# Primer and Probe Design Tools

Shilpa Deshpande Kaistha
Dept of Biotechnology

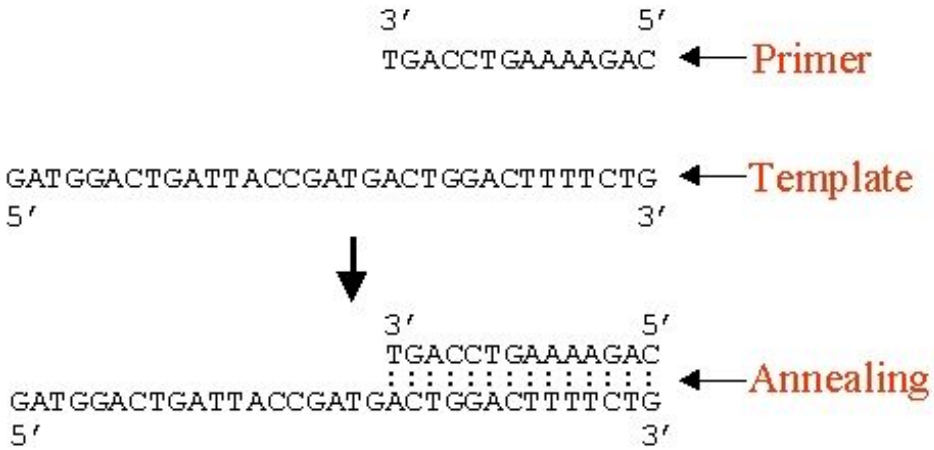
School of Life Sciences & Biotechnology
CSJM Univeristy Kanpur

# Introduction

- Primers **are short stretches of nucleic acids that target unique sequences and help identify a unique part of genome**
- A primer is **a short nucleic acid sequence that provides a starting point for DNA synthesis**. Size 15-25 bases
- **Oligonucleotide probes** are short stretches of single-stranded DNA or RNA used to detect the presence of complementary nucleic acid sequences (target sequences) by hybridization or qPCR. Oligonucleotide probes are usually labelled, for example with radioisotopes, epitopes, biotin or fluorophores to enable their detection- 25-1000 bases.
- Both can be RNA /DNA/cDNA
- While a PCR probe identifies target sequences, the primer serves as a starting point for DNA synthesis and replication. PCR probes and primers also differ in the length of their sequences and the type of DNA they bind

# Primer

- A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing.
- These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal
- DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify




https://microbenotes.com/primer/

# DNA primer and RNA primer

- Living organisms utilize RNA primers while *in vitro* involves DNA primers. However, DNA primers are much preferred due to varied reasons such as stability, easy storage, fewer enzymes required to initiate synthesis.
- The comparison between DNA and RNA primers is listed below.
- **DNA Primers:** 18- 24 base pairs.: PCR amplification, DNA sequencing.
- **RNA Primers:** 10-20 bp; In vivo: DNA replication, cloning.
- DNA primers are synthesized chemically while RNA primers require Primase enzyme

https://microbenotes.com/primer/

# Primer Design Properties

1. primers should be 17-28 bases in length;

2. base composition should be 50-60% (G+C)
3. primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming
4. Tms (Melting temperature ) between 55-80$^{o}$C are preferred
   - Tm (primer)- It measures the least stable primer-template pair.
   - Tm (product)- It measures the melting temperature of the PCR product.
   - The modified step annealing can be performed using gradient PCR where temperature can be set to bind primers.

5. Primer Annealing (Ta): The high Ta results in low PCR product with insufficient primer-template hybridization, while too low Ta will lead to non-specific PCR products caused as a result of a high number of base pairs mismatches.

   Ta= 0.3*Tm (primer ) +0.7 (product) – 14.9, Tm (primer)

1. 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product
2. primer self-complementarity (ability to form 2$^{o}$ structures such as hairpins) should be avoided
3. runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided.

   (adapted from Innis and Gelfand,1991)

# Probe Design Properties

- **Location** – The probes should hybridize with the DNA strand in a close proximity to the reverse or forward primer. It should not overlap with the primer binding sites. Generally, probes hybridize with either strand of the DNA duplex.
- **Melting temperature (Tm)** – The melting temperature of the probe should be 6-8 °C higher than that of the primers.
- **Annealing temperature (Ta)** – The annealing temperature of the experiment should be 5 °C below the melting temperature of primers.
- **GC content** – The GC content of the probe should be 35-65%. The 5′ end of the probe should not contain a G.

