Optimization of Polymerase Chain Reactions

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Optimization of Polymerase Chain Reactions

(PCR) is a powerful method for fast in vitro enzymatic amplification of specific DNA sequence.

It can be grouped into three different categories:

1. **Standard PCR** (amplification of a single DNA sequence &less than 5 kb)
2. **Long PCR** (more than 5 kb and up to 40 kb eg. complete genes)
3. **Multiplex PCR** (less than 5 kb).

Why PCR requires specific optimization?

Lack of optimization often results many problems such as:

- No detectable **PCR product** or low efficiency amplification
- Presence of **nonspecific bands**
- Formation of "**primer-dimers**"
- **Mutations** caused by errors in nucleotide incorporation.

HOW WE ACHIEVE OPTIMAL PCR AMPLIFICATIONS?

Optimization involves various parameters

1. Quality and concentration of ***DNA template***
2. Design and concentration of ***primers***
3. Concentration of ***magnesium ions***
4. Concentration of the four deoxynucleotides ***(dNTPs)***
5. PCR ***buffer*** systems
6. Selection and concentration of ***DNA polymerase***
7. PCR thermal cycling ***conditions***
8. Addition and concentrations of PCR additives/cosolvents
9. Use of the "***hot start***" technique

PCR Materials

1. **Template DNA** (e.g., plasmid DNA, genomic DNA).
2. **Forward and reverse PCR primers.**
3. **MgCl2** (25 m*M*).
4. **dNTPs** (a mixture of 2.5 m*M* dATP, dCTP, dGTP, and dTTP).
5. **10× PCR buffer**: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 25°C.
6. **Thermal stable DNA polymerase** (e.g., *Taq* DNA polymerase).
7. **Water** free nuclease.

Methods for optimization

1. *Setting Up of PCR*
2. *PCR Cycling*
3. *Verifying PCR Amplification*

1. Setting Up of PCR

All of the reaction components can be mixed in together in a 0.5 mL PCR tube except for the DNA polymerase, which should be added last.

For each PCR, the following components are mixed together:

1. Template DNA (1–500 ng).
2. Primers (0.05–1.0 μM).
3. Mg2+ (0.5–5 mM).
4. dNTP (20–200 μM each).
5. 1× PCR buffer: 1 mM Tris-HCl and 5 mM KCl.
6. DNA polymerase (0.5–2.5 U for each 50 μL of PCR).

2. PCR Cycling

- **Initialization** (92-95°C for 2-5 minutes)
- **Denaturation** (90 to 98°C for 10 s to 1 min)
- **Annealing** (55 to 70°C for 30 s to 1 min)
- **Extension** (72 to 74°C for 1 min per kb of expected PCR product)
- **Final Extension** (5 to 15 min at 72°C)
- **Hold** (4°C for an indefinite time)

3. Verifying PCR Amplification

To measure the success of a PCR amplification, 5 to 10 μL of the final PCR product is run on a 1 or 2% agarose gel and visualized by staining with EtBr.

The critical questions are as follows:

Is there *a **band*** on the gel*?*

Is the band at the ***expected size***?

Are there any ***nonspecific bands*** beside the expected PCR band on the gel?

Is there ***smear*** on the gel?

A successful PCR amplification should display a ***single band*** with the ***expected size*** without ***nonspecific bands*** and ***smear*.**

Notes

1. The *quality* and *concentration* of DNA templates.

- For **Long PCR** (>5 kb) use 100 to 500 ng of template DNA.
- For **Multiplex PCR** two- to fivefold more DNA template than a typical PCR should be used.

2. Appropriate primer design and primer concentration

The purpose of primer design is to achieve a balance between the ***specificity*** and ***efficiency*** of an amplification.

Specificity defines how frequently mispriming occurs.

Efficiency represents the increase of the amount of PCR product over a given number of cycles.

The **optimal primer size** is usually between 18 and 28 bases.

Shorter primers are generally less specific but may result in more efficient PCR.

Longer primers improve specificity yet can be less efficient.

primer design

Primers from both directions should have **melting temperatures** Tm, defined as the dissociation temperature of the primer/template duplex.

Avoid ***complementary sequences*** within a primer or between the two primers, this will reduce formation of ***primer-dimers***

Primers with Tm higher than 50°C will generally provide specific and efficient amplifications.

A (GC) content of 40 to 60% is desirable for primers because this assures a ***higher Tm*** and therefore increases specificity.

Primer concentration

Concentrations of primers are influenced amplification ***specificity and efficiency.***
primer concentrations between 0.05 to 1.0 μM (or 0.5–100 pmol) are used in 100 μL of PCR

Higher primer concentrations can result in nonspecific priming and formation of primer-dimers, whereas **lower primer** concentrations may adversely affect PCR efficiency.

Lower concentrations of primers are more desirable for multiplex PCR.

