# 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 ~ 2 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 (Q5 or Phusion are better than Pfu which is better than Taq, in terms of getting large products). Anything larger than ~5 kb 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 µl 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 µl per tube. A PCR that is working well should give you about 50 ng of product per µl of reaction, thus 2.5 µg per 50 µl. For a cloning experiment, if you prepare 4 x 50 µl PCRs, this should give you plenty of insert DNA (~10 µg). 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 µl)**

- 10 x buffer: 2.5 µl (→ 1 x)
- sterile milliQ water: 20 µl
- dNTPs (10 mM): 0.5 µl (→ 200 µM)
- primer #1 (50 µM): 0.25 µl (→ 0.5 µM)
- primer #2 (50 µM): 0.25 µl (→ 0.5 µM)
- polymerase* (5 U/µl): 0.25 µl (→ 0.05 U / µl)

( DNA template: 1 µl )

**FOR 8 REACTIONS** (1 strip of tubes)**

- 10 x buffer: 22.5 µl
- sterile milliQ water: 182 µl
- dNTPs (10 mM): 4.5 µl
- primer #1 (50 µM): 2.3 µl
- primer #2 (50 µM): 2.3 µl
- polymerase (5 U/µl): 2.3 µl

( DNA template: 8 x 1 µl )

* 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 µl reactions were being prepared, to ensure mix doesn't run out.

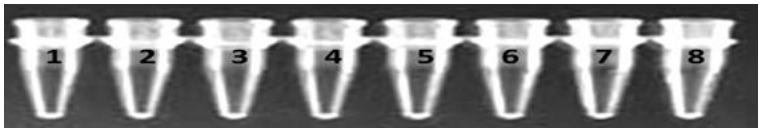
Template considerations: The correct amount of DNA template depends on the type of sample. If you are using a plasmid, you only need a tiny bit (~1 ng) because the ratio of target:non-target sequences is very high. For genomic DNA of a single microbe, a more usual amount would be 10-20 ng per reaction. 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>.

12. Store the PCR products in the cold (4°C) or frozen (-20°C). Cold is better if you are going to use it a lot (repeated freeze/thaw cycles are bad for DNA), but frozen is better for very long-term storage.

**THERMOCYCLING**

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

| | | |
|---|---|---|
| Initial denaturation: | 95°C, 5 min | |
| Denaturation: | 95°C, 15 sec | 30 cycles |
| Annealing: | **55°C**, 30 sec | |
| Extension: | 72°C, **1 min** | |
| Final extension: | 72°C, 5 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 (Tm) of the primers. If these Tm'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 Tm's 60-65°C, which gives a predicted annealing temp 55-60°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%).

## PCR primer design

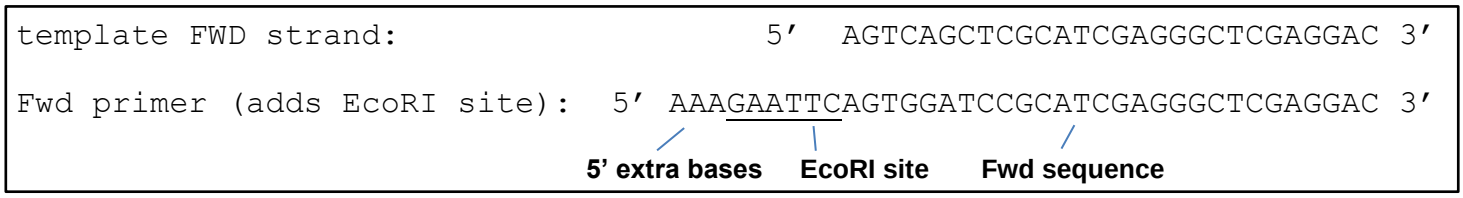
Designing good primers is crucial to the success of PCR. Time spent on primer design can save a lot of time wasted later trying to optimise bad primers. For long PCRs or PCRs from complex templates (eg. soil) the need for excellent primers is even more important.

You need 15-20 bases at the 3' end of the primer that are absolutely conserved in the target sequence, but you can add stuff at the 5' end (up to another approx. 30 bases) that may or may not be found in the target sequence, eg restriction sites, mutations, overhangs to join to things etc.

