Implikasi REPLIKASI DNA DAN PCR

(Polymerase Chain Reaction)

Utut Widyastuti
What is PCR?

PCR is an exponentially progressing synthesis of the defined target DNA sequences *in vitro*.

It was invented in 1983 by Dr. Kary Mullis, for which he received the Nobel Prize in Chemistry in 1993.
What is PCR? : Why “Polymerase”? 

It is called “polymerase” because the only enzyme used in this reaction is DNA polymerase.
What is PCR? : Why “Chain”? 

It is called “chain” because the products of the first reaction become substrates of the following one, and so on.
History

• The Polymerase Chain Reaction (PCR) was not a discovery, but rather an invention

• A special DNA polymerase (*Taq*) is used to make many copies of a short length of DNA (100-10,000 bp) defined by primers
What PCR Can Do

- PCR can be used to make many copies of any DNA that is supplied as a template.
- Starting with one original copy an almost infinite number of copies can be made using PCR.
- “Amplified” fragments of DNA can be sequenced, cloned, probed or sized using electrophoresis.
- Defective genes can be amplified to diagnose any number of illnesses.
- Genes from pathogens can be amplified to identify them (ie. HIV).
- Amplified fragments can act as genetic fingerprints.
How PCR Works

- PCR is an artificial way of doing DNA replication.
- Instead of replicating all the DNA present, only a small segment is replicated, but this small segment is replicated many times.
- As in replication, PCR involves:
  - Melting DNA
  - Priming
  - Polymerization
Initiation - Forming the Replication Eye

Origin of Replication

[Diagram showing the process of initiation and forming the replication eye.]

5’  3’
5’  3’
5’  3’
5’  3’
Extension - The Replication Fork

- Leading Strand
- Laging Strand
- Okazaki fragment
- RNA Primers
- DNA Polymerase
- Helicase
- Primase
- Single strand binding proteins

5' → 3'

3' → 5'
<table>
<thead>
<tr>
<th>Function</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting DNA</td>
<td>èHelicase</td>
</tr>
<tr>
<td></td>
<td>èSSB Proteins</td>
</tr>
<tr>
<td></td>
<td>èTopoisomerase</td>
</tr>
<tr>
<td>Polymerizing DNA</td>
<td>èDNA</td>
</tr>
<tr>
<td>Providing primer</td>
<td>èPrimase</td>
</tr>
<tr>
<td>Joining nicks</td>
<td>èLigase</td>
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Components of a PCR Reaction

- Buffer (containing Mg\(^{++}\))
- Template DNA
- 2 Primers that flank the fragment of DNA to be amplified
- dNTPs
- *Taq* DNA Polymerase (or another thermally stable DNA polymerase)
PCR

The cycling reactions:

There are three major steps in a PCR, which are repeated for 20 to 40 cycles. This is done on an automated **Thermo Cycler**, which can heat and cool the reaction tubes in a very short time.

**Denaturation at around 94°C:**

During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example the extension from a previous cycle).
Annealing at around 54°C:
Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. If the primers exactly fit the template, the hydrogen bonds are so strong that the primer stays attached.

Extension at around 72°C:
The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).
PCR

Exponential increase of the number of copies during PCR
PCR

Melting

94 °C

Temperature

Time

3' 5'

5' 3'
PCR

Melting at 94 °C

Temperature vs. Time

Heat

3' to 5'

5' to 3'
PCR

Melting 94 °C
Annealing Primers 50 °C
Extension 72 °C
Melting 94 °C

Temperature

Time

3' 5'
5' 3'

5' 3'
PCR

Temperature

Melting 94 °C
Annealing 50 °C
Extension 72 °C
Melting 94 °C

Time

Heat

Heat
PCR

Temperature

Time

Melting 94 °C
Annealing Primers 50 °C
Extension 72 °C

Melting 94 °C

Heat

5' 3' 5'

Heat
PCR
Melting
94 °C
Annealing
50 °C
Extension
72 °C
Temperature
Time
30x
Melting
50 °C
Annealing
Primers
Extension
Fragments of defined length
Theoretical Yield Of PCR

Theoretical yield = \(2^n \times y\)

Where \(y\) = the starting number of copies and
\(n\) = the number of thermal cycles

If you start with 100 copies, how many copies are made in 30 cycles?

\[2^n \times y\]

\[= 2^{30} \times 100\]

\[= 1,073,741,824 \times 100\]

\[= 107,374,182,400\]
Verification of PCR product
How The Functions Of Replication Are Achieved During PCR

Function                      PCR

• Melting DNA                 èHeat
• Polymerizing DNA            èTaq DNA Polymerase
• Providing primer            èPrimers are added to the reaction mix
• Joining nicks               èN/A as fragments are short
PCR and Contamination

- The most important consideration in PCR is contamination

- Even the smallest contamination with DNA could affect amplification

- For example, if a technician in a crime lab set up a test reaction (with blood from the crime scene) after setting up a positive control reaction (with blood from the suspect) cross contamination between the samples could result in an erroneous incrimination, even if the technician changed pipette tips between samples. A few blood cells could volitilize in the pipette, stick to the plastic of the pipette, and then get ejected into the test sample.

