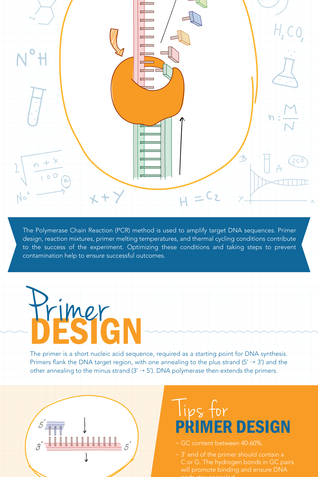
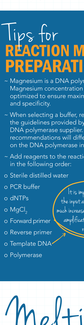
Optimizing PCR EXPERIMENTS



The Polymerase Chain Reaction (PCR) method is used to amplify target DNA sequences. Primer design, reaction mixtures, primer melting temperatures, and thermal cycling conditions contribute to the success of the experiment. Optimizing these conditions and taking steps to prevent contamination help to ensure successful outcomes.

Primer DESIGN

The primer is a short nucleic acid sequence, required as a starting point for DNA synthesis. Primers flank the DNA target region, with one annealing to the plus strand (5′ → 3′) and the other annealing to the minus strand (3′ → 5′). DNA polymerase then extends the primers.



Tips for PRIMER DESIGN

- GC content between 40-60%.
- 3′ end of the primer should contain a C or G. The hydrogen bonds in GC pairs will promote binding and ensure DNA ends stay annealed.
- Avoid primer dimers: ensure the 3′ ends of a primer set are not complimentary.
- Avoid hairpin loops: ensure the 3′ end of a single primer is not complimentary to other sequences in the primer.
- Avoid slipping: do not use single base runs of more than 4, and do not use dinucleotide repeats.

There are many databases and primer design tools available to simplify the process.

Reaction MIXTURE

The PCR reaction mixture contains water, buffer, polymerase, dNTPs, $MgCl_2$, primers, and the DNA template.

To minimize the risk of pipetting errors, prepare a PCR master mix consisting of sterile distilled water, buffer, dNTPs, primers, and polymerase in a single tube. Aliquot the master mix, then add $MgCl_2$ and DNA template.

Tips for REACTION MIXTURE PREPARATION

- Magnesium is a DNA polymerase cofactor. Magnesium concentration must be optimized to ensure maximum yield and specificity.
- When selecting a buffer, refer to the guidelines provided by the DNA polymerase supplier. Buffer recommendations will differ depending on the DNA polymerase in use.
- Add reagents to the reaction mixture in the following order:
  - Sterile distilled water
  - PCR buffer
  - dNTPs
  - $MgCl_2$
  - Forward primer
  - Reverse primer
  - Template DNA
  - Polymerase

It is important to optimize the input amount of DNA as too much increases the risk of nonspecific amplification and too little will reduce yield.

Melting TEMPERATURE

Primer T_m is the temperature at which half the DNA duplex dissociates and becomes single stranded. The primer melting temperatures should differ by no more than 5°C from each other. The conventional calculation for T_m is as follows:

$$2°C\ (A + T) + 4°C\ (G + C)$$

The nearest-neighbor thermodynamic models are superior to the conventional calculation, as they take into consideration the stacking energy of neighboring base pairs.

PCR additives, co-solvents, and modified nucleotides can lower T_m.

There are several online resources for calculating T_m with the nearest-neighbor models.

Thermal Cycling CONDITIONS

Thermal cyclers heat and cool the reaction mixture to facilitate denaturation, annealing, and elongation. Cycle duration and temperature depend on characteristics of the polymerases, buffers, template size, and GC content of the DNA.

Tips for SETTING THERMAL CYCLING CONDITIONS

- The initial denaturation step occurs at 94°C to 98°C, depending on the size of the template and DNA GC content.
  - High GC content (>65%) may require a longer incubation or higher temperature.
  - Buffers containing high salts may also require a higher temperature.
- The annealing step should occur approximately 5°C below the T_m of the primers.
- Extension time depends on the DNA polymerase in use. Taq DNA polymerase requires 1 min/kb and Pfu DNA polymerase requires 2 min/kb. Check manufacturer recommendations for temperature and time parameters specific to the DNA polymerase in use.

Avoiding PCR CONTAMINATION

PCR contamination may result in the production of unexpected amplicons. Taking precautions throughout the experiment helps to ensure accurate results.

Tips for AVOIDING PCR CONTAMINATION

- Use PCR plates made with low binding materials such as polypropylene to increase recovery.
- Ensure plates, tubes, and pipette tips are certified to be free from DNA, DNase, RNase, and inhibitors.
- Employ a unidirectional workflow during setup, separating pre- and post-amplification space.
- Work in a space dedicated to PCR, and work in separate PCR setup and PCR analysis areas.
- Set aside dedicated equipment (pipettes, centrifuge, vortex, etc.) for PCR setup.
- Decontaminate equipment regularly.
- Open tubes carefully and as infrequently as possible to minimize aerosols.