Analytical Biochemistry 441 (2013) 133–139

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Stopped-flow DNA polymerase assay by continuous monitoring of dNTP incorporation by fluorescence

Jesse L. Montgomery, Nick Rejali, Carl T. Wittwer *

Department of Pathology, University of Utah, Salt Lake City, UT 84132, USA

ARTICLE INFO

Article history:
Received 17 April 2013
Received in revised form 3 July 2013
Accepted 6 July 2013
Available online 16 July 2013

Keywords:
Activity assay
Polymerase
Stopped-flow
Intercalating dye

ABSTRACT

DNA polymerase activity was measured by a stopped-flow assay that monitors polymerase extension using an intercalating dye. Double-stranded DNA formation during extension of a hairpin substrate was monitored at 75 °C for 2 min. Rates were determined in nucleotides per second per molecule of polymerase (nt/s) and were linear with time and polymerase concentration from 1 to 50 nM. The concentrations of 15 available polymerases were quantified and their extension rates determined in 50 mM Tris, pH 8.3, 0.5 mg/ml BSA, 2 mM $MgCl_2$, and 200 μM each dNTP as well as their commercially recommended buffers. Native Taq polymerases had similar extension rates of 10–45 nt/s. Three alternative polymerases showed faster speeds, including KOD (76 nt/s), Klentaq I (101 nt/s), and KAPA2G (155 nt/s). Fusion polymerases including Herculase II and Phusion were relatively slow (3–13 nt/s). The pH optimum for Klentaq extension was between 8.5 and 8.7 with no effect of Tris concentration. Activity was directly correlated to the $MgCl_2$ concentration and inversely correlated to the KCl concentration. This continuous assay is relevant to PCR and provides accurate measurement of polymerase activity using a defined template without the need of radiolabeled substrates.

The extension rates of DNA polymerases under PCR conditions have not been characterized. A wide variety of polymerases are available and many are designed for increased fidelity and speed. The conventional way to measure the activity of DNA polymerase is in terms of units, most commonly defined as the number of nanomoles of radiolabeled dNTPs incorporated into activated calf thymus or salmon sperm DNA at 72 to 75 °C for 30 min. This is a time-consuming endpoint assay and does not provide information about the initial extension rates of polymerases. In addition, assay conditions are not standardized and often differ from those used during PCR.

A number of alternative assays have been introduced for DNA and RNA polymerases. These include methods based on atomic force microscopy [1], light microscopy [2], single-molecule optical trapping [3], quartz crystal microbalance [4], and radiometric assays [5]. Others use enzyme-coupled reactions to monitor pyrophosphate release [6,7]. Fluorescence-based methods have monitored the displacement of single-stranded DNA-binding protein [8] or polarization of labeled extension templates [9,10]. Quench–flow [6] has been used and allows kinetic analysis of rapid reactions. However, this method requires stopping the reaction at several time points, followed by analyzing the products on gels or by chromatography methods. Stopped-flow [6,9,11] assays have been developed and enable continuous reaction monitoring, but these use covalent fluorescent labels or nucleotide analogs. Some of these methods are capable of providing extension rates in terms of individual nucleotide incorporation [1–3,8,9,11]. However, they all require template modifications (fluorescent or radioactive) or immobilization of either template or polymerase onto a substrate.

We introduce a fluorescent stopped-flow assay for monitoring polymerase extension that requires no modification of the template or polymerase. This method relies on the increase in fluorescence of double-stranded DNA dyes during nucleotide incorporation. These dyes are frequently used in real-time PCR, eliminating the need to change reaction chemistry. Measured extension rates are directly applicable to PCR. We use this assay to compare the speed of 15 polymerases at equimolar concentrations. Because their activity was strongly dependent on the reaction buffer, we then measured the effects of common buffer conditions, including pH and KCl, $MgCl_2$, and Tris concentration.

Materials and methods

Oligonucleotides

The sequence tagcgaaggatgtgaacctaatcccTGCTCCCGCGGCCG atctgcCGGC-CGCGGGAGCA was used as the extension template and a baseline fluorescence standard (capital letters denote self-complementary sequences). The oligonucleotide forms a hairpin with a 14-bp stem that has a free 3′ end and a 25-base overhang

* Corresponding author. Fax: +1 801 581 6001.
E-mail address: carl.wittwer@path.utah.edu (C.T. Wittwer).

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.07.008

for extension. The fully extended template was also synthesized as a fluorescence standard: TAGCGAAGGATGTGAACCTAATCCCTGCTC CCGC-GGCCGatctgcCGGCCGCGGGAGCAGGGATTAGGTTCACATCCT TCGCTA. Oligonucleotides were ordered from Integrated DNA Technologies with the extension substrate purified by high-pressure liquid chromatography and the fully extended standard purified by polyacrylamide gel electrophoresis. Each was quantified by absorbance at 260 nm following digestion by purified phosphodiesterase [12] for accurate quantification.

DNA polymerases

Fifteen polymerases were included in this study: Amplitaq (Invitrogen), KOD (EMD Millipore), Taq (New England Biolabs), Platinum Taq (Invitrogen), GoTaq (Promega), Titanium Taq (Clontech), Paq5000 (Agilent), Herculase II (Agilent), Phusion (New England Biolabs), KAPA2G (Kapa Biosystems), MyTaq (Bioline), Ex Taq (Clontech), Taq (Roche), SpeedSTAR (Clontech), and Klentaq I (purchased from either AB Peptides or Washington University in St. Louis, MO, USA).

