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For a complete description of primers, PCR programs and a discussion of the PCR conditions please consult: *Andrologia* **26**: 97-106 (1994) and *Biotechniques* **23**: 504-511 (1997). Click [here](#) to get the Biotechniques paper in PDF format.

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Designing PCR programs

Basic Principles (see also Page 01)

The requirement of an optimal PCR reaction is to amplify a specific locus without any unspecific by-products. Therefore, annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA-DNA matches to occur in the reaction. For any given primer pair, the PCR program can be selected based on the composition (GC content) of the primers and the length of the expected PCR product. In the majority of the cases, products expected to be amplified are relatively small (from 0.1 to 2-3 kb). ([For long-range PCR \(amplifying products of 10 to 20-30 kb\) commercial kits are available](#)). The activity of the Taq polymerase is about 2000 nucleotides/minute at optimal temperature (72-78° C) and the extension time in the reaction can be calculated accordingly.

- As the activity of the enzyme may not be always optimal during the reaction, an easy rule applied successfully by the author was to consider an **extension time** (in minutes) equal to the number of kb of the product to be amplified (1 min for a 1 kb product, 2 min for a two kb product etc.). Later on, after the product(s) become "known", extension time may be further reduced.
- Many researchers use a 2-5 minutes **first denaturing step** before the actual cycling starts. This is supposed to help denaturing the target DNA better (especially the hard to denature templates). Also, a final **last extension time**, of 5-10 minutes, is described in many papers (supposedly to help finish the elongation of many or most PCR products initiated during the last cycle). Both these steps have been tested for a number of different loci, and, based on this experience, neither the first denaturing nor the last extension time changed in any way the outcome of the PCR reaction. Therefore, it is the author's habit not to use these steps (light blue in the table below) anymore.
- The **annealing time** can be chosen based on the melting temperature of the primers (which can be calculated using othe many applications, freely available for molecular biologists). This may work, but sometimes the results may not match the expectations. Therefore, a simple procedure used many times by the author was to use an initial annealing temperature of 54 ° C (usually good for most primers with a length around

20 bp or more). If unspecific products result, this temperature should be increased. If the reaction is specific (only the expected product is synthesized) the temperature can be used as is.

- For the seventy or so primers used during this work, a **denaturing time** of 30-60 seconds was sufficient to achieve good PCR products. Too long a denaturing time, will increase the time the Taq polymerase is subjected at high temperatures, and increases the percentage of polymerase molecules that lose their activity.
- **Number of cycles.** In general, 30 cycles should be sufficient for a usual PCR reaction. An increased number of cycles will not dramatically change the amount of product (see below).

Influence of annealing temperature and number of loci amplified

Like any other PCR, multiplex reactions should be done at a stringent enough temperature, allowing amplification of all loci of interest without "background" by-products. Although many individual loci can be specifically amplified at an annealing temperature of 56°-60° C, experiments showed that lowering the annealing temperature by 4-6° C was required for the same loci to be co-amplified in multiplex mixtures. This is demonstrated in Fig. 19 below, showing the same PCR reactions performed in conditions in which the only parameter changed was the annealing temperature. For the multiplex a PCR amplification of mixtures C and C*, an annealing temperature of 54° C seems the most appropriate, although the individual loci (for example "Y") could be amplified at 60° C. At 54° C, although some unspecific amplification probably still occurs in the multiplex reaction, it is overcome by the concurrent amplification of an increased number of specific loci and thus remains invisible.

In PCR, due to differences in base composition, length of product or secondary structure some loci are more efficiently amplified than others. When many loci are simultaneously amplified (multiplexed), the more efficiently amplified loci will negatively influence the yield of product from the less efficient loci. This phenomenon is due in part to the limited supply of enzyme and nucleotides in the PCR reaction. Therefore, in the multiplex procedure the more efficiently amplified loci compete better and take over the less efficiently amplified products, thus rendering them less visible or invisible.

(Figure 19 below, depicts a complex situation in which annealing temperature, number of simultaneously amplified loci and buffer concentration were changed in parallel reactions).

