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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.



# PCR generalities

## Standard PCR reaction mix

Consider the standard PCR reaction mix (25 µL reaction) below. All reactions are run for 30 cycles.

**Table 1.** PCR reaction components

| COMPONENT | VOLUME | FINAL CONCENTRATION |
|---|---|---|
| **1.** autoclaved ultra-filtered water (pH 7.0) | 20.7µL | - |
| **2.** 10x PCR Buffer* | 2.5µL | 1x |
| **3.** dNTPs mix (25 mM each nucleotide) | 0.2µL | 200 µM (each nucleotide) |
| **4.** primer mix (25 pmoles/µL each primer) | 0.4µL | 0.4 µM (each primer) |
| **5.** Taq DNA polymerase (native enzyme) | 0.2µL | 1 Unit/25 µL |
| **6.** genomic DNA template (100 ng/µL) | 1.0µL | 100 ng/25 µL |

* The PCR buffer used was made after the recommendations of the manufacturer/vendor (Perkin Elmer Cetus). The 10x PCR buffer contains: 500 mM KCl; 100 mM Tris-HCl (pH 8.3); 15 mM $MgCl_2$ (the final concentrations of these ingredients in the PCR mix are: 50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$).

It is useful to prepare a larger volume of this buffer (10-15ml), aliquot it and store the vials at -20 C for years.

## Pipetting and DNA template

- It is best to start pipetting water first, followed by the other ingredients. There was no difference in results when various components of the reaction were pipetted in different orders.
- To minimize the chance of primer binding to the DNA template and to prevent the

polymerase from working (even theoretically) prior to the first denaturing step, it is useful to keep the vials on ice while pipetting the ingredients of the reaction.

- Depending on the profile of the laboratory (i.e. current DNA probes in use), pipetting can be done under a laminar flow of sterile air (when plasmids are commonly used in the lab ) or at the bench (when the template DNA is genomic DNA or when a larger amount of DNA is used).
- When plasmids, phages or cosmids are used as templates in PCR, it is very important to use aerosol-resistant pipette tips, otherwise, false positive results are almost always the rule (even trace amounts of these targets provide a sufficient numer of copies to allow amplification to work). When using complex templates like genomic DNA (of which, sometimes, tens or hundreds of nanogrames are taken in one reaction) such precaution may not be necessary. However, to be on the safe side, it is a good idea to use aerosol resistant tips for every PCR reaction.
- Another problem when pipetting small volumes (1-2 µL) of a complex DNA sample (like genomic DNA) is the likelihood of differences in the amount of DNA actually taken in each PCR vial. This is illustrated in Fig. 1 below, where multiplex PCR was performed on two different genomic DNA samples.