3. Magnesium concentration

Magnesium concentation is a crucial factor in PCR amplification. Components in the reaction (**template, chelating agents such as EDTA of citrate, dNTPs and proteins**) affect the amount of free magnesium present in the reaction. DNA polymerases are **inactive** in the **absence of Mg.**

The role of Mg++ in PCR is dual: **promoting DNA/DNA interactions** and **forming complexes with dNTPs** that are the actual substrates for thermal DNA Polymerase.

Common magnesium concentrations used in PCR are between (0.5 to 5 m*M)*.

When Mg++ is too low, **primers fail to anneal** to the target DNA then the result is little or no PCR product.

When Mg++ is too high, the **base pairing becomes too strong** and the **amplicon fails to denature completely** and **mispriming** can result then seen as **multiple bands** on an agarose gel.

4. Concentration of dNTPs

[dNTP] can affect the yield, specificity, and fidelity of amplification. Conc. of **(20 to 200 μ*M)*** of each dNTP used normaly. For long PCR **(250 μM)** of each of dNTPs and for **{ 20 kb ⟶ 400-500 μM}**

Lower concentrations of dNTPs minimize mispriming and reduce the extending misincorporated nucleotides, which in turn increase specificity and fidelity of PCR amplifications.

Higher dNTP concentrations increase the error rate of DNA polymerases, millimolar concentrations of dNTPs inhibit *Taq* DNA polymerase.

5. DNA polymerase

Standard PCR amplifications using *Taq* DNA polymerase are performed in 10 m*M* Tris- HCl (pH 8.3–8.4 at 20–25°C) and 50 m*M* KCl.

The half-life of Taq DNA polymerase is **40 min at 95°C**, which is sufficient to remain active over 30. Taq DNA polymerase has an extension rate of **35 to 100 nucleotides** per **second** at **72°C**.

It was originally purified from the **gram-negative** thermophilic bacterium ***Thermus aquaticus***. The error rate for *Taq* DNA polymerase, which lacks proofreading 3′ → 5′ exonuclease activity

DNA polymerases with ***proofreading activity*** (for example, *Pwo*, *Pfu*, *Tli)*

DNA polymerase

Concentration range for *Taq* DNA polymerase is between 1 - 2.5 units per **100 µL** of PCR.

Increasing the amount of *Taq* DNA polymerase beyond the 2.5 units/reaction can in some cases **increase PCR efficiency** or it can increase the yield of **nonspecific PCR products**.

6. Cycle number

Cycle number has the temperature and incubation time for **template** denaturation, **primer annealing**, and **primer extension**.

Few cycles of PCR result in ***low product*** yield, most PCR amplifications are performed for no more than 20 to 40 cycles.

The first cycling denaturation step is ***an initial dissociation step*** at 92 to 95°C for 2 to 5 min to **ensure the complete separation** of the DNA strands.

Templates with high GC content use **longer incubation** time to achieve complete denaturation.

It can also cause ***depurination*** of the DNA template, which reduces amplification efficiency and also will ***reduce*** the amount of ***active DNA polymerase***. The half-life of *Taq* DNA activity is:

2 h at 92.5°C,

40 min at 95°C,

5 min at 97.5°C.

Primer annealing

Primer annealing temperature for a particular PCR amplification depends on the **base composition**, **nucleotide sequence**, **length**, and **concentration of the primers.**

A typical primer annealing temperature is 5°C below the calculated Tm of the primers. Annealing temperatures from 55 to 70°C generally yield the best results.

A higher annealing temperature increases amplification specificity because it enhances ***discrimination against incorrectly annealed*** primers and ***reduces mis-extension*** of incorrect nucleotides.

Incubation times from (30 s to 1 min) are generally recommended to assure successful primer annealing

Primer extension

It depends on the length and concentration of the target sequence, as well as the extension temperature.

***Taq* DNA polymerase extends at a rate of**
0.25 nucleotides / s. at 22°C,
1.5 nucleotides / s. at 37°C,
24 nucleotides / s. at 55°C,
more than 60 nucleotides / s. at 70°C,
150 nucleotides / s. at 75 to 80°C

Taq DNA polymerase is expected to extend at the rate of greater than **3500 nucleotides per minute.**

Two-step cycling program

It is the combination of primer annealing and extension in one step and generally applied when a high annealing temperature is used, such as 65 to 70°C.

A typical two-step cycling program:
Initial denaturation at (94°C for 2 min)
Denaturation at (92 to 95°C for 10 - 30 s)
Annealing and extension at (65 - 68°C for 1 min) per kb then held at 4°C.

Hot start

Is a technique enhances PCR specificity by eliminating the production of ***nonspecific products*** and ***primer-dimers***.

Hot start Taq DNA polymerase is constructed through the addition of an ***anti-Taq DNA polymerase antibody.***

The ***antibody*** will prevent the ***DNA polymerase activity*** until the temperature rises during the initial denaturation step.

Then increased temperature dissociates and degrades the bound antibody, initiating PCR amplification.

THANKS FOR YOUR QUESTIONS