POLYMERASE CHAIN REACTION

Target DNA

Taq polymerase
Nucleotides
Primers

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• *In vitro* technique for amplification of the specified DNA sequences.

• It enables us to produce enormous numbers of copies of a specified DNA sequence without having to clone it in a living cell.

1. Research applications

   *DNA sequencing, molecular cloning, etc.*

2. Diagnostics and forensic applications
Before the invention of PCR, cloning of DNA in vivo (bacteria or viruses) was used to amplify the desired DNA targets.
POLYMERASE CHAIN REACTION

HISTORY OF PCR

• Invented by Kary Mullis in 1983.
• Won the Nobel prize in chemistry in 1993.
• Simulates the natural DNA replication processes in vitro.
DNA replication in vivo

Extension by DNA polymerase from 5' to 3'

Unwinding of DNA templates (helicase)

RNA primers
POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

1. Template denaturation

heat 94-96 °C
2. Annealing of DNA primers

- Two synthetic oligodeoxyribonucleotide primers bracketing the amplified sequence are used to amplify a short, well-defined part of a DNA strand.
From the sequence of gene of interest, a research needs to amplify a fragment of the gene using PCR. Suppose that one primer has the sequence 5’ CGGACGCGAGCGGG 3’ as underlined, what would be the sequence of the other primer if the desired PCR product size is 250 bp?
2. Annealing of DNA primers

Lower temperature from 95 °C to 50 - 60 °C (annealing temperature)
3. Extension

- dNTPs (dATP, dGTP, dCTP and dTTP)
- DNA polymerase
- Buffer [pH, monovalent (K⁺) and divalent cations (Mg²⁺)]
Originally, Klenow fragment of DNA polymerase I from *E.coli* is used.  
*Optimal temperature ~ 37 °C*

Heat labile → new enzyme needs to be added every cycle  
→ *tedious and expensive*

*Discovery of a DNA polymerase in thermophilic bacteria living in hot springs.*

*Thermus aquaticus*

*Taq* polymerase is stable to heat. *Optimal temperature ~72 °C*  
→ no new enzyme is needed.  
→ less tedious and reduced contamination
The specified DNA region is amplified into DNA fragments of defined sizes (depending on the distance of the two primers).
Why is it called Polymerase Chain Reaction?
POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

• Newly synthesized DNA can serve as templates - chain reaction.

• Theoretical yield starting from one template copy

  \[ 2, 4, 8, 16, 32, 64, 128, 256 \ldots \]

  or \[2^n - 2n\] or \[\sim 2^n\]

  \(n = \text{number of amplification cycles}\)

• After 30 cycles, one DNA template copy can theoretically generate up to \(2^{30}\) or 10,000,000,000 copies of PCR products.
A PCR reaction normally consists of:

1) Reaction buffer

2) Mixtures of all four deoxyribonucleotide triphophates (dNTPs)

3) DNA polymerase

4) Template DNA

5) Two different oligonucleotide primers (forward & reverse)
1) REACTION BUFFER

Standard PCR buffer for *Taq* polymerase

- 10 mM Tris pH 8.3-8.8 (at room temperature)
  ~ 7.0-7.5 at 72 °C (optimal pH for *Taq*)
- 50 mM KCl
- 1.5 mM MgCl₂

- usually prepared using distilled, sterile water as 10x stock (autoclaved)

- effective for a wide range of PCR applications.

- *Taq* is highly hydrophobic protein. Addition of non-ionic detergent (Triton X-100, NP40 or Tween-20) to the buffer helps stabilize the enzyme and maintain full activity.
1) REACTION BUFFER

- Mg$^{2+}$ concentration is important for Taq activity and primer annealing. -> yield and specificity of the reaction.

- Standard range 0.5 - 10 mM (free Mg$^{2+}$ should be 0.5-3.0 mM above the concentration of dNTPs).

excess Mg$^{2+}$ -> non-specific annealing of primers to templates
low Mg$^{2+}$ -> low enzymatic activity
1) REACTION BUFFER

- May need optimization, particularly when the concentration of dNTPs or primers is changed.

<table>
<thead>
<tr>
<th>Mg$^{2+}$ concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 2.0 2.5 3.0 3.5 4.0 mM</td>
</tr>
</tbody>
</table>

[Agarose gel electrophoresis image with PCR product highlighted]
2) DEOXYRIBONUCLEOTIDES (dNTPs)

- **200 μM each** (recommended concentration), ranging from 50-1500 μM

  - Very low (<5 μM each) or high (>1000 μM each) concentration increases error rates.

