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Low temperature cycled PCR protocol for Klenow fragment of DNA polymerase I in the presence of proline

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ABSTRACT

A method for performing cycled PCR at low temperatures, using the thermolabile Klenow fragment of DNA polymerase I, is reported. Application of proline as a buffer additive in the range of 3.0–5.5 M remarkably increases the thermal stability of the polymerase and decreases the denaturation temperature of DNA template. This method might be applicable to a broad spectrum of thermolabile DNA polymerases in cycled PCR and other methods of DNA amplification.

Klenow fragment of DNA polymerase I from *Escherichia coli* (Klenow polymerase) (1), as well as other thermolabile DNA polymerases (2), were used for PCR in the early days of this method by fresh addition to each cycle. Introduction of thermostable enzymes added much convenience to PCR, and Klenow polymerase was not applied further in PCR. Fuller (3), using glycerol and ethylene glycol as buffer components, developed the ideology of low temperature PCR (LT-PCR); the additives should decrease DNA melting temperature (T_m) and stabilize thermolabile polymerases without inhibiting their enzymatic activity. Cycling LT-PCR with a moderately-thermostable Bca DNA polymerase was demonstrated in the presence of 40% glycerol, whereas the stability of Klenow polymerase in the presence of >40% glycerol (half-life of 3 min at 65°C in 40% glycerol) was not sufficient for cycling PCR (3).

We show here that proline, known to decrease DNA melting temperature (4), only slightly decreases Klenow polymerase enzymatic activity (Fig. 1) and is a better stabilizer of Klenow polymerase than glycerol, with a half-life of the enzyme of 21 min at 65°C in 5 M L-proline (Fig. 2). These findings have enabled a successful design of a PCR protocol for a rather GC-rich genomic DNA template as shown in Figure 3 (and Figs 1S and 2S of supplementary material). The amount of Klenow polymerase in the presented protocol (10–15 U) could be further reduced when 7-deaza-dGTP is used instead of dGTP, due to the expected decrease of denaturation temperature. Our results reveal that proline concentration in the range of 3–5.5 M is sufficient to confer stability to Klenow polymerase.

Proline could be used as a sole additive in the protocol or in combination with glycerol or any other DNA-destabilizing agents which the polymerase tolerates. Proline (up to 5.0 M) decreases the T_m of various DNAs (Fig. 4A) and leads to DNA partial 'isostabilization' (a decrease of T_m difference between GC and AT pairs, manifested by an apparent linear decrease of dT_m/dGC factor) (5), while at higher concentrations, proline destabilizes GC and AT pairs evenly (Fig. 4A and B). A complete 'isostabilization' of DNA, as in the case of betaine (6) and 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine [THP(B)] (7) (equal stability of AT and GC pairs, dT_m/dGC = 0), was not reached for proline. The T_m values of the tested natural DNAs (57–78°C) decreased to a narrow range of 28–32°C in the presence of 6.2 M proline (Fig. 4C). The partial 'isostabilization' of DNA by proline at high concentration may cause low specificity of PCR, when 20–25 bp primers are used. Primers of 30–35 bp length, used in the presented PCR protocol, were found to be effective to remedy the decreased priming specificity at high concentrations of proline, and to achieve a good selectivity of amplification.

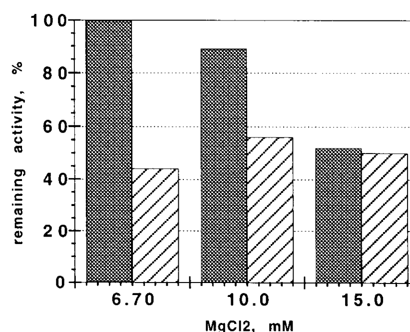

Figure 1. Klenow fragment of DNA polymerase I activity at 37°C in the absence (dark bars) and presence (hatched bars) of 5.0 M proline. The assay was performed at 37°C in 15 µl reaction mixture containing 67 mM Tris–HCl (pH 7.4), 1.0 mM 2-mercaptoethanol, 5.2 nM [α-^{32}P]dATP (3000 Ci/mmol, Amersham), 6.4 µM dATP and 320 µM of each dCTP, dGTP and dTTP, 0.6 mM of activated calf thymus DNA (Sigma), in the presence of different concentrations of $MgCl_2$, as indicated in the figure. No significant differences were found when magnesium acetate was used instead of $MgCl_2$ either in this assay or in the PCR (Fig. 3). Tris–HCl buffer, activated calf thymus DNA, [α-^{32}P]dATP, dNTP and $MgCl_2$ were added to PCR microtubes, evaporated to dryness by speed-vacuum and the respective volumes of water or proline (L-proline, 99.5%+, Fluka, from a 5.5 M stock solution) and 2-mercaptoethanol were added. Klenow polymerase (MBI Fermentas), 0.1 U, was added to the reaction mixture, incubated at 37°C for 7.5 min (a time-point within the region of linear kinetics, determined in a separate experiment is not shown), and the mixture was placed on ice. The reaction was stopped by addition of 12 µl of 50 mM EDTA and then applied on strips of chromatographic paper (Whatman No. 3). Strips were washed three times by 10% TCA, dried, and the radioactivity was counted. Each bar is an average of three experiments from which an average of three control experiments (without polymerase added) was subtracted.

