به نام خدا

دانشگاه علوم پزشکی کرمان
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Primer Design Principles and Fundamentals Online Workshop

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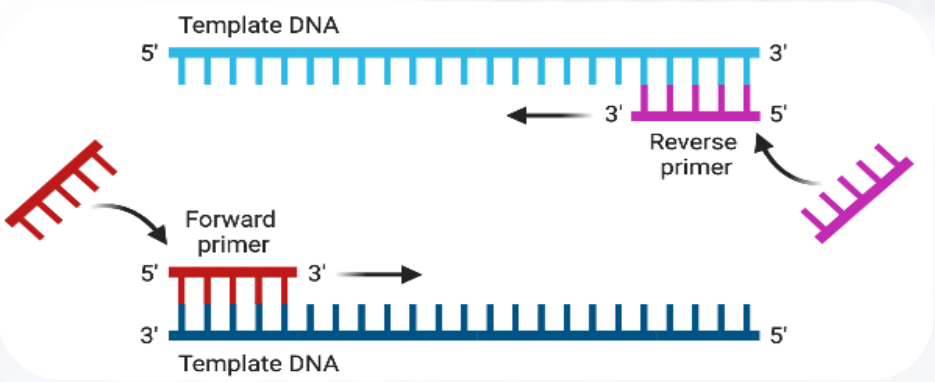
What is a primer?

Definition: A primer is a short, synthetic strand of DNA or RNA.

Function: It serves as a starting point for DNA synthesis in techniques like PCR and DNA sequencing.

Design Principle: Its nucleotide sequence is the reverse complement of the target DNA region, enabling specific annealing.



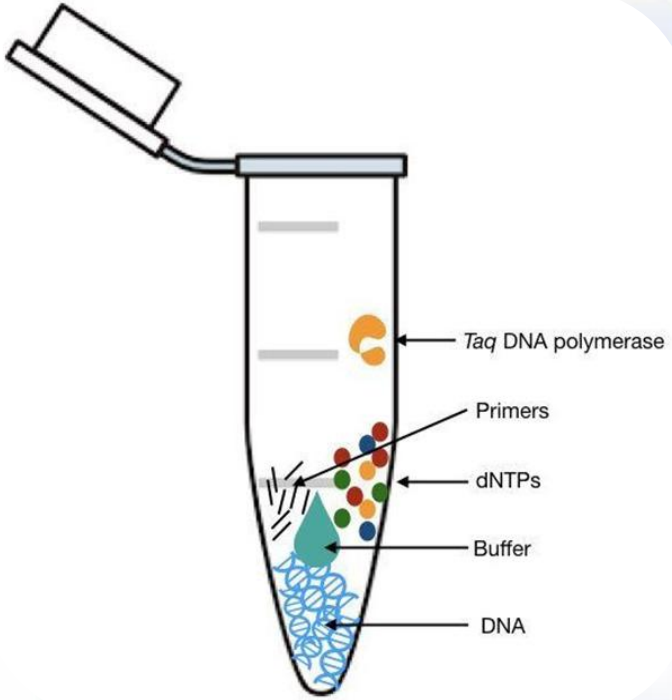
Why are primers important?

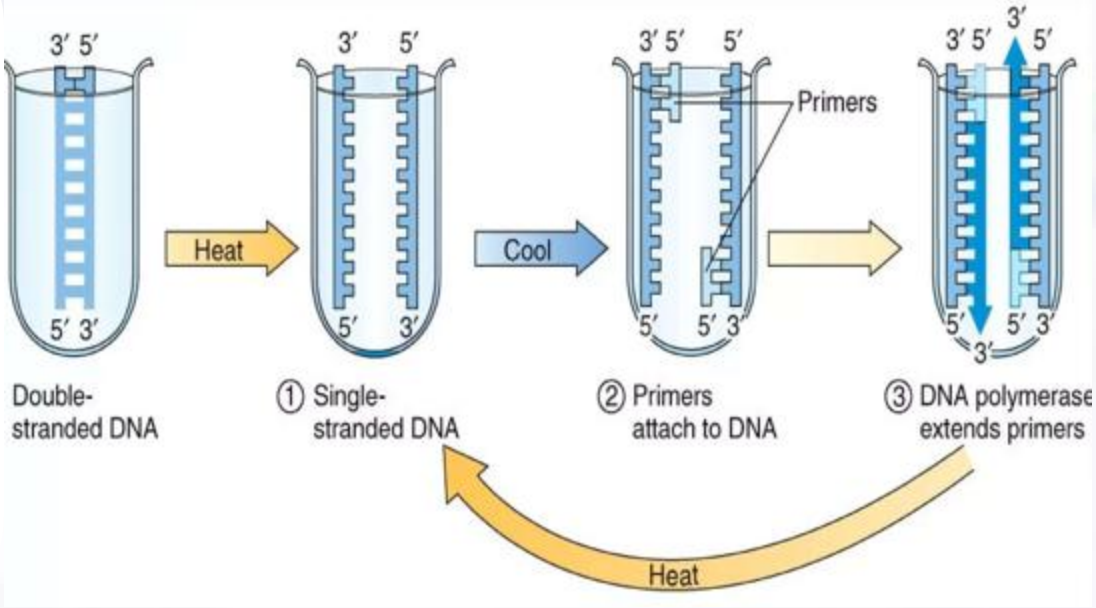
Key to Specificity: Primers are the fundamental component that provides specificity to PCR.

