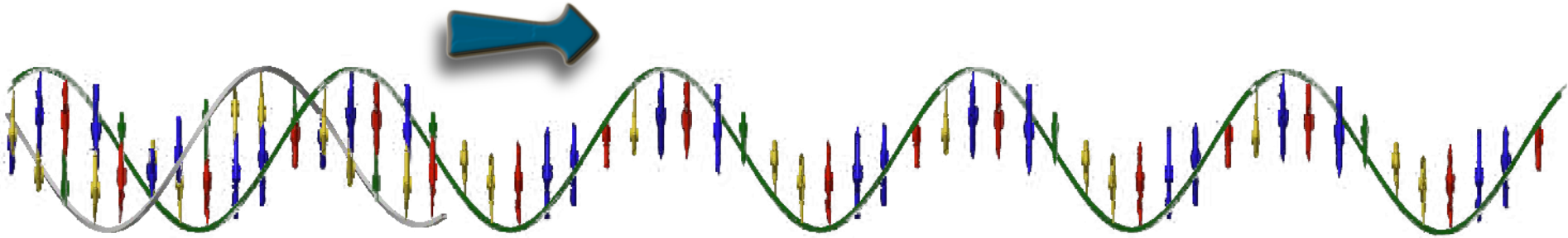
Connective Tissue Diseases Research Center
CTDRC

PCR and Primer Design



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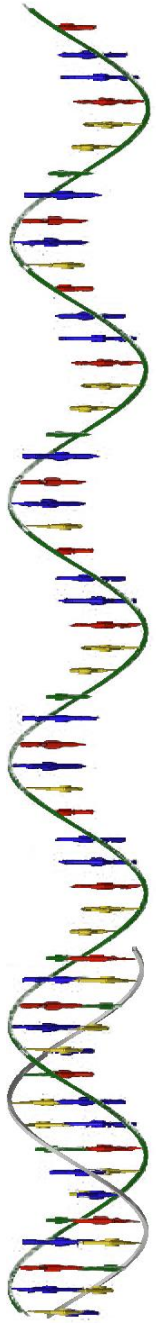
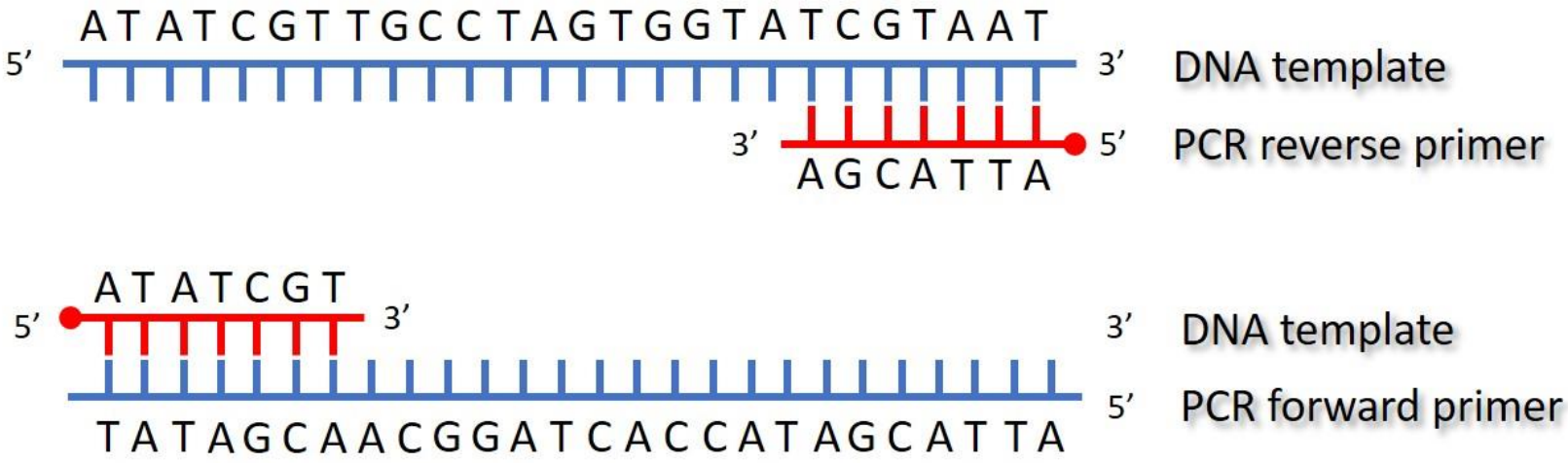
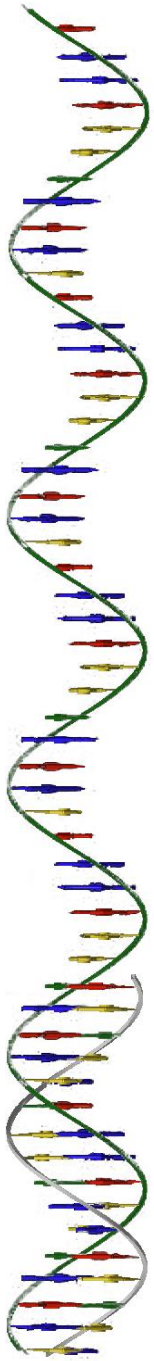


