Polymerase Chain Reaction (PCR)
History

- PCR was first conceived in April, 1983 by Kary Mullis.


- In 1989 PCR was selected as the major scientific development and Taq DNA polymerase as molecule of the year by the science magazine

- In 1993 Kary Mullis was awarded the Nobel Prize in chemistry for this achievements.
PCR is in vitro method for enzymatically amplifying defined sequences of DNA (or RNA) into large quantities in a few hours.

Specifically targets and amplifies a SINGLE sequence from within a complex mixture of DNA.
PCR requirements

- Starting nucleic acid - DNA/RNA
  - Tissue, cells, blood, hair root, saliva, semen
- Heat-stable DNA polymerase
  - *e.g.* Taq Polymerase
    - *Thermus aquaticus* DNA polymerase
    - Thermophilic organism
    - Enzymes resistant to high temperatures 95°C
    - 72-74°C optimum
Thermus aquaticus:
PCR requirements

- Two oligonucleotide primers.

  **Primer design**
  - Both primers should have approximately the same Tm.
  - 50% GC composition.
  - 18-28 bases length.
  - No internal complementarity hairpin loops.
  - No 3' complementarity 'primer-dimers'.
  - Two out of three bases on the 3` end should be G or C to get good hybridization.
PCR requirements

- **Buffer**
  - Tris-HCl (pH 7.6-8.0)
  - Mg2+

- **Deoxynucleotides**
  - dNTPs (dATP, dCTP, dGTP, dTTP)
DNA Structure

Hydrogen Bonds

Cytosine

Adenine

Thymine

Guanine

Deoxyribose (Sugar molecule)

Phosphoric Acid (Phosphate molecule)
How PCR works:

- Begins with DNA containing a sequence to be amplified and a pair of synthetic oligonucleotide primers that flank the sequence.
- Next, denature the DNA to single strands at 94°C.
- Rapidly cool the DNA (37-65°C) and anneal primers to complementary s.s. sequences flanking the target DNA.
- Extend primers at 70-75°C using a heat-resistant DNA polymerase such as *Taq* polymerase derived from *Thermus aquaticus*.
- Repeat the cycle of denaturing, annealing, and extension 20-45 times to produce 1 million (220) to 35 trillion copies (245) of the target DNA.
- Extend the primers at 70-75°C once more to allow incomplete extension products in the reaction mixture to extend completely.
- Cool to 4°C and store or use amplified PCR product for analysis.
PCR Steps

- **Thermal Denaturation**
  Initial denaturation temperature of 94°C for 8 min. For subsequent cycles, 94°C for 1-2 min. is usually adequate.

- **Primer Annealing**
  The temperature and length of time required for primer annealing depends on the base composition and the length and concentration of the primers.

- **Primer Extension**
  Primer Extension is typically carried out at 72°C, which is close to the temperature optimum of the Taq polymerase.

```
GTCATAGCATTATTATTATTTCAGGACTA
CAGTATCGTAATAATAATAATAAGTCCTGAT
```

A template sequence with 5 ATT repeats.
PCR Cycle - Step 1 - Denaturation by Heat

Target Sequence

Target Sequence
PCR Cycle - Step 2 - Biotinylated Primer Pair Anneals to Ends of Target Sequence
PCR Cycle - Step 3 - *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

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PCR Protocol

- mix DNA, primers, dNTPs, Taq, buffer, Mg2+
- program thermocycler for times and temps
  - denaturation
  - annealing
  - extension
- 20-40 cycles
- analyze amplified DNA (amplicons)

Thermal Cycler
Analysis of PCR product

- Agarose gel electrophoresis with ethidium bromide
  - DNA fragments separate according to size, and the dye ethidium bromide forms a brightly fluorescent adduct as it binds to DNA
  - Standard low-molecular weight marker is used
Analysis of PCR product

- Southern blot analysis
  - The DNA is hybridized with a radioactively-labeled DNA (a "probe"), then exposed to X-ray film, DNA fragments appeared as dark bands on the film, known as an autoradiogram.
Southern blot analysis
ELISA PCR
Reverse Transcription (RT-PCR)

