Preparation of master-mix for PCR reaction and Setting of PCR reaction

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Key Topic

- Introduction
- Master mix
- Steps of PCR reaction
- Modifications

Introduction

- **Polymerase chain reaction** (**PCR**) is a method widely used in molecular biology to rapidly make millions to billions of copies of a specific DNA sample allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail.
- PCR was originally developed in 1983 by the American biochemist **Kary Mullis**. He was awarded the Nobel Prize in Chemistry in 1993 for his pioneering work.
- The revolutionary evolutions of the molecular biological research are based on the PCR technique which provides the suitable and specific products especially in the field of the characterization and the conservation of the genetic diversity.
- There are variants of PCR such as real-time PCR, competitive PCR, PCR in situ, RT-PCR, etc.

Five core ‘ingredients’

- The principles behind every PCR, whatever the sample of DNA, are the same.
- Five core ‘ingredients’ are required to set up a PCR. These are:
  - the DNA template to be copied
  - Primers, short stretches of DNA that initiate the PCR reaction, designed to bind to either side of the section of DNA you want to copy
  - DNA nucleotide bases also known as dNTPs. DNA bases (A, C, G and T) are the building blocks of DNA and are needed to construct the new strand of DNA
  - Taq polymerase enzyme to add in the new DNA bases
  - Buffer to ensure the right conditions for the reaction
- PCR involves a process of heating and cooling called thermal cycling which is carried out by machine.

Template DNA/ Matrix DNA

- Matrix DNA can come from any organism and even complex biological materials that include DNAs from different organisms.
- But to ensure the success of a PCR, it is still necessary that the DNA matrix is not too degraded.
- It is also important that the DNA extract is not contaminated with inhibitors (detergents, EDTA, phenol, proteins, etc.) of the PCR.
- The amount of template DNA in the reaction medium initiate that the amplification reaction can be reduced to a single copy.
- The maximum quantity may in no case exceed 2 μg.
- In general, the amounts used are in the range of 10–500 ng of template DNA.

Primers

- Primers serve as the starting point for DNA synthesis.
- Primers are single strands of DNA or RNA sequence that are around 20 to 30 bases in length.
- The two separated strands of DNA are complementary and run in opposite directions (from one end - the 5' end – to the other - the 3' end); as a result, there are two primers – a forward primer and a reverse primer.
- Depending on the reaction volume chosen, the primer concentration may vary between 10 - 50 pmol or 50 ng per sample.
- The primers are designed or selectively synthesized to be complementary in sequence to short sections of DNA on each end of the sequence to be copied.
- Only once the primer has bound can the polymerase enzyme attach and start making the new complementary strand of DNA from the loose DNA bases.

dNTPs (deoxyribonucleoside triphosphates)

- dNTPs (deoxyribonucleoside triphosphates) provide both the energy and the nucleotides needed for DNA synthesis during the chain polymerization.
- They are incorporated in the reaction medium in excess, that is, about 200 μM final.

Taq DNA Polymerase

- Taq DNA polymerase is an enzyme taken from the heat loving bacteria *Thermus aquaticus*. This bacteria normally lives in hot springs so can tolerate temperatures above 80^{0}C.
- The polymerase enzyme can only add DNA bases to a double strand of DNA.
- The amount of Taq polymerase per sample is generally between 1 and 3 units.

Buffer

- 100 mM Tris-HCl, pH 9.0
- 50 mM $MgCl_2$ (Fc - 0.5 mM to 3.0 mM)
- 500 mM KCl (Fc- 50-100 mM)
- Indeed, some pairs of primers work better with solutions enriched with magnesium. On the other hand, with high concentrations of dNTP, the concentration of magnesium should be increased because of stoichiometric interactions between magnesium and dNTPs that reduce the amount of free magnesium in the reaction medium.

