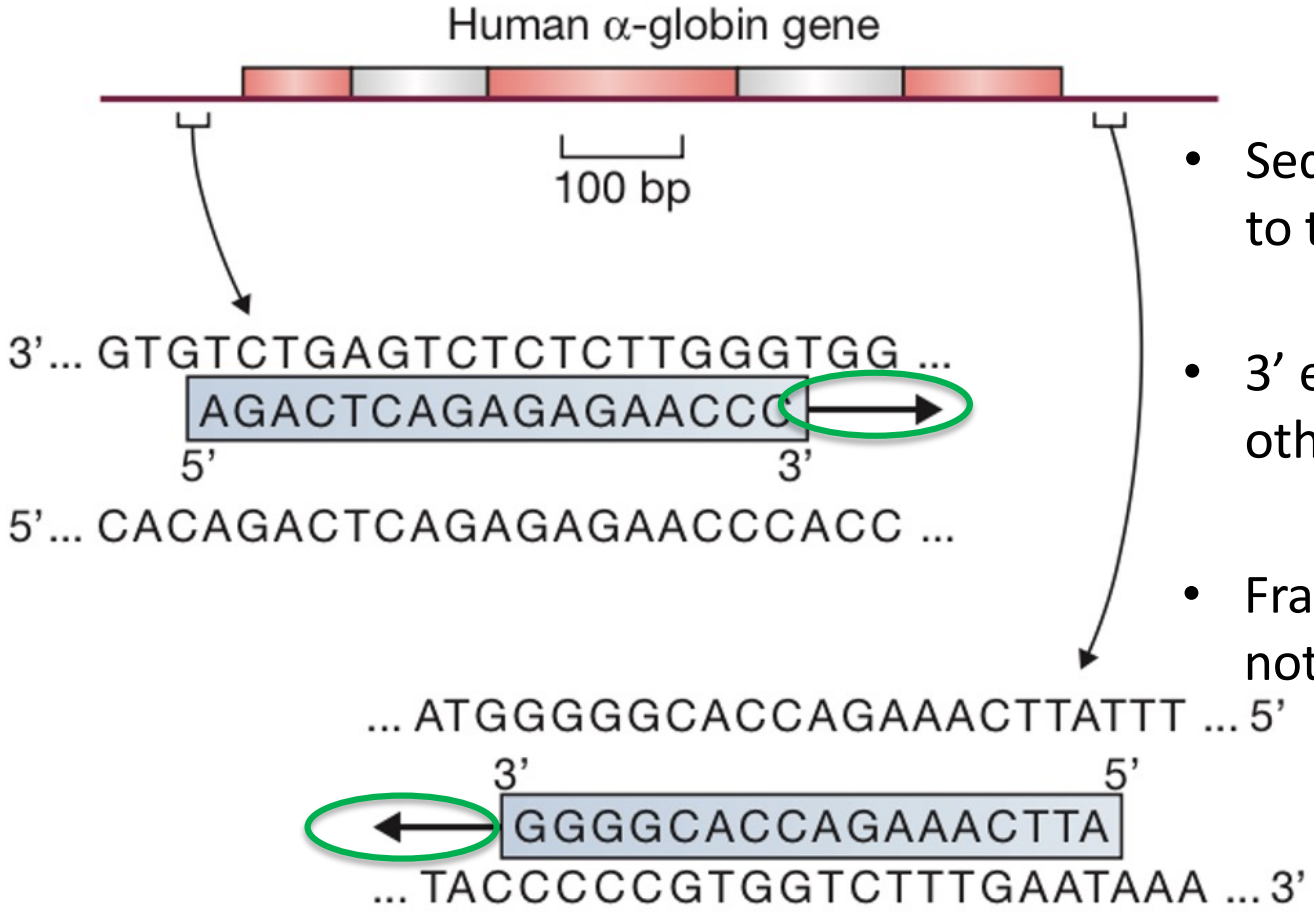
BMC – Bloco Biologia Molecular - 2022

TP4 – PCR “polymerase chain reaction” (part II)

