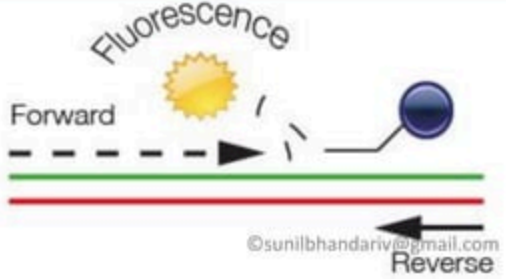
Primer and Probes

[How to]
Design
PRIMER



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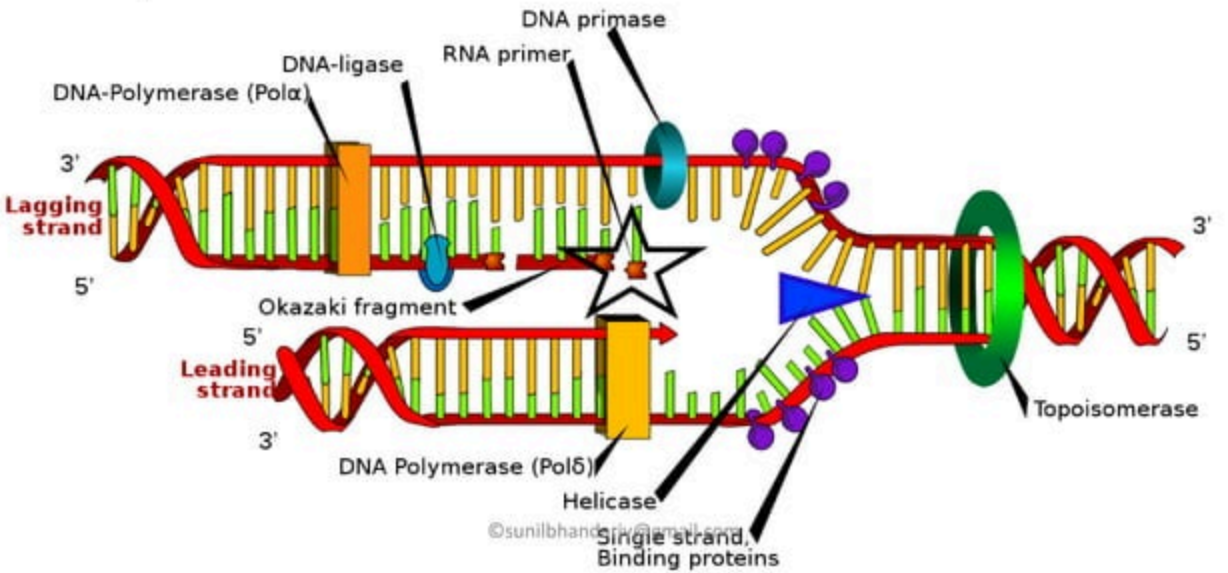
Primer

- A **primer** is a short nucleic acid sequence that provides a starting point for DNA synthesis. In living organisms, **primers** are short strands of **RNA**. A **primer** must be synthesized by an enzyme called primase, which is a type of **RNA** polymerase, before DNA **replication** can occur.

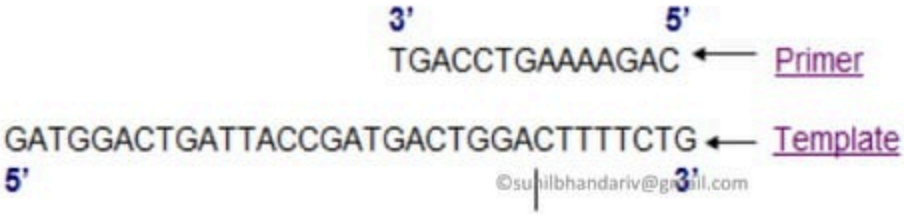


In sense of artificial purpose (Laboratory use)

What is Primer?

- A primer is a **short synthetic oligonucleotide** which is used in many molecular techniques.

