CHAPTER 12B – POLYMERASE CHAIN REACTION (PCR)

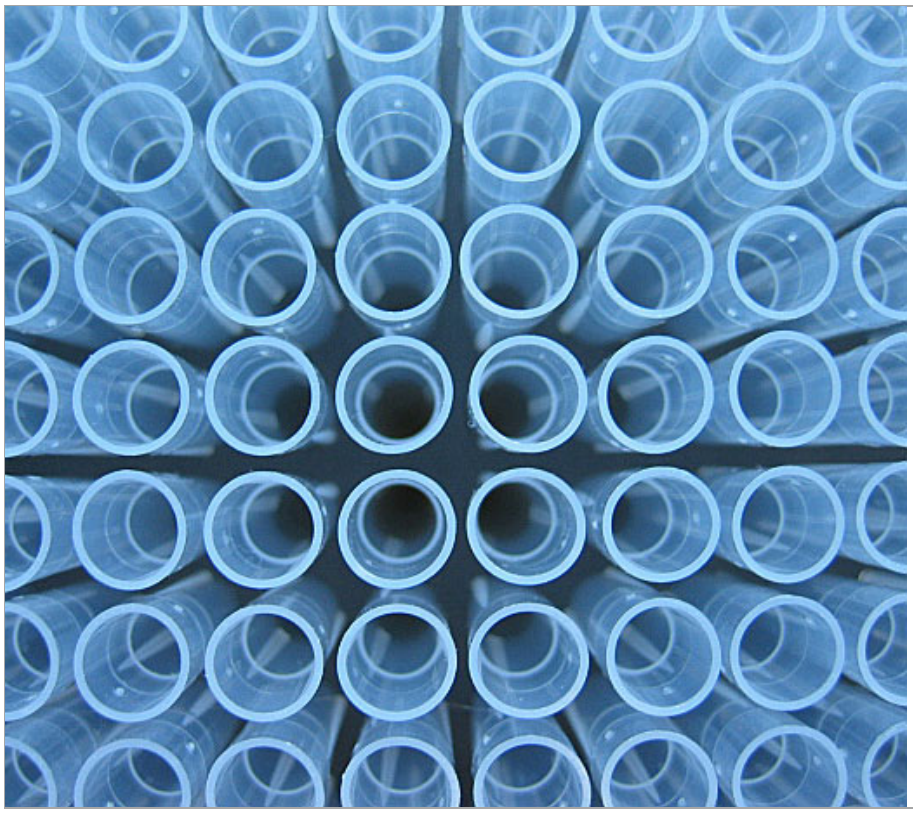

Figure 1.
Plastic disposable tips for a micro-pipettor are used to accurately distribute microliter volumes of liquid in molecular biology.
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INTRODUCTION

While, genetics is the study of the inheritance and variation of biological traits, today, **classical genetics** is often complemented by **molecular biology**, to give **molecular genetics**, which involves the study of DNA and other **macromolecules** that have been isolated from an organism. Usually, molecular genetics experiments involve some combination of techniques to isolate, analyze, and characterize the DNA, RNA, and/or protein transcribed and translated from a particular gene. In some cases, the DNA may be subsequently manipulated by mutation or by recombination with other DNA fragments. Techniques of molecular genetics have wide application in many fields of biology, as well as forensics, biotechnology, and medicine. Polymerase Chain Reaction (PCR) is a widely used technique to amplify and isolate specific DNA sequences. It requires a "template" DNA, which is often genomic DNA. From this template, specific sequences can be amplified and many copies can be produced for analysis or manipulation.

1. ISOLATING GENOMIC DNA

DNA purification strategies rely on the chemical properties of DNA that distinguish it from other molecules in the cell, namely that it is a very long, negatively charged molecule. To extract purified DNA from a tissue sample, cells are broken open by grinding or **lysing** in a solution that contains chemicals that protect the DNA while disrupting other components of the cell (**Figure 2**). These chemicals may include **detergents**, which dissolve lipid membranes and denature proteins. A cation such as Na^+ helps to stabilize the negatively charged DNA and separate it from proteins, such as histones. A **chelating agent**, such as **EDTA**, is added to protect DNA by sequestering Mg^{2+} ions, which can otherwise serve as a necessary co-factor for **nucleases** (enzymes that digest DNA). As a result, free, double-stranded DNA molecules are released from the cell and from chromatin into the extraction

buffer, which also contains proteins and all other cellular components. (The basics of this procedure are simple enough that it can be done with household chemicals as presented on YouTube.)