  - Always use equal concentration of each dNTP to prevent misincorporation.
3) DNA POLYMERASE

- *Taq* is almost universally used (due to its thermostability, reliability and efficiency).
  
  Thermostable, half-life > 2 h at 92.5 °C
  Optimal pH @ 72 °C
  incorporate ~ 50-100 nucleotides/sec

- Use at 2-2.5 U/100 μl of reaction.

  *Too much enzyme -> reduced specificity*
3) DNA POLYMERASE

- *Taq* DNA polymerase lack 3’-5’ exonuclease (proofreading function).

  --> a relatively high error rate (*low fidelity*)

  *AT - GC transition*
  *Deletion (causing framshift mutations)*

- May be fine for many applications, not concerning about fidelity.

- May not be suitable if the PCR products are subsequently cloned or expressed.
3) DNA POLYMERASE

High fidelity, thermostable DNA polymerases are used when high fidelity amplification is required.

*Pfu* (*Pyrococcus furiosus*)

*Deep Vent* (*Pyrococcus* species GB-D)

*Vent* (*Thermococcus litoralis*)

- Possess 3’-5’ exonucleases activity (proofreading function).
- Suitable for high fidelity PCR.

<table>
<thead>
<tr>
<th>Fidelity</th>
<th>Pfu</th>
<th>Deep Vent</th>
<th>Vent</th>
<th>Taq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error rates (errors/base)</td>
<td>1.3x10⁻⁶</td>
<td>2.7x10⁻⁶</td>
<td>2.8x10⁻⁶</td>
<td>2x10⁻⁵</td>
</tr>
</tbody>
</table>

Error rate of *Pfu* ~ 5-10-fold lower than that of *Taq*
4) DNA TEMPLATES

- Virtually all forms of DNA can be templates for PCR.

  \textit{Genomic, plasmid, or phage DNA or previously amplified PCR products}

- PCR does not require highly-purified template DNA. Samples prepared via standard molecular techniques are sufficiently pure for PCR.

  \textit{Even crude DNA preparations are adequate (e.g. in colony PCR).}

- 0.1-1 \(\mu\)g of mammalian genomic DNA
  pico- or nanogram ranges for cloned templates
  (e.g. plasmids, PCR products 100 - 100,000 target copies)

  \textit{Even DNA from a single cell may be sufficient.}
PCR REACTION COMPONENTS

5) PRIMERS

- Primer length and sequence are of critical importance for successful PCR amplification.

*Poor primer design → poor yield or non-specific products*
PRIMER DESIGN TIPS

a) Primer length

- Typically 15-30 bases long, complementary to the DNA targets

- A primer needs to be long enough for specific annealing to the target.

  - A chance of a primer with 15 bases long to appear in human genome (3x10⁹ bp) is one in every 4¹⁵ or 1x10⁹ bp. -> less specific

  - A primer with at least 16 bases long -> one in 4¹⁶ or 4.3 x10⁹
  -> more specific

  - For amplification using *E.coli* genomic DNA (3x10⁶ bp), a primer at least 11 bases long (4¹¹ or ~ 4x10⁶) should be adequate.
B) Melting- and annealing temperature ($T_m$ & $T_a$) of primer

- Annealing temperature is a critical parameter for successful PCR amplification.

**Too low**

- non-specific priming to non-target sites
- non-specific products

```
5' CCTAACG CTC AGCATCTAGTC 3'
3' ATAGGATTGCATTTCGTAGATCAGTACTACTGGACGTACAG 5'
```

**Too high**

- poor annealing due to disruption of H bonds
- low PCR amplification yields
**PCR REACTION COMPONENTS**

**PRIMER DESIGN TIPS**

B) Melting- and annealing temperature ($T_m$ & $T_a$) of primer

<table>
<thead>
<tr>
<th>Annealing temperatures</th>
<th>50</th>
<th>52</th>
<th>54</th>
<th>56</th>
<th>58</th>
<th>60 °C</th>
</tr>
</thead>
</table>

Sometimes, optimal annealing temperatures need to be optimized.

Too low, non-specific priming -> non-specific products
Too high, poor annealing -> low yields
**PRIMER DESIGN TIPS**

B) Melting- and annealing temperature ($T_m$ & $T_a$) of primer

- Optimal $T_a$ should be 5°C below the lowest $T_m$ (melting temperature) of the pair of primers to be used.

\[ T_m = \text{a temperature where 50\% of the DNA duplexes are denatured and become single-stranded.} \]

\[ T_m = 60 \, ^\circ\text{C} \]

\[ T_m = 57 \, ^\circ\text{C} \]

Optimal $T_a$ should be ~ 52°C.