2 most important parameters

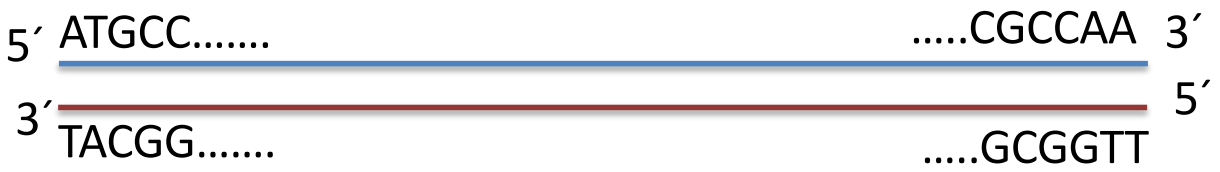
Primer design

Annealing temperature



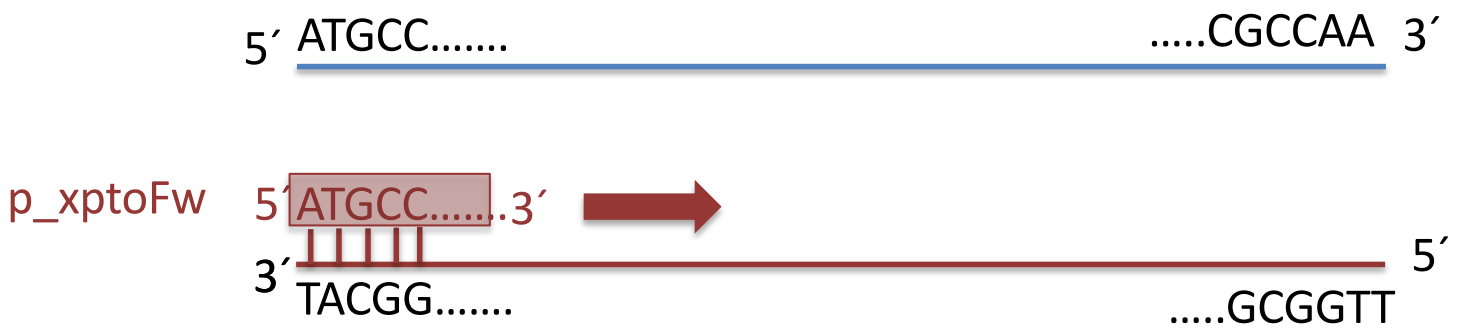
- Sequence complementary to the chain to be replicated
- 3' ends should point to each other
- Fragment to amplify should not exceed 3Kb

PCR – primer design



- Always display seq 5' - 3' and describe the **name** and **characteristics (number of nt; GC%; Tm)** of the primer.

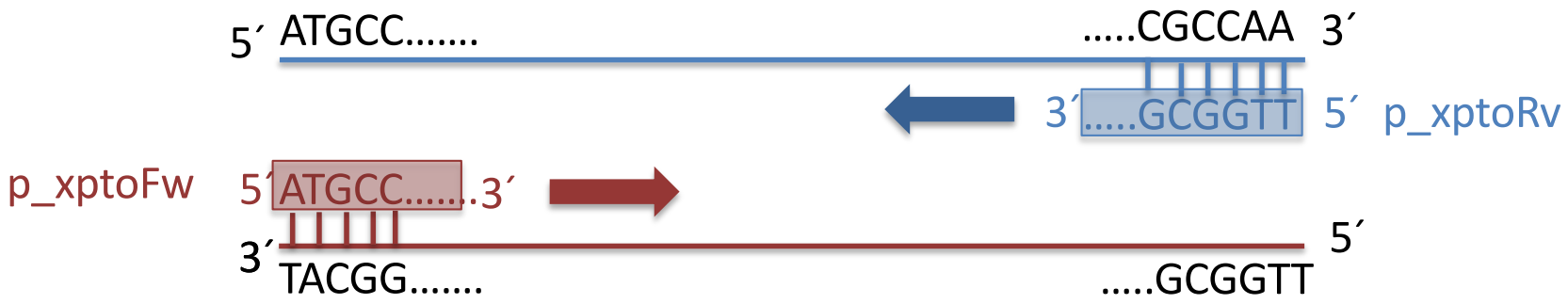
PCR – primer design



Primer p_xptoFw: 5´- ATGCC….-3´ (XX nts; YY GC%; Tm)

- Primer forward (or sense): sequence identical to region 5' of the 5'-3' chain
- Always display seq 5' - 3' and describe the **name** and **characteristics (number of nt; GC%; Tm)** of the primer.

