Biotechnology

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1 Introduction

Biology deals with a lot of small things–many complex fibers are very thin, and those fibers are made of cells, which have in them even smaller organelles. Humans have fat fingers, making it hard to poke around and do experiments. As such, we need biotechnology to explore and discover all that biology has to offer.

2 Gel Electrophoresis

2.1 DNA Gel Electrophoresis

It's often useful to know how long certain fragments of DNA are, especially for comparing pieces of DNA.

A common way to identify genetic similarities is cutting up a sample of DNA with **restriction enzymes**. Restriction enzymes cut DNA at certain sequences (more information in "Molecular Cloning"), so if two samples of DNA are identical, restriction enzymes will cut at the same place, and you end up with equal-sized pieces.

However, since DNA is so small, we can't just measure all the fragments with a ruler. DNA gel electrophoresis gives us a way to compare the length of DNA fragments.

Gel electrophoresis is a general term for when you use electricity to run something through a gel. The point is to take advantage of the charge on your sample (for example, DNA has a negative charge), and use an electric field to pull the sample to the opposite end of the gel. The gel acts as a "molecular sieve," forcing larger molecules to move more slowly than smaller ones. The ending positions of different molecules in your sample, thus, gives you information about those molecules' sizes. Gel electrophoresis is also used for other types of molecules (see "SDS-PAGE"), but each process is slightly different.

The process for DNA gel electrophoresis is as follows:

1. You start with your sample of DNA–maybe it's in your desired fragments already, maybe you need to cut it up with restriction enzymes.
2. Mix your DNA sample with **loading dye**. Because DNA is transparent to your eyes, you can't tell how far it's gone in the gel without treating the gel. Thus, it's possible for the DNA to run off the gel if you have the electric field on for too long. Loading dye moves ahead of your DNA during gel electrophoresis, telling you about your progress and allowing you to stop when you're happy with how long the DNA's run.
3. Load your samples into your **wells**.

Here's a little more information about the setup.

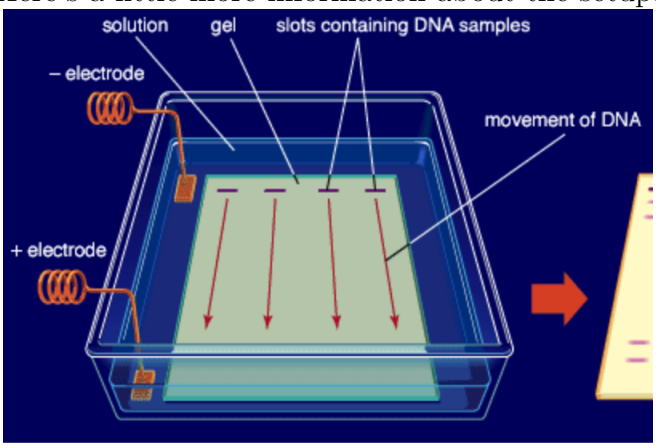


Figure 1: Gel electrophoresis. (Source: Britannica)

Your gel is usually in a tray full of liquid buffer, which maintains a constant pH. This is important since pH affects charge, and a changing pH may mess up our results.

The electric field in the gel is created by an external electric source. There are two electrodes, the positive one (anode) and the negative one (cathode). The wells, where the DNA starts, is at the cathode (-), and when the field is turned on, the negatively charged DNA is drawn towards the anode (+).

The gel is usually made of agarose, a polymer derived from seaweed. A higher concentration of agarose in the gel means the "holes" in the "sieve" are smaller, making higher concentrations of agarose more suitable for smaller DNA samples. Even at higher concentrations, the holes in an agarose gel are still quite large. Polyacrylamide gel is usually used for smaller pieces of DNA. Acrylamide, the monomer used to make it, is a potent neurotoxin, so those working with it have to be careful!

In addition to your DNA samples of interest, it's a good idea to add a **DNA ladder/marker**. This is a sample of DNA with fragments of known size. When your gel is done running, you can compare the distance traveled by fragments in your sample with the ladder to see approximately how long they are.

4. Run your gel. Turn on the electric source, and wait an hour or two for the DNA to make it far enough so you can see a difference.

5. Dye your gel with **ethidium bromide**, and view it under UV light to see your DNA bands. Other dyes are used, but ethidium bromide is by far the most popular.

