

Polymerase chain reaction (PCR)

NOTES: As with all molecular biology and microbiology work, aseptic technique is crucial. Clean your bench, tube racks, and pipettes with 80% ethanol before starting, and work carefully to ensure you don't contaminate the PCR reagents or mixtures with microbes, DNA, or enzymes from your hands or the bench or the ice bucket etc etc. Ensure pipette tips, Eppi tubes and PCR tubes are sterile.

Different PCRs behave differently. Some PCRs are very straightforward, and a trained monkey could make these work, while others are very challenging, and even a highly skilled postdoc might struggle. The factors impacting on the ease / difficulty of PCR include the following:

Type of DNA Template: the easiest template is a plasmid or PCR product containing your sequence of interest. These are like a 'positive control'– they are very clean, and have a very high ratio of target sequence to non-target sequence. A medium difficulty template would be genomic DNA from a single microbe. The most difficult template is metagenomic DNA from a complex mixture of microbes – this has a very low signal:noise ratio, ie. there are lots of places where the primers can bind incorrectly and only a few places they can bind correctly.

Purity of DNA template: highly purified DNA (e.g. FastPrep or CTAB method) will amplify much better than crude DNA (e.g. boiled cells), which will amplify better than whole cells, which will amplify better than a complex and dirty template (e.g whole soil) (this is unlikely to work at all!). That said, you can certainly do PCR on whole cells of most gram negative bacteria, including E.coli (this is known as 'colony PCR'.), and this is very useful for rapidly screening clones.

Type of primers: specific primers work well, degenerate primers work less well, and the higher the degeneracy, the worse they perform. Primers that are designed well (no dimers/hairpins or only weak dimers/hairpins) will work better than primers that are poorly designed (strong dimers/hairpins). Primers that have lots of junk at the 5' end like long non-target sequences or fluorochromes will perform worse than primers that are exactly the same as the template. Primers with mismatches to the template may still work, but the more mismatches present, the worse they will work, and the closer the mismatches are to the 3' end, the more serious the problems will be.

Type of target gene, and its copy number: If your target gene is on a plasmid, it will amplify more easily than a chromosomal gene (all other things being equal). If your gene has many copies in the template DNA, it will amplify better than a gene which is very rare. If your target gene is 16S rDNA or some other highly-conserved gene, and your primers are 'universal' primers, you might expect problems with contamination, since this gene is *everywhere*, including on your hands, on the bench, in your pipette etc. (the negative control with no DNA added is critical in this case!). On the other hand, if your target gene is only found in your particular favourite and unusual organism, then contamination will be less of a concern.

Size of PCR product: The smaller the product, the easier the PCR. Product size should not cause problems up to ~ 1 kb, but as you go larger than this, the PCR will become increasingly challenging. This also depends a lot on the type of polymerase used (Phusion is better than Pfu which is better than Taq, in terms of getting large products). Anything larger than ~5 kb is going to be difficult to amplify, even for an experienced user – success here requires using a fancy polymerase, *excellent* primers (well-designed, no dimers/hairpins), clean and high-quality template with high signal:noise ratio, fresh reagents, and excellent hands-on technique. In theory, PCR products up to ~20 kb are possible, but these are extremely difficult to obtain in practice.

Protocol for setup of PCRs

NOTES: The usual PCR is 25 μ l volume, this is appropriate for “screening” purposes. However, if you are trying to make a lot of PCR product for cloning, then scale reactions up to 50 μ l per tube. A PCR that is working well should give you about 50 ng of product per μ l of reaction, thus 2.5 μ g per 50 μ l. For a cloning experiment, if you prepare 8 x 50 μ l PCRs, this should give you plenty of insert DNA (10-20 μ g), even if your PCR is not working at high efficiency. This is way in excess of the theoretical requirement, but allows for losses due to subsequent purification steps, and the likelihood that you may need to repeat the experiment a few times :-/

EXAMPLE REACTION MIX (per 25 μ l)

