Molecular Detection of *Acinetobacter* bacteria & NDM-1 gene.

Experiment 2: Polymerase Chain Reaction; amplify the gene.

Objective

To amplify a given region of DNA (region of interest).

Theory

Polymerase chain reaction, better known as PCR, is one of the technologies that not only made a tremendous impact on the scientific community. PCR has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed as a results of its simplicity and usefulness. In 1993, Karry Mullis got Nobel Prize in Chemistry for his dedicated work on PCR. It has been used to detect DNA sequences, to diagnose genetic diseases, to carry out DNA fingerprinting, to detect bacteria or viruses (particularly the AIDS virus), and to research human evolution.

PCR is a rapid, inexpensive and simple way of copying specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality. It is the Polymerase enzyme that drives a PCR. A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double stranded starting point. This is very useful because you can choose *which gene* you wish the polymerase to amplify in a mixed DNA sample by adding small pieces of DNA complimentary to your gene of interest. These small pieces of DNA are known as **primers** because they prime the DNA sample ready for the polymerase to bind and begin copying the gene of interest. During a PCR, changes in temperature are used to control the activity of the polymerase and the binding of primers.

- To begin the reaction the temperature is raised to 95^{o}C. At this temperature all double stranded DNA is "denatured" in to single strands.
- The temperature is then lowered to ~50^{o}C. This allows the primers to bind to your gene of interest. Thus the polymerase has somewhere to bind and can begin copying the DNA strand.
- The optimal temperature for the polymerase to operate is 72^{o}C so at this point the temperature is sometimes raised to 72^{o}C to allow the enzyme to work faster. There are now twice as many copies of your gene of interest as when you started.
- The cycle of changing temperatures (95^{o}C, 50^{o}C and 72^{o}C) is then repeated and two copies become four. Another cycle and four become eight, up to 30-35 cycles. After amplifying gene into many millions of copies it is possible to run the amplified DNA out on an **agarose gel** and stain it with a dye to visualize it. The bigger the visible band, contains more copies of gene of interest that have been created.

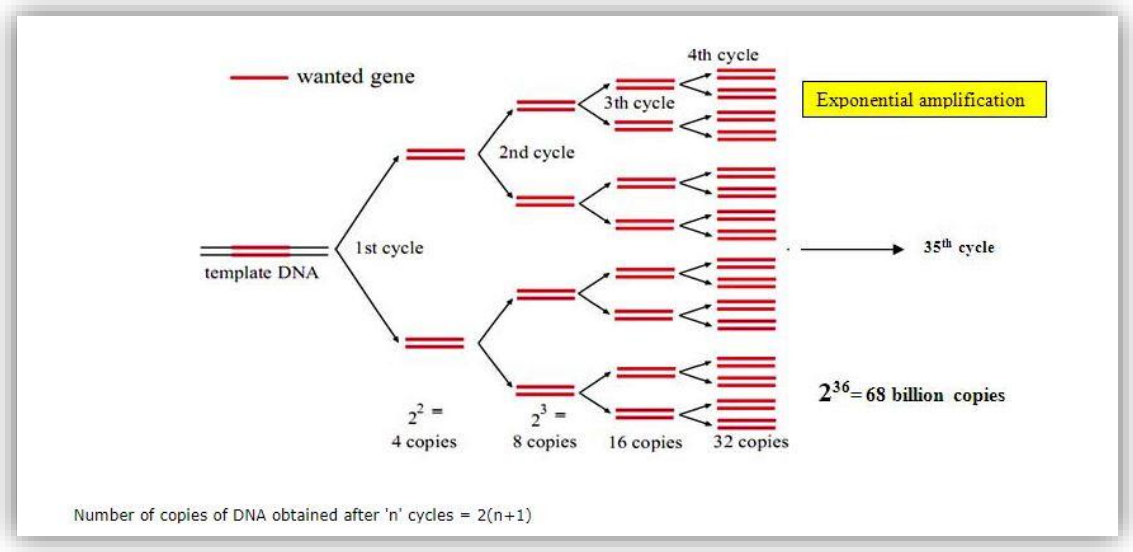

Fig 1.1 Showing amplification of target gene using PCR.

Parameters that affect PCR

Essential components of polymerase chain reactions:

- *A thermostable DNA polymerase to catalyse template-dependent synthesis of DNA:*

  Depending on the ability, fidelity, efficiency to synthesize large DNA products, a wide choice of enzymes is now available. For routine PCRs, Taq polymerase (0.5-2.5 units per standard 25-50 uL reaction) remains the enzyme of choice. The specific activity of most commercial preparations of Taq is ~80,000 units/mg of protein.

- *A pair of synthetic oligonucleotides to prime DNA synthesis:*

  Design of the oligonucleotide primer being the most important factor that influence the efficiency and specificity of the amplification reaction, careful designing of primers is required to obtain the desired products in high yield, to suppress amplification of unwanted sequences and to facilitate subsequent manipulation of the amplified product. Since the primers so heavily influence the success or failure of PCR protocols, it is ironic that the guidelines for their design are largely qualitative and are based more on common sense than on well understood thermodynamic or structural principles.

- *Deoxynucleoside triphosphates(dNTPs)*

  Standard PCRs contain equimolar amounts of all four dNTPs. Concentrations of 200-250µM of each dNTP recommended for Taq polymerase in reactions containing 1.5 mM $MgCl_2$. In a 50 uL reaction, these amounts should allow synthesis of ~6-6.5µg DNA which should be sufficient even for multiplex reaction in which eight or more primer pairs are used at the same time. High concentrations of dNTPs (>4mM) are inhibitory, perhaps because of sequestering of Mg^{2+}. However, a satisfactory amount of amplified product can be produced with dNTP concentrations as low as 20µM- 0.5-1.0pM of an amplified fragment ~1 kb in length.