2.2 SDS-PAGE

SDS-PAGE is another electrophoresis technique, but for proteins. SDS is a compound that's added to prepare your protein sample, and PAGE stands for "polyacrylamide gel electrophoresis" (polyacrylamide is the type of gel that's used). The process looks identical if you squint, but there are some modifications that make SDS-PAGE better suited for proteins.

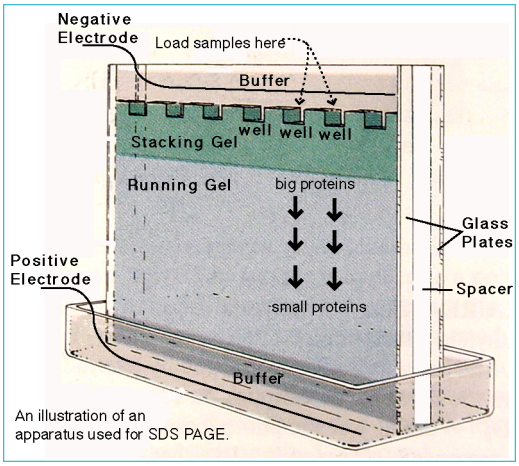


Figure 2: Generic SDS-PAGE set-up. (Source: GAtech)

1. Prepare your sample. It's customary to add **sodium dodecyl sulfate (SDS)** and **β-mercapto-ethanol**, especially since the name of the process contains "SDS".

   SDS is a **detergent**, and it's purpose is to denature your protein, putting it in primary (linear) form, essentially a chain of amino acids. It also coats the protein in negative charge, proportional to its size. This is important because many proteins already have charge, and if we don't even the charge, the proteins that move the furthest in our gel may not be the smallest, but instead the most negatively charged.

   β-mercaptoethanol is a **reducing agent**, which aids by breaking disulfide bonds between cysteines, something SDS can't do.

   Denaturing the protein is important to do since the 3D shape of the protein can cause drag in the gel unrelated to its size (which is what we're trying to investigate). This can mess with our results, so it's best if we make all the proteins linear.

   There are versions of PAGE that don't involve SDS or β-mercaptoethanol. These versions are meant to investigate intrinsic properties of your protein sample that may get taken away, such as the charge. Gel electrophoresis on untreated proteins is called **native PAGE**.

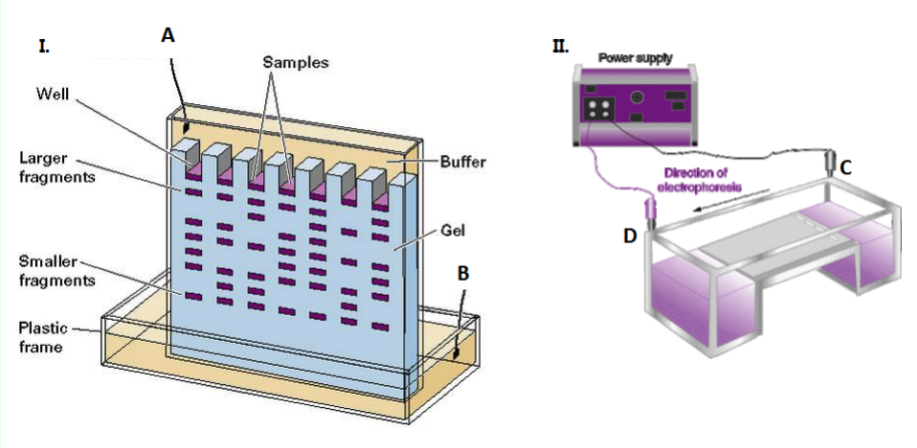
2. Load your samples. The setup is virtually the same as DNA gel electrophoresis, except it's vertical, with the wells on top. The gel is made of polyacrylamide, since proteins are generally smaller than DNA.

3. Run your gel.

4. Stain your gel with **Coomassie blue** to visualize your proteins.

SDS-PAGE is often done to prepare for Western blots (see "Blotting").

Example 2.1 (USABO Semifinal Exam 2017) Below are diagrams of two sets of apparatus. The apparatus in Figure I is used for SDS-PAGE (polyacrylamide gel electrophoresis) and the apparatus in Figure II is used for DNA agarose gel electrophoresis. Which letters indicate the anode on each apparatus?



Solution: The anode is the positive electrode and the one the proteins/DNA move towards. It is opposite the wells. Thus, **BD**.