The free DNA molecules are subsequently isolated by one of several methods. Commonly, proteins are removed by adjusting the salt concentration so they precipitate. The **supernatant**, which contains DNA and other, smaller metabolites, is then mixed with ethanol, which causes the DNA to precipitate. A small **pellet** of DNA can be collected by centrifugation, and after removal of the ethanol, the DNA pellet can be dissolved in water (usually with a small amount of EDTA and a pH buffer) for the use in other reactions. Note that this process has purified all of the DNA from a tissue sample (genomic and mitochondrial DNA); if we want to isolate a specific gene or DNA fragment, we must use additional techniques, such as PCR.

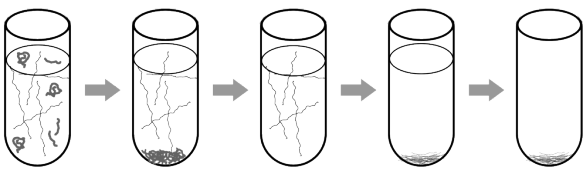

Figure 2.
Extraction of DNA from a mixture of solubilized cellular components by successive precipitations. Proteins are precipitated, then DNA (in the supernatant) is precipitated with ethanol, leaving a pellet of DNA.
(Original-Deyholos-CC BY-NC 3.0)

2. ISOLATING OR DETECTING A SPECIFIC SEQUENCE BY PCR

2.1. COMPONENTS OF THE PCR REACTION

The **Polymerase Chain Reaction (PCR)** is a method of DNA amplification that is performed in a test tube (i.e. *in vitro*). Here "polymerase" refers to a DNA polymerase enzyme extracted and purified from bacteria. The "chain reaction" refers to the ability of this technique produce billions of copies of a specific DNA molecule, by using each newly replicated double helix as a template to synthesize two new DNA double helices. PCR is therefore a very efficient method of amplifying a specific sequence of DNA from a small sample of a large, complex genome.

Besides its ability to make large amounts of DNA, there is a second characteristic of PCR that makes it extremely useful. Recall that most DNA polymerases can only add nucleotides to the end of an existing strand of DNA, and therefore require a **primer** to initiate the process of replication. For PCR, chemically synthesized primers of about 20 nucleotides are used. In an ideal PCR, primers only hybridize to their exact complementary sequence on the template strand (**Figure 3**).

Figure 3.
The primer-template duplex at the top part of the figure is perfectly matched, and will be stable at a higher temperature than the duplex in the bottom part of the figure, which contains many mismatches and therefore fewer hydrogen bonds. If the annealing temperature is sufficiently high, only the perfectly matched primer will be able to initiate extension (grey arrow) from this site on the template.
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The experimenter can therefore control exactly what region of a DNA template is amplified by specifying the sequence of the primers used in the reaction.
To conduct a PCR amplification, an experimenter combines in a small, thin-walled tube (**Figure 4**), all of the necessary components for DNA replication, including:

(1) DNA polymerase and solutions containing
(2) nucleotides (dATP, dCTP, dGTP, dTTP),
(3) a DNA template,
(4) DNA primers,
(5) a pH buffer, and
(6) ions (e.g. Mg^{2+}) required by the polymerase.

Successful PCR reactions have been conducted using only a single DNA molecule as a template, but in

practice, most successful PCR reactions contain many thousands of template molecules. The template DNA (e.g. total genomic DNA) has usually already been purified from cells or tissues using the techniques described above. However, in some situations it is possible to put whole cells directly in a PCR reaction for use as a template.

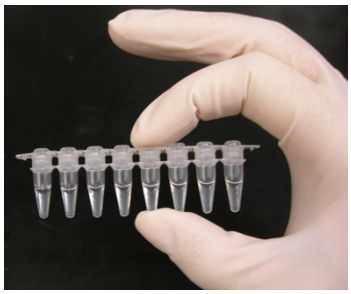

Figure 4.
A strip of PCR tubes
(Wikipedia-madprime- CC BY-SA 3.0)

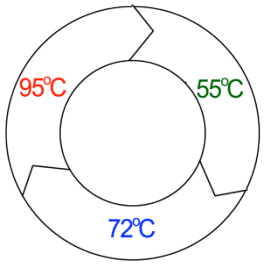

Figure 5.
Example of a thermal-cycle, in which the annealing temperature is 55°C.
(Original-Deyholos-CC BY-NC 3.0)

