PCR Primer Design Guide

Proper PCR primer design is an essential factor contributing to the success of any PCR experiment. Careful attention to detailed PCR assay primer design and PCR assay validation is usually rewarded by a significant reduction in time and effort troubleshooting PCR problems. This guide will highlight some of the most important considerations when designing and validating PCR assays.

General Considerations:

1. The use of online or stand-alone computer software aided primer design programs is highly recommended. These software packages will identify the potential for problematic internal secondary structure and complementarity at the 3' ends of each individual primer, primer pair, and primer/probe combinations.

   ### Online or stand-alone primer design software tools:

   IDT Oligo Analyzer (www.idtdna.com/calc/analyzer)
   Primer Quest (http://www.idtdna.com/Primerquest/Home/Index)
   Primer3 (http://primer3.sourceforge.net/)

   ### Primer3 online web interfaces:

   Primer3 (http://primer3.ut.ee/) or Primer3 Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi)
   Oligo7 (http://www.oligo.net/)

   Design PCR primers that are 18 to 24 bases in length with melting temperatures (T_m) ranging from 57-61°C. It is important to closely match the Tms of primer pairs with no more than 2°C difference. Specific applications may require modifications to primer length and T_m .

   It is best to use the default settings provided in any primer design software rather than changing the settings for Mg and salt concentrations. Keep in mind that the total Mg concentration of a PCR mastermix is meaningless without also knowing the total dNTP concentration since dNTPs will chelate a given amount of Mg. Most commercially available mastermixes also contain proprietary components that will alter the Tm of PCR primers and amplicons.

2. Avoid amplicons of excessively high or low GC content and regions of strong RNA secondary structure both of which can interfere with primer/probe hybridization and impede procession of the taq polymerase and reverse transcriptase (in the case of 1-step RT-PCR). GC content and RNA secondary structure of the target can be assessed using online software tools or stand-alone software packages. In general, the GC content of the primers and probes should match the overall GC content of the template.

   ### RNA folding software:

   RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi)
   UNAFold (http://www.idtdna.com/UNAFold?)

3. For best results with qPCR applications, amplicon size should be between 70 and 150 bp. Design probes of 20-30 bases (or longer if using double quenched probes) with T_m of 6-8°C higher than the PCR primers. Probes can be designed to bind to either strand and should be positioned close to one of the primers without overlapping the primer binding site. Avoid a G at the 5' end to prevent quenching of the 5' fluorophore. Optimal results may require titrations of primers (between 300 and 900 nM) and probe concentrations. A final concentration of 450 nM each primer and 100 to 150 nM probe is effective for most applications.

4. The efficacy and efficiency of any primer/probe set should be validated under both standard cycling and fast cycling (rapid ramp rate) protocols before use in qPCR studies. Prepare a standard curve by titrating the template to calculate PCR efficiency. Perform a Primer Blast to avoid homologies to non-specific targets.

   Primer Blast: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

5. For quantification of mRNAs primers should span intron-exon boundaries to avoid amplification of gDNA. Compare RNA templates treated or un-treated with DNAse I to verify that the primer designs do not amplify gDNA. You can also compare the amplification of equivalent amounts of RNA with first strand cDNA as template.

Summary of PCR Primer Design Parameters:

Parameter	Range
Primer length	18-24 bases
T_m	59°C +/-2°C
GC content of primers and template	40-60%
Amplicon size (qPCR)	70-150 bp without significant secondary structure
Annealing temperature	2°C below the primer T_m
3' End stability	No more than 2 Gs or Cs in the last 5 bases
Primer dimers	Avoid self-complementarity and heterodimer stability
Runs and repeats	Avoid runs of more than 4 single or di-nucleotide repeats
Palindromes and hairpins	Avoid palindromes which can form hairpin structures particularly at the 3' end

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