3 Other DNA Techniques

3.1 Molecular Cloning

The purpose of molecular cloning is to amplify DNA and have copies of the desired segment of DNA at hand.

Molecular cloning takes advantage of bacterial **plasmids**, which are little loops of DNA that replicate independently. The idea is to insert DNA into the plasmid, allow it to replicate inside the bacteria, and then retrieve the DNA from the plasmids.

The process is as follows:

1. Cut the plasmids with restriction enzymes to make them linear. Plasmids are usually in a **supercoiled** form. Cutting one strand makes them nicked, cutting both makes them linear.

   As a side note, the form of a plasmid affects how far it travels during gel electrophoresis. Nicked plasmids travel the least far, followed by linear, then supercoiled. Circular plasmids will travel the furthest.

2. Add the DNA to be cloned.

3. Add ligase, which covalently seals the DNA into the cut part of the plasmid.

4. Reintroduce the plasmids to the bacteria by making the bacterial cells more permeable (see "Transformation and Transfection").

5. Retrieve DNA with the same restriction enzyme from earlier.

A lot goes into designing a plasmid to ensure the DNA is inserted at the right place. Plasmids are planned out with plasmid maps, like so.

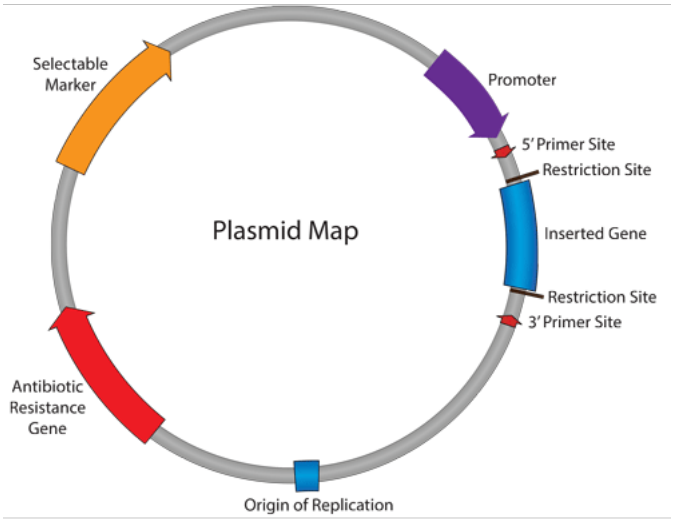
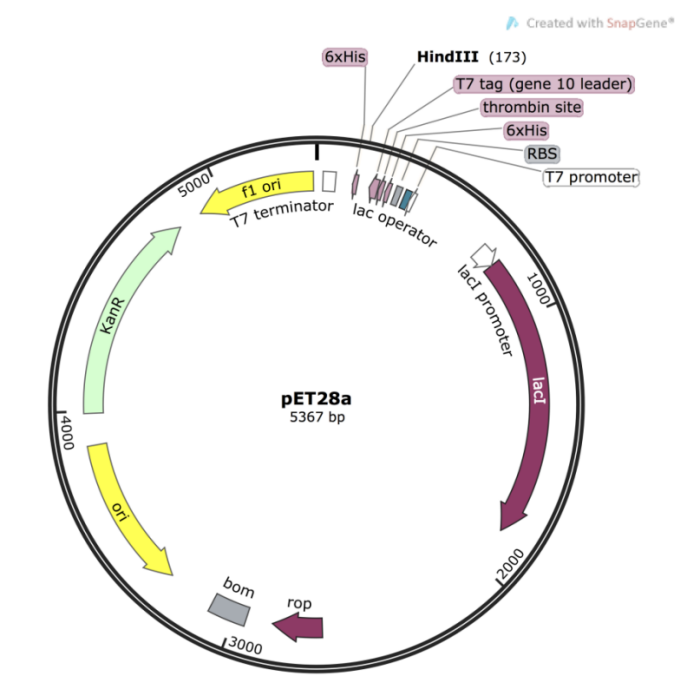


Figure 3: Plasmid map (Source: Addgene)

There are special considerations for what plasmid to use. For example, the F plasmid from E. coli is desirable because it's big, allowing it to contain more DNA, and there are low numbers of it in the cell, making it less likely for there to be recombination with sequences on other copies of the plasmid. Man-made plasmids are also used, like the **bacterial artificial chromosome (BAC)**.

DNA libraries are sometimes made, in which you store a segment of DNA by cutting into small pieces, inserting one fragment into one plasmid, then introduce each plasmid into a separate bacterium so there's only one plasmid per bacterium.

