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High primer concentration improves PCR amplification from random pools

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The success of PCR is partly based on its exponential amplification characteristics. Nevertheless, in practice, the achievable yields are limited. Figure 1A and B shows the quantification of a typical reaction with varying but defined amounts of starting template. Every five cycles an aliquot of the reaction was removed and afterwards the amount of accumulated product quantitated. Although, initially, the amplification rates of all reactions reached the theoretical limit of 32-fold amplification during five cycles (indicated by a dashed line), a sharp decline in amplification rates was observed at later cycles. Interestingly this decline, which occurred in all reactions, did not depend on the total number of cycles in each reaction, but rather seemed to be coupled to the amount of accumulated product. In order to learn more about the basis of this phenomenon, I investigated the effect of primer concentration on PCR. For standard applications a primer concentration between 0.1 and 1 µM is recommended (1), and rarely the primers are completely used up during the reaction. Nevertheless, the primers have to compete with the accumulating product in finding their target sequence, which could become limiting for the reaction at late cycles. To test whether the ratio between primer and product concentration influences the product yield, I performed reactions with increasing amounts of primers but otherwise identical conditions. For all reactions tested, an increase in product yield at late but not early rounds of amplification was observed. Nevertheless, the level of improvement strongly

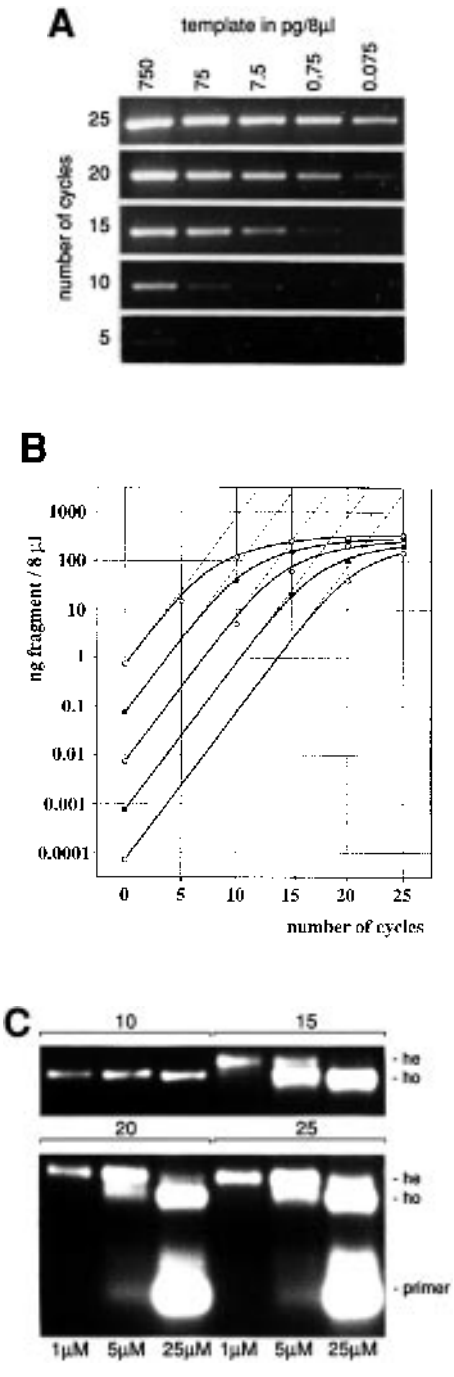

Figure 1. (A) Quantitation of a typical PCR. Between 0.01 and 100 ng of a 5.4 kb-long linearised plasmid containing the indicated amount of a 255 bp template, derived from the *Herpes simplex VP16* gene, were applied to 50 µl reactions. The PCR cycles were performed for 30 s at 94°C, 60 s at 60°C and 60 s at 72°C, with the primers: GCGGAATTCGCCCCCCCGACCGATGTCAGC and CGCGAATTCTACCCACCGTACTCGTCAAT, both at a concentration of 1 µM in a standard PCR buffer (50 mM KCl, 10 mM Tris pH 8.3, 0.01% gelatine and 1.5 mM $MgCl_2$) containing 4 U *Taq* DNA Polymerase (USB). After every five cycles, 8 µl aliquots were removed from the reactions, subsequently separated on an agarose gel and visualised by ethidium bromide staining. Only the relevant parts of the gels for the indicated number of cycles are shown. (**B**) Quantitation of this analysis according to standards present on the same gel. (**C**) PCR from random pools. One ng of a 86mer oligonucleotide containing 35 degenerate positions in the middle were amplified with the following primers matching the flanking sequences: GCGGGATCCACTCCAGGCCGGATGCT and GCGGGATCCGCCTTACACCCTGGTG. The incubation steps for each cycle were: 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. After the cycle number indicated at the top, aliquots were removed and proceeded as described above. The reactions were performed under the same conditions described above, except that three different primer concentrations (1, 5 and 25 µM) were used, as indicated at the bottom. The migration of homoduplexes (ho) and heteroduplexes (he) was verified by mixing experiments between homogeneous fragments (data not shown). Similar results were obtained for annealing temperatures of 55 and 65°C.

varied for the reactions tested: an increase between 2- and almost 20-fold was observed upon a variation in primer concentration from 1 to 20 μM (data not shown and Fig. 1C), indicating that the limiting components largely differ for individual reactions. Although a high primer concentration might increase unspecific priming when applied to complex starting material like genomic DNA (2), the experiments clearly show that primer limitation can make a critical contribution to the attenuation of amplification rates observed for late cycles of PCR.

Selection procedures from random libraries have become a powerful tool to define high affinity interactions with DNA and RNA (3). In these procedures a small amount of selected material is amplified by PCR for the next selection round, and sequential repetition leads to a stepwise enrichment of specifically interacting sequences. I used an oligonucleotide consisting of 86 nt with 35 degenerated positions for an *in vitro* binding site selection (4). Quantitative analysis of PCR from this oligo resulted in an amplification curve similar to Figure 1B. For standard primer concentrations (1 μM) the amplification rate dropped already at 100 ng product/100 μl, whereas the theoretical limit for this primer concentration would be 5.6 μg product/100 μl. Even worse, after only 15 cycles extensive reannealing converted the majority of fragments into heteroduplexes, which results in a mobility shift in the agarose gel (Fig. 1C). Such heteroduplex formation dramatically diminishes the pool of selectable sequences. Upon addition of increasing amounts of primers to the reaction the total product yield increased dramatically, indicating that the primer concentration is the main limiting factor for this reaction. Moreover, the excess of primers in the reaction prevented the formation of heteroduplexes even at late cycles of the reaction, which demonstrates the interdependence between product reannealing and the decrease in the amplification rates. Therefore, for this PCR application, the increase of primer concentration substantially improves the yield as well as the quality of the product.

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