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:

template FWD strand: 5' **AGTCAGCTCGCATCGAGGGCTCGAGGAC** 3'

Fwd primer (adds EcoRI site): 5' **AAAGAATTCAGTGGATCCGCATCGAGGGCTCGAGGAC** 3'

5' extra bases **EcoRI site** **Fwd sequence**

The architecture of a typical RVS primer is shown below:

template FWD strand: 5' **ATCGACTATAGGAGCTGAGATATACC** 3'

Rvs primer (adds BamHI site): 3' **TAGCTGATATCCTCGACTCTATATGGGGATCCAAA** 5'

Rev.compl. sequence **BamHI site** **5' extra bases**

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. 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)