Example 3.1 (USABO Open Exam 2016) Consider the following plasmid, pET28a, a popular expression vector that is used for protein expression in bacteria.



You freshly purified plasmid from overnight bacterial culture using extraction kit, also known as miniprep. You confirmed the quality of plasmids by checking DNA and protein ratio (A260/280) and running them on a DNA agarose gel. They looked pure as they should be. You would now like to digest this vector for gene manipulation (molecular cloning) to generate vectors suitable for molecular cloning. If you cut the above plasmid with restriction enzyme called HindIII, the form of DNA will be changed from

(A) Linear to supercoiled.
(B) Nicked to linear.
(C) Nicked to supercoiled.
(D) Supercoiled to linear.
(E) Supercoiled to nicked.

Solution: The natural state of a plasmid from bacteria is supercoiled. Cutting it with a restriction enzyme cuts both strands of DNA and makes it linear. Thus, **D**.

3.2 Polymerase Chain Reaction (PCR)

PCR is used for amplifying and making copies of DNA. It is incredibly useful for many applications.

PCR occurs in 3 stages:

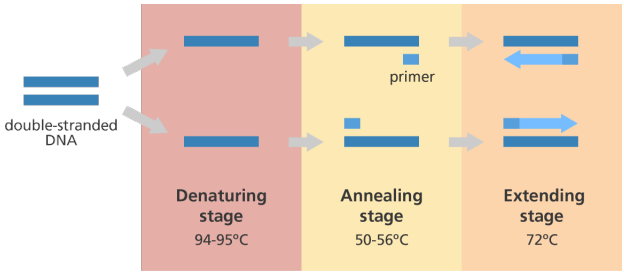


Figure 4: PCR (Source: yourgenome)

1. **Denaturing stage (94-95°C)**
   DNA is heated to denature it and turn it into two separate strands.

2. **Annealing stage (50-56°C)**
   DNA is cooled down and primers are added. The cooler temperature allows for primers to anneal to the DNA.

3. **Extending/Elongation stage (72°C)**
   Nucleotides and DNA polymerase are added to extend the DNA. This process occurs at a higher temperature so the DNA doesn't bind back together. The DNA polymerase used is commonly Taq polymerase, from the bacteria *Thermus aquaticus*, as it tolerates higher temperatures well. Pfu polymerase, from the archaea *Pyrococcus furiosus* may also be used.

It is important to consider what primers to use in PCR. There are 2 types of primers, **forward primers** and **reverse primers**. Forward primers attach to the template/antisense/minus strand, and reverse primers attach to the coding/sense/plus strand.

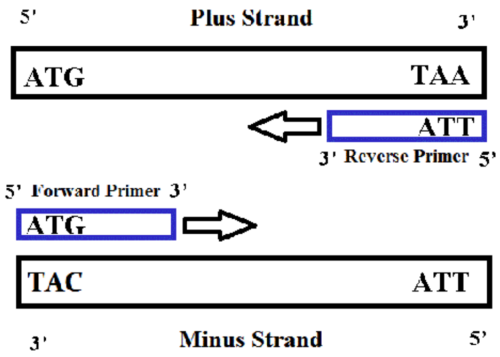


Figure 5: Forward and reverse primers. (Source: Quora)

There are some variations of PCR that are commonly used.

For instance, **quantitative PCR (qPCR)** is used to measure how large a sample of DNA is. Fluorescent reporter molecules are used on the DNA and after each cycle, the fluorescence is measured to determine the amount of DNA.

Another variation is **RT-PCR**, which stands for reverse transcriptase PCR. It's just like PCR, but an additional step is added in which RNA is transcribed into DNA. RT-PCR thus allows biologists to amplify and make copies of RNA. This is often used to examine gene expression. If mRNAs of a certain type are detected using RT-PCR, then biologists can confirm the corresponding gene is being expressed.

Example 3.2 (USABO Open Exam 2015) For forensic inspection, you only obtained red blood cells from an individual and amplified for the D1S80 locus in chromosome 1. Twenty nine different alleles of D1S80 have been identified, and 435 allelic combinations are theoretically possible. Approximately 86% of the population is heterozygous at this locus. You set up a PCR reaction for this locus and ran a DNA gel electrophoresis. What would your expected results be based on the described protocol?