Polymerases were quantified on sodium dodecylsulfate (SDS) polyacrylamide gels stained with Sypro orange (Invitrogen). Gel images were obtained using a Gel Doc XR+ with XcitaBlue (Bio-Rad) conversion screen accessory and analyzed with Image Lab (Bio-Rad) software. Prior to being loaded on the gels, samples were reduced in 30 mM Tris, pH 6.8, 12.5% glycerol, 1% SDS, and 360 mM β-mercaptoethanol at 96 °C for 5 min. Klentaq I (purchased from Washington University in St. Louis) was used as the quantification standard. The standard was quantified by absorbance at 280 nm using an extinction coefficient of $6.91 \times 10^4\ M^{-1}\ cm^{-1}$ calculated from the amino acid content of the published sequence [13]. The purity of this standard was determined by fluorescence integration from polyacrylamide gels and the concentration adjusted proportionately. Two replicates of each quantity standard (50, 100, 200, and 300 ng) and four replicates of each polymerase were included on each gel. Major bands at expected molecular masses were considered to be the polymerase of interest. The integrated fluorescence intensity of these bands was used to calculate the concentration and purity of the polymerases. Molecular masses used in concentration calculations were measured from the gels or taken from the literature [14,15], including vendor product information. Klentaq I was measured by mass spectrometry (Mass Spectrometry and Proteomics core facility at the University of Utah) after dialyzing for 48 h at room temperature in PBS buffer (150 mM NaCl, 2.5 mM KCl, 10 mM disodium phosphate, 1.5 mM dipotassium phosphate, pH 7.4). A molecular mass of 62,596 Da was determined compared to 62,097 Da predicted from the amino acid sequence [13].

The specific activity in units per milligram of polymerase (U/mg) was calculated from the unit concentration provided by the manufacturer and the concentration of polymerase measured on the gels. Most manufacturers define 1 U of polymerase as the amount required to incorporate 10 nmol of dNTPs in 30 min. However, the manufacturers of Klentaq I (ABPeptides) and Taq (NEB) define a unit as the incorporation of 60 and 15 nmol dNTP, respectively. The specific activities of these polymerases were scaled to allow comparison to other polymerases (i.e., the specific activity calculated for Klentaq I was multiplied by 6 and that of Taq (NEB) was multiplied by 1.5). The specific activities for Herculase II and Titanium were not calculated because the manufacturers do not provide the polymerase activities.

Polymerase extension assay

Polymerase extension studies were performed with a stopped-flow instrument (SFM-300, Bio-Logic SAS). Excitation was set at 495 nm with a monochromator and fluorescence collected with a photomultiplier tube and a 530 ± 15 nm discriminating filter. Thermoelectric heaters separately maintained the temperature of the mixing lines and the reaction cuvette. Each line was held at 75 °C. Reactants were added to two separate mixing lines and mixed in a 1:1 ratio at a flow rate of 9 ml/s. The estimated dead time for mixing was 6.6 ms. Extension reactions were carried out in 1× EvaGreen (Biotium) and either the buffer supplied by the manufacturer of each polymerase or a common buffer (50 mM Tris, 0.5 mg/ml bovine serum albumin (BSA), and 2 mM $MgCl_2$, pH 8.3). When $MgCl_2$ was not included in the vendor buffer (KOD and Platinum Taq), a final concentration of 2 mM was used. Preliminary experiments determined maximal extension rates for Klentaq I with 200 μM each dNTP with a K_m of 39 μM. Polymerase extension was initiated by mixing 400 μM each dNTP with 10 nM polymerase and 200 nM oligonucleotide (final concentrations were 200 μM each dNTP, 5 nM polymerase, and 100 nM oligonucleotide). To prevent template degradation, extension experiments for polymerases exhibiting 3′ to 5′ exonuclease activity (Herculase II, KOD, and Phusion) were initiated by mixing the polymerase with dNTP and oligonucleotide. MyTaq includes dNTPs in the vendor buffer at a final concentration of 250 μM each. For this polymerase, extension reactions were initiated by mixing the polymerase with the oligonucleotide.

Polymerase extension curves were calibrated either by allowing the reaction to go to completion or by using fluorescence standards. Except where indicated, calibration was performed with fluorescence standards. Polymerase was omitted from reactions containing fluorescence standards and calibration was repeated for each experiment to account for influences of buffer conditions on absolute fluorescence. Seven to ten stopped-flow shots were repeated for each experiment and the means and standard deviations reported. Data were acquired for 2 min every 50 ms.

Buffer component study

The effects of pH and the concentrations of Tris, KCl, and $MgCl_2$ on extension rate were observed. One parameter was varied while the other three were kept constant. Final conditions were Tris concentrations at 10, 20, 30, 40, and 50 mM, KCl at 0, 12.5, 25, 37.5, 50, and 62.5 mM, and $MgCl_2$ at 1, 1.5, 2, 2.5, 3, 4, 5, and 6 mM, and pH was at 7, 7.5, 8, 8.3, 8.5, 8.7, and 9. Unless varied, Tris concentration was held at 50 mM, KCl at 0 mM, and $MgCl_2$ at 2 mM and the pH at 8.0. Studies were done with Klentaq I at 5 nM, 200 μM each dNTP, 100 nM template, 1× EvaGreen, and 0.5 mg/ml BSA final concentrations. Reaction completion was used to calibrate the data in this study except when the extension was so slow that saturation could not be observedwithin 2 min. This occurred only when KCl concentration was 62.5 mM, the pH was 7, and $MgCl_2$ concentration was 1 mM.

Results

Polymerase quantification

A typical quantification gel and standard curve are shown in Fig. 1. Antibody hot-start polymerases (Platinum, Titanium, MyTaq, ExTaq, and SpeedSTAR) showed characteristic heavy- and light-chain bands at around 50 and 25 kDa. Paq5000 showed a prominent band of unknown identity at 62.5 kDa. Phusion had a diffuse band centered around 150 kDa and a prominent band at 64.5 kDa. Neglecting bands known to be other components, the purity of all polymerases was calculated at greater than 90%, with the exception of KOD (70%), Phusion (50%), and KAPA2G (65%). Measured concentrations for all polymerases are shown in Table 1. Most

