

# Influence of PCR Reagents on DNA Polymerase Extension Rates Measured on Real-Time PCR Instruments

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**BACKGROUND:** Radioactive DNA polymerase activity methods are cumbersome and do not provide initial extension rates. A simple extension rate assay would enable study of basic assumptions about PCR and define the limits of rapid PCR.

**METHODS:** A continuous assay that monitors DNA polymerase extension using noncovalent DNA dyes on common real-time PCR instruments was developed. Extension rates were measured in nucleotides per second per molecule of polymerase. To initiate the reaction, a nucleotide analog was heat activated at 95 °C for 5 min, the temperature decreased to 75 °C, and fluorescence monitored until substrate exhaustion in 30–90 min.

**RESULTS:** The assay was linear with time for over 40% of the reaction and for polymerase concentrations over a 100-fold range (1–100 pmol/L). Extension rates decreased continuously with increasing monovalent cation concentrations (lithium, sodium, potassium, cesium, and ammonium). Melting-temperature depressors had variable effects. DMSO increased rates up to 33%, whereas glycerol had little effect. Betaine, formamide, and 1,2-propanediol decreased rates with increasing concentrations. Four common noncovalent DNA dyes inhibited polymerase extension. Heat-activated nucleotide analogs were 92% activated after 5 min, and hot start DNA polymerases were 73%–90% activated after 20 min.

**CONCLUSIONS:** Simple DNA extension rate assays can be performed on real-time PCR instruments. Activity is decreased by monovalent cations, DNA dyes, and most melting temperature depressors. Rational inclusion of PCR components on the basis of their effects on polymerase extension is likely to be useful in PCR, particularly rapid-cycle or fast PCR.

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Several methods have been developed to measure the activity of DNA polymerases, but complexity, time require-

ments, and specialized instrumentation have prevented their widespread use. Polymerase activity is most often characterized with radiometric assays. These assays measure the incorporation of radiolabelled deoxyribonucleotide triphosphates (dNTPs) into mechanically sheared or enzymatically digested complex genomic DNA. Activity is generally defined as the amount of enzyme required to incorporate 10 nmol of dNTPs in 30 min. However, assay conditions and unit definitions are not standardized, making comparison between measurements difficult. In addition, end-point methods do not provide initial rates and application to PCR kinetics is limited.

Other methods have been used to measure polymerase kinetics, including atomic force microscopy (1), light microscopy (2), single molecule optical trapping (3), quench flow (4), stopped flow (4–7), and quartz crystal microbalance (8). Each of these requires instrumentation not found in most laboratories and has relatively low throughput. Other assays have been adapted for more common instruments, including benchtop fluorimeters and microplate readers (9–11). However, these require covalent fluorescent labels, enzyme-coupled reactions, or saturating amounts of single-stranded DNA binding protein.

Previously we reported a continuous polymerase activity assay that uses a stopped-flow instrument (7). Nucleotide incorporation was monitored with DNA dyes typically used in real-time PCR, eliminating the need to alter reaction chemistry. In the investigation we report here, the assay was modified for use on common real-time PCR instruments. The effects of monovalent cations, melting temperature ( $T_m$ ) depressors, and DNA dyes on polymerase extension rates were measured.

## Materials and Methods

### DNA POLYMERASES

Klentaq I (purchased from Wayne M. Barnes at Washington University in St. Louis), FastStart™ (Roche), Platinum® (Life Technologies), Amplitaq® (Life Tech-

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nologies), *Taq* (New England Biolabs), GoTaq® (Promega), Titanium® *Taq* (Clontech), KAPA2G (Kapa Biosystems), MyTaq™ (Bioline), *Ex Taq*® (Clontech), *Taq* (Roche), SpeedSTAR™ (Clontech), KOD (EMD Millipore), Paq5000 (Agilent), Herculase II (Agilent), Phusion® (New England Biolabs), and Amplitaq® Gold (Life Technologies) were quantified as described previously (7) on SDS gels stained with Sypro® Orange (Life Technologies).

