8 - Polymerase Chain Reaction (PCR)

PCR Reaction Components

The **polymerase chain reaction (PCR)** is essentially DNA replication in a test tube. In the laboratory, PCR can be used to copy or **amplify** any DNA sequence of interest. PCR has a myriad of applications. For example, PCR can be used in forensics to make copies of the DNA molecules left by a suspect at a crime scene, scientists can use PCR to make many copies of a gene to study gene structure and function, and finally, PCR can be used to determine if an individual is infected with a microbe, such as the human immunodeficiency virus (HIV) or SARS-CoV-2 (the virus that causes COVID-19).

Suppose our goal is to study the human insulin gene. We could use PCR to make millions of copies of the insulin gene as part of our research. To accomplish this goal, our PCR reaction would contain the following components:

- **Template (target) DNA.** This double-stranded template DNA molecule includes the gene or segment of DNA that is to be copied (amplified). In our scenario, the template DNA would be a fragment of chromosome 11 that contains the human insulin gene.
- **DNA primers.** These two single-stranded DNA primers (each approximately 20 nucleotides in length) are designed to bind to the template DNA molecule on each side of the gene that will be amplified by PCR. One DNA primer would bind to the left of the insulin gene on one of the two template DNA strands, the other DNA primer would bind to the right of the insulin gene on the other template DNA strand.
- **dNTPs** (dATP, dCTP, dTTP, and dGTP). The dNTPs provide the energy for DNA replication in PCR. Moreover, the nitrogenous base, the deoxyribose sugar, and one of the phosphate groups in each dNTP is incorporated into the growing daughter DNA strand.
- **Thermostable DNA polymerase.** PCR uses a DNA polymerase called ***Taq* polymerase**, isolated from the thermophilic bacterium *Thermus aquaticus. Taq* polymerase is a thermostable DNA polymerase that functions similarly to DNA polymerase I from *E. coli*. A thermostable DNA polymerase is used because PCR involves a series of heating steps (see below) that would denature other DNA polymerases, including all of the DNA polymerases introduced in Part 6.
- **Buffer.** The buffer maintains the proper pH for the PCR reaction and contains enzyme cofactors, such as magnesium ions, necessary for *Taq* polymerase activity.

Key Questions

- What are the functions of the five components of a PCR reaction?

PCR Cycles

Once the PCR reaction is prepared containing each of the five components above, the PCR reaction is subjected to multiple **PCR cycles** (see **figure 8.1**). Each PCR cycle has the following three steps:

1. **The template (target) DNA (i.e., the human insulin gene) is denatured by heat treatment (95°C for 1 minute).** The denaturing step breaks the hydrogen bonds within the target DNA molecule, causing the individual template DNA strands to separate from each other.
2. **The DNA primers anneal (form hydrogen bonds) with complementary sites on the template DNA strands (55°C for 1 minute).**
3. ***Taq* polymerase synthesizes DNA (72°C for 2 - 3 minutes).** *Taq* polymerase synthesizes the daughter DNA strands by adding dNTPs to the free 3'-OH groups provided by the two DNA primers This DNA synthesis step is sometimes called the primer **extension** step.

Each PCR cycle described above is repeated typically 30-35 times. The number of DNA copies (i.e., the number of copies of the human insulin gene) is doubled at the conclusion of each cycle. The total number of template (target) DNA copies made during PCR can be estimated using the following equation:

$$n = a \times 2^b$$

- n = the number of double-stranded template (target) DNA copies made during PCR (i.e., the number of copies of the human insulin gene).
- a = the number of template (target) DNA copies at the beginning of the PCR experiment. Often this is assumed to be a single template DNA molecule (i.e., a single human insulin gene).
- b = the number of PCR cycles that have occurred.

In the lab, the PCR cycles are accomplished by mixing the above five PCR reaction components in a test tube, followed by placing the PCR reaction in a **thermocycler** device. The thermocycler automates the number of cycles, the temperature of each step within a cycle, and the length of each step within a cycle.