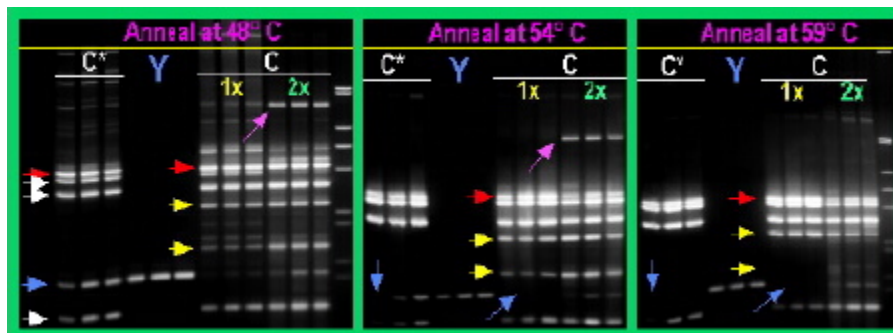


Fig. 19. Multiplex amplification of mixture C* (first three lanes in each gel), primer pair "Y" (lanes 4 to 6, blue arrows) and mixture C (lanes 7 to 12 in 1x or 2x PCR buffer) on three different template DNAs using three PCR programs differing in annealing temperature (48° C, 54° C or 59° C). Lanes 1-9 on each gel show reactions in 1x PCR buffer. Lanes 10-12 on each gel show reactions in 2x PCR buffer. Lanes 7-12 on each gel (under "1x" and "2x") were with primer mixture C. The unmarked lanes are the marker (1 kb ladder). The five

arrows to the left side of the first gel indicate the expected products of mix C* (five products). The longest specific product on each gel is marked by a red arrow. Magenta arrow indicates a strong unspecific product. Yellow arrows indicate the two extra products expected in mix C (total of seven products) compared with C*. Blue arrows indicate position of product Y (either by itself or in the multiplex mixture) in the first gel or the lack of product Y in some of the reactions from the last two gels. Multiplex amplification at 48° C shows many unspecific bands. In 1x PCR buffer, the Y product is stronger when amplified in mixture C* (5 primer pairs) than in mixture C (7 primer pairs) showing that, at least for some products, an increased number of simultaneously amplified loci can influence the yield of some individual loci. Raising the PCR buffer concentration from 1x to 2x allows a more even amplification of all specific products and helps in decrease the intensity of many longer unspecific products (compare lanes 7-9 vs. 10-12). The strong 470-480bp unspecific band (magenta arrow) seen with 2x buffer was eliminated by varying the proportion of different primers in the reaction (compare with C in Fig 1). At 59° C the Y product can be seen only when 2x buffer is used or when the locus is amplified alone.

Number of cycles

Primer mix C* was used to amplify two different genomic DNA templates, stopping the reaction after increasing numbers of cycles (Fig. 20). For the same DNA template, results were reproducible among all vials although one of the two genomic DNAs was better, probably due to the higher quality and/or amount of DNA. The most obvious variation in the amount of products was around 24 cycles (for ethidium bromide stained gels). 28-30 cycles are usually sufficient in a reaction. Little or no quantitative changes (i.e., relative amounts of PCR products) were observed with increasing cycle number up to 45. Little quantitative gain was noticed when increasing the number of cycles up to 60 (Fig. 21)

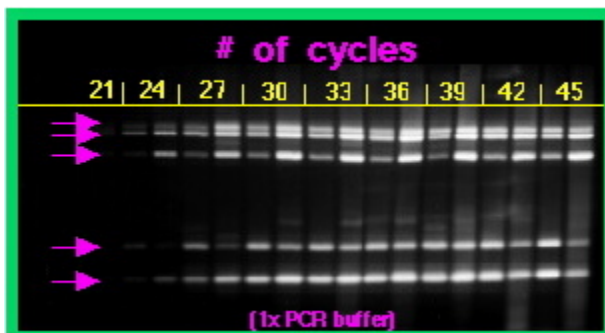


Fig. 21. Multiplex amplification of mixture C* using the same PCR program and increasing the number of cycles by units of ten (up to 60). No additional ingredients were added in the reactions.

Fig. 20. Multiplex amplification of mixture C* using two different DNA templates and increasing the numbers of cycles by units of three.

