Practical 4: PCR in Molecular Diagnosis

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PRINCIPLES

What is PCR

PCR stands for polymerase chain reaction. The PCR method was devised and named by K. Mullis and colleagues at the Cetus Corporation. The PCR technique is a primer extension reaction for amplifying specific nucleic acids in vitro. The use of a thermostable polymerase allows the dissociation of newly formed complimentary DNA and subsequent annealing or hybridization of primers to the target sequence with minimal loss of enzymatic activity.

PCR will allow a stretch of DNA to be amplified to about a million fold so that enough DNA is produced for subsequent analysis. The particular stretch of DNA to be amplified, called the target sequence, is identified by a specific pair of DNA primers, oligonucleotides usually about 20 nucleotides in length.

How PCR is performed:

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

First step - Denaturation at 94°C During the denaturation, the double strand target DNA melts open to single stranded DNA, all enzymatic reactions stop.

Second step - Annealing at ‘annealing temperature’ The reaction mixture is cooled to annealing temperature to allow primers to anneal to the target DNA. Annealing temperature is usually a few degrees below the melting temperature of the primers.

The primers are jiggling around, caused by the Brownian motion. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer, that it does not break anymore.

Third step - Extention at 72°C use an enzyme which can "read" the opposing strand's "sentence" and extend the primer's "sentence" by "hooking" letters together in the order in which they pair across from one another - A:T and C:G. This particular enzyme is called a DNA polymerase (because makes DNA polymers). One such enzyme used in PCR is called Taq polymerase that can withstand the high temperature necessary for DNA-strand separation, and
can be left in the reaction. *Taq* polymerase was originally discovered and isolated from a thermal stable bacterium, *Thermus aquaticus*. This enabled Saiki R. K. and colleagues to synthesize new DNA strands repeatedly, exponentially amplifying a defined region of the starting DNA material, and allowing the birth of a new technology that has virtually exploded into prominence.

The “denaturation”, “annealing” and “extension” steps are typically repeated for 30 - 40 cycles to generate enough DNA for subsequent analysis.

**What are the applications of PCR**

PCR has been successfully used in a wide variety of fields including: molecular biology, environmental science, forensic science, medical science, biotechnology, microbiology, the food industry, diagnostic science, epidemiology, genetics, gene cloning, and many more.

In order to perform PCR, one must already know the exact sequences which flank (lie on either side of) both ends of a given region of interest in DNA. One need not know the DNA sequence in-between. In fact, one can use PCR to determine if the sequence in-between is mutated or deleted in a given disease.

α-Thalassemia is a common recessive genetic disorder. Each individual has 4 α-globin genes, 2 on each of the chromosome 16. Deletion of the entire α-globin genes accounts for most α-Thalassemia mutations. Among them, the most common mutation is the 2 gene deletion which removes both α-globin genes on one of the chromosome 16. One such deletion is the South East Asian (SEA) type 2-gene deletion that involves a 21 kb genomic region. This practical is designed to **test SEA mutation using PCR method.**

[Diagram showing PCR product and deletion]
EXPERIMENT PROCEDURES

(A) PCR reaction to test SEA mutations (2 groups share one set of reagents)

1. Set the program for thermal cycler as the following

\[
\begin{align*}
95 \degree C & \quad 3 \text{ min} \\
95 \degree C & \quad 30 \text{ sec} \\
65 \degree C & \quad 20 \text{ sec} \\
72 \degree C & \quad 20 \text{ sec} \\
72 \degree C & \quad 5 \text{ min}
\end{align*}
\]

2. Label two 0.5 ml PCR tubes as M (Mix), B (blank) followed by your initials. Tubes labeled with N (Normal) and P1 (Patient 1), and P2 (Patient 2) with 2 µl DNA are provided but add your initials after the letter N, P1 or P2.

3. Add 2 µl of dd H\textsubscript{2}O to tube B.

4. Make master mix by adding the following to tube M according to the volume for 5 reactions

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Conc</th>
<th>Volume per rxn(µl)</th>
<th>volume for 5 rxn (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>10 x</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>MgCl\textsubscript{2}</td>
<td>25 mM</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>AFDA primer</td>
<td>20 µM</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>AFDB primer</td>
<td>20 µM</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>AFDC primer</td>
<td>20 µM</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>dd H\textsubscript{2}O</td>
<td>35.8</td>
<td></td>
<td>179</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>5 U/µl</td>
<td>0.2</td>
<td>1</td>
</tr>
</tbody>
</table>

Total 48 240

5. Mix the final master mix well by vortexing at medium setting. Centrifuge briefly. Take care to prevent the formation of bubbles.