Good Design = Success: Well-designed primers lead to efficient and accurate amplification.

Bad Design = Failure: Poorly designed primers result in inefficient or non-specific amplification, causing the PCR to fail.

Brief PCR reminder





PCR Primer Design Guidelines

1. Primer Length
2. Primer Melting Temperature
3. Primer annealing temperature
4. GC Content
5. GC Clamp
6. Primer Secondary Structures
7. Repeats
8. Runs
9. 3' End Stability
10. Avoid Template secondary structure
11. Avoid Cross homology

1. Primer Length

It is generally accepted that the optimal length of PCR primers is 18-22 bp.
This length is long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature.

2. Primer Melting Temperature

Definition of Tm: The temperature at which 50% of the DNA duplex separates into single strands, indicating duplex stability.

Optimal Tm Range: 52-58 °C for the best results.

Accurate Calculation Method: The nearest neighbor thermodynamic theory (considered the most recent, superior, and best available method).

3. Primer annealing temperature

- **Role of Tm:** Estimates DNA-DNA hybrid stability and is vital for selecting the annealing temperature (Ta).
- **High Ta Consequence:** Poor primer binding and low PCR yield.
- **Low Ta Consequence:** Non-specific amplification due to mismatch tolerance.
- **Key to Specificity:** Mismatch tolerance is the most critical factor affecting PCR specificity.
- **If Mismatch Tolerance is HIGH (Ta is too low):** Your primer might bind to these similar, but non-specific, sequences.
- **If Mismatch Tolerance is LOW (Ta is optimized/higher):** The primer will only bind to the intended target.

3. Primer annealing temperature

- **Initial Calculation:** The starting Ta is typically set at 2–5 °C below the calculated Tm of the primer (using the nearest-neighbor method).
- **Empirical Optimization:** Since theoretical calculations are not always perfect, this starting point is optimized using a Temperature Gradient PCR. This experiment tests a range of temperatures (e.g., ±5 °C around the Tm) to empirically determine the temperature that produces the highest yield and the fewest non-specific products.

4. GC Content

- The GC content (the number of G's and C's in the primer as a percentage of the total bases) of the primer should be 40-60%.
- The 40-60% GC content guideline is crucial because it directly impacts the stability, specificity, and reliability of your primer's binding to the target DNA.

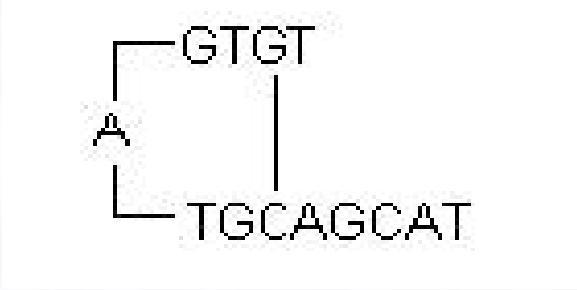
5. GC Clamp

- The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases.
- More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.
- Aim for 1–3 G/C bases in the final 5 nucleotides for a balance between stability and specificity.
- More than 3 Gs or Cs in the last 5 bases may lead to non-specific binding or primer-dimer formation.

6. Primer Secondary Structures

- Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction.

i) Hairpins: It is formed by intramolecular interaction within the primer and should be avoided. Optimally a 3' end hairpin with a ΔG of -2 kcal/mol and an internal hairpin with a ΔG of -3 kcal/mol is tolerated generally.



6. Primer Secondary Structures

ii) Self Dimer: A primer self-dimer is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield. Optimally a 3' end self dimer with a ΔG of -5 kcal/mol and an internal self dimer with a ΔG of -6 kcal/mol is tolerated generally.

6. Primer Secondary Structures

iii) Cross Dimer: Primer cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous. Optimally a 3' end cross dimer with a ΔG of -5 kcal/mol and an internal cross dimer with a ΔG of -6 kcal/mol is tolerated generally.

7. Repeats

- A repeat is a di-nucleotide occurring many times consecutively and should be avoided because they can mispriming.
- For example: ATATATAT
- A maximum number of di-nucleotide repeats acceptable in an oligo is 4 di-nucleotides.

8. Runs

- Primers with long runs of a single base should generally be avoided as they can misprime.
- For example, AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4.
- A maximum number of runs accepted is 4bp.