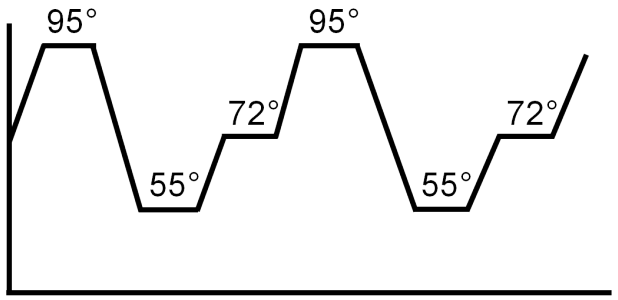

Figure 6.
A temperature vs. time graph showing two cycles of PCR.
(Original-Harrington-CC BY-NC 3.0)

An essential aspect of PCR is **thermal-cycling**, meaning the exposure of the reaction to a series of precisely defined temperatures (**Figure 5**). The reaction mixture is first heated to 95°C. This causes the hydrogen bonds between the strands of the template DNA molecules to melt, or **denature**. This produces two single-stranded DNA molecules from each double helix (**Figure 7**). In the next step (**annealing**), the mixture is cooled to 45-65°C. The exact temperature depends on the primer sequence used and the objectives of the experiment. This allows the formation of double stranded helices between complementary DNA molecules, including the annealing of primers to the template. In the final step (**extension**) the mixture is heated to 72°C. This is the temperature at which the particular DNA polymerase used in PCR is most active. During extension, the new DNA strand is synthesized, starting from the 3' end of the primer, along the length of the template strand. The entire PCR process is very quick, with each temperature phase usually lasting ~30 seconds or less. Each cycle of three temperatures (denaturation, annealing, extension) is usually repeated about 30 times, amplifying the target region approximately 2^{30}-fold. The amount of DNA product reaches a plateau at 20-40 cycles, usually because the nucleotide precursors have been exhausted. Notice from the figure that most of the newly synthesized strands in PCR begin and end with sequences either identical to or complementary to the primer sequences; although a few strands are longer than this, they are in such a small minority that they can almost always be ignored.

After completion of the thermalcycling (amplification), an aliquot from the PCR reaction is usually loaded onto an **electrophoretic agarose gel**