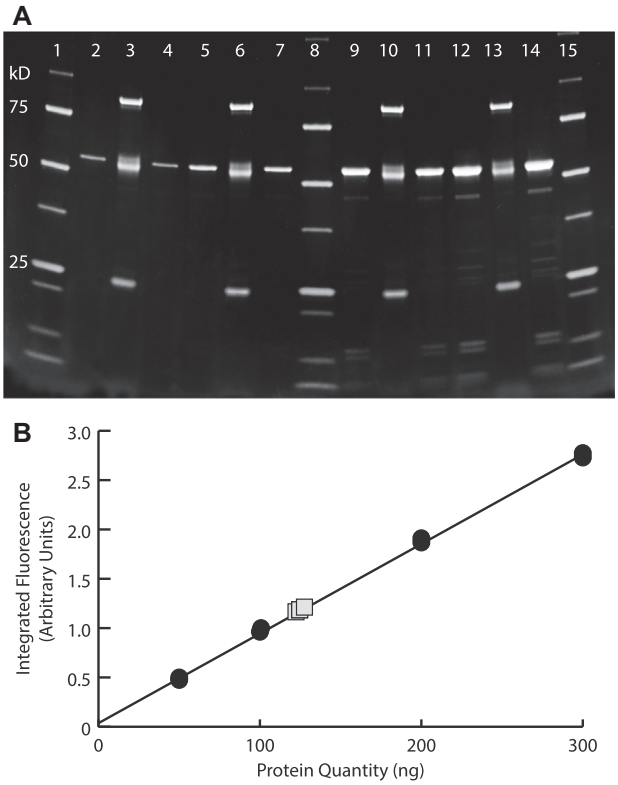

Fig.1. Quantification of polymerases. The purity and size of polymerases were determined on reducing polyacrylamide gels after staining with Sypro orange. (A) Quantification of SpeedStar. Four replicates of the SpeedStar (lanes 3, 6, 10, and 13) were compared to Klentaq I standards at 50 ng (lanes 2 and 4), 100 ng (lanes 5 and 7), 200 ng (lanes 9 and 11), and 300 ng (lanes 12 and 14). Molecular mass markers (Precision Plus Protein, Bio-Rad) are shown in lanes 1, 8, and 15. SpeedStar is an antibody hot-start polymerase and bands corresponding to heavy and light chains are shown near 25 and 50 kDa. The top band near 90 kDa is the polymerase. (B) Quantification of polymerases from a standard curve. The integrated fluorescence intensity of the unknown polymerase (squares) is projected on a regression line through the Klentaq I quantification standards (circles). The R^2 of the regression line is 0.999.

Table 1
Polymerase concentrations of stock solutions purchased from the manufacturers and their recommended concentrations in PCR.

Polymerase	Source	Stock concentration (μM)	Recommended PCR concentration (nM)
Taq (NEB)	Native Taq	1.06 ± 0.01	5.3 ± 0.1
Taq (Roche)	Native Taq	0.26 ± 0.01	5.2 ± 0.1
Amplitaq	Native Taq	0.52 ± 0.02	2.6 ± 0.1
GoTaq	Native Taq	0.98 ± 0.08	4.9 ± 0.4
MyTaq	Native Taq	1.31 ± 0.04	26.1 ± 0.8
ExTaq	Native Taq	1.28 ± 0.08	10.2 ± 0.7
Platinum	Native Taq	0.65 ± 0.02	2.6 ± 0.1
Herculase II	*Pfu* fusion variants	1.9 ± 0.1	19.4 ± 1.3
Phusion	*Pfu* fusion variants	1.01 ± 0.05	10.1 ± 0.5
Klentaq I [13] (ABPeptides)	Deletion variant	39.4 ± 1.5	63 ± 2.5
Titanium [13]	Deletion variant	9.9 ± 0.2	197 ± 3.6
KAPA2G	Engineered Taq	1.11 ± 0.09	4.4 ± 0.4
Paq5000 [16]	*Pfu*	0.85 ± 0.02	8.5 ± 0.2
KOD [15,17]	*Thermococcus kodakaraensis*	4.7 ± 0.2	95 ± 4.1
SpeedStar	Proprietary	1.08 ± 0.06	5.4 ± 0.3

vendors supply polymerases at a concentration around 1 μM. Additionally, the concentration of polymerase in PCR is typically in the range of 5 to 20 nM. Exceptions are KOD (94.5 nM), Klentaq I (63 nM), and Titanium (197 nM), which are supplied and used at considerably higher concentrations.

Assay validation and calibration

Extension rates were derived from the initial slope of the extension curves. Fig. 2 shows that the initial slope is proportional to polymerase concentration from at least 1 to 50 nM. To obtain rates in absolute units, extension curves were calibrated in one of two ways.

Substrate exhaustion

Polymerase extension reactions are allowed to proceed to saturation with complete extension of the template. The maximum and minimum data points of individual extension curves are normalized between 0 and the total number of nucleotides that each polymerase molecule can extend. This is calculated as:

$$[\text{Template}] \times L/[\text{Poly}], \quad (1)$$

where [Template] is the concentration of template, L is the length of extension in base pairs, and [Poly] is the concentration of the polymerase. Normalized this way, the initial slope of extension curves directly yields extension rate in nucleotides per second per molecule of polymerase (nt/s).

Calibration with standards

Extension curves can be normalized using oligonucleotide standards (Fig. 3). The baseline fluorescence is measured from the extension template without polymerase present. A synthetic analog of the fully extended template is used as a maximum fluorescence standard. The average fluorescence of the baseline standard is taken as 0 and the average fluorescence of the maximum standards is scaled to the value calculated by Eq. (1). The same offset and scaling factor are also applied to each experimental curve.

Both analyses were compared using Klentaq I at 75 °C. Ten experiments of 8 to 10 shots each were acquired. Substrate exhaustion yielded an extension rate of 102 ± 4.2 nt/s, whereas calibration with oligonucleotide standards gave 99 ± 8.4 nt/s. The standard deviations of individual shots within an experiment were similar for both methods at 3.8 and 3.4%, respectively. Both

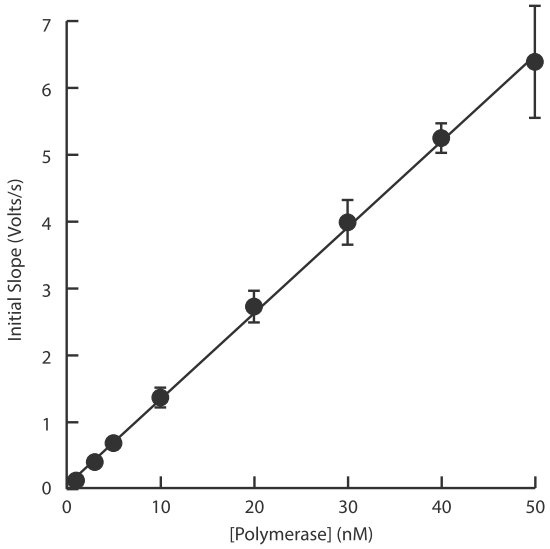

Fig.2. Linearity of extension rates with polymerase concentration. The initial slope of polymerase extension curves is linear with polymerase concentration. A linear regression yields $R^2 = 0.999$. Experiments were performed with Klentaq I.