#### POLYMERASE EXTENSION TEMPLATE

A self-complementary oligonucleotide with the sequence tagcgaaggatgtgaacctaattcccTGCTCCCGCGGC CGatctgcCGGCCGCGGGAGCA was used as the extension template (capital letters denote self-complementary sequences). This forms a hairpin with a 14-bp stem that has a free 3' end and a 25-base overhang for extension. The oligonucleotide was ordered from Integrated DNA Technologies and purified by high-pressure liquid chromatography. Concentrations were determined by absorbance at 260 nm following digestion with purified phosphodiesterase (12).

#### POLYMERASE EXTENSION ASSAY

Extension reactions were performed with a LightCycler® 480 (Roche). Except where otherwise indicated, final concentrations were 50 mmol/L Tris (pH 8.3), 3 mmol/L MgCl<sub>2</sub>, 1× LCGreen Plus, 50 pmol/L KlenTaq I, 100 nmol/L oligonucleotide template, and 200 μmol/L of each nucleotide. CleanAmp™ dGTP (TriLink BioTechnologies) was mixed with unmodified dATP, dCTP (deoxycytidine triphosphate), and dTTP (deoxythymidine triphosphate) (Bioline) to limit extension of the template before temperature equilibration. Preliminary studies showed that the use of a single heat-activated nucleotide with 3 unmodified nucleotides increased extension rates by a mean of 14% compared to using all 4 heat-activated nucleotides. Reduced extension rates were likely caused by lower available dNTPs due to incomplete conversion of the heat-activated nucleotides.

The concentration of polymerase was reduced to 50 pmol/L to lengthen the reaction time and ensure initial rates were observed. This is at least 100-fold below typical PCR concentrations of 5–20 nmol/L (7). To reduce protein loss with serial dilutions, polymerases were diluted from the commercial stock solution immediately before extension reactions in 50 mmol/L Tris (pH 8.3), 300 μg/mL BSA, and 0.03% Tween® 20. The reaction was initiated by activating the CleanAmp dGTP at 95 °C for 5 min, followed by fluorescence monitoring of nucleotide incorporation at 75 °C. This was accomplished by programming the LightCycler 480 for repeated holds at 75 °C for 1 s with a single acquisition. Reactions were allowed to continue to ex-

haustion (30–90 min). Four replicates of each reaction were performed and the SDs reported.

#### PCR DYES AND ADDITIVES

Monovalent cations, T<sub>m</sub> depressors, DNA dyes, and MgCl<sub>2</sub> were titrated into extension reactions to determine their effects on extension rates. LiCl, NaCl, KCl, CeCl, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were included at final monovalent cation concentrations up to 50 mmol/L. Final concentrations of betaine and 1,2-propanediol up to 2.5 mol/L, DMSO and glycerol up to 10%, and formamide up to 7.5% (v/v) were examined. LCGreen® Plus (BioFire Diagnostics), EvaGreen® (Biotium), and SYBR® Green I (Life Technologies) were studied from 0.1 to 5× (approximately 0.1–5 μmol/L) (13), and Syto® 9 (Life Technologies) was examined from 0.4 to 10.0 μmol/L. MgCl<sub>2</sub> was studied at concentrations up to 6 mmol/L. In addition to KlenTaq I, MgCl<sub>2</sub> titrations were also performed for Platinum, Amplitaq, *Taq* (NEB), GoTaq, Titanium, KAPA2G, MyTaq, *Ex Taq*, *Taq* (Roche), and SpeedSTAR.

#### HOT START ACTIVATION

The activation times of 2 chemical hot start polymerases (FastStart and Amplitaq Gold) and heat-activated nucleotide analogs (CleanAmp dNTPs) were assessed. Extension reactions were performed as described above, except that unmodified dNTPs were used with the hot start polymerases and all 4 heat-activated dNTPs were used with KlenTaq I. Activation times between 5 s and 60 min at 95 °C were investigated. The concentration of polymerase was increased to 100 pmol/L with 60-min activation times to compensate for low extension rates.