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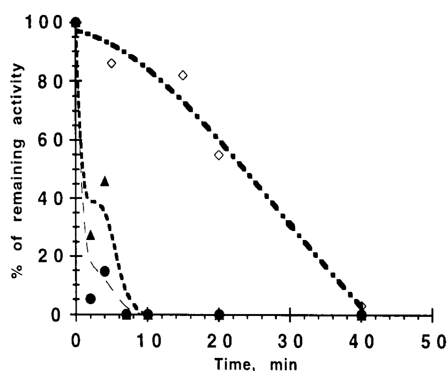

Figure 2. The time course of thermal inactivation of Klenow fragment of DNA polymerase I at 65°C in the absence (filled circles) and presence of either 5 M proline (open diamonds) or 5 M glycerol (filled triangles), expressed as percent of remaining activity. Klenow polymerase (0.5 U) was incubated at 65°C in 50 µl buffer containing: 67 mM Tris–HCl (pH 7.4 at 25°C), 2.5 ng of *Halobacterium marismortui* genomic DNA template, 4 µM of each of the dNTPs, 0.12 nM of each 30mer oligonucleotide primers (see Fig. 3), 6.7 mM $MgCl_2$ and either without or in the presence of a 5.0 M concentration of glycerol or proline. Tris–HCl buffer, template DNA, dNTP, primers and $MgCl_2$ were added to PCR mixtures, evaporated to dryness by speed-vacuum and the respective volumes of water, proline (from a 5.5 M stock solution) or glycerol (from a 5.5 M stock solution) were added. The microtubes were vortexed and Klenow polymerase was added to the samples. Aliquots (5 µl) were taken for polymerase activity assay at different periods of time as indicated in the figure and assayed as described in Figure 1 in a total reaction volume of 25 µl. Each point on the plots is an average of at least two experiments from which an average of three control experiments (without polymerase added) was subtracted. All data were normalized to the respective polymerase activities without thermal inactivation.

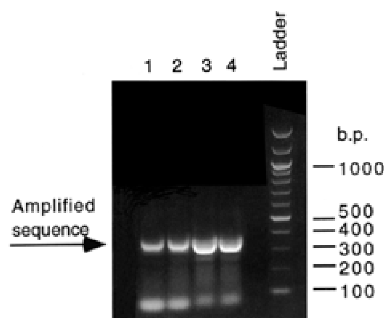

Figure 3. PCR amplification of a 349 bp fragment (66.5% GC) of *H.marismortui* genomic DNA (from position 2546 to 2843) was performed in a 25 µl reaction mixture containing 100 ng of the DNA template, 0.12 nM of each 30mer oligonucleotide primers: 5′-ATG GAA TAC GTA TAC GCT GCA CTC ATC CTG-3′ and 5′-TTA GCC GAA GAG TTC GCC GAG GCC CTC ACC-3′, 0.9 mM of each dNTP, 10 mM Tris–HCl (pH 7.4 at 25°C) and 15 mM of magnesium acetate. Tris–HCl buffer, template DNA, dNTP, primers and magnesium acetate were added to PCR microtubes from stock solutions, evaporated to dryness by speed-vacuum and dissolved in 22 µl of a proline–glycerol solution, containing 5.5 M of L-proline in a 12.5% w/v solution of glycerol in water. Klenow polymerase (10 U/µl, storage buffer contains 50% w/v glycerol) and, in order to keep constant glycerol concentration in the PCR mixtures, aliquots of glycerol solution in water (50% w/v glycerol) were added during the first primer annealing step. Lanes 1 and 2, 1.0 µl of Klenow polymerase (10 U) and 2.0 µl of the glycerol solution were added, respectively; lanes 3 and 4, 1.5 µl of Klenow polymerase (15 U) and 1.5 µl of the glycerol were added, respectively. The final concentration of L-proline in all PCR mixtures was 4.85 M and of glycerol was 17% w/v. All PCR reactions were run on an MJ Research PTC-100 machine equipped with a normal block (ramping rate is 1°C/s). Reaction mixtures were preheated for 3 min at 75°C, and then subjected to 35 thermal cycles as follows: (i) 20 s incubation at 70°C; (ii) 4 min incubation at 37°C. Reaction products were run on a 2% agarose gel and stained by ethidium bromide.