- RT-PCR (Reverse Transcription-PCR) is a modification of PCR in which an RNA strand is first reverse transcribed into its DNA complement or cDNA, followed by amplification of the resulting DNA using PCR.
- Any type of RNA (mRNA, tRNA, rRNA, total, viruses) can be analyzed.
- RNA is reversed transcribed by reverse transcriptase enzymes. 
  e.g. AMV : Avian Myeloblastosis Virus.
  MMULV: Molony Murine Leukemia Virus
Reverse Transcription (RT-PCR)

- For priming of the reverse transcription, a number of primers can be used:
  
a) Oligo (dT) 12-18 primer (binds to poly A+ tail) producing full length cDNA.
  
b) Random hexanucleotides (hexamers): randomly bind at complementary sites in the RNA molecule and give partial length cDNAs.
  
c) Specific template primer: for selective transcription of the RNA of interest.
RT-PCR

3 basic strategies

Specific Priming

Oligo(dT) Priming

Random Priming
Reverse Transcription - Step 1 - Biotinylated Primer Anneals to Target RNA Sequence
Reverse Transcription - Step 2 - \( rTth \) DNA Polymerase Catalysing Primer Extension by Incorporating Complementary Nucleotides
End of Reverse Transcription - Step 3 - Results in Synthesis of Complementary DNA (cDNA) to the RNA Target Sequence
Nested PCR

- Two consecutive rounds of amplification with the second using an inner primer set to amplify the first round product.
- It is usually necessary when amplifying RNA viruses like HCV.
Hot Start PCR

- Highly specific and robust PCR amplification.
- A simple modification of the original PCR process in which the amplification reaction is initiated at an elevated temperature.
- This Hot Start process is facilitated by using Gold DNA polymerase.
- Gold DNA polymerase is a modified version of Taq DNA polymerase. This new enzyme is inactive at room temperature which allows premixing of all reagents without worry of primer-dimer formation or pre-PCR mispriming.
There are three main sources of PCR contamination:

1- PCR product from previous amplifications (carryover contamination).
2- Cloned DNA previously handled in the lab.
3- Sample to sample contamination.
Principles of contamination avoidance

1- Strict physical separation of individual PCR-related maneuvers:

- Sample preparation stage.
- PCR setup stage.
- Post PCR stage (No equipments should leave this room, this location is considered the most likely source of contaminating amplicons).
Principles of contamination avoidance

2- Laboratory practice designed to minimize the risk of contamination.

- All PCR reagents should be aliquoted.
- Use and change gloves frequently.
- Positive displacement pipettes or aerosol resistant tips should be used.
- Re-usable glassware, plasticware should be acid decontaminated.
- If possible, different personnel should be allocated to the pre-PCR and post PCR parts.
- Lab coats must be worn in all area, the coat worn in post-PCR must never be worn in pre-PCR.
- Always work in one way direction from pre-PCR to post-PCR.
- Recently, closed systems for PCR product analysis have been developed, such as TaqMan system.
Principles of contamination avoidance

3- Use of specific anti-contamination measures:
- UV irradiation to damage any contaminating DNA prior to the addition of DNA template.
- Restriction enzyme treatment to restrict any contaminating sequence prior to the addition of DNA template.
  e.g. MspI, DNase I
- Incorporation of dUTP instead of dTTP and treatment with uracil N glycosylase (UNG).
Detection of contamination

To facilitate the monitoring of contamination positive and negative controls should be included.

**Positive Control:** to reduce the possibility of false negative arising as a result of inadequate extraction of nucleic acid.

**Negative Control:** to monitor for false positive due to contamination.
Applications of PCR

- **Genetic fingerprinting**
  - Forensic analysis at scene of crime to identify a person who suspected of committing a crime by comparing his or her DNA with a given sample (blood, hair, semen, etc) obtained from a crime scene.