#### ASSAY CALIBRATION

Linearity between fluorescence and dNTP incorporation was assumed. The first 15 s of data were excluded to eliminate artifacts of initial temperature equilibration. Polymerization was allowed to proceed to substrate exhaustion, apparent as a maximum plateau and taken as the fluorescence equivalent of 100% extension.

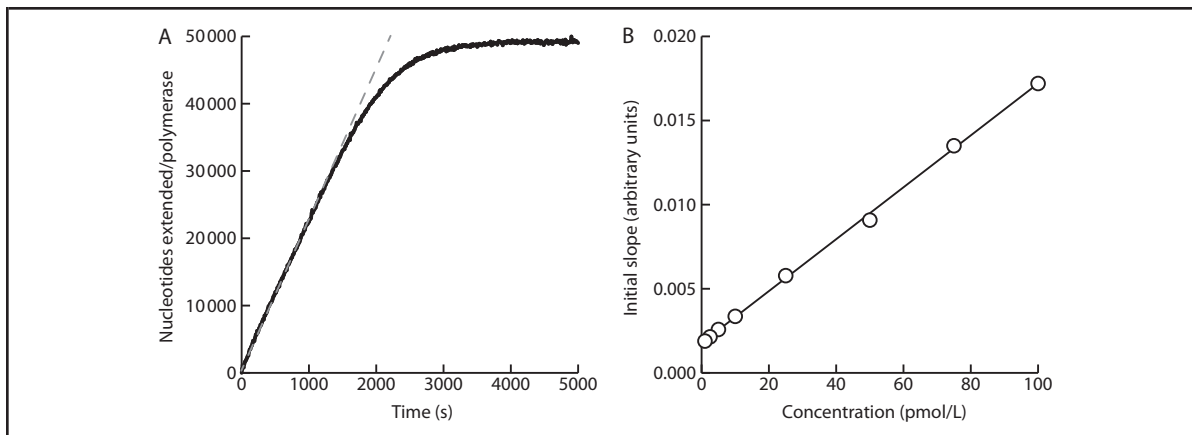
Calibration of fluorescence data allows measurement of polymerase activity. Additionally, specific activity in terms of extension rates can be calculated if polymerase quantification is performed.

#### POLYMERASE ACTIVITY

Extension curves were normalized between zero and the total number of nucleotides that can be extended, given by:

$$[\text{Template}] \times L \times V, \quad (\text{Eq. 1})$$

where [Template] is the concentration of template in nanomoles per liter, L is the extension length of the



**Fig. 1. Validation of a polymerase extension rate assay that mimics PCR with increased fluorescence from dye incorporation performed on a real-time PCR instrument.**

Increase of fluorescence signal during nucleotide incorporation is linear with time for at least 40% of reaction completion (A). Initial slope of fluorescence data is linear with polymerase concentrations from 1 to at least 100 pmol/L with  $R^2 = 0.999$  (B).

substrate in bases, and  $V$  is the volume of the reaction in liters. The initial slope of the normalized extension curves yields polymerase activity in nanomoles of nucleotides per second.

#### EXTENSION RATES

Extension curves were normalized between zero and the total number of nucleotides that each polymerase molecule can extend, given by:

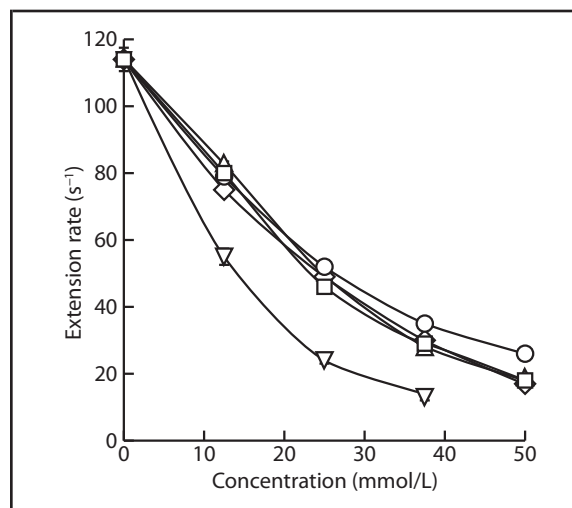
$$[\text{Template}] \times L / [\text{Poly}], \quad (\text{Eq. 2})$$

where  $[\text{Poly}]$  is the concentration of the polymerase in nanomoles per liter. With time in seconds as the  $x$  axis, the initial slope is the extension rate in nucleotides per second per molecule of polymerase, or simply seconds<sup>-1</sup>.