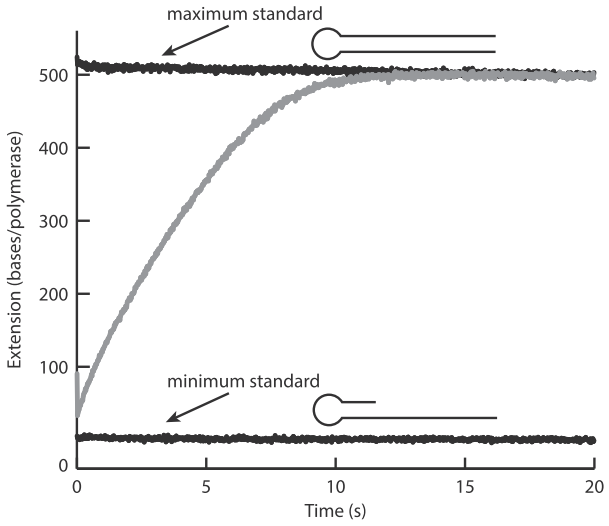

Fig.3. Quantitative analysis of polymerase extension curves. Extension curves are analyzed in one of two ways: (1) by measuring the fluorescence of oligonucleotide standards without polymerase (the minimum standard is the extension template and the maximum standard is a synthetic oligonucleotide identical to the sequence of the fully extended template) or (2) by substrate exhaustion, using time 0 as the fluorescence minimum. In both cases, the maxima and minima are scaled between 0 and the total number of bases extended by each polymerase calculated using Eq. (1). The initial slope is then the extension rate in nucleotides per second per molecule of polymerase (nt/s). Both approaches yield extension rates concordant within 3%.

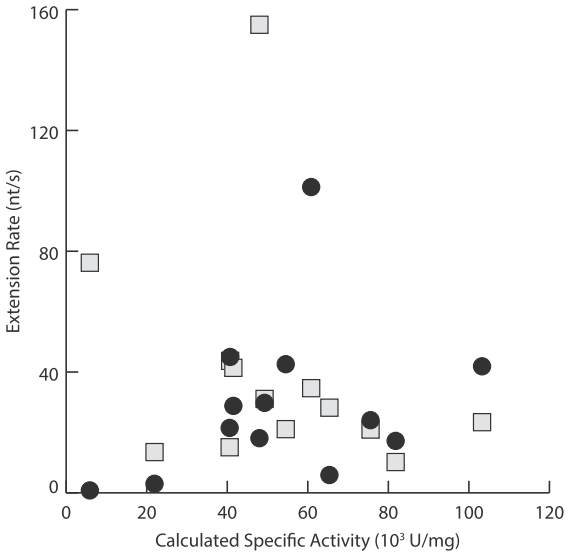

Fig.5. Measured extension rates (nt/s) versus calculated specific activity (U/mg). The specific activity of each polymerase was calculated from the unit concentration supplied by the manufacturer and the mass of polymerase measured from polyacrylamide gels. There is little relationship between the two measurements of specific activity. The correlation is positive in the common buffer (circles), with a Pearson's *r* coefficient of 0.32, and negative in the vendor buffers (squares), with a Pearson's *r* coefficient of −0.30.

analysis methods were concordant to within 3%. The advantage of using standards is that reactions need not proceed to exhaustion, greatly reducing acquisition time when the activity is low. However, increased precision makes substrate exhaustion preferable when the activity is high.

Polymerase comparison

The extension rates of various polymerases were measured in their corresponding vendor buffers as well as in a common buffer composed of 50 mM Tris, pH 8.3, 0.5 mg/ml BSA, 2 mM $MgCl_2$, and 200 μM each dNTP (Fig. 4). Most native Taq polymerases varied in extension rate within a factor of 4. MyTaq showed the fastest performance in either buffer, whereas Platinum Taq was the slowest. Overall, rates for the native polymerases were faster in the common buffer with an average of 31.3 nt/s compared to 25.8 nt/s for the vendor buffers. The fusion variants had the slowest extension rates. These are *Pyroccocus furiosis* (*Pfu*) polymerases fused to a double-stranded DNA binding domain intended to improve fidelity. Comparing these rates to that of PAQ5000, an unmodified *Pfu* polymerase [16], the fused domains hinder extension. The deletion