**e.g.**

Forward primer $T_m = 60 \, ^\circ\text{C}$

Reverse primer $T_m = 57 \, ^\circ\text{C}$

Optimal $T_a$ should be ~ 52°C.
PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

B) Melting- and annealing temperature (Tm & Ta) of primer

• Melting temperature depends on the length and composition of a primer.

  Long, GC-rich -> high Tm

• Tm can be calculated from the following formulas:

  Tm for primers <= 20 bps = [4(G + C) + 2(A + T)]

  Tm for primers > 20 bps = Tm = 81.5 + 16.6log[salt] + 41(GC%/100) - 0.65(%formamide) - 675/length

PCR primers should be designed to have:

- $T_m$ between 55-80 °C (% GC ~50-60%)
- $T_m$ of a primer pair not very different (balanced).

(Acceptable differences of 4°C - 6°C between primers)
C) Avoiding any complementary and secondary structures

2º or complementary structures can cause PCR artifacts, especially at the 3’ end.

Primer A 5’ gtatgccgattcagttcagcatatgctgaa 3’
Primer B 5’ cgatcagtaacctactcagcat 3’

Self dimer (primer A - primer A)
5’ GTATGCCGATTACAGTTCAGCATATGCTGAA 3’
3’ AAGTCGTATACGACTTGACATTTAGCCGTATG 5’

Primer dimer (primer A - primer B)
5’ GTATGCCGATTACAGTTCAGCATATGCTGAA 3’
3’ TACGACTTCCATCCATGACTAGC 5’

Hairpin (primer A)
5’ GTATGCCGATTACAGTTCAGCAT 3’
3’ AAGTCGTA
C) Avoiding any complementary and secondary structures
PRIMER DESIGN TIPS

• The behavior of DNA polymerase focuses on the 3' ends of double stranded nucleic acid sequence.

5’ extension

5’ CCGATTACAGTTC AGCATCTAGTC 3’
3’ ATAGGATTGCATTTCGTAGATCAGTACTACTGGACGTACAG 5’

mismatch

5’ CCTAACG AGCATCTAGTC 3’
3’ ATAGGATTGCATTTCGTAGATCAGTACTACTGGACGTACAG 5’

Advantages - able to add restriction sites, promoter or other extra sequences at the 5’ end of PCR products.
- generate mutated PCR products (PCR-directed mutagenesis).

Therefore the most calculations in primer designs are focusing on the 3' ends.
• Some of the desirable characteristics of the 3' ends:
  - unique
  - pair with relatively low stability.
  - have little internal complementary and secondary structures.

• Primer design softwares ->  - facilitate the design of primers.
  - help find primers with the desired characteristics e.g. length, Tm, size of PCR products, complementary structures, etc.
PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

Primer3: WWW primer tool

Primer3: pick primers from a DNA sequence
Paste source sequence below (5'->3', string of ACGTAcgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINES, etc.) or use a

Pick left primer or use left primer below.
Pick hybridization probe (internal oligo) or use oligo below.
Pick right primer or use right primer below (5'->3' on opposite strand).

Sequence Id:

Targets:

Excluded Regions:

Product Size Min: Opt: Max:

Number To Return:

Max 3' Stability:

Max Mispriming:

Pair Max Mispriming:

http://molbiol-tools.ca/PCR.htm
http://biotools.umassmed.edu/bioapps/primer3_www.cgi
SETTNG UP PCR REACTIONS

1. Prepare a 25 μl* PCR reaction

- 10 x PCR buffer: 2.5 μl
- dNTP stock (2 mM each): 2.5 μl (0.2 mM each)
- forward primer (10 μM): 1.0 μl (0.4 μM)**
- reverse primer (10 μM): 1.0 μl (0.4 μM)
- Genomic DNA (as templates): 1.0 μl (0.1 μg)
- Taq polymerase (5 U/μl): 0.1 μl (0.5 U)
- distilled water: to 25 μl

* reaction volume 5-100 μl
** 0.1- 1.0 μM of primers, ~ 10^7-fold excess of DNA templates
too much -> possible primer dimers, mispriming
too little -> poor yield
2. PCR thermal profile:

- 94 °C for 5 min, initially
- Followed by 35 cycles of
  - 94 °C for 30 sec (denaturation step)
  - 55 °C for 30 sec (annealing step)
  - 72 °C for 1 min (extension step)
- 72 °C for 10 min at the end
- Hold at 4 °C
2. PCR thermal profile:

The invention of automated PCR machines, which heat and cool the reaction tubes to the precise temperatures required for each step of the reaction, has a great impact on the widespread use of PCR.