#### Results

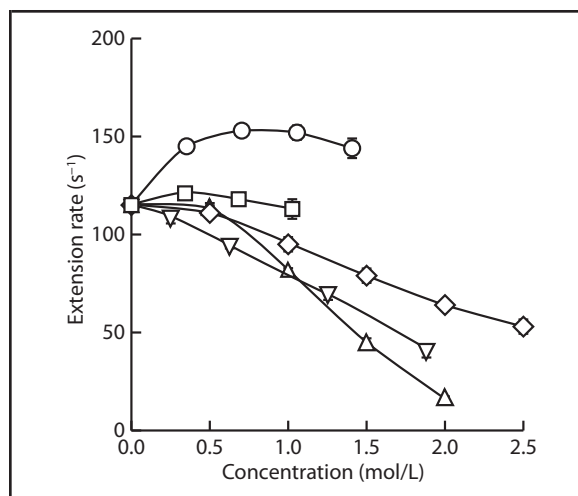
Polymerase extension was linear with time for at least 40% of the reaction (Fig. 1A). The initial slope of extension was proportional to the polymerase concentrations from 1 pmol/L to at least 100 pmol/L (Fig. 1B). Polymerases were diluted in a buffer containing detergent and BSA. When diluted without these components, variable decreases in activity were observed. This was presumably due to loss of polymerase by adsorption onto surfaces during serial dilutions. Tween 20 was used here, but similar retention of activity was obtained with IGEPAL® CA-630, Triton™ X-100, and Brij® 58. The highest extension rates were observed with detergent at 0.03% and BSA at 0.3 g/L (data not shown).

All monovalent cations decreased extension rates (Fig. 2). Lithium, sodium, potassium, and cesium had a similar effect, with a mean decrease of 57% at 25 mmol/L. Ammonium most strongly reduced rates, with a decrease of 79% at 25 mmol/L. Extension assays were also performed with divalent cations replacing magnesium. Calcium, manganese, cobalt, and zinc



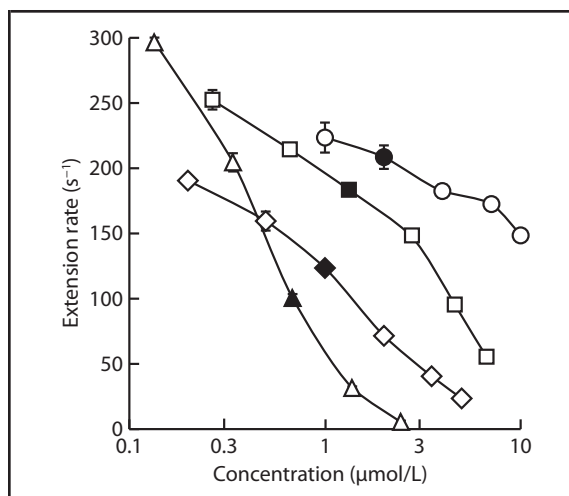
**Fig. 2. Effect of monovalent cations on extension rates.**

Cations of lithium (circles), sodium (squares), potassium (diamonds), and cesium (triangles) all produce similar decreases in rates. Cations of ammonium (inverted triangles) show the strongest inhibition.



**Fig. 3. Effect of  $T_m$  depressors on extension rates.**

DMSO (circles), glycerol (squares), betaine (diamonds), propanediol (triangles), formamide (inverted triangles). DMSO increased extension rates at concentrations up to 10% (1.4 mol/L). Glycerol had little effect on rate. Betaine, propanediol, and formamide each decreased rates with increasing concentration. Propanediol at 2.5 mol/L was not measured because of low activity.