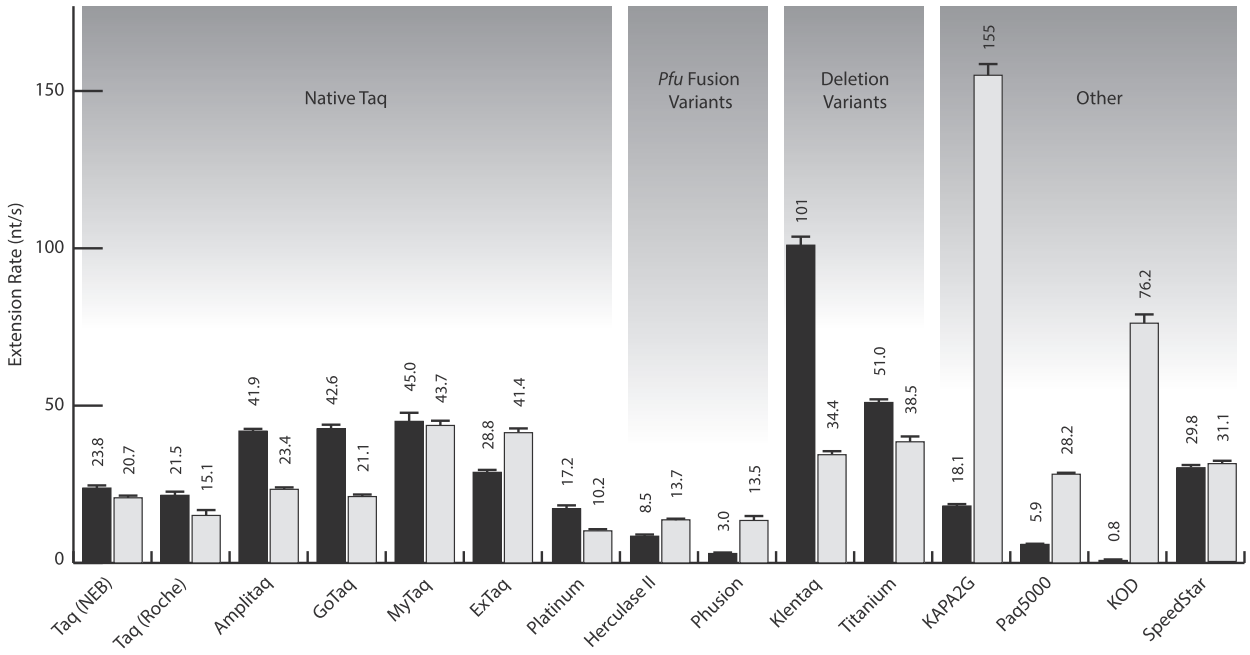

Fig.4. Extension rates of polymerases in the common (black bars) and vendor (gray bars) buffers. Extension rates were strongly influenced by buffer conditions. KOD, Klentaq I, and KAPA2G were the fastest polymerases.

variants are mutants of Taq with a deletion of the 5′ exonuclease domain [13]. These showed faster extension, especially in the common buffer. KAPA2G, an engineered variant of Taq, showed the fastest extension rates. KOD is a polymerase from the *Thermococcus kodakaraensis* KOD1 archae [15,17]. In the vendor buffer, it was the third fastest polymerase. SpeedStar is a polymerase from an organism undisclosed by the manufacturer, but extension rates are similar to those observed for the native Taq polymerases.

The specific activity was calculated from unit concentrations provided by the vendor and the mass of polymerase measured from gels. For most polymerases the specific activity was between 40,000 and 65,000 U/mg. Specific activities were higher for Amplitaq (103,300 U/mg), Platinum Taq (81,800 U/mg), and Taq (NEB) (75,600 U/mg). The specific activities for Phusion (21,900 U/mg) and KOD (5900 U/mg) were lower.

Fig. 5 contrasts the measured extension rates to calculated specific activities. These are analogous measurements of polymerase speed. Both are nucleotide incorporation rates normalized to the amount of polymerase used in the assay. In this study, extension rate is a measurement of the initial rate of nucleotide incorporation using a defined template and is expressed per molecule of polymerase. Specific activity is the rate of nucleotide incorporation into activated DNA and is expressed per milligram of the polymerase. Pearson correlation coefficients were calculated to assess the linear relationship between these two measurements. The relationship is weakly positive when measured in the common buffer and weakly negative when measured in the vendor buffers.

Buffer components

The difference between extension rates in the common versus the vendor buffers for many polymerases is striking. Nearly a 3-fold increase with the common buffer was observed for Klentaq I. The enhancement of KOD and KAPA2G in the vendor buffer approached 100-fold. It is apparent that buffer components strongly influence extension rates.