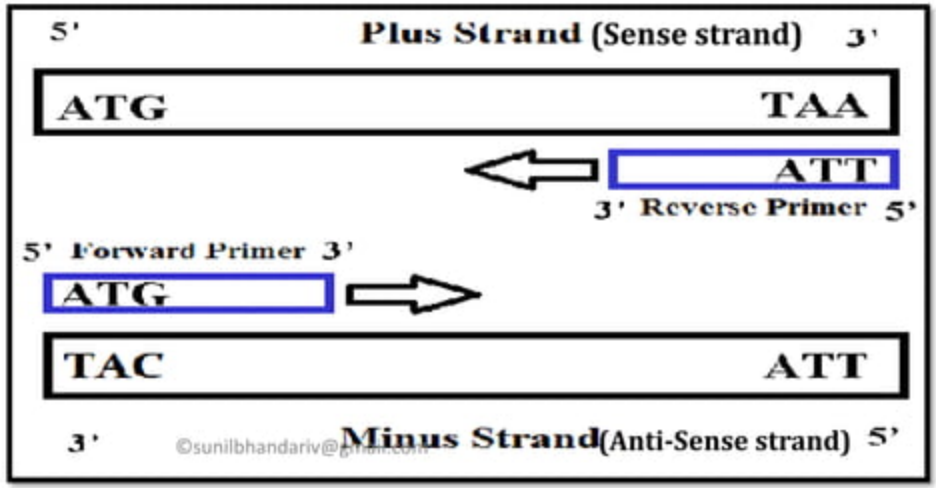
- These primers are designed **to have a sequence which is compliment of a region of template** or target DNA to which we wish the primer to anneal.



Types of Primer..

A. **Forward Primer:** It attaches to the start codon of the template DNA (the anti-sense strand)

B. **Reverse primer:** It attaches to the stop codon of the complementary strand of DNA (the sense strand). The 5' ends of both primers bind to the 3' end of each DNA strand



Role of Primer.....(initiate the Polymerization reaction)