- Paternity testing

- Analysis of ancient DNA.
Applications of PCR

- Cloning genes.
- Genetic diagnosis - Mutation detection
  - PCR facilitates the advancement of prenatal diagnosis of genetic defects such as:
    - Cystic fibrosis
    - Duchenne muscular dystrophy
    - Haemoglobinopathies.
- Mutagenesis to investigate protein function.
- Quantitate differences in gene expression by Reverse Transcription (RT)-PCR.
Applications of PCR

Detection of pathogens especially when applied to those which are:

- Difficult or costly to culture.
- Slow growing.
- Present in low concentration.
- Hazardous to propagate in the lab.
Real-Time PCR

- Quantitative PCR technique allows the simultaneous amplification and detection of the target.
- Is called "real-time PCR" because it allows the scientist to actually view the increase in the amount of DNA as it is amplified.
Real-Time PCR

- TaqMan real-time PCR.
- SYBR Green real-time PCR.
- Molecular beacon real-time PCR.
**TaqMan® real-time PCR**

**Principle:** This technology is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. A probe is designed to anneal to the target sequence between the forward and reverse primers. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.
TaqMan real-time PCR

TaqMan Probe

- The probe consists of two types of fluorophores, the reporter at the 5’ end of the probe and the quencher at the 3’ end of the probe.
- During PCR, the 5’ nuclease activity of the polymerase degrades the probe into fragments and separates the quencher from the reporter, allowing the reporter to emit a strongly fluorescent signal.
TaqMan Assay Principle: 5’ Nuclease
**TaqMan real-time PCR**

**Data analysis:**

- The detected fluorescence is captured and displayed as an amplification plot.
- **Critical threshold line or assigned fluorescence level (AFL):** is the point at which a reaction reaches a statistically significant increase of fluorescence over background.
- **Threshold cycle (CT) or Elbow:** is the cycle at which the sample reaches the threshold level.
- The CT is inversely proportional to the copy number of the target template, the higher the template concentration, the fewer the CT measured.
- **Quantitation standard (QS):** is a synthetic DNA or RNA designed to closely resemble the actual target, is included in a known amount in each and every test.
- Quantitation of the target nucleic acid is accurately accomplished by comparing the amplification plot values of the standard with the target.
TaqMan amplification of HCV

HCV signal from 0 to $10^8$ HCV-RNA copies/mL

Relative fluorescence from HCV
COBAS TaqMan 48 Analyzer
SYBR Green Real-time PCR

- SYBR Green dye binds to dDNA and emits light when excited.
- SYBR Green detection of the PCR product is monitored by measuring the increase of fluorescence caused by the binding of SYBR Green to the increasing amounts of dDNA.
SYBR Green Real-time PCR

Advantages:

- Inexpensive approach as it is not necessary to design specific probe dyes.
- Easy to use and sensitive.

Disadvantages:

- SYBR Green binds to any dDNA in the reaction and leads to false positive results.
Molecular beacon Real-time PCR

- Utilizes a reporter that is wrapped around into a hairpin
- It also has a quencher dye that must be in close contact to the reporter to work.
- The probe remains intact through the PCR product, and is rebound to the target at every cycle.
Molecular beacon Real-time PCR

PCR Product

Primer 1

Primer 2

PCR Product-Specific Nucleotides

Fluorescent Reporter Dye

Quencher Dye

Specific Nucleotides
Branched DNA assay (bDNA)

- Branched DNA (bDNA) Signal Amplification Assay is an alternative hybridization based system for the sensitive and rapid detection of agents of infectious disease. This molecular technique amplifies the signal from a target molecule rather than the target itself.
Branched DNA assay (bDNA)

Principle

• This assay is a sandwich nucleic acid hybridization procedure for the direct quantitation of nucleic acid. The nucleic acid is captured to a microwell by specific probes. A set of target probes hybridizes to both the nucleic acid and the pre-amplifier probes. The amplifier probe subsequently hybridizes to the pre-amplifier forming a branched DNA complex.

• Detection is by chemiluminescence using an alkaline phosphates -specific substrate. The amount of light detected is directly proportional to the amount of bound nucleic acid.
Branched DNA assay (bDNA)
Advantages of (bDNA)

- One room technology.
- No risk of contamination, as target is not amplified (signal amplification).
- RNA extraction not required.
- High throughput of 168 samples/run.