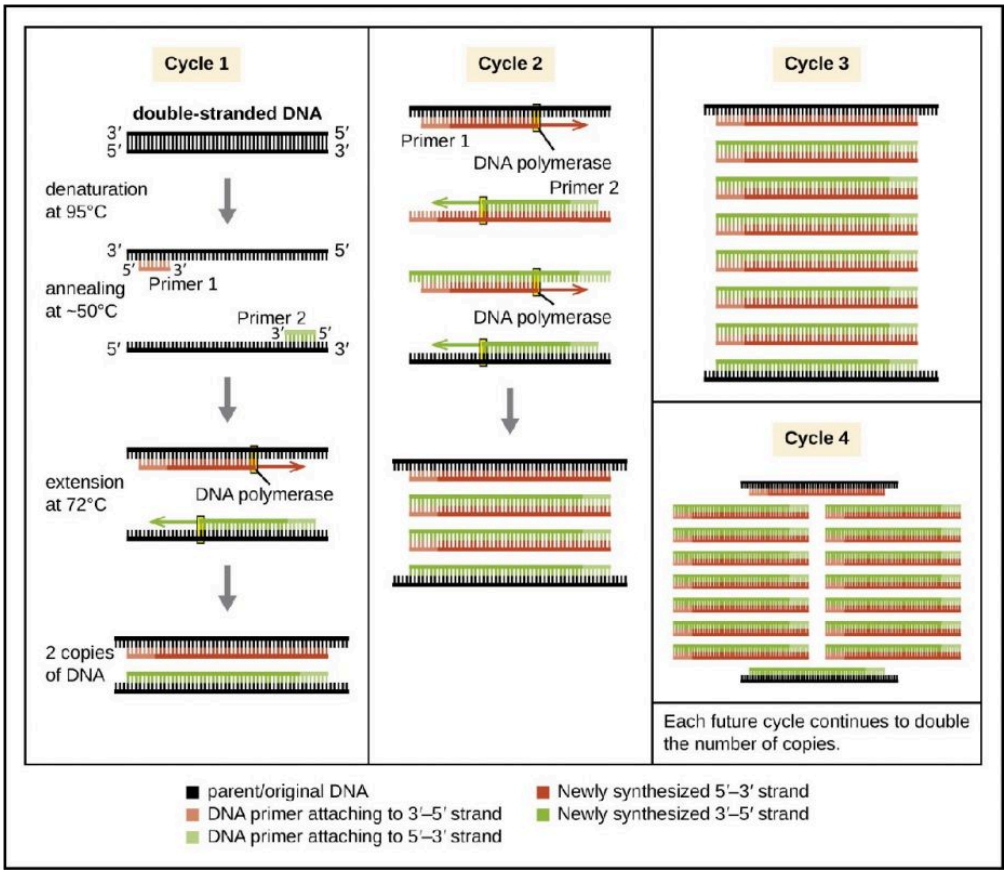

Figure 8.1 PCR Cycles. *Each PCR cycle includes a denaturing step in which the template DNA strands separate, a primer annealing step, and a DNA synthesis (extension) step in which the daughter DNA strands are synthesized by Taq polymerase. The number of double-stranded DNA copies is doubled at the end of each cycle. --- Image used from OpenStax (access for free at https://openstax.org/books/biology-2e/pages/1-introduction*

Key Questions

- What is happening in each of the three steps in a typical PCR cycle?
- Describe how PCR can be used to amplify a gene of interest.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is used in the laboratory to visualize DNA. For our purposes, agarose gel electrophoresis can be used to determine whether the PCR experiment successfully copied (amplified) the human insulin gene. Since agarose gel electorphoresis separates DNA molecules from each other based upon size, agarose gel electrophoresis can also be used to analyze the size (in base pairs) of the insulin PCR products (see **Figure 8.2**).

To perform an agarose gel electrophoresis experiment:

1. **The agarose gel is prepared.** To prepare an agarose gel, agarose (a polysaccharide powder purified from marine algae) is dissolved in a buffer solution by heating in a microwave. This melted agarose solution is then poured into a plastic mold and allowed to cool to form a gel. The resulting agarose gel contains a meshwork (matrix) of agarose polymers. The consistency of the matrix depends on the concentration of agarose used. High agarose concentrations produce a matrix with small pores, useful for separating small DNA molecules. Low agarose concentrations produce a matrix with larger pores, useful for separating large DNA molecules. Moreover, a fluorescent dye called **ethidium bromide** is added to the agarose gel before it solidifies. Ethidium bromide binds to the DNA within the agarose gel and fluoresces orange in the presence of ultraviolet light. Thus, ethidium bromide provides a convenient way to visualize the DNA molecules as they separate within the agarose gel.
2. **The PCR products are loaded into depressions (wells) created at one end of the gel.** A sample called a **molecular marker (DNA ladder)** is also loaded into one well of the gel. This molecular marker sample contains DNA fragments of known size for comparison to the PCR product.
3. **An electric field is applied across the agarose gel.** This electric field causes charged molecules to migrate from one end of the agarose gel to another. Typically, the end of the gel that contains the wells is placed near the negative electrode; the opposite end of the gel is placed near the positive electrode. Since DNA is negatively charged, DNA will migrate from the wells into the agarose gel. DNA migration continues through the agarose gel matrix towards the positive electrode.
4. **The DNA samples separate according to size.** Smaller DNA molecules migrate farther through the agarose gel matrix than larger DNA molecules.
5. **The DNA is visualized using ultraviolet light**. The ethidium bromide in the agarose gel binds to the DNA as the DNA migrates through the gel. The ethidium bromide bound to the DNA fluoresces orange in the presence of ultraviolet light.