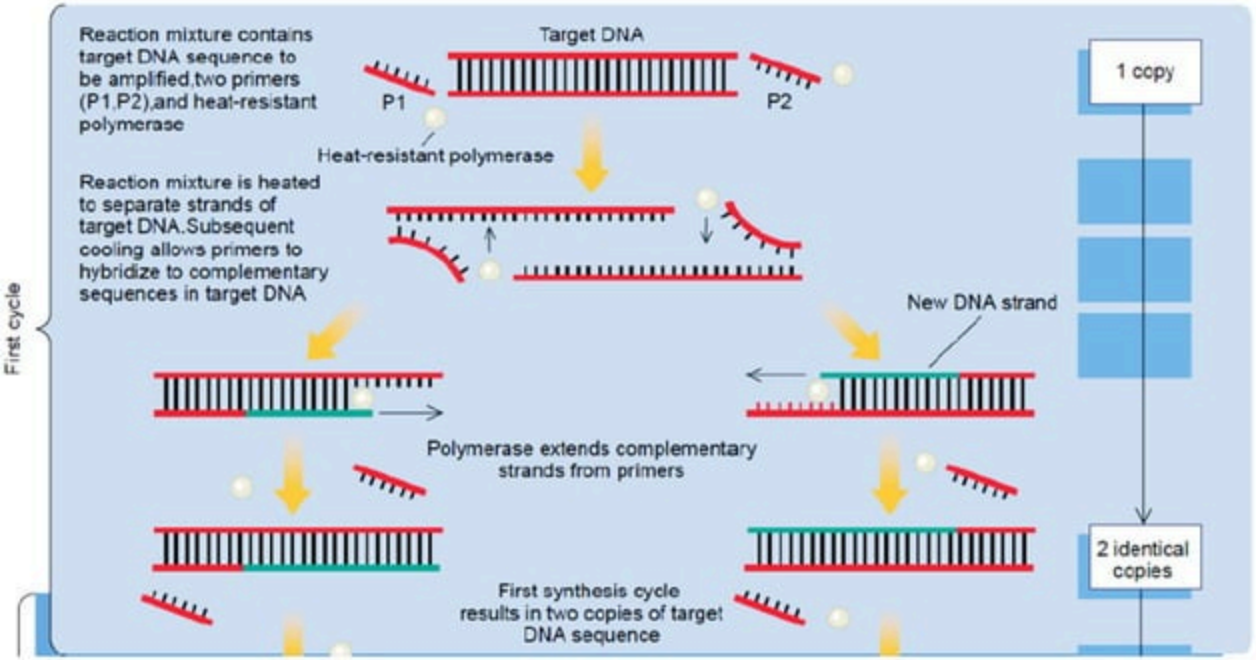


Fig: Polymerase chain reaction (PCR). Procedure takes the advantage of heat resistant DNA polymerase whose activity is not destroyed when temperature is raised to separate the double strand of DNA helix. With each cycle of duplication, the strands are separated, flanking segments (primers) bind to the ends of the selected region, and the polymerase copies the intervening segment.

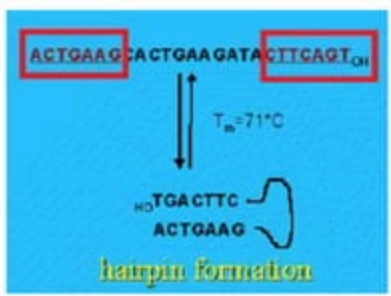
Source: Gerald Karp, 'Cell and Molecular Biology: Concepts And Experiments', 6th edition.

General rules for primer design

1. Primer length:

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

- **Optimal primer length :**
  - 18-24 bp for general applications
  - 30-35 bp for multiplex PCR



2. Melting temperature (T_m)

- T_m is the temperature at which 50% of the DNA duplex dissociates to become single stranded
- Determined by primer length, base composition and concentration.
- Working approximation: $T_m=2(A+T)+4(G+C)$ (*suitable only for 18mer or shorter*).
- Optimal melting temperature: 52°C-- 60°C
- Desirable T_m difference < 5°C between the primer pair

3. **Avoid non-specific amplification**
   - BLASTing PCR primers against NCBI non-redundant sequence database is a common way to avoid designing primers that may amplify non-targeted homologous regions.
4. **Primer G/C content**
   - GC-rich regions of the target DNA are difficult to amplify
   - Optimal G/C content: 45-55%
5. **Repeats**
   - Repeats increases mis-priming potential
   - The maximum acceptable number of repeats is 4 di-nucleotide

6. Primer secondary structures:

- Hairpins
  - Formed via intra-molecular interactions
- Self-Dimer (homodimer)
  - Formed by inter-molecular interactions between the two same primers
- Cross-Dimer (heterodimer)
  - Formed by inter-molecular interactions between the sense and antisense primers

7. GC clamp

- 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's or C's near the 3' end. This may promote mis priming at G or C-rich sequences (because of stability of annealing)

Primer design: Manual

- Take 18-24 sequences

- For forward primer, take sequence as it is and for reverse primer, reverse complement the sequence

http://www.bioinformatics.org/sms2/rev_comp.html

- Include start codon (ATG) (in case of forward Primer and Remove stop codon (TGA, TAA, TAG) in case of reverse Primer

- Tm temperature of two primers should not vary more than 5.

Example: Manual

- Here is the ORF sequence for a gene called bin1

  5'-ATGGCAGAGATGGGCAGTAAGGGGGTGACG.....
  CCCGAGAACTTCACTGAGAGGGTCCCATGA-3'

- Put the sequence through a restriction site analysis (NEB Cutter)
- This sequence contains neither BamHI nor NotI

To design the **forward primer**,

1. select the first 21 bases (functional primer):
   5'- ATG GCA GAG ATG GGC AGT AAG

2. Add the BamHI site (**GGA TCC**) to the 5' end of this sequence
   5'- gga tcc ATG GCA GAG ATG GGC AGT AAG

3. Add a couple of bases to the 5' end of the primer to preserve restriction site during PCR
   BIN1-F: 5'- gcaa gga tcc gtc ATG GCA GAG ATG GGC AGT AAG

- **For the reverse primer:**

1. Start in a similar way by selecting the last 21 bases in this sequence

CCCGAGAACTTCACTGAGAGGGTCCCATGA-3

2. EXCLUDE Stop codon (TGA)

AACTTCACTGAGAGGGTCCCA

3. Reverse complement the 21 bases in the sequence

TTGAAGTGACTCTCCCAGGGT (Complement sequence)

.....