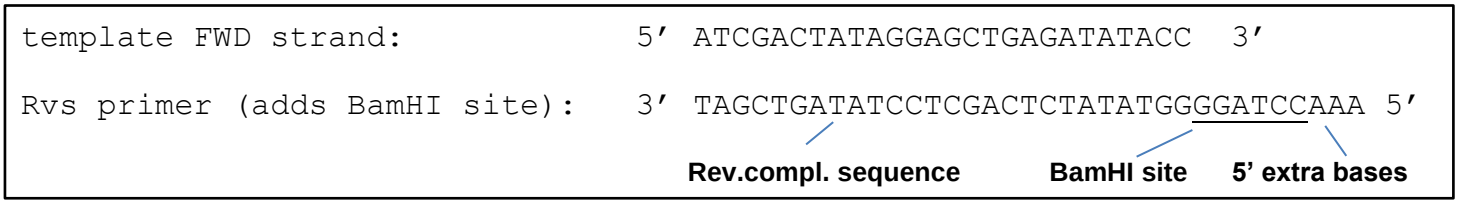
Your forward primer should have the same sequence as the target site on the forward strand of the target DNA, but your reverse primer has the same sequence as the reverse complement of the target site. In both cases, the primer goes in the 5' → 3' direction. A simple tool to use for reverse complementing sequences is this: http://bioinformatics.org/sms/

If you are adding a restriction site at the 5' end of the primer, you also need a few extra bases at the 5' end to allow the RE to bind properly. You can look up for each restriction enzyme how many extra bases are needed (see NEB catalog appendix table "Cleavage close to the end of DNA fragments"). If you add 3 extra bases, that is pretty safe for most enzymes. NdeI is annoying and needs > 7 extra bases. You can make the extra bases match the template at that position if you like, but we usually just add AAA or TTT, whichever is least problematic for dimers and hairpins. (see below).

The architecture of a typical FWD primer is shown below:




The architecture of a typical RVS primer is shown below:




Aim to have primers with a GC content approx 50-60%, and if possible avoid repeat regions (e.g. AAAAAA). High GC regions are also problematic for primer design, since any dimers or hairpins in these regions will be much stronger than in 'normal' DNA region. Aim to end the primer on at least one G or C at the 3' end – this helps keep the 3' end of the primer firmly anchored on the template.

Aim to get melting temperatures (Tm) for the primers around 60°C. You want both primers to have very similar melting temps +/- about 3 degrees C, so they work well at the same annealing temp. You can use the IDT website or Snapgene to check melting temps, and the IDT site also gives info on secondary structures (dimers etc).

Note that the predicted primer melting temperature varies depending on the solution chemistry – the values printed on the primer spec sheets are for DNA in 50 mM NaCl. If PCR is performed using Taq, the optimal annealing temperature for the PCR reaction is about 5°C below this Tm in 50 mM NaCl. For newer PCR polymerases, e.g Phusion or Q5, the optimal annealing temperature is higher due to a processivity domain which makes the enzyme bind more tightly to the DNA – in these cases, the optimum annealing temp could be same or even higher than the Tm value.

The real art of primer design is in making primers that don't make self-dimers, pair-dimers and hairpins - all of these structures will take the primer away from its intended target site in the template. Software eg. Primer3 or PrimerSelect will help you find these. The dimers should have deltaG values higher than approx -5 kcal/mol to be acceptable. eg. a primer where the worst self-dimers are -8 kcal/mol would not work well, but a primer where the worst self-dimer is -3 kcal/mol should be OK. For hairpins, the cutoff is about -2 kcal/mol. You need to check for both self-dimers and pair dimers.

If your primer has bad dimers or hairpins, there are a number of ways to deal with this problem. The first step in all cases is to identify which particular bases are problematic (using the software/websites mentioned above). Then you can try the below:

- slide the primer location up or down a little bit to move it away from those problematic bases. this only works if the problem bases are near the ends of the primer. if the problem bases are in the middle of the primer, you will probably need to choose a new priming location

- if the problem bases are towards the 5' end, you can sometimes mutate them to another base which doesn't cause the dimer/hairpin to form. you need to be very careful with this approach though that the mutations you introduce don't cause problems later.

- you can (at your own peril) ignore the hairpins and dimers and just order the primer and see what happens… sometimes primers that look bad in silico work OK enough in the lab.
- if the problems are arising from the bases in a restriction site, can you change this to a different restriction site?

- if the problems are arising from your extra bases at the 5' end that are added to facilitate digestion, you can change these to any bases you like (they get cleaved off after digestion)