PCR – primer design



Primer p_xptoFw: 5´- ATGCC….-3´ (XX nts; YY GC%; Tm)

Primer p_xptoRv: 5´- TTGGCG….-3´ (XX nts; YY GC%; Tm)

- Reverse primer (or anti-sense): reversed complementary sequence of the 3' chain region 5'-3'
- Always display seq 5' - 3' and describe the **name** and **characteristics (number of nt; GC%; Tm)** of the primer.

PCR – primer design

- **Sequence size**

- Annealing temperature
- Melting temperature
- GC Content

- GC clamps
- Possible secondary structures
- Repeats of the same nucleotide

- Avoid secondary structures in the template chain
- Avoid regions of frequent homology

PCR – primer design

Primer size

Primers too short

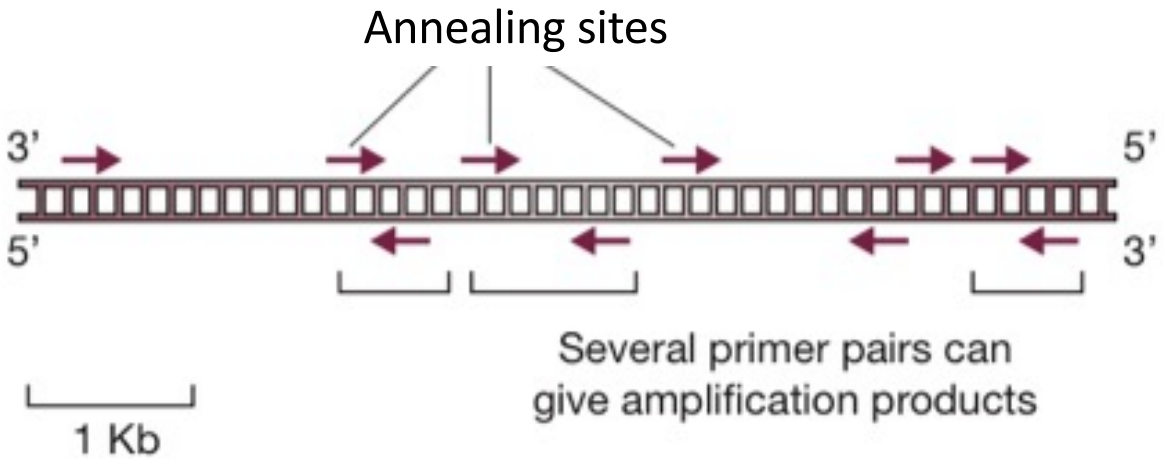
Probability of the sequence to occur:

1 per 4^8 = 65 536 pbs

Human Genome:3 200 000 000 pbs

3 200 000 000 / 65 536 = 49 000

Primers with 8 nucleotides in length



Primer size

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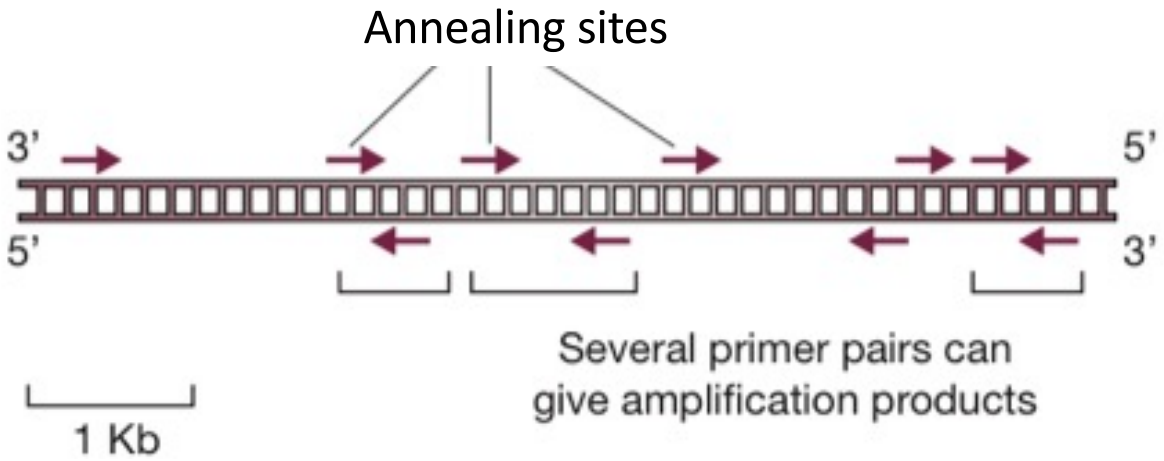
Primers with 8 nucleotides in length

