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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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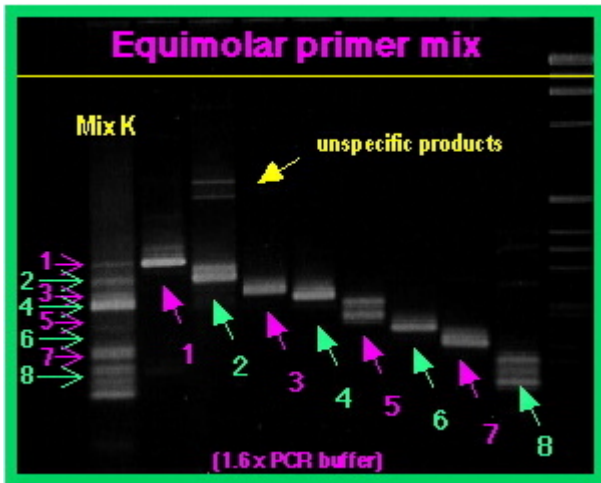
## Choosing/designing PCR primers

In designing primers for PCR, the following steps/rules were tested and proven to be useful:

- **length** of individual primers between 18-24 bases. Longer primers (30-35 bp) seem to work in more similar cycling conditions compared with shorter primers, and can make multiplexing easier (see picuters below).
- it is desirable (but not absolutely necessary) that the two primers have a close melting temperature or  $T_m$  (say, within 5° C or so). If  $T_m$  difference between the two primers is high, the lower  $T_m$  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.
- **purine:pyrimidine content** around 1:1 (maybe 40-60%)
- if possible, primer sequence should **start and end** with 1-2 GC pairs
- each primer pair should be tested for **primer-primer interactions**. For this purpose a useful Macintosh program is "CPrimer", a freeware available at <ftp.bio.indiana.edu>. This program also provides the **melting** temperature for the sequences entered, thus helping in designing PCR programs. Very convenient, some web sites offer programs that can be used directly on those sites to do the same functions: (search for optimal primers, melting temperatures).
- primer sequences should be aligned with all DNA sequences entered in the databases (using **BLAST** programs) and checked for similarities with repetitive sequences or with other loci, elsewhere in the genome. If two loci are very similar (for example across species) it is useful to design the primers so that at least 1-2 bases at the 3' end are specific for the locus to be amplified
- **cycling** conditions and **buffer** concentrations should be adjusted for each primer pair, so that amplification of the desired locus is specific, with no secondary products (see other pages). If this is not possible, the sequences of the primers should be either elongated with 4-5 bases or simply, changed entirely.

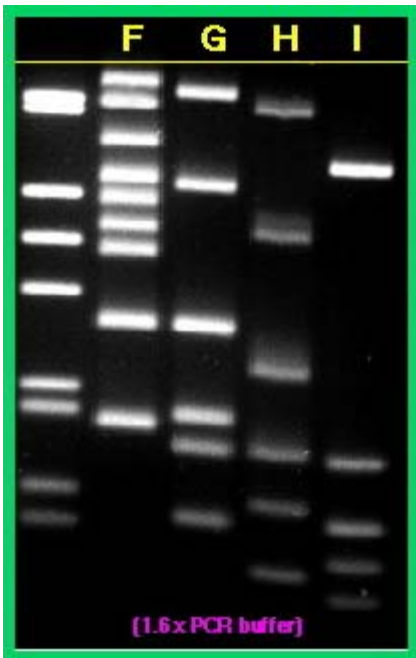
**Fig. 7. Multiplex PCR using primers 18-24 bp long**

When PCR reaction Eight individual loci are amplified with similar intensities when the primer



pairs are used separately. When equimolar amount of these primers are mixed together for a multiplex reaction (Mix K), some of the products are much weaker (#1, #2, #5, #6) than other. In this case, primers had "usual" length, between 18-24bp.

(primers used in this case amplify polymorphic loci, explaining the "double" or "triple" bands as seen on a regular agarose gel)



**Fig. 8. Multiplex PCR using primers 30-35 bp long**

Compared to the figure above, in this case the primers used for multiplexing were longer than 30 bp (up to 37 bp). Equimolar amounts of primer were used and all loci were amplified with comparable intensities in each reaction.