- *Divalent cations*

  All thermostable DNA polymerases require free divalent cations- usually Mg^{2+} for activity. Because dNTPs and oligonucleotides bind Mg^{2+}, the molar concentration of the cation must exceed the molar concentration of phosphate groups contributed by dNTPs and primers. A concentration of 1.5 mM of Mg^{2+} is routinely used.

- *Buffer to maintain pH*

  Tris -Cl , adjusted to a between **8.3 and 8.8** at room temperature is included in standard PCRs at a concentration of 10mM. When incubated at 72°C (extension phase of PCR), the pH of the reaction mixture drops by more than a full unit, producing a buffer whose pH is ~7.2.

- *Monovalent cations*

  Standard PCR buffer contains 50mM KCl and works well for amplification of segments of DNA >500bp in length. Raising the KCl concentration to ~70-100mM often improves the yield of shorter DNA segments.

Programming PCRs

PCR is an iterative process, consisting of 3 elements; denaturation of the template by heat, annealing of the oligonucleotide primers to the single stranded target sequence(s), and extension of the annealed primers by a thermostable DNA polymerase.

- ✓ Denaturation

  Double stranded DNA templates denature at a temperature that is determined in part by their G+C content. *The higher the proportion of G+C, the higher the temperature required to separate the strands of template DNA.* The longer the DNA molecules, the greater the time required at the chosen denaturation temperature to separate the two strands completely. In PCRs catalyzed by Taq polymerase, denaturation is carried out at 94-95°C, which is the highest temperature that the enzyme can endure for 30 or more cycles without sustaining excessive damage. In the first cycle of PCR, denaturation is sometimes carried out for 5 minutes to increase the probability that long molecules of template DNA are fully denatured. Denaturation for 45 seconds at 94-95°C is routinely used to amplify linear DNA molecules whose GC content is <55% and higher temperature for template and/or target DNAs whose GC content is >55%. So much more heat tolerant polymerases are preferred in such cases.

- ✓ Annealing of primers to template DNA

  The temperature used for the annealing step is critical. If the annealing temperature is too high, the oligonucleotide primers anneal poorly. If the annealing temperature is too low, non-specific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA.

- ✓ Extension

  Extension of oligonucleotide primers is carried out at or near the optimal temperature for DNA synthesis catalyzed by the thermostable polymerase, which in the case of Taq polymerase is 72-78°C. In the first two cycles, extension from one primer proceeds beyond the sequence complementary to the binding site of the other primer. In the next cycle, the first molecules are produced whose length is equal to the segment of DNA delimited by the binding sites of the primers. From the third cycle onwards, this segment of DNA is amplified geometrically, whereas longer amplification products accumulate arithmetically. The polymerization rate of Taq polymerase is ~2000 nucleotides /minute at the optimal temperature (72-78°C) and as a rule of thumb, extension is carried out for 1 minute for every 1000bp of product.

- ✓ Number of cycles

  The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction, the efficiency of primer extension and amplification. Once established in the geometric phase, the reaction proceeds until one of the components becomes limiting. At this point, the yield of specific amplification products should be maximal, whereas nonspecific amplification products should be barely detectable, if at all. This is generally the case after ~30 cycles in PCRs containing ~105 copies of the target sequence and Taq DNA polymerase (efficiency~0.7). At least 25 cycles are required to achieve acceptable levels of amplification of single copy target sequences in mammalian DNA templates.

Procedure:

The protocol describes how to amplify a segment of double-stranded DNA in a chain reaction catalyzed by a thermostable DNA polymerase. It is the foundation for all subsequent variations of the polymerase chain reaction.

Materials

Buffers and Solutions

- 2x PCR master mix
- Chloroform
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

Enzymes and Buffers

- Thermostable DNA polymerase
- Nucleic Acids and Oligonucleotides
- Forward primer (20 µM) in H2O
- Reverse primer (20 µM) in H2O
- Template DNA.

  Dissolve template DNA in 10 mM Tris-Cl (pH 7.6) containing a low concentration of EDTA (<0.1 mM) at the following concentrations: mammalian genomic DNA, 100 µg/ml; yeast genomic DNA, 1 µg/ml; bacterial genomic DNA, 0.1 µg/ml; and plasmid DNA, 1-5 ng/ml.

Method

1. In a sterile 0.5-ml microfuge tube, mix in the following order:

REAGENTS	AMOUNT (µl)
PCR master mix (2x)	6.25 µl
Forward primer	0.50 µl
Reverse primer	0.50 µl
$MgCl_2$	0.50 µl
Nuclease free water	1.75 µl
Template DNA	3.00 µl
Total volume	**12.50 µl**

   Standard reaction conditions for PCR. Mg^{2+} (1.5 mM) ;KCl (50 mM) ;Primers(1 µM);DNA polymerase (1-5 units); Template DNA(1 pg to 1 µg).

   Typical amounts of yeast, bacterial, and plasmid DNAs used per reaction are 10 ng, 1 ng, and 10 pg, respectively.

2. Prepare a negative control adding 3.0µl of sterile water instead of template DNA.
3. Set PCR amplification reactions to pre-denaturation step of 5 minutes at 94˚C, followed by 35 cycles of amplification (30seconds denaturation at 94˚C, 30seconds Annealing 56.4°C and 45seconds extension at 72 ˚C) and final extension of 5min at 72 ˚C
4. Store the sample for “Analysis of PCR products”