A Primer is a …

- Short nucleic acid sequences
- Serves as a starting point for DNA synthesis
- It is required for DNA replication.
- The polymerase starts replication at the 3'-end of the primer

Primer annealing in PCR



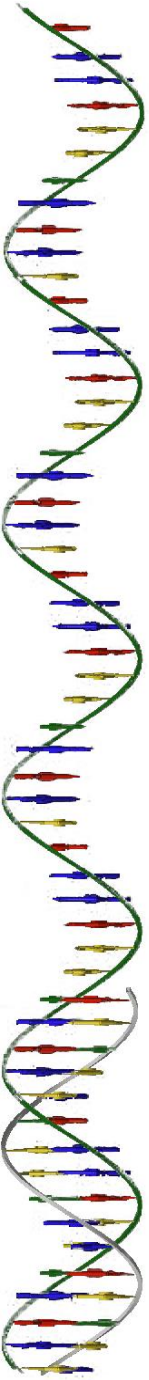


Before you start designing primers…!

What are the purposes of primers design?

- General purpose amplification?
- SNPs detection/validation?
- Methylation study?
- Real-time PCR?
- Nested PCR?
- Degenerate PCR?
- Multiplex PCR?

Good Primer's Characteristic



- Uniqueness
- Typically 20-30 bases in length
- A melting temperature (T_m) in the range of 52 ◦C to 65 ◦C
- Annealing temperature
- Avoid secondary structure, particularly 3'
- Avoid primer complementarity (primer dimer)
- Absence of significant hairpin formation (>3 bp)
- The last 3 nucleotides at the 3` end is the substrate for DNA polymerase

Uniqueness

- There shall be one and only one target site in the template DNA where the primer binds, which means the primer sequence shall be unique in the template DNA.

https://blast.ncbi.nlm.nih.gov/Blast.cgi

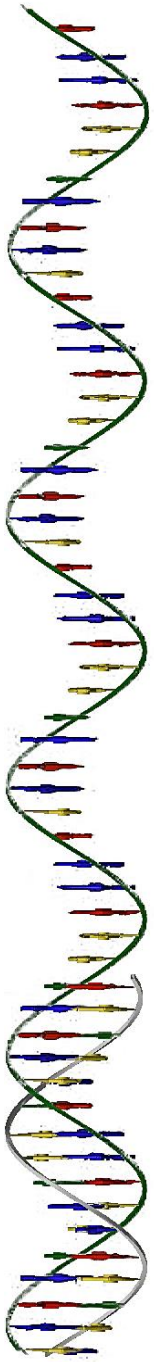
Template DNA

```
5′...TCAACTTAGCATGATCGGGTA...GTAGCAGTTGACTGTACAACTCAGCAA...3′
     TGCTAAGTTG                CAGTCAACTGCTAC  TGCT AGTTG
                                               A
```

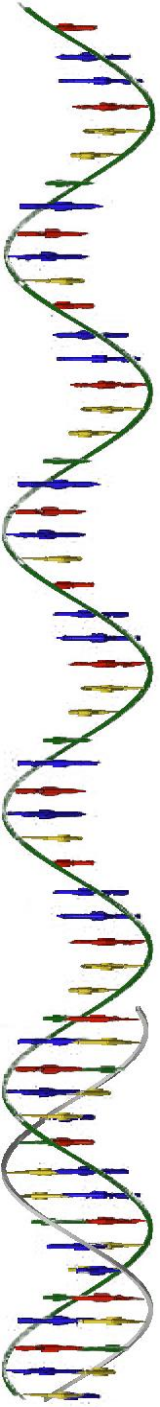
Primer candidate 1 5′-TGCTAAGTTG-3′ NOT UNIQUE!