SETTING UP PCR REACTIONS

Denaturation step

• 30-60 sec at 92-96 °C for targets 1 kb or less.
• add roughly 30-60 sec every 1 kb.

   too short -> incomplete denaturation, poor yield
   too long  -> losing polymerase activity

   (Taq half-life at 95 °C ~ 40 min)

Initial denaturation step (10 min) - to ensure a complete template denaturation.
   - may inactivate harmful proteases or nucleases in the reaction.
SETTIMG UP PCR REACTIONS

Annealing step

• Optimal $T_a$ should be 5°C below the lowest $T_m$ (melting temperature) of the pair of primers or obtained from the optimization experiments.

• 30-60 sec

Too low -> non-specific priming -> non-specific products
Too high -> poor annealing -> low yields
SETTLE UP PCR REACTIONS

Extension step

- *Taq* is highly processive (incorporates 50-100 nucleotides/sec).
  - 30-60 sec at 72 °C for targets 1 kb or less
  - add roughly 30-60 sec every 1 kb

  *may be as short as 15 sec for products less than 400 bp.*

- A longer extension may be used at the final cycle to make sure complete extension of PCR products.
**Cycle Number**

- Theoretical yield starting from one template copy
  \[ \sim 2^n \] (n = number of amplification cycles)

  If PCR starts with N template copies, the yield will be \[ \sim N2^n \].

- Therefore, the number of PCR cycles necessary to produce a band visible on a gel depends largely on the starting copy number of the template.
  - 40 - 45 cycles to amplify 50 target molecules
  - 25 - 30 to amplify 3x10^5 molecules

- too few cycles -> not enough products
- too many cycles -> nonspecific products – why?
**SETTLING UP PCR REACTIONS**

**Cycle Number**

- Increasing the number of cycles does not increase specificity or efficiency of the PCR reaction because the plateau effect encourages nonspecific amplification.

**'Plateau Effect' in PCR Amplification**

- Not enough of primers or dNTPs in late cycles through utilization.

- The stability of the reactants (enzyme, primers, dNTPs).

- Reannealing of product at higher concentrations, preventing the extension process.

- Accumulation of nonspecific products.
VARIATIONS OF PCR METHODS

Hot Start PCR
RT-PCR
PCR cloning
Real-time quantitative PCR (SI BC512)

Allele-specific PCR
Anchored PCR
Asymmetric PCR
in-situ PCR
Inverse PCR
Ligation-mediated PCR
Multiplex PCR
Nested PCR
Overlap-extension PCR
Recombinant PCR
Touchdown PCR
VARIATIONS OF PCR METHODS

Hot Start PCR

During sample preparation at room temperature, complexes of nonspecific primer-template may be generated.
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VARIATIONS OF PCR METHODS

Hot Start PCR

• In hot start PCR, the essential components (e.g., primers, Mg\(^{2+}\), dNTP, or polymerase) are added after the reaction temperature has reached the annealing temperature.

  1. Manually added
  2. Commercially available products

• *Amplitaq\(^{TM}\) Gold*
  Chemically modified *Taq* which can be activated at high temperature.

• *TaqStart\(^{TM}\) antibody*
  The antibody inhibits polymerase activity before the onset of thermal cycling, preventing nonspecific amplification and primer dimer formation. When the temperature is raised, the antibody is quickly inactivated and PCR proceeds.
VARIATIONS OF PCR METHODS

Reverse Transcriptase-PCR (RT-PCR)

- Amplify and quantify RNA by producing cDNA from mRNA.
  
  *mRNA, viral RNA, etc*

- An RNA template is copied onto a complementary DNA transcript (cDNA) using a reverse transcriptase (RNA-dependent DNA polymerase), followed by amplification of the cDNA using PCR.

  Moloney murine leukemia virus (MMLV) RT

  Avian myeloblastosis virus (AMV) RT

  Thermostable reverse transcriptases (*Thermus thermophilus* and *Thermus flavus*)
Reverse Transcriptase-PCR (RT-PCR)

**Reverse Transcription**

- mRNA
- primer
- reverse transcription
- reverse transcriptase
- denaturation
- cDNA

Amplify cDNA using conventional PCR
• *Taq* usually adds an adenine nucleotide at the 3’ ends of PCR products.

-> generating 3’-A-overhangs
Cloning of PCR products

- *Taq*-amplified PCR products can anneal with a specialized vector with 3’-T-overhangs

  -> convenient cloning of PCR products. No restriction digestions required.
Suggested Readings


• วัชรี อัตถทิพพหลคุณ และ มนตรี อัตถทิพพหลคุณ. ทฤษฎีการประยุกต์ใช้ประโยชน์ PCR technology (2536)