If PCR amplification of the insulin gene was successful, a band will be seen in the agarose gel when exposed to ultraviolet light.

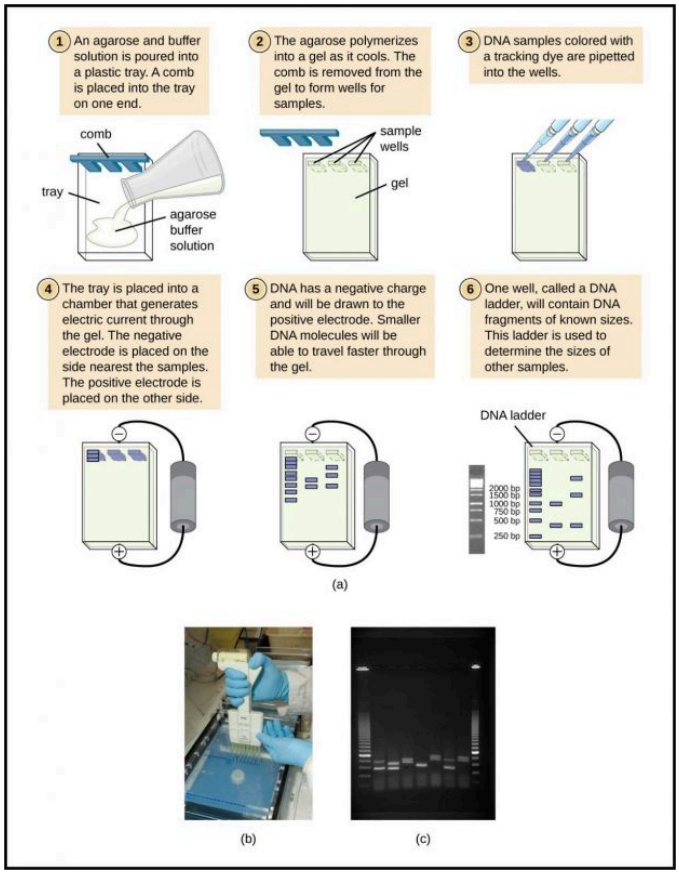

Figure 8.2 ***Agarose Gel Electrophoresis*** *--- Image used from OpenStax (access for free at https://openstax.org/books/biology-2e/pages/1-introduction*

Key Questions

- What is an agarose gel?
- Describe how DNA molecules separate in an agarose gel.
- Describe how agarose gel electrophoresis can be used to determine if the insulin gene was successfully amplified by PCR.

Reverse Transcription PCR (Future Content)

PCR can also be used to amplify a particular mRNA molecule; however, mRNAs are not used directly as templates for the PCR reaction. To amplify a mRNA molecule, **reverse transcription** is done prior to PCR. Reverse transcription converts the mRNA molecules within a cell into a collection of DNA molecules called **complementary DNAs (cDNAs)**. These cDNAs can then be used as templates in PCR.

Reverse transcription to produce cDNAs involves the following steps:

1. Purify the mRNA molecules from a cell.
2. Mix the mRNA molecules with a **poly-dT primer**. The mRNA molecules produced from eukaryotic genes contain approximately 250 adenine bases at the 3' end of the mRNA (see the Part 10). This sequence of 250 A bases is called a **poly-A tail.** The poly-dT primer is composed of a series of T nucleotides that form hydrogen bonds with the poly-A tails on these mRNA molecules. The poly-dT primer also provides the free 3'-OH group required for DNA synthesis.
3. Add **reverse transcriptase** and **dNTPs**. Reverse transcriptase is a DNA polymerase isolated from certain types of viruses, including the human immunodeficiency virus. Reverse transcriptase is a unique DNA polymerase because it uses an RNA template to synthesize DNA (the other DNA polymerases we have studied use a DNA template to synthesize DNA). The newly synthesized strand of DNA generated by reverse transcriptase is complementary (forms hydrogen bonds) to the mRNA. Thus, after reverse transcription, an RNA:DNA double-helix is present in the reaction.
4. Add **ribonuclease H (RNase H)** to digest portions of the mRNA. RNase H is a viral endonuclease that cuts phosphodiester bonds within the RNA component of an RNA:DNA double-helix . One consequence of RNase H treatment is that the mRNA is cleaved into fragments; each mRNA fragment is essentially a primer, containing the free 3'-OH groups required to synthesize a second DNA strand.
5. Add **DNA polymerase I** and **DNA ligase** from *E. coli* to synthesize the second DNA strand to form a double-stranded cDNA molecule. Recall that DNA polymerase I has both 5' to 3' exonuclease and 5' to 3' polymerase activities that replace the RNA bases with DNA bases, while DNA ligase forms the final phosphodiester bonds to convert Okazaki fragments into a continuous DNA strand.

