is expensive or available only in small amounts.

Comparable by sensitivity to the traditional radioisotopic technique, the proposed approach to the determination of DNA polymerase activity is much safer (it does not require work with radioactivity) and easier (continuous measuring the dNTP incorporation without taking aliquots and the possibility of parallel determination of activity of 32-96 samples using modern amplifiers for the real-time PCR). The flexible temperature schedule (unlike that in the PCR-based methods [9, 10]) and the time scale of high resolution (unlike that in "end-point" measurements [5, 6]) enable estimation of the temperature-dependent inhibition of enzyme activity. According to our data, the possibility of simultaneous detection of polymerase and 5′-3′-exonuclease activities, not possible using existing methods, allows one to modulate exhibition of any activity depending on reaction conditions. This may help researchers involved in the development of new or optimization of already existing methods based on the use of DNA polymerases (which is impossible with methods based on the use of modified dNTP [7, 8], because the efficiency of incorporation of the latter may depend on reaction conditions as well). Besides, the proposed method is rather inexpensive: the cost of the fluo-

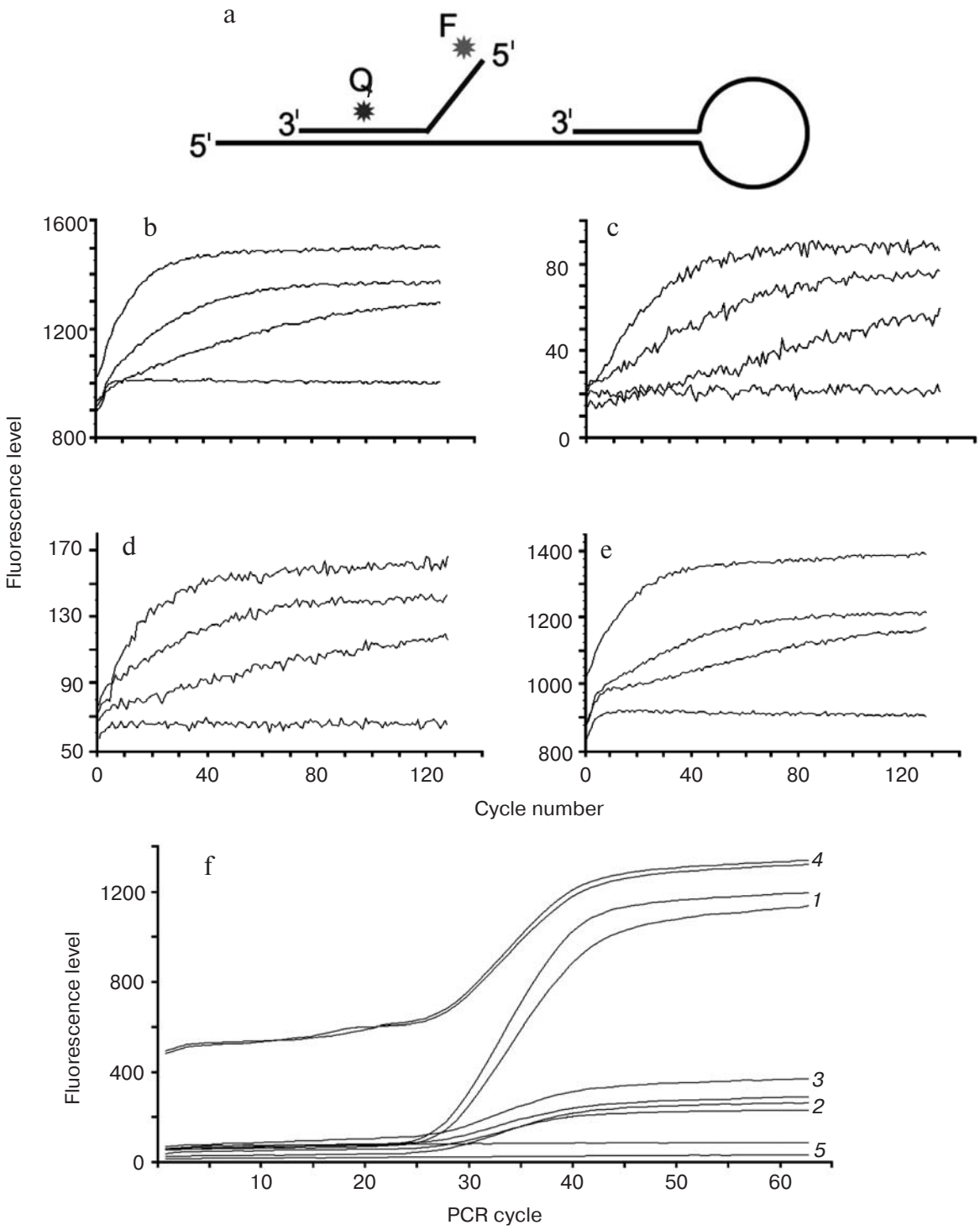

Fig. 5. a) Scheme of endonuclease activity determination in Taq polymerase. In the presence of endonuclease activity, the cleavage of the oligonucleotide probe occurs at a branching point and is accompanied by separation of the fluorophore and quencher and consequent increase in the level of fluorescence. b-e) Isothermal detection of the Taq polymerase endonuclease activity in real time (cycle duration is 10 sec). Probes 24d, endo24d(4/9), endo24d(10/15), and endo24d(10/24) (b-e, respectively) were used. Each diagram shows polymerase concentrations from the top down: 4, 2, 1, and 0 mU/μl. f) Use of probes 24d, endo24d(4/9), endo24d(10/15), and endo24d(10/24) (*1-4*, respectively) under real time conditions; *5*) negative control with the template-free probe 24d (indicates the absence from the reaction mixture of contaminant 5′-3′-exonuclease activity). An amplicon, in which the probe binding site coincides with that in 24d, was used as template. Reactions with each probe were measured in duplicate.

rophore-labeled oligonucleotide probes is much lower than that of α-^{32}P-labeled nucleoside triphosphate (in addition, the latter requires special transport and storage conditions and is susceptible to rapid decay, decreasing the reproducibility of results), and amplifiers for the real-time PCR are used everywhere both in research and in diagnostic laboratories.

The use of similar protocols seems promising for searching for new polymerase and nuclease activities as well of alternative activities of known polymerases, including also the RNA-dependent DNA polymerases, RNA polymerases, and enzymes of template-less polynucleotide synthesis.

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