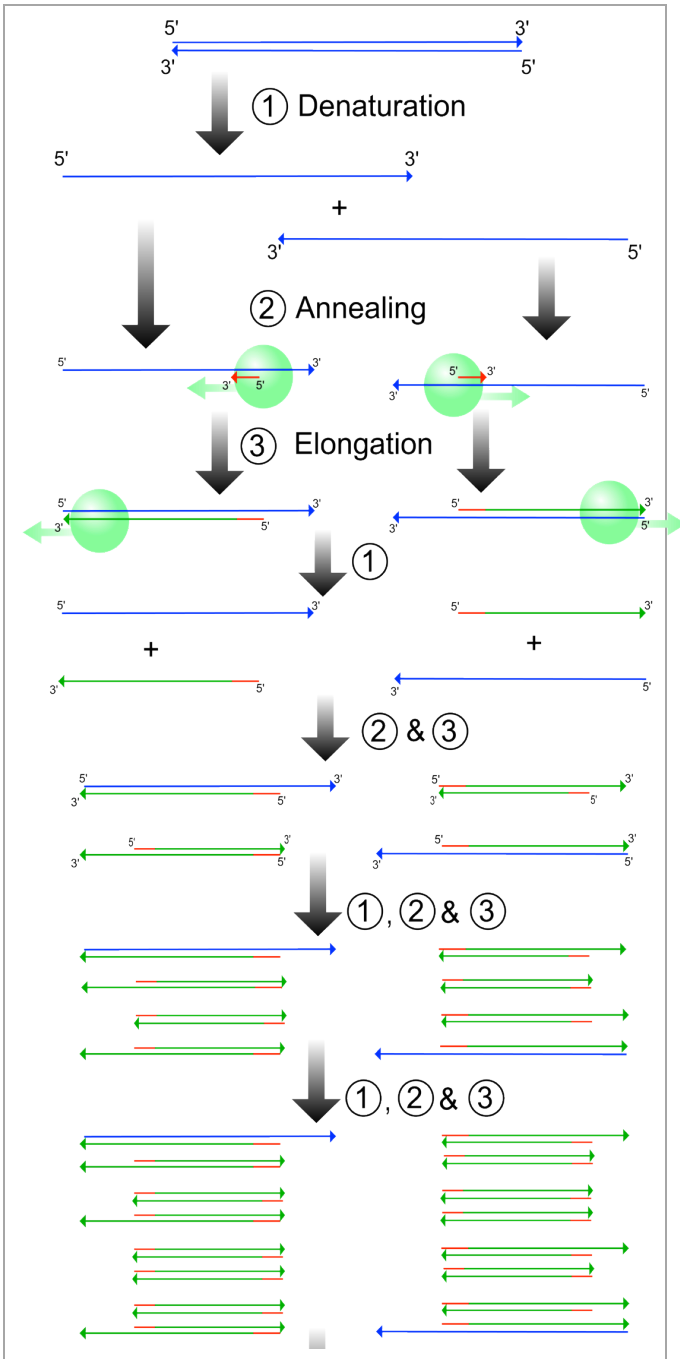

Figure 7.
PCR with the three phases of the thermal cycle numbered. The template strand (blue) is replicated using primers (red), to prime the newly synthesized strands (green). The green strands, which are flanked by the two primer sites, will increase in abundance exponentially through successive PCR cycles.
(Wikipedia-madprime- CC0 1.0)

(described in chapter 28) to determine whether a DNA fragment of the expected length was successfully amplified or not. Usually, the original template DNA will be so dilute that it will not be visible on the gel, only the amplified PCR product. The presence of a sharp band of the expected length indicates that PCR was able to amplify its target. If the purpose of the PCR was to test for the presence of a particular template sequence, this is the end of the experiment. Otherwise, the remaining PCR product can be used as starting material for a variety of other techniques such as sequencing or cloning.

2.2. REAL TIME PCR /QUANTITATIVE PCR (QPCR)

In a standard PCR reaction, the DNA molecule of interest is amplified and then the products are typically visualized at the end of the reaction on an electrophoresis gel. On the other hand, a procedure known as real-time PCR or quantitative PCR (qPCR) detects the replicated DNA molecules during the amplification process. qPCR uses florescent molecules and relies on the fluorescence of the amplified product measured over a number of cycles. However, the procedure of amplifying the DNA molecule is identical to the standard PCR procedures. There are two ways of processing qPCR.

(1) Using florescent chemical molecules known as **fluorochrome** that binds to all double stranded DNA molecule (Nonspecific).

In the first method, the florescent dye molecule binds to any double stranded DNA molecule. After each cycle of amplification, the amount of ds-DNA molecules synthesized can be quantified by measuring the florescence. The intensity of the florescence would indicate the amount of DNA molecule present.

(2) Using florescent reporter probe (Specific).

The second method is using a florescent reporter probe that hybridizes with the DNA sequence of interest. When the taq polymerase replicates the DNA molecule, it degrades the probe and the florescent molecule is released to the solution. This increases the intensity of florescence. The florescence is measured by the real-time PCR

machine and quantifies the DNA molecules being synthesized.

2.3. REVERSE TRANSCRIPTASE PCR (RT-PCR)

Reverse Transcriptase PCR (RT-PCR) can detect both the quality and quantity of mRNA molecules (gene transcription). As a result, we are able to find out the spatial (where the gene is expressed) and temporal (when the gene is expressed) level of gene expression.

Here is how it works (**Figure 8**):

(1) mRNA is extracted from the cell, tissue, or organism.

(2) An enzyme called **reverse transcriptase** (obtained from a retrovirus – see Chapter 30) is added, along with oligo-DT, which anneals to the poly-A tail and acts as a primer, to synthesize complementary DNA (cDNA) to the mRNA.

(3) mRNA template is degraded, and cDNA is added to a PCR reaction to amplify a specific gene sequence. If amplification occurs, the mRNA is present; if not, then it is absent. This permits the quantitation of a specific mRNA (gene) sequence.

The amplified products visualized on a gel verify the existence and the quantity of the gene of interest. By extracting mRNA at different stages, we can figure out the **temporal level** of gene expression. If we extract mRNAs from different cell types, we can figure out the **spatial level** of gene expression.