Modern labs take account of this fact and devote tremendous effort to avoiding cross-contamination.
Optimizing PCR protocols

PCR can be very tricky

While PCR is a very powerful technique, often enough it is not possible to achieve optimum results without optimizing the protocol

Critical PCR parameters:

- Concentration of DNA template, nucleotides, divalent cations (especially Mg\(^{2+}\)) and polymerase
- Error rate of the polymerase (Taq, Vent exo, Pfu)
- Primer design
DNA Sequencing

Sequencing methods

- The process of determining the order of the nucleotide bases along a DNA strand is called **DNA sequencing**

- In 1977 two separate methods for sequencing DNA were developed: the **chain termination method** or **cycle sequencing** (Sanger et al.) and the **chemical degradation** method or **Maxam-Gilbert sequencing** (Maxam and Gilbert)

- Both methods were equally popular to begin with, but, for many reasons, the cycle sequencing method is the method more commonly used today

- This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis
**Cycle Sequencing**

*Concept*: If we know the distance of each type of base from a known origin, then it is possible to deduce the sequence of the DNA.

Obtaining this information is conceptually quite simple. The idea is to cause a termination of a growing DNA chain at a *known base* (A,G,C or T) and at a *known location* in the DNA.

In practice, chain termination is caused by the inclusion of a small amount of a single dideoxynucleotide base in the mixture of *all four* normal bases (e.g. dATP, dTTP, dCTP, dGTP and ddATP). The small amount of ddATP would cause chain termination whenever it would be incorporated into the DNA. The incorporation of ddATP would be random and thus all possible chains that end in 'A' will exist.
Deoxy versus dideoxy
DNA synthesis

5'-to-3' direction of chain growth
Metode dalam sekuensing:

Diseoksinukleotida
Sintesa berhenti

DNA Sequencing

The different steps in cycle sequencing

30 cycles of 3 steps:

1. **Step 1: denaturation**
   - 1 min at 94°C

2. **Step 2: annealing**
   - 15 sec at 50°C
   - 1 primer !!!!

3. **Step 3: extension**
   - 4 min at 60°C
   - Mixture of dNTP's and ddNTP's

(Andy Vierstraete 1999)
DNA Sequencing

Cycle sequencing

chain termination

Taken from:
http://www.mbio.ncsu.edu/JWB/MB409/lecture/lecture04/lecture04.html
DNA Sequencing

The separation of the sequencing fragments

To measure the sizes of the fragments, each of the four reactions would be loaded into a separate well on a gel, and the fragments would be separated by gel electrophoresis.
Figure 20.28  Photograph of an autoradiograph of a 2′,3′-dideoxynucleotide chain-terminator sequencing gel. The sequence defined by the lower portion of the gel is shown on the right.
DNA Sequencing

Sequencing systems

LICOR DNA 4300

ABI 3100
Automated DNA sequencing

Figure 20.29 (a) The standard slab-gel method. (b) DNA sequencing. (c) Computer plot of the results of an automatic sequencing run, showing nucleotide sequence of a segment of DNA.
DNA Sequencing

Snapshots of the detection of the fragments on the sequencer

four-dye system

single-dye system
DNA Sequencing

Chromatogram file
DNA Sequencing

The linear amplification of the gene in sequencing

- Primer fits only on one strand
- On incorporation of a fluorescently labelled ddNTP (complementary with the base on the template) the elongation stops

Linear amplification

30 cycles: 180 complementary strands

mixture of strands with different length which end on a fluorescently labelled ddNTP

(Andy Vierstraete 1999)
Maxam dan Gilbert (1977):

Prinsip: degradasi struktur kimia DNA

Gambar 1. Sekuensing dengan metode Maxam-Gilbert

5'  32p  G  (A+G)  C  (C+T)  A>C  3'  

Tambahkan senyawa pendegradasi

32p ACACTGAAACGTTTCATGTCGA..........  

me

32p ACACTGAAACGTTTCATGTCGA..........  

me

32p ACACTGAAACGTTTCATGTCGA..........  

me

32p ACACTGAAACGTTTCATGTCGA..........  

me

32p ACACT

32p ACACTGAAC  

32p ACACTGAACGTTCATGTCGA  

Penghilangann senyawa modifikasi dan hidrolisa ikatan fosfat dengan piperidine

Pisahkan fragmen dengan elektroforesis

Gambar 1. Sekuensing dengan metode Maxam-Gilbert