9. 3' End Stability

- **Core Concept:** The ΔG (Free Energy) of the last 5 bases at the primer's 3' end is a critical measure of its stability.
- **Key Rule:** An unstable 3' end (characterized by a less negative ΔG value) promotes higher PCR specificity.
- **Mechanism:** A stable 3' end can bind strongly even to non-target sequences with mismatches, causing false priming. An unstable 3' end requires a perfect match to initiate synthesis, thus reducing non-specific amplification.
- **Design Goal:** Ensure the 5-base 3' end does not have a very negative ΔG value (i.e., avoid a very stable sequence).

10. Avoid template secondary structure

- **Fundamental Problem:** Single-stranded nucleic acid templates are unstable and tend to fold into secondary structures (e.g., hairpins).
- **Key Drivers:** The stability of these structures is determined by their free energy (ΔG) and melting temperature (Tm).
- **Impact on PCR:** If a primer is designed to bind to a region where a stable secondary structure forms, the primer cannot access the template.
- **Consequence:** This inaccessibility significantly reduces or prevents amplification, leading to poor PCR yield. This is especially critical in qPCR.
- **Design Solution:** Primers must be designed to target regions of the template that do not form stable secondary structures during the annealing step.

11. Avoid Cross homology

- ✓ **Primary Goal:** Design highly specific primers that only amplify the intended target gene and not other sequences in the sample.
- ✓ **Main Challenge:** Avoid designing primers in regions of the template that have significant homology (similarity) to other genes.
- ✓ **Traditional Method:** Designing primers first and then checking their specificity using a BLAST search against a genomic database.

Practical Steps for Primer Design (Primer Design Workflow)

1. **Select the Target Gene or Sequence**

- ✓ Identify the gene or DNA region of interest (gene name or accession number).
- ✓ Retrieve the sequence from NCBI GenBank or a similar database.
- ✓ Choose the region to be amplified:
- ✓ For conventional PCR, select a region of about 100–1000 bp.
- ✓ Tip: Avoid repetitive or highly GC-rich/AT-rich regions.

Primer Design Workflow (2)

2. **Copy the Target Sequence**
Copy the desired region in FASTA format.
Include flanking sequences (≈200–300 bp upstream and downstream) to allow flexible primer placement.

Primer Design Workflow (3)

3. **Input the Sequence into Primer Design Software**

✅ Common tools: Primer3Plus, Primer-BLAST (NCBI), SnapGene, Geneunner

Steps:

- ✓ Paste the target sequence into the software.
- ✓ Set the desired product size (e.g., 150–300 bp).
- ✓ Define primer design parameters

Primer Design Workflow (4)

🧬 4. Evaluate the Suggested Primers

For each primer pair, check:

Parameter	Ideal range	Note
Primer length	18–25 bp	Affects Tm and specificity
GC content	40–60%	Balance stability and binding
Tm	55–65 °C	Forward and reverse Tm should be within ±2 °C
GC clamp	1–2 G/C at 3′ end	Improves primer binding
Self-complementarity	Low	Avoids hairpin or primer-dimer

📌 Tip: Keep the Tm difference between forward and reverse primers ≤ 2 °C.

Primer Design Workflow (5)

🧪 **5. Check Specificity**

✓ Run the selected primer sequences through Primer-BLAST or BLAST.

✓ Confirm that they bind only to the target region and not to non-specific regions in the genome.

🎯 **Goal:** Prevent off-target amplification and non-specific bands.

Primer Design Workflow (6)

🧰 **6. Analyze Secondary Structures**

Use OligoAnalyzer (IDT) or PrimerStat to check for:

✓ Self-dimers

✓ Hairpins

✓ Cross-dimers between forward and reverse primers

❌ If the ΔG value is very negative (e.g., < –9 kcal/mol), redesign the primer.

Primer Design Workflow (7)

🧬 **7. Final Selection and Documentation**

Choose the best primer pair based on all criteria.

Record the following information:

- ✓ Forward and reverse sequences
- ✓ Primer length, GC content, Tm
- ✓ Position on the gene
- ✓ Expected product size

Primer Design Workflow (8)

8. Order Primer Synthesis

- Send the sequences (5’ → 3’) to an oligo synthesis company.
- Typical synthesis scale: 25–100 nmol.

Primer Design Workflow (9)

💧 **9. Prepare Primer Working Solutions**

After receiving primers (lyophilized):

- Reconstitute with nuclease-free water or TE buffer.
- Stock solution: usually 100 µM
- Working solution: usually 10 µM

Primer Design Workflow (10)

🧪 **10. Test and Validate**

Run a test PCR to confirm the primer works as expected.

Analyze the PCR product using agarose gel electrophoresis:

✅ Clear single band at the correct size → primer is good.

❌ Non-specific bands or smears → redesign or optimize.

🧪 Practical Primer Design Session

✨ **What we will do next:**

🧬 Choose a target gene

📥 Retrieve the sequence from NCBI

🧪 Design primer pairs using Primer-BLAST /GeneRunner

🔍 Check Tm, GC content, secondary structure, specificity

✅ Finalize and document our primer pair

🧬 **Good primer design = Reliable PCR results**

🧠 **“Design carefully today to avoid troubleshooting tomorrow.”**