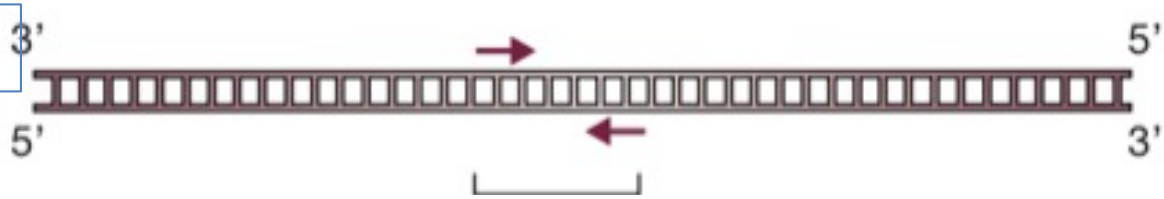


Probability of the sequence to occur:

1 per 4^{17}=17 179 869 184 pbs

3 200 000 000 / 17 179 869 184 < 1

Primers with 17 nucleotides in lenght



Only one fragment is amplified

PCR – primer design

- Sequence size

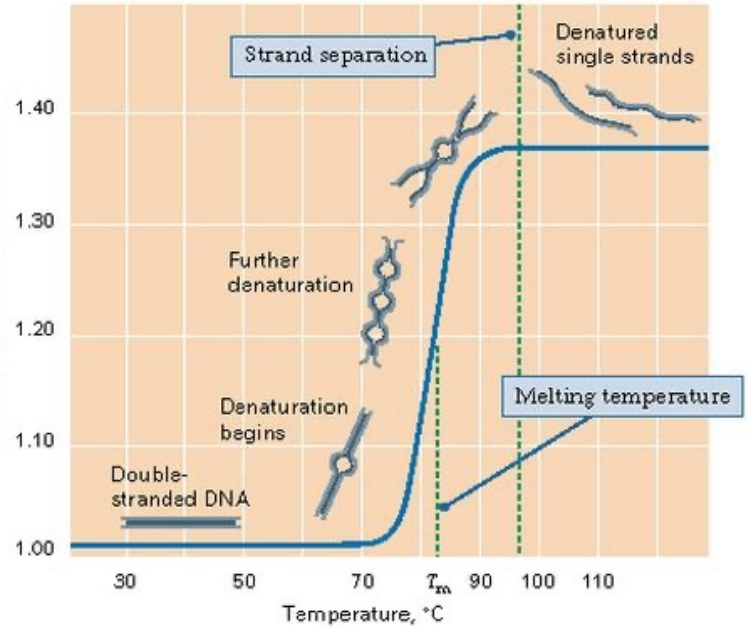
- **Annealing temperature**
- **Melting temperature**
- **GC Content**

- GC clamps
- Possible secondary structures
- Repeats of the same nucleotide

- Avoid secondary structures in the template chain
- Avoid regions of frequent homology

PCR – melting temperature

Melting temperature: temperature at which 50% of the chains in solution are denatured



$$Tm = (4 \times (G+C) + 2 \times (A+T)) \text{ ºC}$$

Primer sequence: 5' AGACTCAGAGAGAACCC 3'

4 Gs 5 Cs 7 As 1 T

$$T_m = (4 \times 9) + (2 \times 8)$$
$$= 36 + 16$$
$$= 52°C$$

PCR – annealing temperature

Tº anneal (Ta)= Tm - 4ºC

Low Ta:	non-specific products
High Ta:	avoids non-specific products lowers de amount of product formed