Primer candidate 2 5′-CAGTCAACTGCTAC-3′ UNIQUE!

Primer length



- **Primer length determines the specificity and significantly affect its annealing to the template**
  - **Too short primers:** low specificity, resulting in non-specific amplification
  - **Too long primers:** decrease the template-binding efficiency at normal annealing temperature due to the higher probability of forming secondary structures such as hairpins.
- **Optimal primer length**
  - 18-30 bp for general applications



General rules for primer design

GC content; repeats and runs

Primer G/C content

- Optimal G/C content: 45-55%
- Common G/C content range: 40-60%

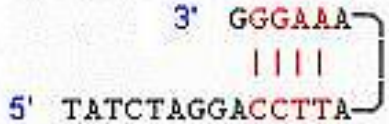
Runs (single base stretches)

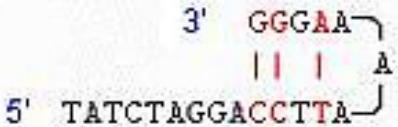
- Long runs increases miss-priming (non-specific annealing).
- The maximum acceptable number of runs is 4 bp.

General rules for primer design

Primer secondary structures

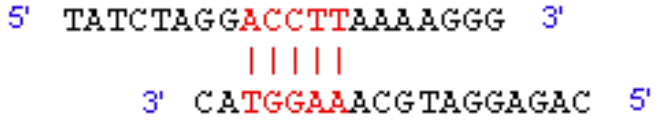
Hairpin





Dimer

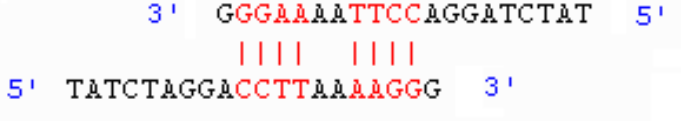
forward primer



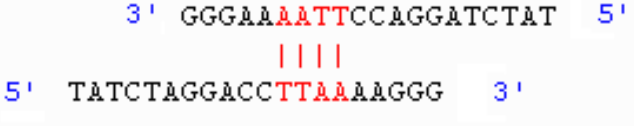
reverse primer

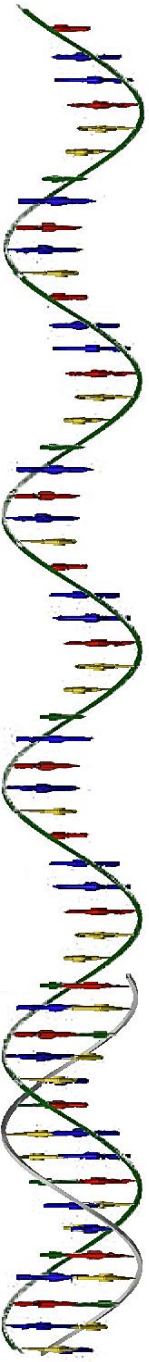
Self-Dimer

8 bp



4 bp





General rules for primer design

Melting temperature (T_m)

T_m

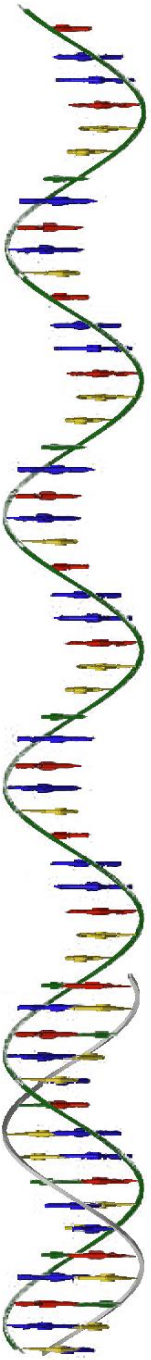
- Determined by primer length and GC content
- Working approximation: $T_m=2(A+T)+4(G+C)$

