DNA AMPLIFICATION BY Polymerase Chain Reaction (PCR)

- The Polymerase Chain Reaction (PCR) is a method to replicate a specific DNA sequence.
- It is an in-vitro technique for amplification of a region of DNA whose sequence is known or which lies between two regions of known sequence.
- PCR iteratively cycles between different temperatures to achieve amplification.
- The product grows exponentially with the number of cycles (roughly as $2^{t/2}$ where $t$ is the number of cycles).
HISTORY OF PCR

• 1966, Thomas Brock discovered Thermus Aquaticus, a thermostable bacteria in the hot springs of Yellowstone National Park
• 1983, Kary Mullis postulated the concept of PCR ( Nobel Prize in 1993)
• 1985, Saiki publishes the first application of PCR ( beta-Globin)
• 1985, Cetus Corp. Scientists isolate Thermostable Taq Polymerase (from T.Aquaticus), which revolutionized PCR
PCR Reagents

- **Target DNA strand to be amplified (template)**
  - Usually DNA
  - Can be RNA if an extra step is added
- **Two Primers (may be specific or random)**
- **Thermostable polymerase**
- **Other Materials:**
  - buffer
  - dNTPs
  - Mg$^{2+}$

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DNA template

- DNA containing region to be sequenced
- Size of target DNA to be amplified: up to 3 Kb
Primers

- 2 sets of primers
- Generally 20-30 nucleotides long
- Synthetically produced
- Complementary to the 3’ ends of target DNA
- Not complimentary to each other
Thermostable Polymerase Enzyme

• Usually Taq Polymerase or anyone of the natural or Recombinant thermostable polymerases
• Stable at T\textsuperscript{0} up to 95\textdegree{}C
• High processivity
• Taq Pol has 5’-3’ exo only, no proofreading
Thermostable Polymerases

- **Taq:** *Thermus aquaticus* (most commonly used)
  - Sequenase: *T. aquaticus* YT-1
  - Restorase (*Taq* + repair enzyme)
- **Tfl:** *T. flavus*
- **Tth:** *T. thermophilus* HB-8
- **Tli:** *Thermococcus litoralis*
- Carboxysothermus hydreniformans (RT-PCR)
- **P. kodakaraensis** (Thermococcus) (rapid synthesis)
- **Pfu:** *Pyrococcus furiosus* (fidelity)
  - Fused to DNA binding protein for processivity
Performing PCR

• Assemble a reaction mix containing all components necessary for DNA synthesis.

• Subject the reaction mix to amplification via PCR Cycles.

• Analyze the product of the PCR reaction (the amplicon).
The PCR Cycle

• Comprised of 3 steps:
  
  • (1) Denaturation of DNA at 95°C
  • (2) Primer hybridization (called annealing) at 40-50°C
  • (3) DNA synthesis (Primer extension) at 72°C
The PCR Cycle

- (1) Denaturation of target (template)
  - Usually 95°C

- (2) Annealing of primers
  - Temperature of annealing is dependent on the G+C content
  - May be high (no mismatch allowed) or low (allows some mismatch) stringency

- (3) Primer Extension – gives synthesis of new product strand
PCR Cycle - Step 1 - Denaturation Template DNA by Heat (95°C)
PCR Cycle - Step 2 –

Temperature is lowered ($T_m$) and primers anneal to target sequences.
At 72 °C Taq DNA polymerase catalyses primer extension as complementary nucleotides are incorporated.
End of the 1st PCR Cycle –

Results in two copies of target sequence
Target Amplification

<table>
<thead>
<tr>
<th>No. of Cycles</th>
<th>No. of Amplicon Copies of Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
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<td>4</td>
<td>16</td>
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<td>6</td>
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<td>20</td>
<td>1,048,576</td>
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<tr>
<td>30</td>
<td>1,073,741,824</td>
</tr>
</tbody>
</table>

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AMPLIFICATION BY PCR

1. Denature
2. Anneal primers
3. Extend primers

Target

Two copies of target

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PCR: First 4 Cycles

The first 4 cycles of PCR in detail

wanted gene

template DNA

number of double strands with the right length:

<table>
<thead>
<tr>
<th></th>
<th>1st cycle</th>
<th>2nd cycle</th>
<th>3rd cycle</th>
<th>4th cycle</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

(Andy Vierstraete 2001)

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PCR: Completed Amplification Cycle

Exponential amplification

(template DNA) 1st cycle 2nd cycle 3rd cycle 4th cycle

2¹ = 2 copies 2² = 4 copies 2³ = 8 copies 2⁴ = 16 copies

2³⁵ = 34 billion copies

(Andy Vierstraete 2001)

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Standard thermocycle

Step 1: Denature Template
Step 2: Anneal Primer
Step 3: Primer Extension

One "Cycle"