**Fig. 4. Effect of DNA dyes on extension rates.**

Syto 9 (circles), EvaGreen (squares), LCGreen Plus (diamonds), SYBR Green I (triangles). Typical  $1\times$  concentrations used in PCR are indicated by filled markers. Extension rates were not measured for SYBR Green I at  $5\times$  (3.4  $\mu\text{mol/L}$ ) because of low activity and for Syto 9 at 0.4  $\mu\text{mol/L}$  because of low signal. Dye concentrations are plotted on a log scale.

were tested at concentrations ranging from 0.3 to 10 mmol/L. Each of these produced artifacts in fluorescence (i.e., quenching or enhancement) that precluded accurate analysis (data not shown).

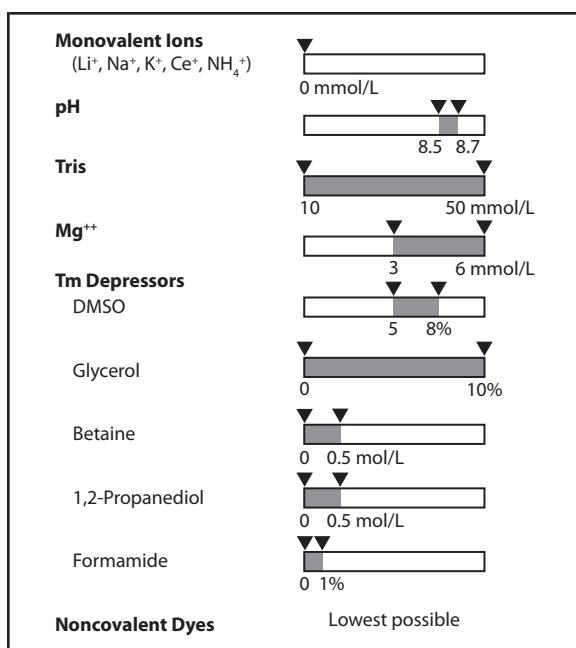
The effect of  $T_m$  depressors on extension rates is shown in Fig. 3. DMSO enhanced rates at concentrations up to 10% (1.4 mol/L), with an optimum between 5 and 7.5% (0.7 and 1.1 mol/L). Glycerol had very little effect on extension, with a small increase of 5% at 2.5% (0.3 mol/L) and a decrease of 6% at 10% (1.4 mol/L). Betaine and propanediol did not influence rates at 0.5 mol/L but showed linear decreases above this concentration. Extension rates decreased with betaine at a rate of 16% with every increase of 0.5 mol/L beyond 0.5 mol/L. Propanediol showed twice the inhibition, with a decrease of 33% per 0.5 mol/L. A small decrease in rate of 6% was observed with formamide at a 1% concentration (0.3 mol/L). At higher concentrations, extension rates also declined linearly. The rates decreased 10% for every 1% increase of the formamide concentration.

Each of the DNA dyes studied decreased polymerase extension rates, but to varying degrees (Fig. 4). SYBR Green I showed the greatest inhibition, followed by LCGreen Plus, EvaGreen, and Syto 9. Extension rates for the dyes at typical  $1\times$  concentrations varied over a 2-fold range with SYBR Green I at  $101\text{ s}^{-1}$ ,

LCGreen Plus at  $124\text{ s}^{-1}$ , EvaGreen at  $184\text{ s}^{-1}$ , and Syto 9 at  $209\text{ s}^{-1}$ .

Extension rates increased with increasing concentrations of  $\text{MgCl}_2$  up to 6 mmol/L for 9 of the 11 polymerases studied (see Fig. 1 in the Data Supplement that accompanies the online version of this report at <http://www.clinchem.org/content/vol60/issue2>).  $\text{MgCl}_2$  between 4 and 5 mmol/L produced the fastest extension rates for Titanium and KlenTaq I, 2 deletion variants of *Taq* polymerase, with decreasing rates at higher concentrations. Data from 4 additional polymerases (KOD, Paq5000, Herculaase II, and Phusion) could not be analyzed because the template was degraded by 3' to 5' exonuclease activity before acquisition. Optimal conditions for fast polymerase extension found here and in our previous study (7) are summarized in Fig. 5.