Preparation of Master-mix

Reagent	Stock solution of reagent	Final Concentration in PCR	Requried volume for reaction (10 µl)	Requried volume for (10 ml)
dNTPs	100mM	1.5-2 mM	.3 µl	300 µl
Taq DNA polymerase	5 U	1U	.2 µl	200 µl
Tris-HCl	100mM	1X	.5 µl	500 µl
MgCl2	50mM	0.5-3.0 mM	.8 µl	800 µl
KCl	500mM	50-100 mM	2.0 µl	2000 µl
Autoclaved ddH_2O			6.2 µl	6200 µl

- Spin ⟶ Vortex ⟶ Spin

Preparation of Reaction Mixture

- The volumes of reaction medium vary between 10 and 100 µl.

Reagent	Final Concentration in PCR	Requried volume for reaction
Matrix DNA	200 ng	2-5 µl
Forward Primer	50 ng	1 µl
Reverse Primer	50 ng	1 µl
Master-mix	1X	10 µl
Autoclaved ddH_2O	-	Balance to make up (µl)
Total		20 µl

- Spin ⟶ Vortex ⟶ Spin

Modification

- It is possible to add detergents (Tween 20, Triton X-100) or glycerol in order to increase the conditions of stringency that make it harder and therefore more selective hybridization of the primers. This approach is generally used to reduce the level of nonspecific amplifications due to the hybridization of the primers on sequences without relationship with the sequence of interest.
- We can also reduce the concentration of KCl until eliminated or increase the concentration of $MgCl_2$.

Three main stages of PCR

- **Denaturing** – when the double-stranded template DNA is heated to separate it into two single strands.
- **Annealing** – when the temperature is lowered to enable the DNA primers to attach to the template DNA.
- **Extending** – when the temperature is raised and the new strand of DNA is made by the Taq polymerase enzyme.
- These three stages are repeated 20-40 times, doubling the number of DNA copies each time.