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RT-PCR

- Reverse Transcriptase PCR
- Uses RNA as the initial template
- RNA-directed DNA polymerase (rTh)
- Yields ds cDNA
Reverse Transcription - Step 1 –
Primer Anneals to Target RNA Sequence
Reverse Transcription - Step 2 –

*rTth* DNA Polymerase also has RT activity Catalyses Primer Extension by Incorporating Complementary Nucleotides
PCR Step 1 - Denaturation by Heat
PCR Step 2 - Annealing of Primer to cDNA
PCR Step 3 - \( rTth \) DNA Polymerase Catalyses Primer Extension
End of 1st PCR Cycle - Yields a Double-Stranded DNA Copy (Amplicon) of the Target Sequence

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Detection of amplification products

- Gel electrophoresis
- Sequencing of amplified fragment
- Southern blot
- etc...
PCR Primers

• Primers are single-stranded 18–30 b DNA fragments complementary to sequences flanking the region to be amplified.
• Primers determine the specificity of the PCR reaction.
• The distance between the primer binding sites will determine the size of the PCR product.
Primer Design

• Types of primers
  – Random
  – Specific

• Primer length
  – Annealing temperature
  – Specificity

• Nucleotide composition

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Primer Design

• Not containing inverted repeat sequences to avoid formation of internal structures
• 40-60% GC content preferred for better annealing
• avoid repeated Gs longer than GGGG
Primer Design

• Melting Temperature: $T_m$ of forward primer = $T_m$ of reverse primer

• $T_m$ of primers can be calculated to determine annealing $T^0$

• $T_m = 0.41(\%G+C) + 16.6\log(J^+) + 81.5$ where $J^+$ is the concentration of monovalent ions

• For short (14–20 bp) oligomers: Melting Temperature:
  $T_m = 4^\circ (GC) + 2^\circ (AT)$
A Standard PCR Reaction Mix

0.25 mM each primer
0.2 mM each dATP, dCTP, dGTP, dTTP
50 mM KCl
10 mM Tris, pH 8.4
1.5 mM MgCl$_2$
2.5 units polymerase
$10^2$ - $10^5$ copies of template
50 ml reaction volume

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PCR Cycle: Temperatures

• Denaturation temperature
  – Reduce double stranded molecules to single stranded molecules
  – 90–96°C, 20 seconds

• Annealing temperature
  – Controls specificity of hybridization
  – 40–68°C, 20 seconds

• Extension temperature
  – Optimized for individual polymerases
  – 70–75°C, 30 seconds
### Combinations Of Cycle Temperatures

<table>
<thead>
<tr>
<th>TEMP</th>
<th>FOR</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>94-60-72</td>
<td>Perfect, long primers</td>
<td>Higher temp can be used; maximum annealling temp</td>
</tr>
<tr>
<td>94-55-72</td>
<td>Good or perfectly matched primers between 19-24 nt</td>
<td>Standard conditions</td>
</tr>
<tr>
<td>94-50-72</td>
<td>Adequate primers</td>
<td>Allows 1-3 mismatches/20 nt</td>
</tr>
<tr>
<td>94-48-68</td>
<td>Poorly matched primers</td>
<td>Allows 4-5 mismatches/20 nt</td>
</tr>
<tr>
<td>94-45-65</td>
<td>Unknown match, likely poor</td>
<td>Primers of questionable quality, long-shot PCR</td>
</tr>
<tr>
<td>94-37-65</td>
<td>Hail Mary</td>
<td>Uncontrolled results</td>
</tr>
</tbody>
</table>

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Amplification Reaction

- Amplification takes place as the reaction mix is subjected to an amplification program.
- The amplification program consists of a series of 20–50 PCR cycles.
Automation of PCR

• PCR requires repeated temperature changes.
• The thermal cycler changes temperatures in a block or chamber holding the samples.
• Thermostable polymerases are used to withstand the repeated high denaturation temperatures.
Avoiding Misprimers

- Use proper annealing temperature.
- Design primers carefully.
- Adjust monovalent cation concentration.
- Use hot-start: prepare reaction mixes on ice, place in preheated cycler or use a sequestered enzyme that requires an initial heat activation.
  - Platinum Taq
  - AmpliTaq Gold
  - HotStarTaq
Product Cleanup

• Gel elution
  – Removes all reaction components as well as misprimers and primer dimers
• Solid phase isolation of PCR product (e.g., spin columns)
• DNA precipitation
Contamination Control

• Any molecule of DNA containing the intended target sequence is a potential source of contamination.

• The most dangerous contaminant is PCR product from a previous reaction.

• Laboratories are designed to prevent exposure of pre-PCR reagents and materials to post-PCR contaminants.