Fig. 6 shows extension rates as a function of activation time at 95 °C for heat-activated nucleotide analogs (CleanAmp dNTPs) and 2 chemical hot start polymerases (FastStart and AmpliTaq Gold). The heat-activated nucleotides were maximally active after 20 min with an extension rate of  $110\text{ s}^{-1}$ , but activation was 92% complete after 5 min. Maximal activity of the hot start polymerases required 40 min, with extension rates of  $45\text{ s}^{-1}$  for FastStart and  $28\text{ s}^{-1}$  for AmpliTaq Gold. After 20 min, activation was 90% complete for FastStart and 73% complete for AmpliTaq Gold. Consistent with prior findings (7), the maximal extension



**Fig. 5. Optimal conditions for fast polymerase extension.**

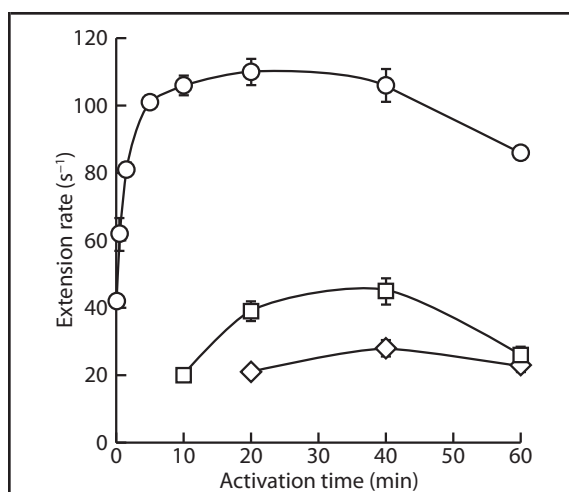
Fastest rates are obtained by eliminating monovalent ions, using high  $MgCl_2$  between 3 and 6 mmol/L, and including DMSO between 5% and 8%. Optimal pH is narrow, between 8.5 and 8.7, and Tris has little effect (7). Betaine and 1,2-propanediol are best kept below 0.5 mol/L and formamide below 1%. Noncovalent dyes are best used at the lowest concentration that produces the desired fluorescence intensity.

rate of native Taqs was lower than with deletion variants.

Compared to the previously described stopped-flow assay (7), convenience and throughput are greatly improved, with a 96/384 well plate format without compromise of accuracy or precision (data not shown). The instrument expense and setup requirements of a temperature-controlled stopped-flow apparatus also greatly exceed those of real-time PCR machines.

## Discussion

Measurement of polymerase extension rates on common real-time PCR instruments enables systematic study of numerous PCR reagents and conditions. Prior work has been hindered by laborious radiolabeled assays or expensive instrumentation. Our previously reported stopped-flow assay conveniently used noncovalent DNA dyes to measure polymerase extension (7). Conversion of the stopped-flow assay to real-time PCR instruments required (a) decreasing the concentration



**Fig. 6. Extension rates after activation with hot start polymerases and nucleotides.**

FastStart (squares), Amplitaq Gold (diamonds), hot-start nucleotide analogs (circles). FastStart showed nearly double the extension rates of Amplitaq Gold with the same activation time. The hot start nucleotides required half the activation time as the hot start polymerases for maximum activity.

of polymerase to increase the reaction time to 30–90 min, (b) using a heat-activated nucleotide analog to prevent extension during sample preparation, and (c) activating the nucleotides at 95 °C for 5 min, followed by rapid cooling to the desired extension temperature (75 °C) to ensure that initial velocities are observed. It was also critical to dilute the polymerase in detergent and BSA, presumably to prevent polymerase adsorption on vessel walls during dilution. Although we used a LightCycler 480, any instrument capable of exporting fluorescence data as a function of time can be used. We have developed an online tool to simplify analysis of the kinetic data (<https://www.dna.utah.edu/ext/ExtensionCalc.php>).