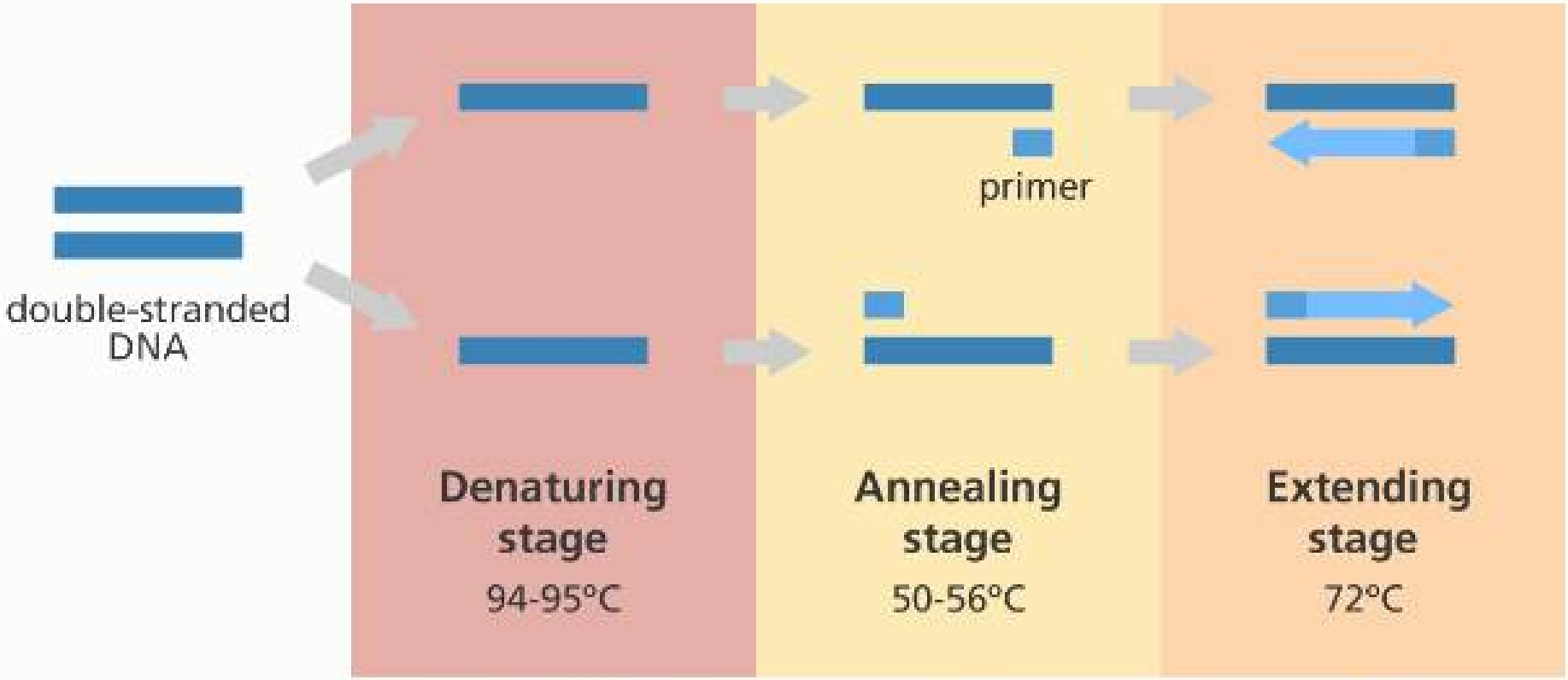

Illustration showing the main steps in the polymerase chain reaction (PCR).

Image credit: Genome Research Limited

Steps

Step 1: Denaturation by Heat:

Heat is normally more than 90 degrees Celsius at separates double-stranded DNA into two single strands. This process is known as "denaturation". The hydrogen bonds linking the bases to one another are weak, therefore denaturation is possible. This usually takes between 15-30 seconds.

Step 2: Annealing Primer to Target Sequence:

The beginning of the DNA target sequence of interest is marked by the primers that anneal (bind) to the complementary sequence. Annealing usually takes place between 40 degrees Celsius and 65 degrees Celsius, depending on the length and base sequence of the primers. This step usually takes about 10-30 seconds.

Step 3: Extension:

The temperature is raised to approximately 72 degrees Celsius after the primers anneal to the complementary DNA sequences. Also, the enzyme Taq DNA polymerase is used to replicate the DNA strands. The duration of this step depends on the length of DNA sequence being amplified but usually takes around one minute to copy 1,000 DNA bases (1Kb).

End of the First PCR Cycle:

There are now two new DNA strands identical to the original target at the end of the first PCR cycle. The newly formed strands have a beginning, which is precisely defined by the 5′ end of the primer, but the 3′ end is not precisely defined.

- The DNA strand synthesized from such a template then has a precisely defined length that is limited at either end by the 5′ end of each of the two primers. These DNA strands are known as AMPLICON.

After PCR has been completed, a method called electrophoresis can be used to check the quantity and size of the DNA fragments produced.

PCR Thermal Cycling Steps

- Program the PCR machine as fellow:

Steps	Temperture (˚C)	Time	Role
Initial denaturetion	92-95	2-10 min	For linerization
Denaturation	94-95	30 sec	30-45 Repeat Cycle
Anealing	Depending on the Tm of Primer	15-60 sec	
Extension (Ta)	68-72	30-60 sec (1000 bp/min)	
Final Extension	68-72	5-10 min	
Hold	4	∞	

Applications

- PCR is a technique of purification or cloning.
- PCR is widely used for diagnostic purposes to detect the presence of a specific DNA sequence of this or that organism in a biological fluid.
- It is also used to make genetic fingerprints, whether it is the genetic identification of a person in the context of a judicial inquiry, or the identification of animal varieties, plant, or microbial for food quality testing, diagnostics, or varietal selection.
- PCR is still essential for performing sequencing or site-directed mutagenesis.

Tips

- The choice of the duration of the temperature cycles and the number of cycles depends on the size of the sequence of interest as well as the size and the complementarity of the primers.
- The durations should be reduced to a minimum not only to save time but also to prevent risk of nonspecific amplification.

Thanks