Troubleshooting Forum

Molecular Biology Techniques Q & A

DNA and General PCR Methods: PCR Primer Design

This month's questions from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) come from the "DNA and General PCR Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but they do not represent endorsements by, or the opinions of, *BioTechniques*.

Is it acceptable to use PCR primers that form hairpins or dimers and will this influence restriction digest efficiency on the products? (Thread 22185)

Q My results from primer design analysis with the Oligo Analyzer software show that my primers will form various hairpins and primer dimers. What is an acceptable ΔG value for primer hairpins and primer dimers? Can anyone recommend a handbook or some literature that explains how to determine what ΔG values are acceptable for genomic and plasmid PCR amplification?

A Does the program give you a melting temperature (T_m) for the hairpin and primer dimer? If the T_m for the hairpin or dimer is at least 10 degrees lower than the annealing temperature for the primers, then you should have no problems with those primers. Even so, if it is possible, you should move the location of your primers a few bases up- or downstream to avoid the problematic sequence region. Sometimes one base change can make a big difference.

A I have used primers with ΔG values of -9 for dimers successfully. It seems that hairpins usually have higher ΔG values than that, so they are of less concern.

A I'm usually more concerned with the stability of the hairpins, the ability of the primer dimers to make a product, and the T_m than with ΔG values.

Can you tell if the hairpins are stable? How many base pairs are involved?

Can you tell if the 3' end of your primer dimers will anneal and be extended to make a product?

If you're only interested in the amino acid sequence and not the exact nucleotide sequence, you could change a base or two to disrupt either the hairpin or the dimers.

A You very rarely need to make mutations. Usually you can just move the primer site and eliminate the problem since hairpins are sequence-dependent. Anything larger than a 6-nucleotide sequence that forms a 3-bp hairpin will cause a problem.

In my experience, the 3' end of the primer from which extension originates is the most important. A primer dimer can initiate extension twice as easily as a hairpin. A hairpin within the primer should not create artifacts and only under specific circumstances would it interfere with cloning. It might affect PCR efficiency, though.

A With primer design, there is plenty room for paranoia. Just aim for a primer that's a nice mix of bases, 20 bases or more, and no chances of primer dimer formation. I always try for a 50°C annealing temperature and never bother calculating the melting temperature. Primers don't cost much. On the very rare occasion that I don't get the PCR product I need, I order a different primer rather than wasting time optimizing. My best advice is to not get too hung up on designing the perfect primer. Regardless of what any computer tells you, the only test is to actually run the PCR.

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Q My PCR works. My worry is about the restriction digest afterwards. If a hairpin loop forms on the 5' end, will the enzyme still attach and cut at its site?

A All you need is enough to cut so that you get colonies after cloning. You can determine this empirically, so just give it a try. My guess is that it will work. Even if one stretch forms a hairpin, some will also anneal correctly and there will be fluctuation between the hairpin and the correct pairing states.

A The restriction digest should work fine. At 37°C, the ends of the DNA products will "breathe" (dissociate temporarily and re-associate). They will spend more time double-stranded and paired with their perfect matches than as imperfect hairpins. The restriction enzyme will find a way except in some cases with extreme hairpin issues. But even in these cases, you can always warm and very slowly chill the amplicon to facilitate perfect matches.

What type of purification is best for primers? (Thread 22404)

Q I spent many months trying to optimize my PCR. Eventually, I ran my primers in a 15% neutral PAGE gel and saw multiple bands. The primers were HPLC-purified, which I thought should result in primers with superior quality, but I think that the primers were such low quality that they jeopardized my work.

What type of primer purification is the best: HPLC or PAGE?

Can anyone suggest a good primer synthesis company in the U.S. or Europe?

A I have had good experience with IDT. I order standard desalting purification for my PCR primers used for subcloning and mutagenesis. I believe they have other purification choices like PAGE and HPLC, too.

A If your primers are ~21 nucleotides in length, no purification is needed. For longer primers, PAGE purification may be desirable. I have successfully used 30-nucleotide primers without purification.

A If you run your primers on a non-denaturing PAGE (without urea or a denaturation step in formamide), you might see multiple bands even with good primers.

I use Proligo primers in Europe and they work well.

A Unlike biosynthesis, oligonucleotides are made from the 3' end. This means that they are likely to work in PCR even if they are a few bases short. As long as you have chosen a reputable company to make your oligonucleotides, purity is never an issue. It's much more likely that you've designed poor oligonucleotides or that your cycling parameters don't match your oligonucleotides.

A I agree with the previous statements. Primers are cheap at this point. It's often much simpler to design and order 2 new sets of primers if an attempt at optimization does not work.

Selected by Kristie Nybo, Ph.D.

BioTechniques 46:505-507 (June 2009) doi 10.2144/000113179

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