TGG GAC CCT CTC AGT GAA GTT (Reverse complement)

4. Writing it in the 5' to 3' orientation (required when we're ordering the primer)

5'- TGG GAC CCT CTC AGT GAA GTT

5. Add a NotI (GCG GCC GC) restriction site

5'- gcg gcc gc TGG GAC CCT CTC AGT GAA GTT

6. This will shift the reading frame by one base (between the restriction site and the complementary sequence). So, add a base to shift the reading frame back in to place:

– 5'- gcg gcc gct TGG GAC CCT CTC AGT GAA GTT 3'

7. Final primer:

BIN1-R: 5'- gaa gcg gcc gct TGG GAC CCT CTC AGT GAA GTT -3'

Online Primer Designing Tools

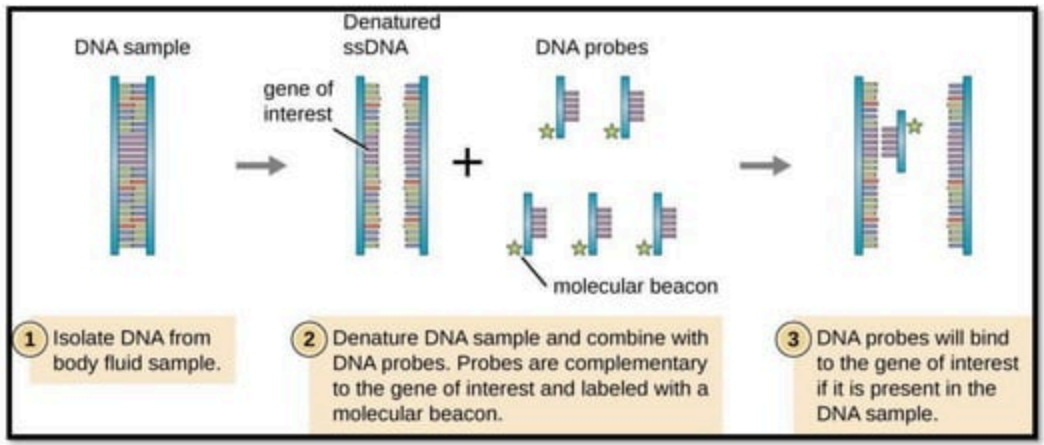
- NCBI/ Primer-BLAST
- PrimeTime® Predesigned qPCR Assay Database
- GeneFisher - Interactive PCR Primer Design
- Primer3Plus

Molecular Probes

- DNA or RNA probes are stretches of single-stranded DNA or RNA used to detect the presence of complementary nucleic acid sequences (target sequences) by hybridization.

- Probes are usually labelled, for example with radioisotopes, epitopes, biotin or fluorophores to enable their detection.

Role of Probe……



- Mostly, **Probes are labelled** with radioisotopes or any other fluorescence dyes.
- When mixed with target (template) DNA/RNA, **probes detects the complementary sites in template and binds** to it.
- This probe-template bonded state is **called hybridized** condition and process is called **hybridization**.
- When Probe bind to the target, labelled the radio/fluorescent dyes **emits the signal which can be detected and it ensure the hybridization** or detection of target part/sites in template DNA/RNA.

Probes vs Primer

- **Probe is** used to **detect the presence of a specific DNA fragment in the mixture through the hybridization with a double-stranded DNA.**

- **Primer is used in the initiation of the polymerase chain reaction by hybridization with single-stranded DNA.**