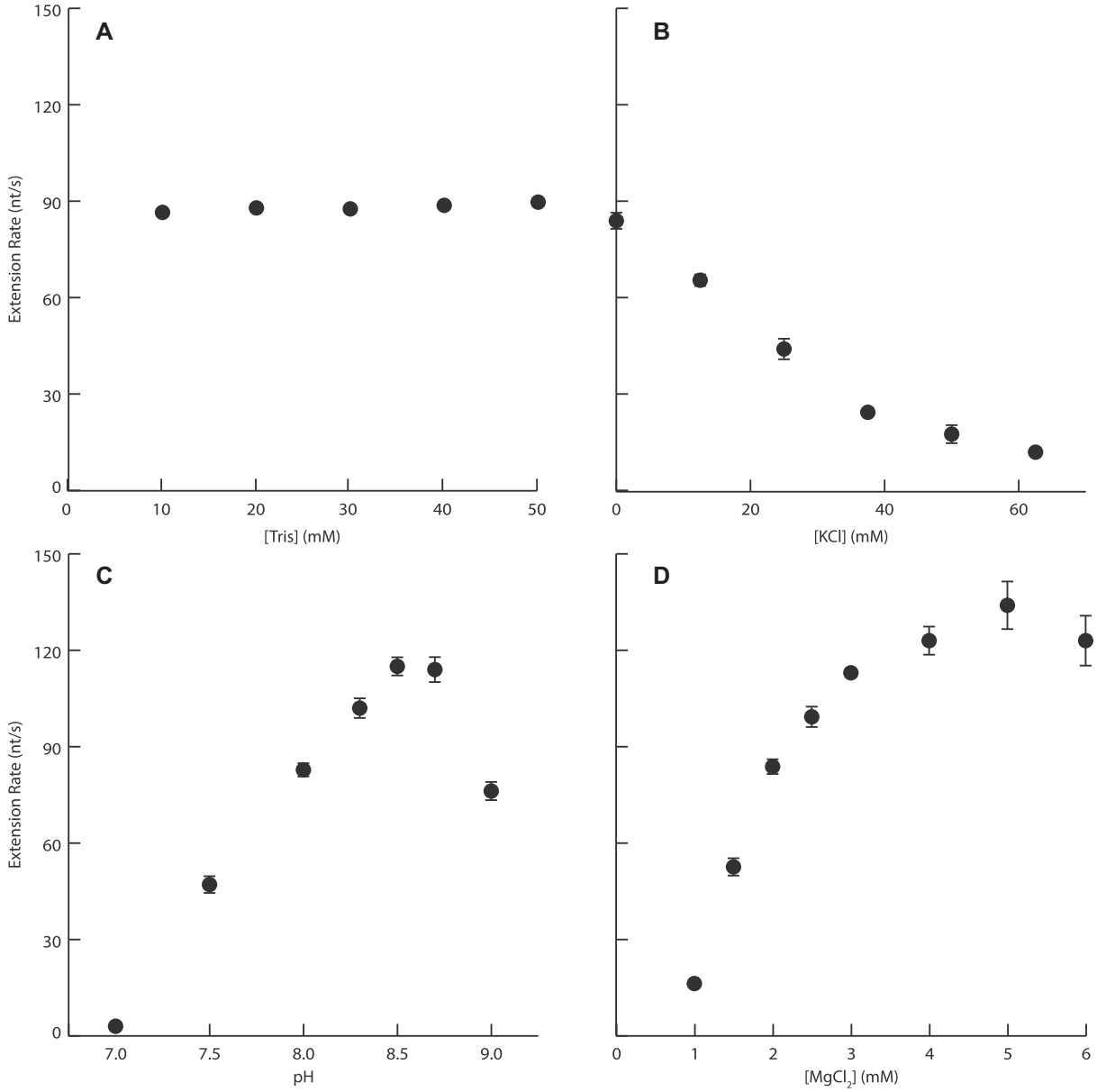

Fig.6. Effects of buffer components on KlenTaq extension rates. (A) Tris has little effect. (B) KCl strongly inhibits extension. (C) Optimal pH for extension is between 8.5 and 8.7, with rapid decreases outside these values. (D) Magnesium increases extension rates with saturation near 5 mM.

The effects of four common components of PCR buffers on extension rates were studied for Klentaq I (Fig. 6). The concentration of Tris has very little influence on extension rates (Fig. 6A). KCl concentration inhibits polymerase activity (Fig. 6B). Extension rates decline linearly between 0 and 37.5 mM with over a 70% decrease. Only 21% of total activity was measured at 50 mM. Optimal pH is between 8.5 and 8.7, with rapid decreases outside of this value (Fig. 6C). Extension is almost entirely inhibited at pH 7. Rates quickly increase with total $MgCl_2$ concentration (Fig. 6D), saturating at 5 mM. At 1.5 mM $MgCl_2$, extension rates were 43% of the maximum at 5 mM.

Discussion

The homogeneous stopped-flow assay presented here provides a simple and precise measurement of polymerase activity. The use of double-stranded DNA dyes allows continuous monitoring of extension. These dyes are commonly used in real-time PCR and eliminate the need for template modifications including covalent labels, radioactivity, or nucleotide analogs that are used in other assays. Template and buffer conditions reflect those found in PCR. Performance of polymerases can easily be tested under a variety of conditions and can aid in screening polymerases and buffer conditions for various applications.

EvaGreen was used in these studies and inhibits PCR with increasing concentration [18]. The same effect has been observed for SYBR Green I [19] and Syto 9 [20]. Comparative studies have shown that the degree of inhibition varies across dyes [18,20,21]. The effect of DNA dyes on polymerase activity has not yet been studied.

In these experiments, template was in 20-fold excess of the polymerase and each polymerase molecule bound and extended multiple templates just as in PCR. It has been shown that template binding is not a limiting step in polymerase extension [22] and is not expected to contribute to the rates measured here. Extension rates are measured in nt/s and have greater relevance to PCR than the standard unit definition. PCR amplifies templates of defined length and knowledge of the extension rates in nt/s provides better insight into the speeds obtainable during PCR. For example, this could guide optimization of thermal cycling protocols for faster and more efficient PCR.

As shown in Fig. 5, vendor-claimed specific activities correlate poorly with measured extension rate per molecule. These are both normalized measurements of the rate of nucleotide incorporation into a template and should be directly comparable. Extension rate is expressed per molecule of polymerase and specific activity is expressed per milligram of polymerase. However, these normalization approaches are similar because the molecular masses of all the polymerases in this study other than Klentaq and Titanium are within about 4%. Poor correlation between the two measurements of activity can be attributed to differences in buffer conditions and extension templates. Buffers used in traditional radiometric assays for polymerase activity vary widely and differences in pH, denaturants, and $MgCl_2$, KCl, template, and dNTP concentration may contribute to disagreement in specific activities reported for polymerases. The average specific activity calculated for the native Taq polymerases in this study is nearly fivefold lower than in a study that measured the specific activity of Taq polymerase at 292,000 U/mg [23] under different conditions. Wide variance in assay conditions complicates comparison of specific activities across studies.

Different templates also introduce variability. Radiometric assays use activated DNA, which is prepared with a variety of techniques including enzymatic digestion and mechanical shearing. This results in a heterogeneous template that does not reflect PCR conditions. PCR amplifies defined templates and is processive rather than random. One study compared the activity of polymerase with activated salmon sperm DNA and a defined template using single-stranded M13 with a primer [23]. The activity differed between the templates by about 60% at 70 °C with Taq polymerase. Activity measurements of DNA polymerases will have greater relevance to their intended use if assay conditions are similar.

Manufacturers claim superior speed for 7 of the 15 polymerases that were studied. Of the native Taq polymerases, fast extension rates are claimed only for MyTaq. This polymerase was the fastest in the category of native Taq polymerases and the fifth fastest polymerase overall (Fig. 4). Fast extension rates are claimed for both the fusion polymerases, Herculase II and Phusion, though they were among the slowest polymerases studied. Phusion claims to be 10-fold faster than unmodified *Pfu* polymerase; however, Phusion was 13.5 nt/s while 28.2 nt/s was observed for Paq5000, a native *Pfu* polymerase. Speed claims are also made for Paq5000, though this polymerase exhibited only moderate activity. KAPA2G and KOD both have fast extension rates as indicated by the manufacturer, but this is dependent on the buffer used. SpeedStar did not demonstrate superior speed as claimed, with a maximum extension rate (31.1 nt/s) only marginally faster than the average extension rate of all native Taq polymerases (28.3 nt/s). The second fastest extension rate was observed with Klentaq I, though this is not generally considered a fast polymerase.