- **The Tm of the 2 primers should not differ by more than 3-5 degrees**

PCR – annealing temperature

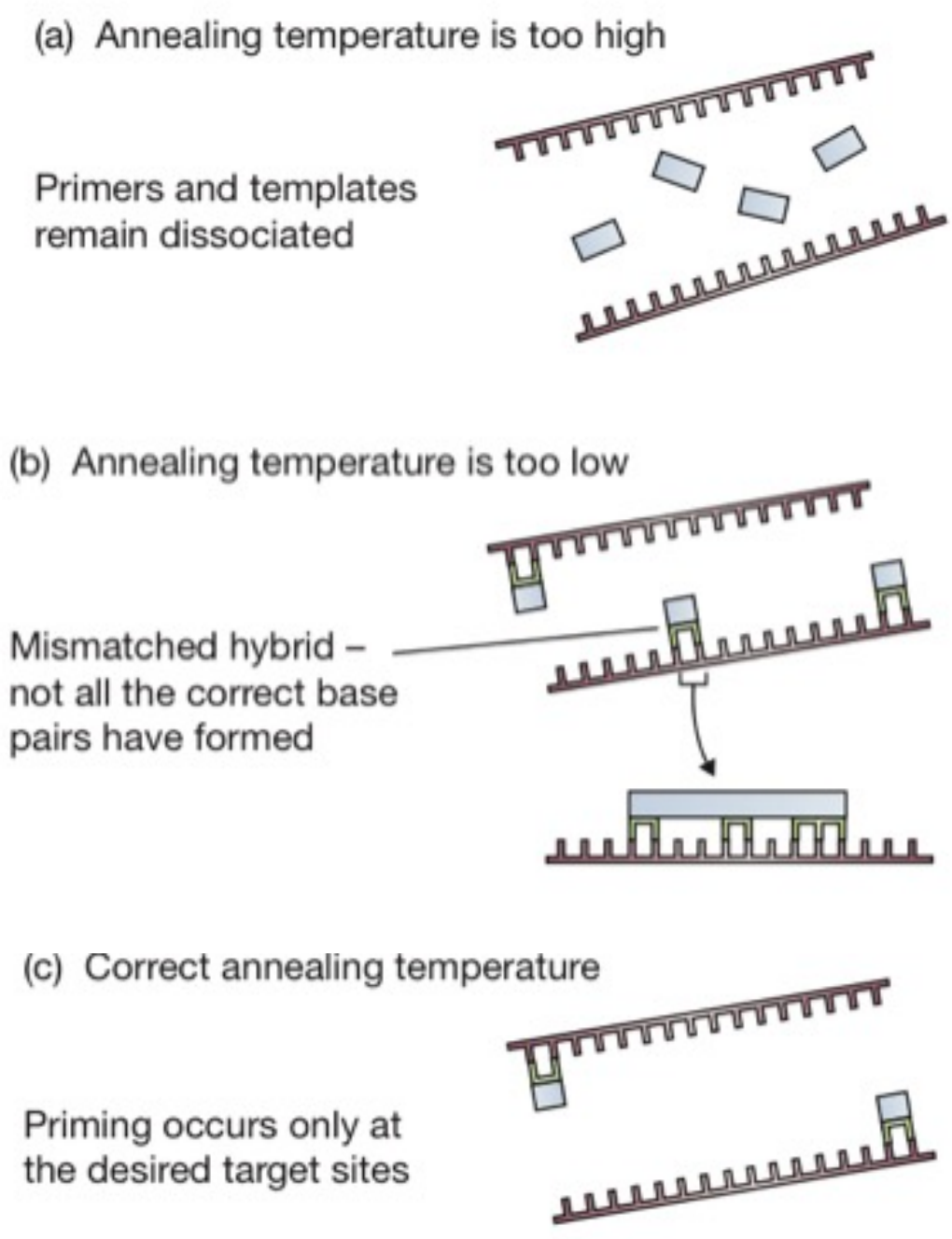
Annealing temperature determines the specificity of amplification.

