

General guidelines for primer design

Good primer design is essential for successful PCR reactions. A number of primer design tools are available, such as NCBI Primer-Blast and Primer3. The following considerations should be taken into account when designing primers.

1) *Primer sequence*

The 3'-end of the primer molecule is especially critical for the specificity and sensitivity of PCR. It is recommended **not** to have:

- 3 or more **G** or **C** bases at this position. This may stabilize nonspecific annealing of the primer.
- A 3' **thymidine**, since it is more prone to mispriming than the other nucleotides. Primer pairs should be checked for complementarity at the 3'-end. This often leads to primer-dimer formation and should be avoided.

Bases at the 5'-end of the primer are less critical for primer annealing. Therefore, it is possible to add sequence elements, like restriction sites, to the 5'-end of the primer molecule.

2) *Primer length*

Usually a primer length of **18-22 bases** is optimal for most PCR applications. Shorter primers could lead to amplification of non-specific PCR products.

3) *Melting temperature (T_m)*

The **specificity** of PCR depends strongly on the melting temperature (T_m) of the primers (the temperature at which half of the primer has annealed to the template). Usually good results are obtained when the T_m 's for both primers are similar (within 2-4 °C) and in the range of 55-65°C.

4) *GC content*

The GC content of a primer should be between **40** and **60%**.

Primer design for general cloning

Design of the 5'-end primer

The 5'-end primer overlaps with the 5'-end of the gene of interest and should contain the following elements:

1) *Restriction site*

The restriction site should be the same or provide the same sticky end to the first of the restriction enzymes in the multiple cloning site of the vector chosen to clone the gene of interest.

2) *5'-extension to the restriction site*

Restriction enzymes cleave DNA much less efficiently towards the end of a fragment. A 5' extension of the restriction site with 4 to 8 random bases greatly increases the cleavage efficiency of most enzymes. Data on the effect of the extension length and sequence on the cleavage efficiencies of the most used restriction enzymes can be found in the reference appendix of the New England Biolabs catalogue (page 330).

3) *Start codon*

A start codon (usually **ATG**) should be included when the gene of interest is not expressed with an N-terminal tag or fusion partner. The start codon and the gene of interest sequence should be in frame with an eventual N-terminal tag and/or fusion partner.

4) *Overlap with the gene of interest.*

The overlap between the primer and the gene of interest should be long enough to give a T_m of 60°C or more (calculated as shown above).

Design of the 3'-end primer

The 3'-end primer overlaps with the DNA strand complementary to the 3'-end of the gene of interest and should contain the following elements:

1) *Restriction site*

The restriction site should be the same or provide the same sticky end to the second of the restriction enzymes in the multiple cloning site of the vector chosen to clone the gene of interest.

2) *5'-extension to the restriction site*

Restriction enzymes cleave DNA much less efficient towards the end of a fragment. A 5' extension of the restriction site with 4 to 8 random bases greatly increases the cleavage efficiency of most enzymes.

3) *Stop codon(s)*

A stop codon should be included when no C-terminal tag is used. **TAA** is preferred because it is less prone to read-through than **TAG** and **TGA**. To increase the efficiency of termination it is possible to use 2 or 3 stop codons in series.

4) *Overlap with the strand complement to the 3'-end of the gene of interest.* The overlap between the primer and the strand complement to the 3'-end of the gene of interest should be long enough to give a T_m of 60°C or more. If including a C-terminal tag in the final construct, it is important to check that the gene of interest is in frame with the C-terminal tag provided by the vector.

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