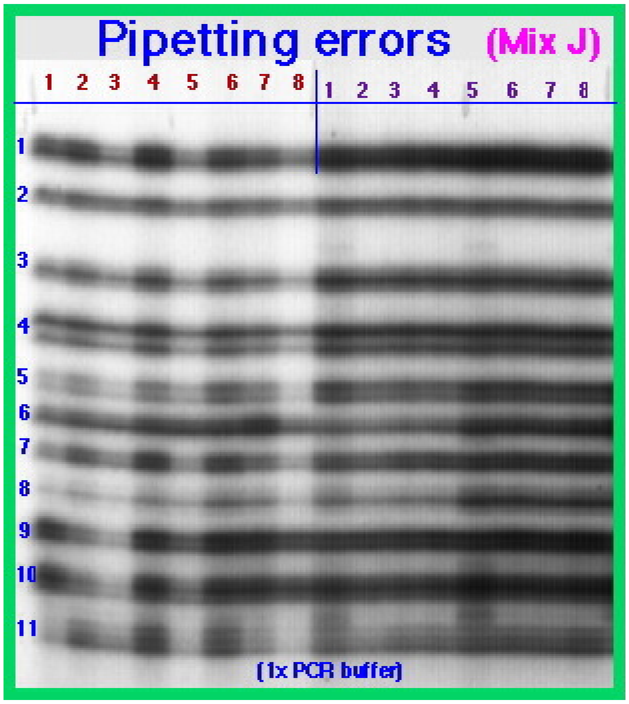


**Fig. 5. Multiplex PCR test reaction for pipetting errors.** Two genomic DNA samples (each 100 ng/ml) were used in multiplex PCR reactions with mix J, simultaneously amplifying eleven different loci (between 165 and 85 bp long). Labeling was done by adding radioactive dCTP to the reaction mix and separation of products was done on a sequencing PAA gel.
One microliter each of DNA sample A was taken in vials 1-4, and of DNA sample B in vials 5-8. On the left side, the DNA was pipetted separately in each vial. On the right side, the DNA was mixed with all other PCR ingredients and the mixture was split in equal parts in the vials.
The uneven amplification on the left side indicates that, even after thourough mixing, 1 microliter of genomic DNA may contain variable amounts of DNA. This may negatively influence interpretation of the data, especially in quantitative PCR and multiplex PCR reactions. On the right side, amplifications are much more consistent (compare 1-4 and 5-8). Small differences may be due to slight temperature differences in various places in the metal block of the

thermocycler.

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## **First PCR program** (see also Page 08)

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$^{o}$ 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 numer 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.
- An **annealing time** of 30-60 seconds was sufficient for all primer pairs tested so far. The **annealing temperature** 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 $^{o}$ C (usually good for most primers with a length around 20 bp or more). If unspecific products result, this temperature should be inccreased. If the reaction is specific (only the expected product is synthesized) the temperature can be used as is. It is desirable (but not absolutely necessary) that the two primers have a close melting temperature or Tm (say, within 5$^{o}$ C or so). If Tm difference between the two primers is high, the lower Tm can be increased by increasing the length of that primer at the 3' end (this can also keep the size of the amplified locus constant) or the 5' end.
- For the seventy or so primers used during this work, a **denaturing time** of 30-60 seconds at 94 $^{o}$ C 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).

Table 2. Designing a first PCR program.

| Name | Temperature | Time |
|---|---|---|
| First denaturing | 94 º C | optional |
| Denaturing | 94 º C | 30-60 sec |
| Annealing | 54º C | 30-60 sec |
| Extension | 72 º C | 30-90 sec |
| Last extension | 72 º C | optional |

## Thermocyclers and PCR vials

A number of different types of thermocyclers and PCR vials were used and tested in time. Some potentially useful observations were made:

- The same PCR program will work slightly different on different thermocyclers (temperature and time profiles may be different, depending on the construction), therefore the PCR results using the same primer pair may vary. However, with proper cycling adjustments, the same results can be obtained on most (or any) thermocyclers.
- Many new PCR machines accomodate the thin-walled 0.2 ml PCR vials (and/or 96 wells microtiter dishes) in their metal block. For this type of vials, the differences in results from vial to vial are usually negligable (contact between the metal and plastic is very good and aided by the downward pressure from the heated lid). Older machine type could accomodate 0.5 or 1.5 ml vials. Because of slight differences in shape and wall thickness among manufacturers, contact between the vials and the metal block of the thermocycler was not always perfect, often resulting in reduced or no amplification. Such a case is exemplified in the figure below. Some manufacturers offer machines controlling the temperature of a small waterbath in which the PCR vials rest during the reaction. In such cases, due to the good thermal change between water and plastic, variations in PCR results are very little (if any).

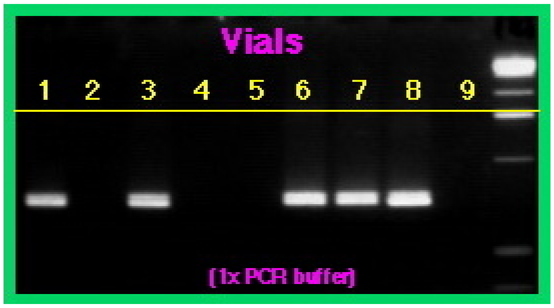


**Fig. 6. Variation in amplification due to lack of proper contact between the metal block and some vials.** A PCR mixture containing all ingredients was split in nine equal parts in the same typ/brand of vials, and the tubes were placed in different wells of the metal block of a thermocycler. Reactions 2, 4, 5 and 9 were negative. The same aspect was not reproducible: in another experiment, reactions in other positions could become negative. This was explained by slight variations in vial construction (wall shape or thickness) but not by temperature variations in the metal block (when the aspect should have been reproducible).