The hybridization of 2 single chain DNA molecules depends on the temperature.

Nonspecific hybridization

Low enough – increases the efficiency of the PCR reaction

High enough - minimizes mismatchs



PCR – primer design

- Sequence size

- Annealing temperature
- Melting temperature
- GC Content

- **GC clamps**
- **Possible secondary structures**
- **Repeats of the same nucleotide**

- Avoid secondary structures in the template chain
- Avoid regions of frequent homology

PCR – primer design

GC content approx 50-60% - hybridization efficiency of PCR reaction 

GC Clamp

The presence of G or C bases in the last 5 bases at the 3'-OH end of the primer (GC Clamp) promotes higher specificity of the bonds at this extremity and increases duplex stability.

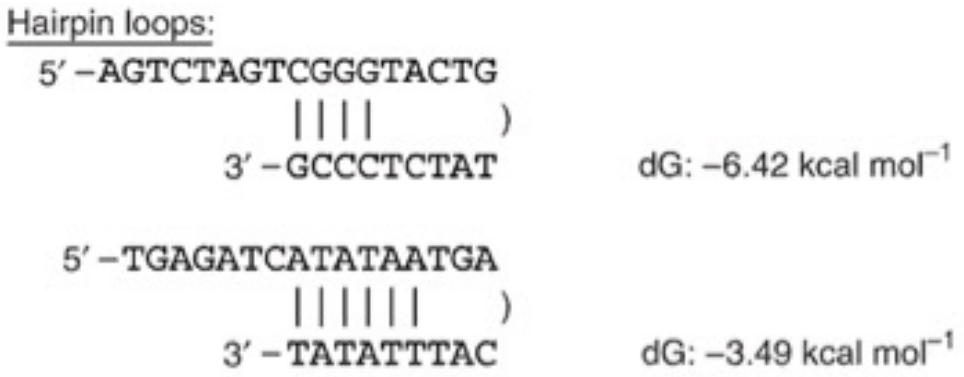
PCR – primer design

Possible secondary structures

Presence of secondary inter- or intramolecular structures can decrease efficiency, or even prevent product formation.

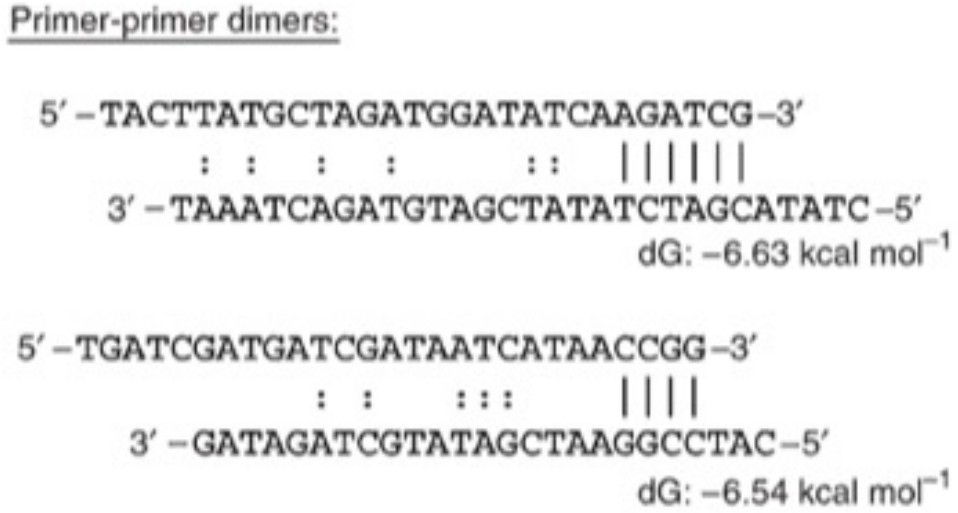
Formation of loops (hairpins)

hybridization efficiency in pcr reaction



Formation of dimers

hybridization efficiency in PCR reaction



ΔG closer to zero as possible

Formation of hairpins/dimers must be avoided especially at 3′ (prevents polymerase binding)

