

Setting Up PCR Reactions

1) The PCR procedure is very sensitive to contaminants which requires that care is taken in preventing contamination of stock solutions and that proper controls be run for each experiment to ensure that the band one sees on the gel is what you expect it to be. The first time a given set of primers are used together you should include the following set of controls which are run on the gel along with the rest of the reactions. After you are sure that your product band is due to the presence of both primers, you may get away with only the "no template" control which should **always** be included in any PCR experiment to check for contamination of any of the reagents:

- a) Primer #1 with template
- b) Primer #2 with template
- c) Both primers, no template
- d) Template with both primers

2) PCR is highly sensitive to trace contamination. Therefore, it is very important that care is taken to avoid contaminating reagents that go into the PCR reactions. The most important thing not to contaminate is the oligonucleotide stocks themselves--since these are expensive to replace (i.e. resynthesize). When using oligonucleotide stock solutions use a set of pipetman which is unlikely to have come in contact with your template DNA (or the target sequence),. It is best to make a working stock dilution at a convenient concentration which can be replaced if contaminated (see below).

3) A typical reaction with non-degenerate oligonucleotides of 17-30 nt in length is shown below. The final reaction volume is 100 μ l in a sterile 0.5 ml eppendorf tube. The contents include:

- a) 10 μ l 10X Taq Polymerase Buffer (in small box in freezer)
- b) 10 μ l of 2 mM dNTPs (this is 10X concentration)
- c) 200 ng of each oligo (see below for degenerate oligos)
- d) Template DNA: 1-10 ng of cloned DNA (aprox. 1 μ l of miniprep DNA) or 1 μ g of genomic yeast DNA (Jiang's stock at 200 ng/ μ l)
- e) Add ddH₂O to 100 μ l (add this first).

- f) Add 0.8 μ l of Taq Polymerase (Boehringer 5 U/ μ l). See below about when to add this.

4) Degenerate oligos are normally used at 400-1000 ng per oligo per 100 μ l reaction, depending on the degeneracy of the oligos and their tendency to form "primer dimers". It is best to try two concentrations and determine which works best empirically.

4) In all cases it is best to use what is known as the "hot start" technique: this simply means adding the Taq polymerase while the tubes are in the thermal cycler, during the first annealing cycle after the initial denaturing cycle. This minimizes the possibility of getting junk priming (including primer dimers) during the setup of the reactions. In order to use a hot start the thermal cycler must be programmed to give you time to add the polymerase during the first annealing cycle. On Levinson's thermal cycler we use program #14 for most applications. This is set for a hot start and varies according to who used it last as to the annealing temperature and the number of cycles. It starts by denaturing for 5 min at 96°C and then goes to the initial annealing temperature for 10 min to allow time to add the polymerase. For cloned DNA the a minimum number of cycles is best, so as to avoid introducing mutations; Usually 20-25 cycles is sufficient in this case. For genomic DNA it is better to use 30-40 cycles. If you are going to clone the products of the reaction it is important that you do at least two independent reactions and carry the subcloning through in duplicate. In this way if you ever suspect any PCR-derived mutation was introduced you can always check the other subclone.

5) After finishing, remove 15 μ l from each tube for gel analysis and keep the remainder at 4 °C until you know the results. Make sure that you have good size standards in the region that you expect your product to be. Also make sure you have the right type and percentage of agarose gel to run them on. For example a 1% gel is good for products between 0.5 and 2.0 Kb while a 2% gel is best for things 0.2-0.5 Kb.

If the reactions look good then dilute the remaining sample to 200 μ l for each tube with TE. Extract the diluted samples once with PCI and once with chloroform:isoamyl alcohol and remove the aqueous layer to clean sterile tubes after each extraction. Precipitate the DNA by adding 0.4 volume of 10M NH₄OAc and then 2 volumes (including the added NH₄OAc) of 100% ice cold EtOH and. Let sit 30 min at -20°C (or overnight) and then pellet the DNA by spinning for 15 min at 4°C in an eppendorf microfuge. Wash the pellet once with 75% ice cold EtOH and let the EtOH evaporate. Resuspend in 50 μ l of TE pH 8.0.

5) Digest 20 μ l of the resuspended DNA with appropriate restriction enzymes to give the correct sticky ends for ligation. Many restriction enzymes do not cut as effeciently when they are close to the end of a DNA fragment (see NEB catalog appendix) and therefore may require more time and/or enzyme to give complete cutting. Purify the correct fragments by gel purification (remember to do this in duplicate with two identical but independent PCR reactions if possible) using the freeze-squeeze method. Subclone into the appropriate vector as usual. Since PCR fragments are sometimes difficult to subclone you may want to put them into a plasmid with a blue/white selection (such as pUC18 or Bluescript) first and then subclone them out of this; however, this is usually not necessary if the restriction sites on each end of the PCR fragment are different and the vector has been digested completely with both enzymes prior to gel-purification. Always do a control ligation without any insert to determine the background of non-recombinants. If the PCR fragment has ligated into the vector well there should be a 2-10 fold more colonies from the the ligation with insert than the one without.