(A) You would most likely see no bands.
(B) You would see more than two bands.
(C) You would see two bands for the heterozygous while one band for the homozygous.
(D) You would see two bands for the homozygous while two bands for the heterozygous.

Solution: It's a trick question–red blood cells have no DNA. No DNA means no amount of PCR can amplify the DNA, and there will be no results on your gel electrophoresis. Thus, **A**.

3.3 Hybridization Techniques

Hybridization in this context is the pairing of complementary nucleic acids with each other. Biologists design **nucleic acid probes**, fluorescently-labeled, short single-stranded lengths of

RNA/DNA to the nucleic acid sequence of interest. We can tell whether these probes are bound to a sample of nucleic acids based on whether or not there is fluorescence. This is a powerful technique that allows us to tell whether or not a certain nucleic sequence is present in a cell or body part.

***In situ* hybridization** is a technique that allows us to evaluate mRNA expression in an organism, such as in a *Drosophila* embryo. Fluorescent nucleic acid probes complementary to the mRNA sequence of interest is made, and incubating the organism with the probes results in something like this:

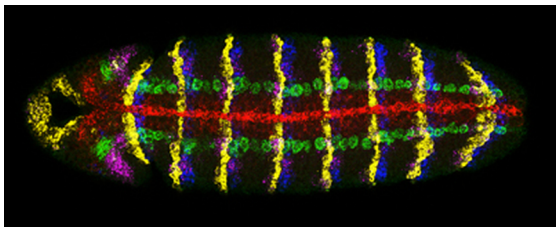


Figure 6: *In situ* hybridization in *Drosophila* embryo. (Source: Bier Lab)

Based on the color and presence of fluorescence, biologists can tell where particular mRNA sequences are present.

Hybridization is also used in **microarrays**. Although they are less powerful than some gene expression techniques, like **RNA seq**, which sequences mRNAs from tissues, they are still used in some cases to look at entire genomes. A microarray is a glass slide with an array of dots. Each of them has a single-stranded DNA fragment attached to it. mRNAs from cells/tissues of interest are reversed-transcribed into **cDNA** (essentially DNA without introns, since it comes from processed mRNA), which is turned into nucleic acid probes. These probes are fluorescently labeled, and after washing off unhybridized probes, biologists can tell which genes are being expressed in the cells/tissues the mRNAs came from. Oftentimes, 2 cells/tissues are examined, and the probes from each are labeled red and green, respectively. When only one cell/tissue expresses the gene, the corresponding dot is that color, when both express it, the dot is yellow, and when neither do, the dot is black.

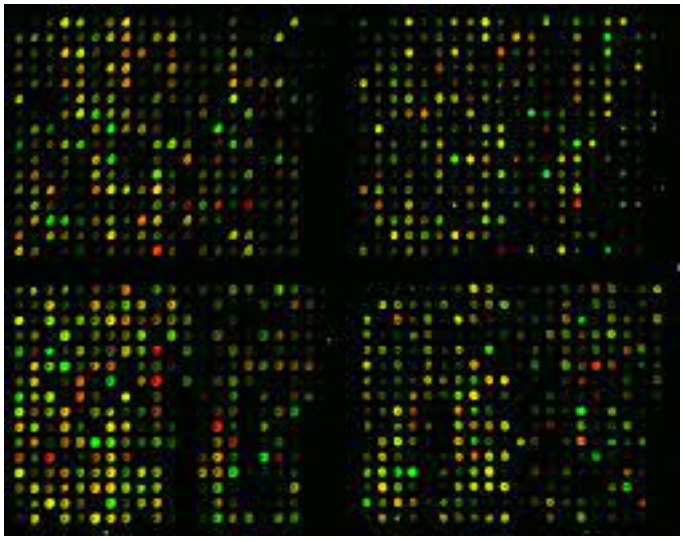


Figure 7: Microarray. (Source: NHGRI)

4 Sequencing DNA

The methods listed are in the order of discovery.