6. Dispense 48 µl to tube B, N, P1 and P2 containing either dd H\textsubscript{2}O or DNA, respectively. Mix by vortexing.

7. Place tubes B, N, P1 and P2 to PCR thermal cycler and run program 2. The PCR reaction will take approximately one hour to complete.
(B) Agarose Gel Electrophoresis to analyze PCR products

1. Prepare 1.5% agarose gel in 100 conical flask (one gel per group)

   Agarose       0.375 g
   0.5X TBE      25 ml

   Swirl to mix and boil in microwave till the solution is clear.

   Cool the agarose solution to 55°C (it will take 3 – 5 min), add 1 µl ethedium bromide (10 mg/ml), swirl to mix and pour to the gel tray.

2. After PCR cycles are completed, load 10 µl of each PCR rxn with 2 µl 6 x loading dye. Load 10 µl of 100 bp DNA ladder (see Appendix II for size information) in the first well for size reference.

3. Run the gel at 100 V for 35 min.

4. Keep the rest of the reaction at – 20°C for the practical of molecular cloning.

(C) Visualization of PCR products using Gene Wizard gel documentation system

1. Carry the gel in the holding tray to the area with Gene Wizard documentation system. Double click ‘GeneSnap’ icon on desktop and an image acquisition window will appear.

2. Click on the open draw icon and the sample draw will open.

3. Lift the gel carefully out of the tray, tip it slightly so that the excess liquid will drain off, and transfer it to the sample tray. Gently close the door.

4. Click on the auto-expose button to get the best non saturated image.

5. Click on ‘Print’ to get a copy of the image on a thermal paper. Print one image per student and keep it in your notebook.

6. Remove your gel from the light box and wipe the surface of the box thoroughly with hand-towels.

Gel electrophoresis will resolve the PCR fragments into their respective sizes:

   PCR products:          Normal 314 bp
                         SEA Deletion 195 bp
Appendix  Photograph of 100 bp DNA ladder

Description: The 100 bp DNA Ladder has a number of proprietary plasmids which are digested to completion with appropriate restriction enzymes to yield 12 bands suitable for use as molecular weight standards for agarose gel electrophoresis. The digested DNA includes fragments ranging from 100–1,517 base pairs. The 500 and 1,000 base pair bands have increased intensity to serve as reference points.

<table>
<thead>
<tr>
<th>Base Pairs</th>
<th>DNA Mass (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 1,517</td>
<td>45</td>
</tr>
<tr>
<td>- 1,200</td>
<td>35</td>
</tr>
<tr>
<td>- 1,000</td>
<td>95</td>
</tr>
<tr>
<td>- 900</td>
<td>27</td>
</tr>
<tr>
<td>- 800</td>
<td>24</td>
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<tr>
<td>- 700</td>
<td>21</td>
</tr>
<tr>
<td>- 600</td>
<td>18</td>
</tr>
<tr>
<td>- 500/517</td>
<td>97</td>
</tr>
<tr>
<td>- 400</td>
<td>38</td>
</tr>
<tr>
<td>- 300</td>
<td>29</td>
</tr>
<tr>
<td>- 200</td>
<td>25</td>
</tr>
<tr>
<td>- 100</td>
<td>48</td>
</tr>
</tbody>
</table>

Equipments: PCR thermal cycler
Gene Wizard gel documentation system
Microwave oven,
Microfuge and adapters for 0.5 ml tubes
Gel electrophoresis apparatus and Power Pack
Measuring cylinders (100 ml), and conical flasks (50 ml)
Weighing balance and spatula
Weighing boots
0.5 ml PCR tube stand
Ice container (big foam cup) and ice
Tips (10, 20 ul, 200ul) and tips containers
Pipettes: P10, P20, and P200

Reagents: PCR reagents: Taq DNA polymerase, 10 X Mg²⁺-free PCR buffer,
NTPs mix, MgCl₂, normal and patient DNA
PCR primers: AFDA/AFDB/AFBC
Normal and Patient DNA
100 bp DNA ladder
Agarose, 0.5X TBE buffer
Ethidium bromide, 5X loading dye, Autoclaved dd H₂O
Report

1. Describe the objectives of this practical using the space provided.

2. Paste your gel photo on the left. Explain and discuss your result using the space on the right of the gel photo.

3. Imagine you are working in a clinical lab and an anemic patient wants to know if he has SEA mutation. Draw a flow chart illustrating how you would carry out the test for the patient.