Troubleshooting Forum

Molecular Biology Techniques Q&A

Primer design

This month’s question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the “Real-Time qPCR/qRT-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 do not represent endorsements by, or the opinions of, BioTechniques.

What characteristics are most important when designing RT-PCR primers? (Thread 32802)

Q I would like to study changes in the expression of a target gene using SYBR technology. I began by using Clone Manager to design primers based on NCBI sequence data. To meet the Clone Manager criteria for Tm, percentage of GC content, stability, etc., it looks like I need to make the forward and reverse primers 29 bp long. I read that primers for RT-PCR should be about 20 bp, but primers this short do not meet the criteria thresholds. Will using these long primers cause problems?

A I am not familiar with Clone Manager, but in my experience, the priorities of several primer design programs don’t make much sense. You can modify the tolerances of the program to permit it to return something more reasonable. For example, primer length and percentage of GC don’t matter much.

It is important that the primers have similar Tm so they function properly in the same PCR program. Your Tm should be several degrees above the annealing temperature to leave room for error. Primers of 20 bp in length are common because they usually have around 50% GC and a Tm of 60°C. Twenty-nine bp in length is not a problem if you have higher AT percentages to prevent the annealing temperature from becoming too high. Tm calculators assume 50 mM salts, so you may also change the salt concentrations in your buffer to alter Tm.

You should avoid primers with a high percentage of either AT or GC since these might non-specifically prime repetitive sequences in the template. The 3’ end is especially important since that is where the extension occurs. You should also avoid repetitive sequences or primers that may form dimers.

Q I am using an ABI StepOnePlus device with ABI fast SYBR mix. I have the ABI primer design software, but the instructions were not specific. For example, it reads “choose a primer with a low penalty score,” but doesn’t mention how the scores were calculated. The primers I designed have a Tm of 62°C. Clone Manager does not consider salt concentrations, so I thought it would be better to have primers with a slightly higher Tm than the desired annealing temperature.

The GC content is low: 34% and 31%, respectively, for the forward and reverse primers.

Forward: ACAAGGGTTACAGGACTATTACTTAAATC
Reverse: GAGAAAGTTGAAATTGCGTGTTTAAATAC

A Your primers look fine for length and GC content. For conventional PCR, those primers might work, but they leave little room for error with a 60°C annealing temperature.

I see a possible dimer problem with the reverse primer. There’s only 1 mismatch in the 12 nucleotides at the 3’ end and it is a GT pair, the most tolerated of mismatches.

I checked NCBI’s PrimerBLAST; it shows the 3’ self complementarity of the reverse primer at the maximum level it permits and indicates a Tm of 59.3°C by the Santa Lucia calculation, which I think tends to come out slightly low. This will probably work fine at a 60°C, especially if the qPCR mix raises it somewhat. They look like they are worth trying, but you might want to order a second pair so you don’t lose much time if they don’t work.

Q I also tried using PrimerBLAST to design primers, but was confused by the complementarity values. I assume that you want these values to be as low as possible, but I couldn’t find any critical values or cutoffs recommended for that score.

I initially designed primers with NCBI, but when I put them into Clone Manager, the primers didn’t meet the criteria for GC clamp and 5’ versus 3’ stability. GC clamp refers to the number of G or C residues at the 3’ end of the primer. If the 3’ end of the primer does
not match the target, some polymerases, especially proofreading polymerases, will remove the mismatch and prime from the wrong base. Clone Manager recommends 1 or 2 G or C nucleotides at the 3’ end of the primer.

5’ versus 3’ stability depends on the thermal stability of pentamers within the primer, with stable sections returning negative kcal values. The stability score comes from a comparison of the 3’ end pentamer with the most stable pentamer in the rest of the primer. The software requires the 5’ end to be at least 1.2 kcal more stable than the 3’ end.

Do you think it is better to use the PrimerBLAST primers that didn’t meet the Clone Manager criteria, or the Clone Manager primers that are too long?

A There are a lot of rules out there that you can follow, but all you really want is primers that work. I often choose primers that are far from ideal on the Primer3/BLAST list, even after I weaken the parameters. The primers usually still work.

That’s interesting about the GC clamp. That explains why I’ve heard that the last nucleotide of a primer should never be a mismatch. However, I have seen primer databases that ignore this rule and claim good results. I have also successfully used primers without a GC clamp.

Overall, my preference is to look for a primer with a random mix of bases and moderate GC content. I like to see mixed GC content in the last 5 or 6 nucleotides, with one of the last two nucleotides being a G or C. That said, there are often constraints for particular template targets and I don’t follow all the rules.

Try one primer from each method to see which gives a single clear band with good efficiency (low Cp) from a positive control. It’s the empirical results that matter, not the preliminary calculations.

Q I ran an NCBI primer set through Clone Manager. The forward primer missed the stability threshold and the reverse had no GC clamp and too many nucleotide runs.

I believe amplicon length is more important than primer length, so I think I will order the 29 nucleotide primer set to see if it works. The GC content is within the 30%-80% range recommended, the T_m is about 2°C above the annealing temperature, and there is a GC clamp.

The presence of Mg in the buffer decreases the binding specificity (or increases the binding capacity of polymerase), essentially dropping the annealing temperature, correct? So should a primer with a T_m at the annealing temperature bind in the presence of an MgCl_2-containing PCR buffer? The ABI product guide for Primer Express claims to design primers with a T_m of 60°C, which is also the recommended annealing temperature. Does this mean that their buffer is Mg rich?

A Primer length is only a problem if you have a fixed T_m, requiring you to extend the length of the primer while increasing the AT percentage to keep the low T_m. This will weaken the primer and increase random priming. In general, long primers are more specific, as long as they maintain a good GC percentage and have a high T_m and appropriate annealing temperature.

High salt and Mg^{++} raise the primer T_m. I don’t know about the polymerase binding. All PCR buffers contain MgCl_2 since it is necessary for the polymerase and interacts with the dNTPs.

A Higher concentrations of Mg^{++} increase DNA-dependent DNA polymerase activity at the expense of specificity, whereas lower Mg^{++} decreases DNA-dependent DNA polymerase activity but increases its specificity. I agree that high salt and high Mg^{++} would tend to increase the T_m, since it provides a lower stringency environment that makes it easier for duplexes to form.

T_m is defined as the point at which 50% of primers are still annealed to their target sequence. If the annealing temperature infers the point at which 100% of primers are bound, then the 2 temperatures should never converge, no matter how much salt or Mg^{++} is added.

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