PCR – primer design

Repeats of the same nucleotide

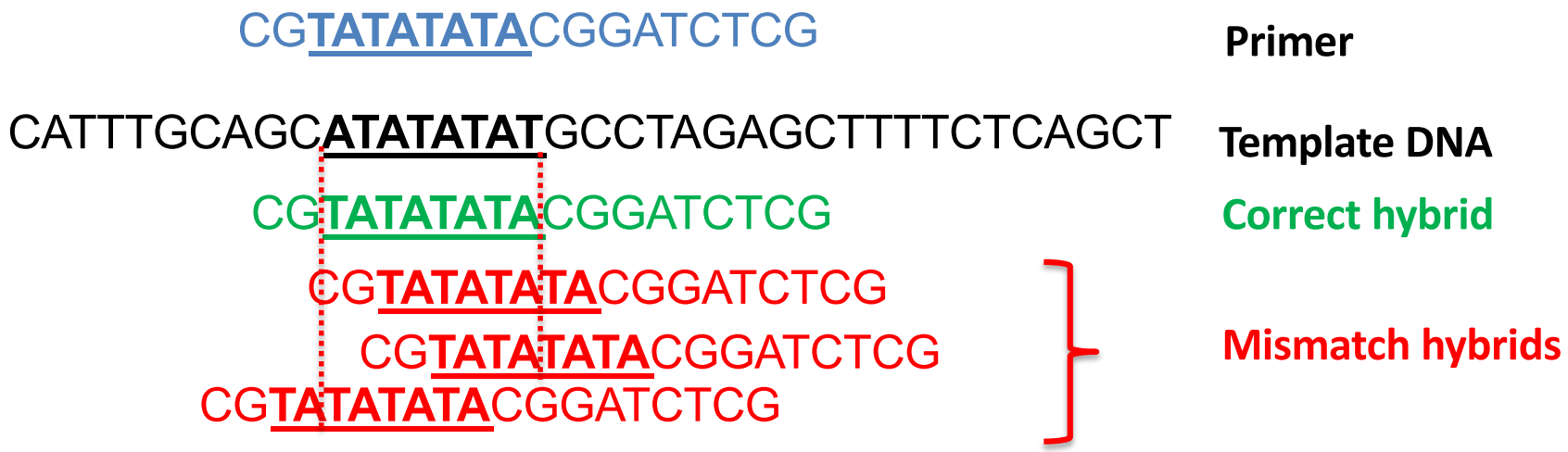
Primers with many repeats of a nucleotide can pair unspecifically and should be avoided.

For example AGCGGGGGATGGGG has 5 repetitions of G and 4 more repetitions of this same nucleotide. Maximum number of repetitions = 4 nt.

AGC**GGGGG**AT**GGGG**

Dinucleotide repeats

Many dinucleotide repeats (for example ATATATAT) should be avoided as they lead to nonspecific pairings, and formation of secondary structures (primer dimers, hairpins). The maximum reference number for dinucleotide repeats should be 2 (2 repetitions of AT = 4 nts: ATAT).



PCR – primer design

- Sequence size

- Annealing temperature
- Melting temperature
- GC Content

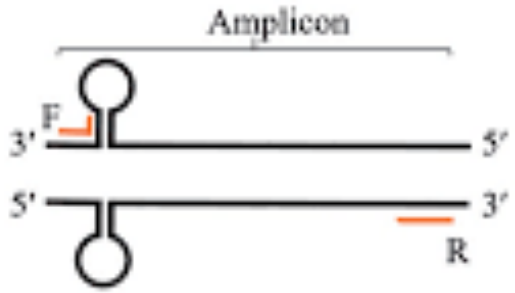
- GC clamps
- Possible secondary structures
- Repeats of the same nucleotide

- **Avoid secondary structures in the template chain**
- **Avoid regions of frequent homology**

PCR – primer design

Avoid secondary structures in the template chain

It is important that the primers do not form stable secondary structures with the template chain.



Avoid homology regions

In order to increase the specificity of primers, avoid regions of frequent homology. Primers designed for a specific area should not hybridize other zones at the same locus, or at different loci. Typically, primers are designed and aligned by BLAST to test specificity with the target.

Primer design video: https://www.youtube.com/watch?v=c-f1H07D_70