- 10 x buffer: 2.5 μ l (\rightarrow 1 x)
- sterile milliQ water: 20 μ l
- dNTPs (10 mM): 0.5 μ l (\rightarrow 200 μ M)
- primer #1 (50 μ M): 0.25 μ l (\rightarrow 0.5 μ M)
- primer #2 (50 μ M): 0.25 μ l (\rightarrow 0.5 μ M)
- polymerase* (5 U/ μ l): 0.25 μ l (\rightarrow 0.05 U / μ l)
(DNA template: 1 μ l)

FOR 8 REACTIONS** (1 strip of tubes)

- 10 x buffer: 22.5 μ l
- sterile milliQ water: 182 μ l
- dNTPs (10 mM): 4.5 μ l
- primer #1 (50 μ M): 2.3 μ l
- primer #2 (50 μ M): 2.3 μ l
- polymerase (5 U/ μ l): 2.3 μ l
(DNA template: 8 x 1 μ l)

* This means thermostable DNA pol e.g. Taq, Pfu, Phusion, Q5, etc. but NOT Klenow or T4 pol.

** Calculations done as if 9 x 25 μ l reactions were being prepared, to ensure mix doesn't run out.

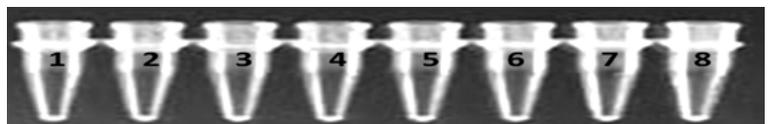
Template considerations: The correct amount of DNA template depends on the type of sample. If you are using a plasmid, you only need a tiny bit (~1 ng) because the ratio of target:non-target sequences is very high. For genomic DNA of a single microbe, a more usual amount would be 10-20 ng per reaction. For metagenomic DNA, you may need to go even higher (100 ng per reaction), although note that there are diminishing returns here due to the presence of PCR inhibitors in many templates that will interfere with PCR. For complex templates like soil DNA, the optimum concentration of template often needs to be determined experimentally. More is not always better; in some cases, diluting the template (e.g. 1/10) will yield a band when undiluted template gives no band at all.

SETUP OF MASTER MIX AND REACTIONS

1. Exact setup of PCR depends on which element is the variable in the reaction. Usually this is the DNA template, but sometimes it is the primer. The ‘Master Mix’ should be made to include everything except the variable being tested. The below protocol and above recipes are written assuming that this variable is the DNA; you need to modify if the same template is being tested with multiple primers.

2. Calculate how many PCRs you need altogether, and thus the total volume of master mix required. Its important to make more than you need (~10% more), as pipetting errors will always change the expected volumes a little bit, e.g. the above recipe for 8 PCRs is calculated as if it were 9 PCRs. Write up the exact recipe that you need in your lab book.

3. Label your strip tubes on the side, at the top, as shown below. If you label the lids or the bottom of the tube, the labels tend to come off during thermocycling. Place the tubes in a tube-rack (a 96-well microtitre plate works well). Leave the lids off the tubes – you are more likely to contaminate the reactions via excessive opening and closing of tubes than from stuff falling in from the air.



4. Retrieve the PCR reagents from the -20°C freezer and thaw them (except polymerase, this remains liquid even at -20°C due to glycerol in buffer). Its important that the 10x buffer, dNTPs, and primers are thoroughly thawed out before use. Give them a brief vortex or flick to mix. These reagents don't need to be kept on ice during the time needed to set up the PCR, but don't leave them on the bench longer than needed.

5. Prepare the master mix in a sterile Eppi tube, containing everything except the DNA template. Add ingredients in the order listed in the recipes above. Some folks will insist that this must be done on ice, opinions differ. It is true that you will minimise premature polymerase activity by setting up on ice, but whether this really makes a difference for most PCRs is arguable. Setup on ice is supposedly more important for Pfu and Phusion which contain 3'-5' exonuclease activity which can 'eat' your primers during setup. If you set up on ice, risks of contamination from the ice itself must be managed.

6. Aliquot out the master mix between all of the PCR tubes, putting 25 µl in each tube.

7a. (DNA template in solution) Add DNA template (1 µl) to all the tubes, being very careful to match the sample #'s to the labels on the tubes. You need to concentrate your attention here and really focus in order to avoid missing a tube or putting two templates in the same tube. Be careful also to change tips between every DNA template.