Extension rates are normalized to a single polymerase molecule and are analogous to specific activity. However, unlike prior radiometric assays, initial velocities are measured, templates are standardized, and the buffers mimic those in PCR. As a result, extension rates better reflect the kinetics seen in PCR with processive extension of a defined template for more reproducible activity measurements.

Polymerase quantification is not necessary when only activity measurements are desired. The initial slope of calibrated curves yields polymerase activity in nanomoles of nucleotides per second. This is analogous to the common definition of activity, except that

initial velocities rather than end-point rates are measured.

All monovalent cations studied decreased extension rates in a concentration-dependent manner (Fig. 2). Oddly enough, potassium chloride and ammonium sulfate are frequently found in PCR buffers. With these components, amplification appeared more specific with higher yields in some reported studies (14–16). Fig. 2 indicates that any benefit obtained from inclusion of monovalent cations in PCR does not result from enhanced extension rates.

Tm depressors are often added for PCR of GC-rich templates. We found that DMSO increases extension rates and glycerol has little effect at concentrations up to 10%. Other studies found that activity decreased 50% in the presence of 10% DMSO and 30% with 10% glycerol (17, 18). These prior studies used radioactive assays and activated salmon sperm DNA as a template. We observed a linear decrease in extension rates with formamide concentrations above 1%. A previous study showed no effect up to 10% (17). Another showed a 50% decrease in activity at 10%. Our study showed greater inhibition, with a 65% decrease in the presence of 7.5% formamide. The discrepancies in these studies suggest that greater uniformity in assay conditions and standardization of the template may improve the reproducibility of activity assays.

Betaine and propanediol both produced linear decreases in extension rates. When maximal polymerase extension rates are a concern, betaine, propanediol, and formamide should be used at the lowest concentrations possible for successful amplification.

The noncovalent DNA dyes studied here decrease polymerase extension rates (Fig. 4). Selection of the appropriate dye and concentration will depend on a number of factors, such as instrument optical requirements, desired extension rates, and post-PCR processing. For example, the fastest extension rate was observed for SYBR Green I at  $0.2\times$  ( $0.14\ \mu\text{mol/L}$ ). However, SYBR Green I does not detect heteroduplexes in high-resolution melting analysis (19, 20). Faster extension rates can be obtained with each dye by lowering the concentration, but this is also accompanied by a lower signal and may be limiting, depending on the sensitivity of the instrument.

The choice of a hot start method determines the speed of activation before PCR. Chemical hot starts show low extension rates despite very long activation times, though FastStart appears to require about half the activation time of Amplitaq Gold for the same extension rate. Rates were faster for the heat-activated nucleotide analogs at all activation times, indicating a large difference in the specific activity of chemically modified hot start polymerases and Klentaq I. For

faster PCR, heat-activated nucleotide analogs are more desirable than modified polymerases.

Routine measurements of activity and extension rates are enabled by this continuous fluorescence assay. High throughput is attained with microtiter plates, allowing simultaneous comparison of several polymerases and conditions. Components are easily optimized to identify suitable PCR reagents and storage buffers. Engineered polymerase variants can be screened for desired activity. Polymerase preparation lots can be assayed for consistent activity to ensure reproducible PCR efficiency. Polymerases screened for high extension rates are needed for rapid PCR applications. Because the extension rate is measured under PCR conditions, insight into the speed of extension obtainable during PCR can guide optimization of thermal cycling protocols for faster, more efficient amplification.

Is PCR constructed rationally, or are we following the initial choices of PCR pioneers and reluctant to change familiar reagents and ingrained protocols? Considering the data obtained from this and our prior stopped-flow study (7), extension rates are improved by high  $\text{Mg}^{2+}$  (3–6 mmol/L) and DMSO (5%–10%) in a narrow pH range (8.5–8.7) and decreased by  $\text{K}^+$ ,  $(\text{NH}_4)_2\text{SO}_4$ , dyes, and most Tm suppressors. Of course, PCR is much more than just polymerase extension. Fidelity and specificity are also crucial. Nevertheless, polymerase extension is a central factor in understanding PCR and paramount to efforts to increase its speed.

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