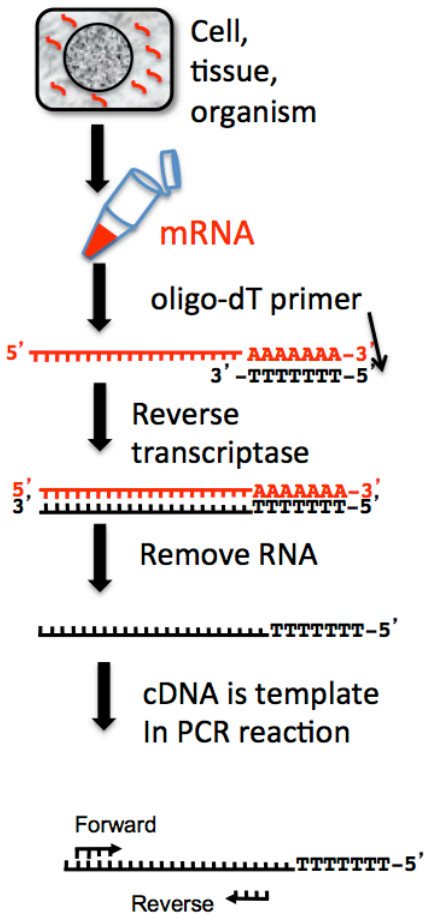

Figure 8.
The process of RT-PCR. Isolated mRNA can be reverse transcribed, and then used as a template in a PCR reaction to amplify one, or more, gene specific primers.
(Original-J. Locke-CC BY-NC 3.0)

2.4. AN APPLICATION OF PCR: THE STARLINK AFFAIR

PCR is very sensitive (meaning it can detect very small starting amounts of DNA), and specific (meaning it can amplify only the target sequence from a mixture of many DNA sequences). Due to these characteristic, PCR has many practical applications. For example, PCR can detect trace DNA contaminants in food, air, water or cells. The presence or absence and the type or species of the contaminant can be identified.

As an example, PCR was used as a tool to test whether genetically modified corn was present in consumer products on supermarket shelves. Although currently (2013) 85% of corn in the United States is genetically modified, and contains genes that government regulators have approved for human consumption, back in 2000, environmental groups showed that a strain of genetically modified corn, which had only been approved for use as animal feed, had been mixed in with corn used in producing human food, like taco shells. To do this, the groups purchased taco shells from stores in the Washington DC area, extracted DNA from the taco shells and used it as a template in a PCR reaction with primers specific for the unauthorized gene (*Cry9C*). Their suspicions were confirmed when they ran this PCR product on an agarose gel and saw a band of size expected for

Cry9C. The PCR test was sensitive enough to detect one transgenic kernel in a whole bushel of corn (1 per 100,000). The company (Aventis) that sold the transgenic seed to farmers had to pay for the destruction of large amounts of corn, and was the target of a class action law-suit by angry consumers who claimed they had been made sick by the taco shells. While no legitimate cases of harm were ever proven, and the plaintiffs were awarded $9 million, of which $3 million went to the legal fees, and the remainder of the judgment went to the consumers in the form of coupons for taco shells. The affair damaged the company, and exposed a weakness in the way the genetically modified crops were handled in the United States at the time.

PCR can be also used in medical diagnostic tests for detection of pathogens in blood, tissues and body fluids. More recently PCR has been used in the genotyping of patients to match their care with specific treatments for better outcomes.

PCR is also used for DNA genotyping of biological samples in forensic or criminal investigations. . People can be genotyped for identification purposes, so as to match with samples present at a crime scene or to establish family relationships in paternity/maternity cases. Genotypes also establish identity of people for future comparisons, much like taking fingerprints.

SUMMARY:

- Molecular biology involves the isolation and analysis of DNA and other macromolecules
- Isolation of total genomic DNA involves separating DNA from protein and other cellular components, for example by ethanol precipitation of DNA.
- PCR can be used as part of a sensitive method to detect the presence of a particular DNA sequence
- PCR can also be used as part of a method to isolate and prepare large quantities of a particular DNA sequence
- qPCR methodology allows the quantity of DNA product to be measured.
- RT-PCR methodology detects the quantity and quality of the mRNA, which indicates the spatial and temporal level gene expression.

KEY TERMS:

classical genetics
molecular biology
molecular genetics
macromolecules
lysis
detergent
chelating agent
EDTA
nuclease
supernatant
pellet
PCR
primer
thermalcycle
denature
anneal
extention
thermostable
Taq DNApol
electropheretic agarose gel
fluorochrome
Reverse Transcriptase PCR (RT-PCR)
Temporal level
Spacial level
StarLink affair
Cry9C gene

STUDY QUESTIONS:

1) What information, and what reagents would you need to use PCR to detect HIV in a blood sample?

2) If you started with 10 molecules of double stranded DNA template, what is the maximum number of molecules you would you have after 10 PCR cycles?

3) What is present in a PCR tube at the end of a successful amplification reaction? With this in mind, why do you usually only see a single, sharp band on a gel when it is analyzed by electrophoresis?