❖ Optimal melting temperature

- 52 °C- 60 °C

❖ Primer pair T_m mismatch

- Primer pair T_m mismatch can lead to poor amplification
- Desirable T_m difference < 5°C between the primer pair

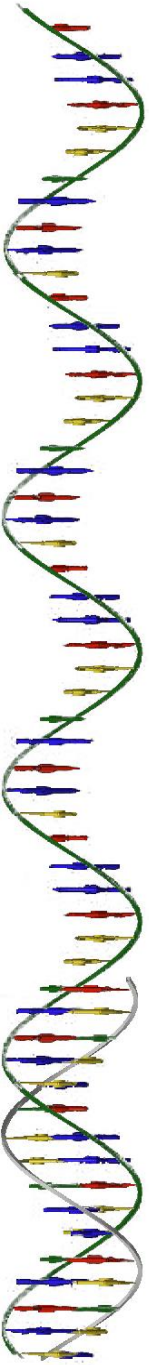


General rules for primer design

Annealing temperatures

❖ T_a (Annealing temperature) vs. T_m

- T_a is determined by the T_m of both primers (F and R).
- General rule: T_a is 5 °C lower than T_m
- Higher T_a enhances specific amplification but may lower yields.

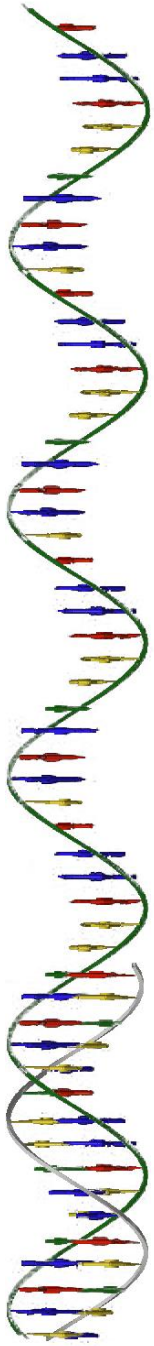


General rules for primer design

Primer Pair Matching

- Primers work in pairs: **Forward primer** and **Reverse primer**. Since they are used in the same PCR reaction, it shall be ensured that the PCR condition is suitable for both of them.

- One critical feature is their annealing temperatures, which shall be compatible with each other. The maximum difference allowed is **3 °C**. The closer their T_{anneal} are, the better.