# Specific vs degenerate primer

- A primer, or more generally any DNA sequence, is called specific if it represents a unique sequence
- Degenerate if it represents a collection of unique sequences
- Two primers, forward primer and reverse primer, are used in each PCR reaction, which are designed to flank the target region for amplification. Two complementary single strands of DNA are released during denaturation

# Degenerate Primers

- A mix of oligonucleotide sequences in which some positions contain a number of possible bases, giving a population of primers with similar sequences that cover all possible nucleotide combinations for a given protein sequence” (Iserte 2013)
- These are used simultaneously in the hope that one of the sequences of the oligonucleotides will be perfectly complementary to a target DNA sequence.

# Degenerate Primer

- For example, the amino acid sequence shown below could be encoded by the following codons.
- AspGluGlyPheLeuSerTyrCysTrpLeuProHisGln
  GATGAAGGTTTTCTTTCTTATTGTTGGCTTCCTCATCAA
  C G C CT CAGC C C T C C C G
  A A A A A
  G G G G G

One could then select the 14 base sequence (in blue) to generate a smaller set of degenerate oligonucleotides. Each oligonucleotide in the set would have one base changed at a time (shown in purple below). A total of 32 unique oligonucleotides would be generated.

TATTGTTGGCTTCC

TACTGTTGGCTTCC

TATTGCTGGCTTCC

# Challenges encountered with primer design

- **Hairpins:** The loop structure formed by the intramolecular interactions within the primer which optimally 3’ end with -2kcal/m and internal hairpin with -3kcal/m can be tolerated.
- **Dimers:** A structure forming ds DNA by intermolecular interactions between 2 primers. Likewise, if the interaction formed between 2 homologous or the same sense of primer, – called as self-dimers while the opposite primers are called as cross dimers.
- **Primer- Template Cross Homology:** Primers should be designed in such a way that no homology within the template is been noticed other than the target site which resulted in non-specific binding and amplification.
- This can be categorized into 2 types: a) **Intra-primer homology:** The complementary bases within the same pair in the region of more than 3 bases can cause intramolecular bonding b)**Inter-primer homology:** Forward and reverse primers with complementary sequences are responsible for intermolecular bonding.

# Best Primer design online tools

- Primer designing tool (nih.gov)
- Primer3 Input
- Primer3Plus (bioinformatics. NL)
- PrimerQuest – design qPCR assays | IDT (idtdna.com)
- PerlPrimer (sourceforge.net)
- Primer Design with Oligo Primer Analysis Software v. 7
- Real-Time PCR Primer Design – Real-Time PCR Probe Design – GenScript
- www.autoprime.de

# Probe Design Tools

- PROBER is an oligonucleotide primer design software application that designs multiple primer pairs for generating PCR probes useful for fluorescence *in situ* hybridization (FISH)
- Oligo Design Software
- OligoAnalyzer

# Assignment

- Design primers 15-25 bp long that will amplify both of the sequences below.
- After you have identified the sequence of your primers, check the primers with the **programs** used to calculate melting temperature ($T_m$) and the formation of primer dimers.
- If the $T_m$ is less than 55$^oC$ or bad hairpins or dimers form, try another region of sequence.

>CCTTGGCCTCTGCCTAATCACACAGATTCTAACAGGATTATTTCTCGCAATACACTACACAGCTGACA
TCTCAACAGCCTTCTCCTCCGTCGCCCACATCTGTCGAGATGTTAACTACGGATGACTAATTCGAAAC
ATTCATGCAAACGGAGCCTCCTTTTTCTTCATCTGCCTCTACCTTCACGTAGCCCGAGGCATATACTA
TGGCTCATACCTCTACAAAGAAACCTGAAACATCGGAGTAGTTCTCCTACTCCTAACTATAATAACCG
CCTTCGTAGGATATGTGCTCCCATGAGGACAGATATCCTTCTGAGGAGCCACCGTAATTACCAACCTT
CTTTCCGCCTTCCCCTACATCGGGGACACCCTAGTACAATGAATCTGAGGTGGTTTCTCAGTAGACAA
CGCCACCCTAACC