Primer Design

ACGCTGCACGTGTCACGTGCTATACGATGC

IMBB 2014
You must know this!
The reverse PCR primer is the reverse complement of the sequence on the forwards (sense) strand

Forwards
5’ - **TCTCAACCAACCAAAAGACATCGG**TACCTTATACCTAATCTTCGCCGCATGAGCCGGTATCCTCTACTGGTTTAGATGTTACATTAGCAGTAACG - 3’

TAGGAGATGACCCAAATCTACAATGTAATCGTCATTGCCCCATGCCTTCGTAATGATCTTCTTCT - 3’
ATCCTCTACTGGTTTAGATGTTACATTAGCAGTAACG**GGTACGGAAGCATTACTAGAAGA** - 5’

← Reverse
General rules

- Typical primers are 18-28 nucleotides in length
- GC composition of 40-60%
- Have a balanced distribution of G/C and A/T domains
- No long strings of a single base (<4 is good)
- Primers with annealing temp (Ta) between 50-65°C are preferred

**Good**

5’ ATGCACTCAGACGTACAACGTGAC 3’
24 bases
AT: 12
GC: 12 (50% GC)
Balanced distribution
Ta ~60°C

**Bad**

5’ AAAACAAAAACGATTTTTTT 3’
17 bases
AT: 14
GC: 3 (18% GC)
Unbalanced distribution
Ta ~35°C
Unique

- Unique (Lack of secondary priming sites). Only one target site in the template DNA where the primer binds, which means the primer sequence shall be unique in the template DNA.

- Sometimes uniqueness can be determined by BLAST if the genome sequence is available (Bioinformatics)
Length

• Primer length has effects on uniqueness and melting/annealing temperature.

• The longer the primer, the more chance that it’s unique; the longer the primer, the higher melting/annealing temperature.

• The length of primer should be at least 18 bases to ensure uniqueness.

• Usually primers of 18-28 bases are used for PCR.
Melting temperature (Tm)

Melting Temperature (Tm): the temperature at which half the DNA strands are single stranded and half are double-stranded.

Tm is characteristic of the DNA composition: Higher G+C content DNA has a higher Tm due to more H bonds.

Calculation

Shorter than 21: $Tm = (A+T) \times 2 + (G+C) \times 4$

Longer than 21: $Tm = 64.9 + \frac{41(G+C-16.4)}{(A+T+G+C)}$

To calculate Tm go to [http://www.basic.northwestern.edu/biotools/oligocalc.html](http://www.basic.northwestern.edu/biotools/oligocalc.html)

In practice, because the Tm is variously affected by the individual buffer components and even the primer and template concentrations, any calculated Tm value should be regarded as an approximation.
Annealing Temperature (Ta)

Annealing Temperature (Ta) is the temperature used in the PCR to anneal the primers to the template DNA. It can be estimated from Tm.

\[ T_a = T_m \pm 5^\circ C \]

i.e. Ta is usually within 5°C of the Tm

- Primers with Ta between 50-65°C are preferred
- Ta is determined empirically
- Try testing Ta in the range Tm -5°C to Tm +5°C, at 1-2°C intervals (gradient PCR)
Internal Structure

If primers can anneal to themselves, or anneal to each other rather than anneal to the template, the PCR efficiency will be decreased dramatically. They shall be avoided.

However, sometimes these 2° structures are harmless when the annealing temperature does not allow them to take form. For example, some dimers or hairpins form at 30°C while during PCR cycle, the lowest temperature only drops to 60°C.
Primer compatibility

- Primers work in pairs – forward primer and reverse primer.

- Since they are used in the same PCR reaction, the PCR conditions should be suitable for both.

- One critical feature is their Ta, which should be compatible with each other.

- Tm of primer pair should have a maximum difference of 3°C. The closer their Tm, the better.
Summary

1. Uniqueness: ensure priming site is unique within the DNA.
2. Primer length: 18-28 bases is usual.
3. Base composition: G+C content between 40-60%.
4. Avoid long (A+T) and (G+C) rich regions if possible (balanced base composition).
5. Annealing temp (Ta) between 50-65°C are preferred and is determined empirically. Ta is based on the Tm.
6. The primer pair should have Tm within 3°C of each other.
7. Minimize internal secondary structure: hairpins and dimers should be avoided if possible.
8. The reverse primer is the reverse complement of the sequence on the forwards (sense) strand.
If optimisation fails to give good results then redesign your primers!
Designing Degenerate Oligonucleotides

• A group of degenerate oligonucleotides contain related sequences with differences at specific locations.
• These are used simultaneously in the hope that one of the sequences of the oligonucleotides will be perfectly complementary to a target DNA sequence.
• For example, the amino acid sequence shown below could be encoded by the following codons.

AspGluGlyPheLeuSerTyrCysTrpLeuProHisGln
GATGAAGGTTTTTCTTTTCTTATTGGCTTCCTCATCAA
  C  G  C  CT CAGC  C  C  T  C  C  C  G
  A  A  A  A  A  G  G  G  G  G
Standard Mixed Base Definitions

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primer design software

CLC Main Workbench: Primer Design

Primer3web version 4.0.0
http://primer3.wi.mit.edu/

GeneFisher2 Interactive PCR Primer Design
http://bibiserv.techfak.uni-bielefeld.de/genefisher2/
Primer design

https://bioweb.uwlax.edu/GenWeb/Molecular/seq_anal/primer_design/primer_design.htm
Thank you