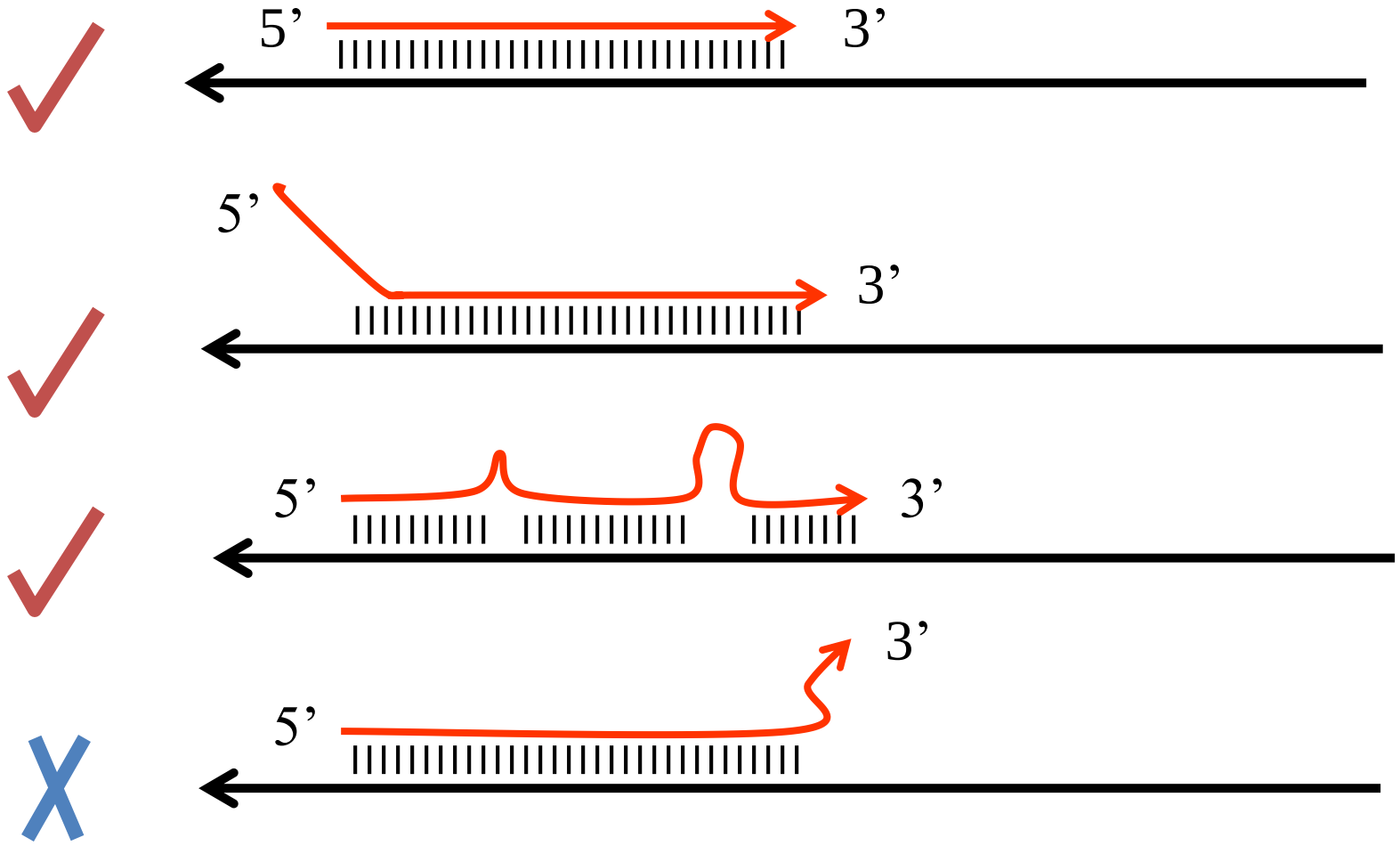
General rules for primer design

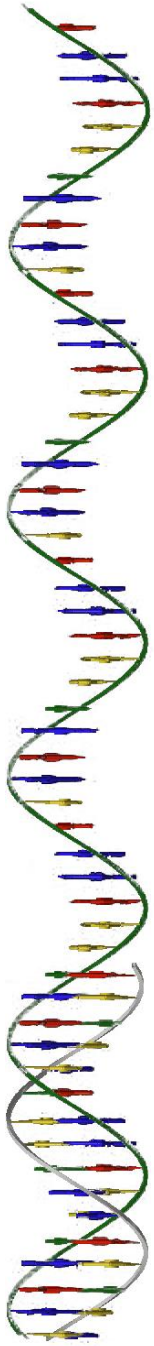
GC clamp and max 3’ end stability

❖GC clamp

- Refers to the presence of G or C within the last 4 bases from the 3’ end of primers
- Essential for preventing mis-priming and enhancing specific primer-template binding
- Avoid >3 G or C near the 3’ end of primers

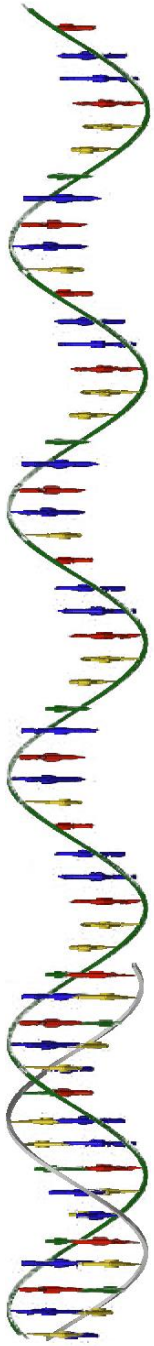
Summary: When is a "primer" a primer?





Online PCR Primer Design

- NCBI primer design
- Primer3
- Primer3Plus
- PrimerZ



Primer Design Software

- **OLIGO**
- **PRIMER**
- **PrimerQuest**

Melting temperature calculation software:

- BioMath: http://www.promega.com/biomath/calc11.htm

منابع

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