Workshop 4: PCR, Gel Electrophoresis, & Transformation Notes

Polymerase Chain Reaction (PCR):

- An important technology used to amplify DNA. The technology makes use of DNA's natural cycle of replication, thus doubling the amount of DNA with each cycle.
- A PCR reaction occurs in a PCR tube within a thermocycler. The components and reagents placed in the PCR tube include the following:
    - DNA template (A piece of DNA including the sequence you want to amplify. Note that you do not need to know the exact sequence, just the sequence of the flanking regions - this allows you to make complementary primers.)
    - Two flanking primers (Single-stranded DNA strands of 18-22 bases which are complementary to the 5' ends of both strands of the DNA target sequence).
    - Taq polymerase (The enzyme that copies the DNA from 5' to 3' in order to amplify it. This specific polymerase is used as normal enzyme proteins will denature at 70 degrees Celsius, but the reaction must occur at much higher temperature. Taq polymerase, on the other hand, is isolated from bacteria able to survive at high temperatures, and thus is not denatured.)
    - Deoxynucleoside triphosphates (dNTPs - nucleotides bound to triphosphate groups. These are used by the Taq polymerase to build the new DNA strand.)
    - Buffer solution (allows for optimal polymerase function)
- The PCR Reaction:
    - Denaturation - High temperature (~95 degrees, known as the denaturation temperature) allows the DNA to "unzip."
    - Annealing - Lower temperature (~60 degrees, known as the melting temperature) allows the primers to anneal to the DNA strands.
    - Elongation - Higher temperature (~75 degrees) allows the TAQ polymerase to copy the template strands, starting at the primers.
    - Repeat!
    - Result - Can have 30-35 cycles in 1.5 hrs or so, giving a huge amplification of the DNA. 2^(n-1) amplification, where n is the number of cycles (because the first cycle does not make the DNA region of interest, but instead copies the entire plasmid).
- Primer Design: Primer sequences must anneal to template DNA and contain the restriction site of interest. Their optimal length is 18-22 base pairs. Primers are designed using the Wallace Rule, which states that the melting temperature of the primer can be determined by adding 4 degrees for every C or G in the primer sequence, adding 2 degrees for every A or T, and subtracting two degrees. Ultimately, the melting temperature should be above 55 degrees, usually between 55 and 60 degrees Celsius. The 3' nucleotide of the primer should ideally be a C or G so that the primer is more likely to anneal at this last base pair (because C and G bind with 3 hydrogen bonds, rather than with only 2 like A and T).

Gel Electrophoresis:

- A technique used to confirm the size of a piece of DNA, the size of a piece of RNA, or the charge of a protein. With regard to DNA fragment size, the process uses an electrical current to move DNA fragments through an agarose gel, separating them by size.
- A polysaccharide gel, agarose has a cross-linked matrix that acts like a sieve, allowing smaller molecules to move through the gel at a faster rate than larger molecules. To make the agarose gel, agarose powder and TAE buffer are heated to dissolve the agarose. EtBr is added to the hot mixture before it is poured into a casting tray for cooling. A comb is inserted to form small wells as the gel cools.
- The agarose gel is placed into a gel box and covered with TAE buffer. The DNA samples are added to the wells before an electrical current is applied to the gel. A standard DNA ladder is also added as a reference (the ladder contains DNA fragments of known sizes, allowing for size comparison with the sample fragments). The end with the DNA samples is negatively charged (black anode) and the other end is positively charged (red cathode), allowing the negatively charged DNA to migrate through the agarose. Ultimately, the smaller fragments move more rapidly than the larger ones, separating the DNA by size.
- This process may also be done with RNA to separate it by size. To determine the charge of proteins, proteins may be placed in wells in the center of the gel. If the protein is positive, it will migrate to the negative end of the gel, and if the protein is negative, it will migrate to the positive end of the gel.
- For iGEM purposes, gel electrophoresis is often used to confirm PCR products. However, can be practically applied in paternity testing and criminal investigations.

DNA Sequencing:

- DNA sequencing refers to the process used to determine the order of the four nucleotide bases, adenine, guanine, cytosine, and thymine, within a strand of DNA. Since its invention in 1977, the Sanger sequencing method has been the preferred sequencing protocol, perhaps most publically utilized within the Human Genome Project in 2001. It is especially useful for sequences greater than 500 base pairs.
- Sanger sequencing follows a similar protocol to PCR, using a thermocycler to replicate DNA. Unlike PCR, however, the method employs chain-terminating dideoxynucleotides in addition to normal, non-chain-terminating nucleotides. Each dideoxynucleotide (A, G, C, and T) fluoresce a different color (green, yellow, blue, and red).
    - The PCR reaction runs as per usual, but whenever it adds a dideoxynucleotide to the daughter DNA strand rather than a nucleotide, the polymerase terminates replication activity. Ultimately, DNA strands of a variety of different lengths are obtained.
    - The DNA is run through an agarose gel, separating the various strands according to size. As the each different dideoxynucleotide fluoresces a different color, the colors of the gel image may be “read” bottom to top to give the DNA sequence.

Transformation:

- Transformation refers to the uptake and expression of exogenous DNA. In general, transformation refers to the insertion of recombinant DNA into bacterial cells.
- Methods of Transformation: While some bacteria will transform naturally, many require treatment with one of the following lab techniques.
    - 1. Electroporation- A brief pulse of electricity creates pores in the cell's membrane, which allows for the uptake of foreign DNA.
    - 2. Calcium Chloride / Heat Shock- Chemically competent cells (made through treatment with $CaCl_2$) uptake DNA after heat shock. The cells are incubated on ice to slow the fluid membrane before they are shocked with heat to increase the permeability of the cell membrane.
- Luria-Bertani (LB) Broth - A media that contains nutrients for bacterial growth and gene expression, including carbohydrates, amino acids, nucleotides, salts, and vitamins.
- After bacteria are transformed, they are incubated in LB media for 1 hour at 37 degrees Celsius in a shaker, allowing the culture to grow and express the plasmid genes. The bacteria may then be plated on media with an antibiotic to select for transformed bacteria. As the transformed bacteria express the antibiotic resistance gene on the plasmid, these transformed bacteria contain a plasmid conferring resistance, while non-transformed bacteria die. After incubating the plates overnight, transformed colonies may be identified.
- Transformation may be accomplished in other organisms, but it is most efficient in bacteria and other single-celled organisms. In order for a multicellular organism to be completely transformed, every cell must uptake, integrate, and express the plasmid.