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Contamination of PCR Reactions

- Most common cause is carelessness and bad technique.
- Separate pre- and post-PCR facilities.
- Dedicated pipettes and reagents.
- Change gloves.
- Aerosol barrier pipette tips.
- Meticulous technique
- 10% bleach, acid baths, UV light
- Dilute extracted DNA.
Real-Time (or Quantitative) PCR

• Monitors the quantity of amplification of a targeted DNA strand during the PCR

• Methods used for the detection of PCR products in real-time PCR:
  – (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA and
  – (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence

- fluorescence chart produced in real-time PCR
- Melting curve produced at the end of real-time PCR
**Nested PCR**

- Repeat PCR using a second pair of inner primers
- Reduces primer matching errors
Isothermal PCR Amplification Protocols

Isothermal: Do not require changing the reaction.

- So do not require thermocyclers.

- Usually very fast.

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Isothermal PCR Amplification Protocol: Rolling Circle PCR

- Circular dsDNA (double-stranded) is synthesized
- Circular dsDNA is "nicked" by a nicking enzyme.
- The 3’ end is elongated using "unnicked" DNA as leading strand (template)
- 5’ end of ssDNA product is displaced, forming ssDNA product

Diagram:

1. Circular dsDNA
2. Nicking by a nicking enzyme
3. Elongation of 3’ end with unnicked DNA
4. Displacement of 5’ end of ssDNA product
5. Synthesis of second strand
6. dsDNA and DNA ligase
7. ssDNA, synthesis of second strand
Isothermal PCR Amplification Protocol:

Strand displacement amplification (SDA)

• Nicks are created by a strand-limited restriction endonuclease or nicking enzyme at a site contained in a primer.

• Uses a strand-displacing DNA polymerase, typically Bst, to initiate replication at nicking sites, so target DNA is regenerated with each polymerase displacement step, resulting in exponential amplification.

• Used in clinical diagnostics.
Strand Displacement Polymerases

Target Generated containing engineered restriction enzyme site

Bumper Primer binds and displaces strand generated by restriction engineered primer

Restriction Enzyme cleaves primer

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Strand displacement amplification (SDA)

**Target Production**

SDA\textsubscript{F}

- Bump primer
- DNA polymerase

Target region to be amplified

**Exponential Target Amplification**

Nicking enzyme

- Cycle

* Target amplification, shown above for SDA\textsubscript{F}, will also occur simultaneously with SDA\textsubscript{R}.

New England BioLabs Website
The Amplification Phase

Step 1

• The exponential amplification process begins with altered targets (single-stranded partial DNA strands with restricted enzyme recognition sites) from the target generation phase.
Step 2

- An amplification primer binds to each strand at its complimentary DNA sequence.
Step 3

- DNA polymerase uses the primer to identify a location to extend the primer from its 3' end, using the altered target as a template for adding individual nucleotides.
Step 4

- The extended primer forms a double-stranded DNA segment containing a complete restriction enzyme recognition site at each end.
Step 5

• The restriction enzyme binds to the double stranded DNA segment at its recognition site.
Step 6

• The restriction enzyme dissociates from the recognition site after having cleaved only one strand of the double-sided segment, forming a nick.
Step 7

- DNA polymerase recognizes the nick and extends the strand from the site, displacing the previously created strand.
Step 8

- The recognition site is repeatedly nicked and restored by the restriction enzyme and DNA polymerase with continuous displacement of DNA strands containing the target segment.
Step 9

- Each displaced strand is then available to anneal with amplification primers similar to the action in step 2. The process continues with repeated nicking, extension and displacement of new DNA strands, resulting in exponential amplification of the original DNA target.
Isothermal PCR Amplification Protocol:

Nicking enzyme amplification reaction (NEAR)

• Uses a strand-displacing DNA polymerase initiating replication at a nick created by a nicking enzyme,

• Produces many short nucleic acids from the target sequence.

• Used for pathogen detection in clinical and biosafety applications.
Loop-mediated isothermal amplification (LAMP)

- Uses 4-6 primers recognizing 6-8 distinct regions of target DNA.
- A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification.
- LAMP is rapid, sensitive
- magnesium pyrophosphate produced during the reaction can be seen by eye, making LAMP well-suited for field diagnostics.
Loop-mediated isothermal amplification (LAMP) uses 4-6 primers recognizing 6-8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification.
Isothermal PCR Amplification Protocol:

Helicase-dependent amplification (HDA)

• Employs the double-stranded DNA unwinding activity of a helicase to separate strands, enabling primer annealing and extension by a strand-displacing DNA polymerase.

• Like PCR, this system requires only two primers.

• Used in diagnostic devices and FDA-approved tests.