Although the native Taq polymerases should be molecularly similar, their extension rates vary by nearly a factor of 3 in the common buffer. These differences indicate there is some variability in the activity of the same polymerase prepared under different conditions. Also, polymerase extension rates are strongly dependent on buffer conditions and the vendor buffer is not always optimal (Fig. 4). Faster speeds were observed in the common buffer (with 95% confidence) for both of the Taq polymerase deletion variants and five of the seven native Taq polymerases—Taq (NEB), Taq (Roche), Amplitaq, GoTaq, and Platinum Taq. In contrast, faster extension rates were observed in vendor buffers for KAPA2G and KOD.

KCL and $MgCl_2$ concentration and pH greatly influence extension rates. The range of optimal pH is narrow (Fig. 6C). Below the optimum of pH 8.5 to 8.7, extension rates declined about 60% with each pH unit. Above the optimum, the decline was nearly twice as rapid. Another study using a radiometric assay found that optimal pH was dependent on the buffer system [24]. Optimums for Tris, glycine, and potassium phosphate buffers ranged between pH 7.0 and 8.0. For each buffer system, rapid decreases in activity were also observed outside the optimal pH. The highest activity for the Tris buffer was at pH 7.8 and was lower than the pH optimum in our study. This buffer contained components not included in the Tris buffer we used, including 2-mercaptoethanol, KCl, and fivefold higher $MgCl_2$ concentration. This suggests that other components in addition to the buffer system may also influence the optimal pH for extension.

Extension rates continued to increase with $MgCl_2$ concentration until saturating at 5 mM (Fig. 6D). This is a higher concentration than is typically used in PCR, often because of concerns with nonspecific amplification. Greater specificity is achieved with faster thermal cycling [25–27] and higher $MgCl_2$ concentration may be most appropriate in rapid PCR.

KCl strongly inhibits extension (Fig. 6B). A number of methods have been used to study the effect of KCl concentration on polymerase activity, including sequencing [28] and measuring the rate of incorporation of radiolabeled dNTPs [23,24]. The outcome of these studies varied with optimal activity at 0 mM [28], 60 mM [24], or either 10 or 55 mM KCl depending on the template [23]. Two studies used a defined template with primers as opposed to activated DNA [23,28]. These also showed KCl inhibition with

activity greatest in the absence of KCl or at the lowest concentration studied.

The manufacturers of Taq (NEB), Taq (Roche), and Amplitaq disclose the contents of their PCR buffers. Each has 10 mM Tris, 50 mM KCl, and 1.5 mM $MgCl_2$ at pH 8.3. Speeds were similar in the vendor buffer, with extension rates between 15.1 and 23.4 nt/s. These polymerases were faster in the common buffer, presumably because of lower KCl concentration (0 mM) and higher $MgCl_2$ concentration (2 mM). In the common buffer, Klentaq is nearly twofold faster than Titanium but has slightly lower activity in the vendor buffers. This behavior is not adequately explained by the influence of the components studied. Both vendor buffers have identical $MgCl_2$ concentration. The pH of the Klentaq buffer is 9.1 and that of the Titanium buffer is 8.0. Our results in Fig. 6Cindicate that the pH's of both buffers are suboptimal. In addition, the Titanium buffer contains KCl at 16 mM, whereas the Klentaq buffer does not. Also included in the Klentaq buffer is ammonium sulfate at 16 mM, which was not studied here. Further studies of this component as well as other PCR additives will allow more complete elucidation of optimal buffer components.

Buffer conditions affect the fidelity of nucleotide incorporation. For example, the rate of base substitution error increases fivefold for Taq polymerase when increasing $MgCl_2$ concentrationfrom 1 to 5 mM [29]. In contrast, the error decreases threefold for *Pfu* polymerase over the same concentration range [30]. The pH of buffers has also been shown to positively and negatively affect fidelity [29–31]. For applications sensitive to nucleotide misincorporation, additional methods should be used to verify adequate fidelity.

Accurate measurement of polymerase activity under PCR conditions has strong implications in achieving rapid PCR. Advancements in instrumentation continue to decrease thermal cycling times, allowing amplification within a few minutes [32–34]. Realizing the full potential of PCR will require optimization of both instrumentation and chemistry. Conditions that are sufficient for standard PCR may not be well suited to very fast PCR. As cycling times are reduced, even small differences in activity may have an impact on the success of amplification. Measurements of activity are more relevant when defined in terms of nucleotides per second per molecule of polymerase rather than units per milligram. Accurate quantification of polymerase activity under optimal reaction conditions will facilitate PCR with maximum speed and efficiency.

Acknowledgment

This work was supported by a Grant from BioFire Diagnostics.