Reverse transcription produces a collection of double-stranded cDNA molecule that correspond to the entire mRNA collection within a cell. This collection of various cDNA molecules can then serve as the templates for PCR. Since the primers used in PCR are typically designed to be specific for a particular gene sequence, PCR amplification copies of a single cDNA type, corresponding to a single type of mRNA. In essence, this **reverse transcription PCR** process has amplified a single type of mRNA from the cell.

Insert a figure here that illustrates reverse transcription PCR.

Key Questions

- What is the starting material for reverse transcription?
- What are cDNAs?
- Describe the function of the poly-dT primer, reverse transcriptase, RNase H, DNA polymerase I, and DNA ligase during reverse transcription.

Real-time PCR (Future Content)

Often, the goal of PCR is to make many copies of a particular DNA sequence. The success of the PCR experiment is determined by analyzing the PCR product on an agarose gel, as described above. In other cases, the goal of PCR is to determine how many copies of the template DNA molecule are present at the beginning of the PCR experiment, before PCR amplification occurs. To determine the number of template DNA molecules present in a sample, a modification of PCR, called **real-time PCR** or **quantitative PCR (qPCR)** is used.

Real-time PCR allows a scientist to monitor the production of PCR products in real-time (i.e., as the reaction is occurring in the thermocycler). If the concentration of template DNA molecules in the reaction is low prior to the start of PCR, then it takes more PCR cycles to produce the number of PCR products required for detection. Alternatively, if the concentration of template DNA molecules in the reaction is high prior to the start of PCR, then detectable products are

formed in earlier PCR cycles. Thus, the real-time PCR technique is especially powerful, as it allows researchers to quantitatively measure the concentration of template DNA sequences. Moreover, if the template molecules are cDNA molecules produced from mRNA by reverse transcription, then real-time PCR can provide a quantitative measure of how actively a gene is transcribed (the more active the gene, the more mRNA molecules are produced by the gene).

Key Questions

- What is an advantage of using real-time PCR?

TaqMan (Future Content)

Real-time PCR involves a modification to the conventional PCR approach. In addition to the five PCR components described earlier, a real-time PCR reaction contains a **probe** molecule. A common probe that is used in many real-time PCR reactions is **TaqMan**. The TaqMan probe is a short DNA molecule that is designed to form hydrogen bonds to one of the two template DNA strands downstream (in the 3' direction) from one of the two DNA primers. The 5’ and 3’ ends of the TaqMan molecule have been modified; the 5’ end of TaqMan is attached to a fluorescent **reporter** molecule, while the 3’ end of TaqMan is attached to a **quencher** molecule. When the reporter and the quencher are in close proximity (i.e., within the same probe molecule), the quencher molecule inhibits the fluorescence produced by the reporter molecule. When the reporter molecule is separated from the quencher molecule (i.e., when the probe is degraded by the 5' to 3' exonuclease activity of *Taq* DNA polymerase), fluorescence occurs.

During the primer annealing step in a real-time PCR reaction, both a primer and the TaqMan probe binds to one of the two template DNA strands. During the DNA synthesis step, *Taq* polymerase synthesizes the daughter DNA strand toward the TaqMan probe bound to the template DNA strand. When *Taq* polymerase encounters the TaqMan probe, *Taq* polymerase uses its 5’ to 3’ exonuclease activity to begin digesting TaqMan. As the TaqMan probe is digested, the reporter molecule is separated from the quencher molecule and fluorescence occurs. If the number of template DNA molecules in the reaction is low, fluorescence is low in early PCR cycles and begins to be detectable in later PCR cycles. If the number template DNA molecules in the reaction is high, fluorescence is detectable in the earlier PCR cycles.

The thermocycler used in real-time PCR is modified to detect the fluorescence emitted by the reporter molecule as it is released from the quencher molecule during DNA replication.

Insert a figure here that illustrates a real-time PCR cycle.

Key Questions

- What are the six major components of a real-time PCR reaction?
- What are the functions of the two parts of a TaqMan probe?
- Describe how real-time PCR using a TaqMan probe can be used to determine the concentration of template DNA molecules in a reaction.