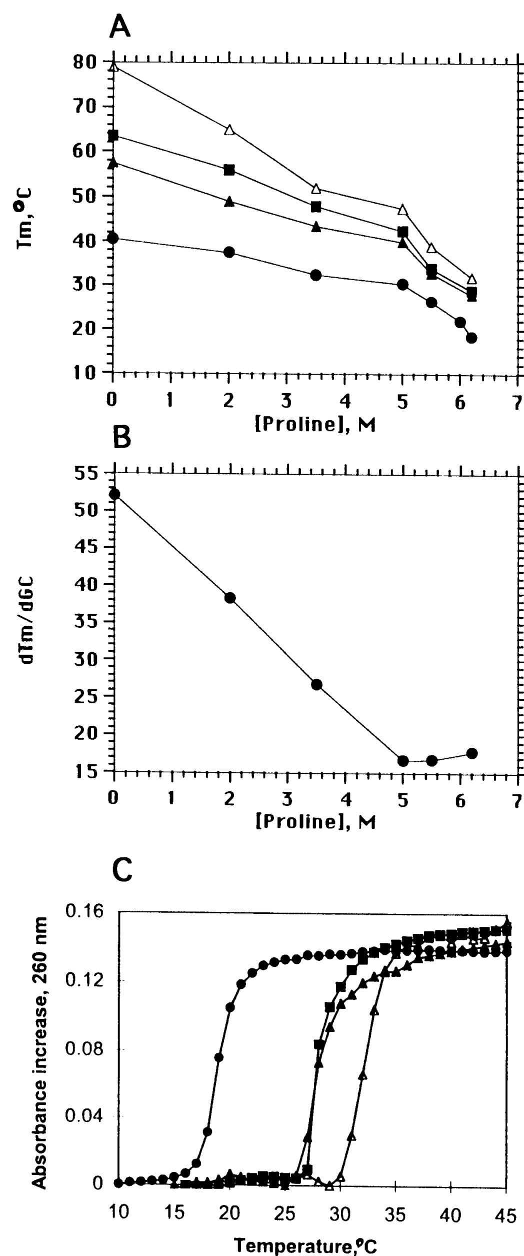

Figure 4. Variations of T_m of DNAs with different GC-content and of dT_m/dGC factor with increasing proline concentration. The DNAs were purchased from Sigma and treated as described (6). All measurements were conducted at pH 7.5 in solutions, containing 5 mM K_2HPO_4, 0.1 mM Na_2EDTA and the indicated concentration of proline (Fluka, 99.5%+). DNA samples in the presence of high concentration of proline were handled on ice to prevent melting prior to experiment. The DNA melting profiles were recorded at 260 nm on a Cary-500 Varian spectrometer equipped with a thermal controller and a thermal probe. Sample and reference quartz curvettes (1 cm path length) were heated at a rate of 0.5°C/min, and the net absorbance curves were calculated by subtraction of the sample data from the reference. Each T_m presented is an average of at least two experiments. (**A**) Changes of T_m with proline concentration for DNAs of varying GC-content. Open triangles, *Micrococcus lysodeikticus* DNA (72% GC); filled squares, calf thymus DNA (42% GC); filled triangles, *Clostridium perfringens* DNA (26% GC); filled circles, poly(dA-dT) (0% GC). (**B**) Variation of dT_m/dGC with proline concentration. T_m was taken in °C, and GC as GC(%)/100, and calculated as described (5). (**C**) Melting profiles of various DNAs in the presence of 6.2 M proline. The experiments were carried out as described in Figure 4A. The DNAs (as in Fig. 4A) were taken as solutions with OD_{260} 0.35–0.40, and their melting profiles are presented without normalization and corrections.

Besides standard PCR and DNA sequencing, the protocol could be interesting for the following methods: (i) use of Klenow polymerase in combination with contiguous hexamer primers and single-stranded DNA binding protein for a specific primer formation (8) utilizing a rather low amount of a source DNA; and (ii) low denaturation temperature cycling might enable usage of less thermostable labels for DNA sequencing or PCR. We hope that our approach will be useful for other thermolabile polymerases in PCR and other DNA amplification methods. For example, T7 DNA polymerase and its modifications, able to amplify GC-rich DNA and regions with stable secondary structures (9), could provide solutions to the cases still remaining beyond today's practical PCR and DNA sequencing capabilities, such as amplification of long CGG triplet repeat sequences (10). Introduction of T4 polymerase to cycled PCR might be of interest for the cases requiring high fidelity, e.g. for amplification of sequences present at a very low frequency requiring many cycles of amplification to be detected.

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See supplementary material available in NAR Online.

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