Primer Design

Protocol author: Wendy Phillips
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Annealing temperature, GC content, length

If you are designing two primers to be used with each other, or if you are designing a primer to be used with an existing primer, try to design them so they will have a similar annealing temperature.

There are many formulas to predict annealing temps. Some may give better estimates than others, but in the end it all needs to be determined empirically anyway. That is why I use the simplest formula. An A or T = 2°, a G or C = 4°. Count up all As and Ts and multiply by 2°. Count up all Gs and Cs and multiply by 4°. Add up those two products and you'll get a predicted annealing temp. This works for a certain size range – I don't know what it is – but your average primer should fall in that range. If you have degenerate bases in your primer, you should either put in an average value for the possible nucleotides (ex. If it could be A or G, use 4°, if it could be A,T, or G use 2.7°) or you could determine the lowest and highest possible temps for that primer, i.e. one time assume any degenerate position is the lowest temp choice and one time assume any degenerate position is the highest temp choice. Note if you go with the averaging option, you'll probably want to start out using that primer a few degrees lower than what you calculated to be sure it has a chance to stick.

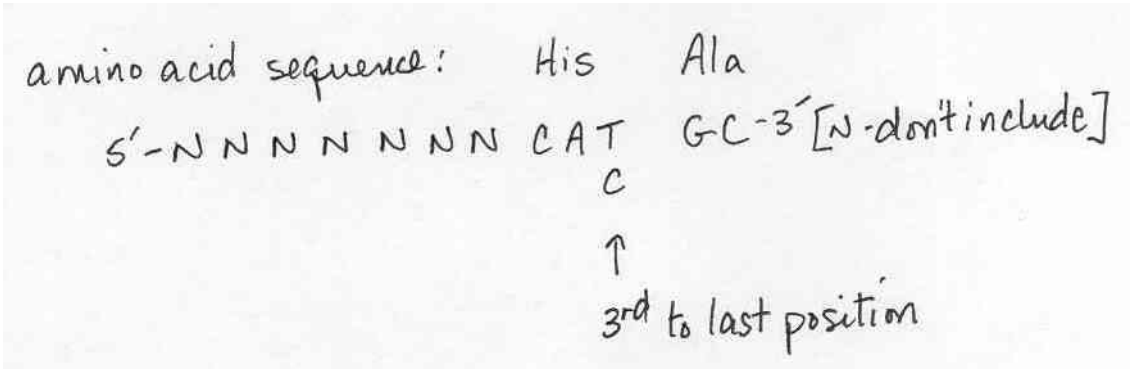
I think the ideal primer is one with 50% AT and 50% GC. Having lots of GCs may seem good because they are sticky, but if you have too many you are increasing the likelihood that it will stick somewhere it shouldn't because it may make the A and T positions less meaningful. If you have too many As and Ts, you might not get as good of a stick. If at all possible, I definitely try to keep the ratio in the 60:40 range either way. I like primers 18-20 nts the most. My ideal primer is a 20-mer with 10 ATs, 10GCs, annealing temp 60°.

3' end stickiness

Along those same lines, you want to pay attention to the GC content of the 3' end. The 3' end is much more important than the 5' end. If the 5' end doesn't stick perfectly, the primer can still work. If the 3' end doesn't stick, the primer will not work. Some people really like to have the last two (3' most) nts be G or C to give the primer more 3' end stick. I try to end with one G or C if possible, but if I can't, I can't. Beware though, you don't want too many Gs or Cs at the 3'end – 4 out of 5 of the last nts are G or C – that is getting to be too many. Again, that could give you artificial stick at a wrong annealing site.

Another note on needing the 3'end to stick. I have read that the last 3 nucleotides must stick. If the 4th nt in from the 3' end is a mismatch, you may still be ok. Since those last three must stick, I sometimes consider the placement in terms of amino acid codons. I try to end the primer on the 2nd nt of an amino acid that is only degenerate in the 3rd position.

If I really want to be sure it will stick, I make the primer degenerate at the third to last position – trying to put that on an amino acid with only two possible codons. Example:

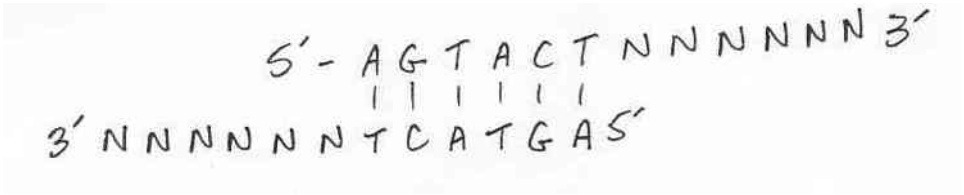


Design of reverse primer

VERY IMPORTANT. Be sure you reverse primer is the reverse complement of the sense strand. If you want a reverse primer for the sense strand 5'-ACCGTACAATGG-3', the primer you order should be 5'-CCATTGTACGGT -3'. Triple check that you are doing this right if you are new to designing primers.

Primer dimers

Beware of primer dimers. A primer dimer happens when two primers anneal to each other. It can be the same primer annealing to itself or if could be two primers sticking to each other. I eyeball the primers to see if this may occur. It will happen when the primer's forward and reverse sequences are complementary to each other. Example:



Primer dimers will generally be worse if the complementary sequences are at either end of the primer. If it is only 2 nucleotides long, maybe even 4, I wouldn't worry about it.

Degenerate primers

If you need to design degenerate primers, keep them as least degenerate as possible. Again, it is more important to keep the 3' end less degenerate. For me, the jury is still out on whether to use inosine or N at a 40way degenerate base. Inosine usually costs $10 extra with each one you add. Apparently inosine doesn't actually bind to anything – it is really more neutral to actually repellant of the other bases. But it does decrease you degeneracy. Because it doesn't actually bind, don't use inosine in you third to last base, instead use N. But preferably you would have the second to last amino acid be one with only two possible codons. In the ideal world, you could put the primer where the second to last amino acid has only one possible codon.

You need to be more cautious when designing primers if you know the genes has repeating domains. If it does, make sure your primer will not stick very well to another of the domains – if it can, you will get multiple products. Try for a ≤ 60% match at the second site.

Ordering purified primers

I usually order primers at the desalted purification scale OPC or column purification gives you a much purer primer, but it is more expensive. Usually a 40-50 nmol scale or a 5 OD scale will give you plenty of primer. If it is a primer you know will need frequently forever, for example vector primers – consider ordering at 0.2 μmol scale.