7b. (colony PCR: DNA in cells). Touch a white tip onto a colony or patch of growth, and pick up a *small* amount of cells. It should be just enough so that you can see there are cells there, not much more than this. Then dip the tip in and out of the master mix in the first PCR tube approx 5 times, and discard the tip. You don't need to wipe the whole chunk of growth on the inside of the tube, just the dipping in and out action will dislodge enough cells to give you enough template DNA. Less is more in this case ! Repeat for other colonies/patches.

8. Put lids on tubes, ensure they are snapped on tight, place immediately in thermocycler. Double check your program parameters before starting. See below for detailed thermocycling instructions.

9. Return all reagents to the freezer.

< thermocycling – see below >

10. After thermocycling is completed, run out your PCRs on an agarose gel to check their size and yield. Typically, we would load 5 µl of PCR mixture. This should give a very strong band if the PCR has been successful. This amount (5 µl) may be hard to quantify if using GelRed in the gel due to smearing; if you are using this staining method, its not a bad idea to run a few different amounts (e.g. 2 µl and 5 µl) to ensure you can accurately size the product.

11. If you are intending to use the PCR product for cloning, check for the presence of non-specific bands (usually fainter, smaller bands than the expected one) – if these are numerous and/or strong, you may need to try a higher annealing temp, or revisit primer design, before the product can be cloned. Alternatively, you can cut out the desired band from the agarose <see other protocol>.

THERMOCYCLING

A standard thermocycling protocol is given below (for Taq polymerase)

Initial denaturation:	95°C, 5 min	
Denaturation:	95°C, 30 sec	} 30 cycles
Annealing:	<u>60°C</u> , 30 sec	
Extension:	72°C, <u>1 min</u>	
Final extension:	72°C, 10 min	
Hold:	15°C	

The numbers that are underlined and in bold indicate variables that need to be optimised for every individual PCR. Sometimes, other variables may also need to be modified too, as below

Annealing temperature: This is typically set at 5°C lower than the melting temperature (T_m) of the primers. If these T_m 's are different, use the lower one for this calculation. This calculation is very crude, and it is best to optimise annealing temperature experimentally by doing a 'gradient PCR', e.g. testing an annealing temp range from 5° lower to 5° higher than the predicted best temp. Aim to make primers with T_m 's around 65°C, which gives a predicted annealing temp around 60°C. The usable range for annealing temp's is approx 50°C – 70°C.

Extension time: This is set primarily by the length of the desired PCR product. For Taq polymerase, this is 1 minute of extension per kb of product. However, note that different polymerases require different extension times: Pfu needs 1.5 min per kb, and Phusion needs 0.5 min per kb. If you use a longer extension time than necessary, you risk increasing the yield of non-specific products; if you use a shorter extension time than necessary, you risk a low yield of the desired product.

Denaturation: The optimal denaturation conditions for Phusion pol are different than for Taq or Pfu. Change the initial denaturation to 98°C - 30 sec, and the denaturation in each cycle to 98°C - 10 sec.

Number of cycles: This can be increased to 35 or even 40 cycles to give a higher yield of product, but this risks introducing more mutations into the PCR amplicons, and also gives a higher chance of secondary non-specific products being formed. Conversely, the # of cycles can be reduced to 25, which will minimise mutations and non-specific products, but will also lower yields.

Hold temperature: Its OK to leave your PCRs in the machine overnight. While some protocols recommend fridge temp (4°C) for this 'Hold' step, this is actually bad for the machine – it will accumulate condensation on the block, which will degrade the block over time. A good compromise is to set the hold temp to 15°C, which keeps the samples cool(-ish), maintaining them in good condition, but is not cool enough to cause condensation to form.

PCR Enhancer Additions: Two enhancers that might be worth testing for problematic PCRs are Bovine Serum Albumin (BSA) and dimethylsulfoxide (DMSO). BSA is good for binding inhibitors, and is typically added at 0.5 mg/ml final conc. from a 10 mg/ml stock (=1.25 µl per 25 µl reaction). DMSO is good for enabling amplification of GC-rich templates, and is typically added at 1 µl of neat DMSO per 25 µl reaction (=4%).