References

[1] S. Kasas, N.H. Thomson, B.L. Smith, H.G. Hansma, X. Zhu, M. Guthold, C. Bustamante, E.T. Kool, M. Kashlev, P.K. Hansma, Escherichia coli RNA polymerase activity observed using atomic force microscopy, Biochemistry 36 (1997) 461–468.
[2] D.A. Schafer, J. Gelles, M.P. Sheetz, R. Landick, Transcription by single molecules of RNA polymerase observed by light microscopy, Nature 352 (1991) 444–448.
[3] G.J. Wuite, S.B. Smith, M. Young, D. Keller, C. Bustamante, Single-molecule studies of the effect of template tension on T7 DNA polymerase activity, Nature 404 (2000) 103–106.
[4] H. Matsuno, K. Niikura, Y. Okahata, Direct monitoring kinetic studies of DNA polymerase reactions on a DNA-immobilized quartz-crystal microbalance, Chemistry 7 (2001) 3305–3312.
[5] D.L. Earnshaw, A.J. Pope, FlashPlate scintillation proximity assays for characterization and screening of DNA polymerase, primase, and helicase activities, J. Biomol. Screen. 6 (2001) 39–46.
[6] R.S. Johnson, M. Strausbauch, R. Cooper, J.K. Register, Rapid kinetic analysis of transcription elongation by *Escherichia coli* RNA polymerase, J. Mol. Biol. 381 (2008) 1106–1113.
[7] A.S. Suarez, A. Stefan, S. Lemma, E. Conte, A. Hochkoeppler, Continuous enzyme-coupled assay of phosphate- or pyrophosphate-releasing enzymes, Biotechniques 53 (2012) 99–103.
[8] M.A. Griep, Fluorescence recovery assay: a continuous assay for processive DNA polymerases applied specifically to DNA polymerase III holoenzyme, Anal. Biochem. 232 (1995) 180–189.
[9] P. Gong, G. Campagnola, O.B. Peersen, A quantitative stopped-flow fluorescence assay for measuring polymerase elongation rates, Anal. Biochem. 391 (2009) 45–55.
[10] S.P. Mestas, A.J. Sholders, O.B. Peersen, A fluorescence polarization-based screening assay for nucleic acid polymerase elongation activity, Anal. Biochem. 365 (2007) 194–200.
[11] G.Q. Tang, V.S. Anand, S.S. Patel, Fluorescence-based assay to measure the real-time kinetics of nucleotide incorporation during transcription elongation, J. Mol. Biol. 405 (2011) 666–678.
[12] G. Kallansrud, B. Ward, A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients, Anal. Biochem. 236 (1996) 134–138.
[13] W.M. Barnes, Thermostable DNA polymerase with enhanced thermostability and enhanced length and efficiency of primer extension. Official Gazette of the United States Patent and Trademark Office Patents 1176 (1995) 2601–2601.
[14] F.C. Lawyer, S. Stoffel, R.K. Saiki, K. Myambo, R. Drummond, D.H. Gelfand, Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from Thermus aquaticus, J. Biol. Chem. 264 (1989) 6427–6437.
[15] M. Takagi, M. Nishioka, H. Kakihara, M. Kitabayashi, H. Inoue, B. Kawakami, M. Oka, T. Imanaka, Characterization of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR, Appl. Environ. Microbiol. 63 (1997) 4504–4510.
[16] K.S. Lundberg, D.D. Shoemaker, M.W. Adams, J.M. Short, J.A. Sorge, E.J. Mathur, High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*, Gene 108 (1991) 1–6.
[17] M. Nishioka, H. Mizuguchi, S. Fujiwara, S. Komatsubara, M. Kitabayashi, H. Uemura, M. Takagi, T. Imanaka, Long and accurate PCR with a mixture of KOD DNA polymerase and its exonuclease deficient mutant enzyme, J. Biotechnol. 88 (2001) 141–149.
[18] F. Mao, W.Y. Leung, X. Xin, Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications, BMC Biotechnol. 7 (2007) 76.
[19] C.T. Wittwer, M.G. Herrmann, A.A. Moss, R.P. Rasmussen, Continuous fluorescence monitoring of rapid cycle DNA amplification, Biotechniques 22 (1997) 130–131. 134–138.
[20] P.T. Monis, S. Giglio, C.P. Saint, Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis, Anal. Biochem. 340 (2005) 24–34.
[21] H. Gudnason, M. Dufva, D.D. Bang, A. Wolff, Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature, Nucleic Acids Res. 35 (2007) e127.
[22] R.D. Kuchta, V. Mizrahi, P.A. Benkovic, K.A. Johnson, S.J. Benkovic, Kinetic mechanism of DNA polymerase I (Klenow), Biochemistry 26 (1987) 8410–8417.
[23] F.C. Lawyer, S. Stoffel, R.K. Saiki, S.Y. Chang, P.A. Landre, R.D. Abramson, D.H. Gelfand, High-level expression, purification, and enzymatic characterization of full-length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5′ to 3′ exonuclease activity, PCR Methods Appl. 2 (1993) 275–287.
[24] A. Chien, D.B. Edgar, J.M. Trela, Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*, J. Bacteriol. 127 (1976) 1550–1557.
[25] T.G. Mamedov, E. Pienaar, S.E. Whitney, J.R. TerMaat, G. Carvill, R. Goliath, A. Subramanian, H.J. Viljoen, A fundamental study of the PCR amplification of GC-rich DNA templates, Comput. Biol. Chem. 32 (2008) 452–457.
[26] C.T. Wittwer, D.J. Garling, Rapid cycle DNA amplification: time and temperature optimization, Biotechniques 10 (1991) 76–83.
[27] C.T. Wittwer, B.C. Marshall, G.H. Reed, J.L. Cherry, Rapid cycle allele-specific amplification: studies with the cystic fibrosis delta F508 locus, Clin. Chem. 39 (1993) 804–809.
[28] M.A. Innis, K.B. Myambo, D.H. Gelfand, M.A. Brow, DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 9436–9440.
[29] K.A. Eckert, T.A. Kunkel, High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase, Nucleic Acids Res. 18 (1990) 3739–3744.
[30] J. Cline, J.C. Braman, H.H. Hogrefe, PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases, Nucleic Acids Res. 24 (1996) 3546–3551.
[31] K.A. Eckert, T.A. Kunkel, Effect of reaction pH on the fidelity and processivity of exonuclease-deficient Klenow polymerase, J. Biol. Chem. 268 (1993) 13462–13471.
[32] M. Hashimoto, P.C. Chen, M.W. Mitchell, D.E. Nikitopoulos, S.A. Soper, M.C. Murphy, Rapid PCR in a continuous flow device, Lab Chip 4 (2004) 638–645.
[33] E.K. Wheeler, C.A. Hara, J. Frank, J. Deotte, S.B. Hall, W. Benett, C. Spadaccini, N.R. Beer, Under-three minute PCR: probing the limits of fast amplification, Analyst 136 (2011) 3707–3712.
[34] C.T. Wittwer, R.P. Rasmussen, K.M. Ririe, Rapid polymerase chain reaction and melting analysis, in: S.A. Bustin (Ed.), The PCR Revolution: Basic Technologies and Applications, Cambridge Univ. Press, New York, 2010, pp. 48–69.