4.1 Sanger Sequencing

One of the earliest forms of sequencing, discovered by Frederick Sanger.
The process is as follows:

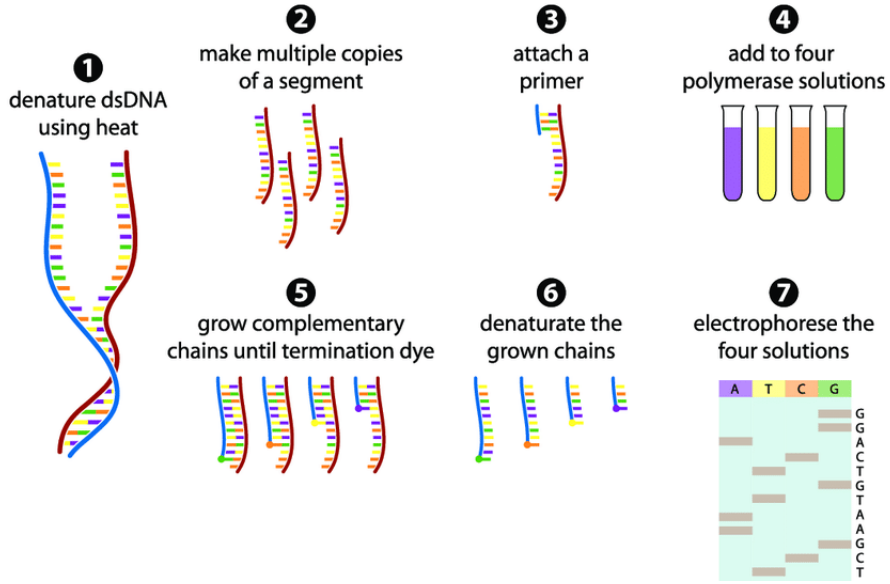


Figure 8: Sanger sequencing. (Source: Gauthier)

1. Amplify DNA with PCR.
2. Denature DNA into single strands.
3. Add dNTPs (normal nucleotides) and fluorescently labeled ddNTPs (dideoxynuclueotides). ddNTPs terminate the chain of DNA they are added to and prevent it from getting any longer.
4. Conduct **capillary gel electrophoresis** with a laser. The idea is that there are so many copies of DNA made that there will be some of each length, and the fluorescence of the last ddNTP in each length of DNA can be read to determine the sequence. The laser causes fluorescence as the DNA moves through the capillary, sorted by size. The fluorescence is read by a sensor and recorded in peaks, which can be analyzed to determine the sequence.

Sanger sequencing is now considered to be slow in the midst of modern biotech.

4.2 Shotgun Sequencing

Shotgun sequencing was developed during the Human Genome project, and was designed to be faster than Sanger sequencing. Its accuracy is questioned, especially in repeating sequences. Still, it is useful for improving on accuracy for already-sequenced DNA.
The process is as follows:

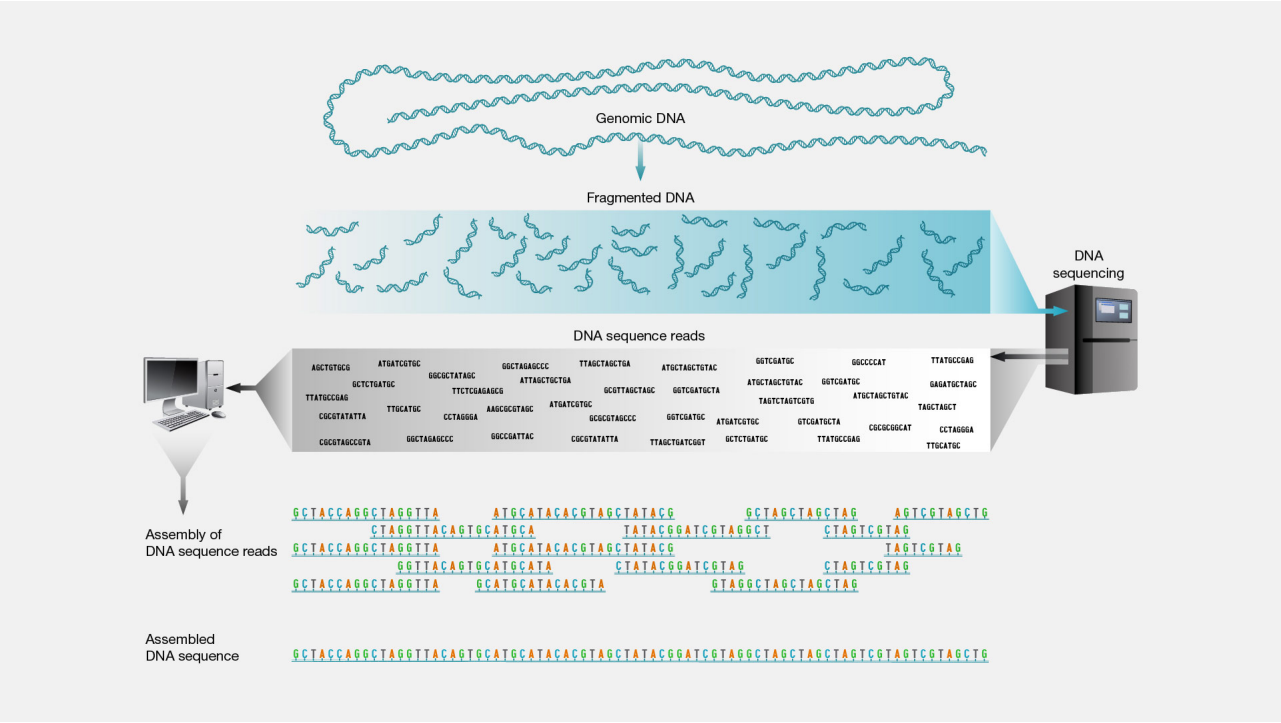


Figure 9: Shotgun sequencing. (Source: NHGRI)

1. Randomly chop up DNA.
2. Sequence the small segments.
3. Stitch together the sequence *in silico* (with a computer).

5 Blotting

The purpose of blotting is to look for specific DNA/RNA/proteins in a mixture by probing.

Blots are given different names based on what they look for. The most important ones to remember are southern, northern, and western blots. Memorize what they are for with this mnemonic:

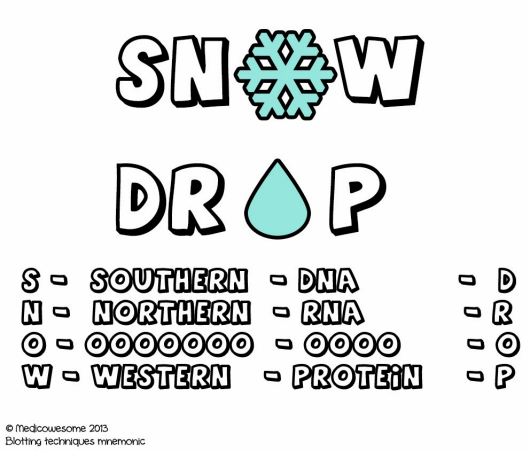


Figure 10: Blot mnemonic. (Source: Medicowesome)

The process is relatively simple:

1. Gel electrophoresis for the molecule of interest.
2. Transfer to a membrane.
3. Probe for specific DNA/RNA/proteins.

This table gives more information about differences in different blots.

	Southern Blot	Northern Blot	Western Blot
Target molecule	DNA	RNA	Protein
Sample preparation	DNA extraction enzymatic digestion	RNA isolation	Protein extraction
Separation	Electrophoresis	Electrophoresis	Electrophoresis
Membrane material	Nylon	Nylon	Nitrocellulose or PVDF
Probe	Nucleic acid probe with sequence homologous to target	RNA, DNA, or oligodeoxynucleotide	Primary antibody
Probe label	Radiolabel, enzyme	Radiolabel, enzyme	Enzyme
Detection methods	X-ray film, chemiluminescence	X-ray film, chemiluminescence	Film, cooled CCD, camera, LED, or infrared imaging system

Figure 11: Blotting comparison. (Source: Lab Manager)

6 Transformation and Transfection

The purpose of transformation and transfection is to insert DNA into organisms. Transformation and transfection mean the same thing, but the word transfection is used for animals because transformation in animals means the conversion of a normal cell to a cancer cell.

Biotechnology methods are used to put cells in a state of **competence**, a state where DNA can be taken up.

Here are some common methods:

- **Electroporation**
  Cells are shocked with electricity to create holes that are later closed by cell-membrane repair.
- **Biolistics**
  Gold or tungsten particles are coated with DNA and shot into cells.
- ***Agrobacterium tumefaciens***
  This bacterium infects plants and inserts its DNA into their cells. Biologists can insert desired DNA into the bacterium, and then have the bacterium infect plants to transfer the DNA to them.

7 Microscopy

7.1 Light Microscopy

Light microscopy is the type of microscopy you generally think of. There are a variety of light microscopy techniques, but the unifying concept is using light, as opposed to other types of waves, to visualize cells and tissues. Cells can either be kept alive, which has the benefit of allowing biologists to observe their behavior, or be fixed and stained (killing them in the process) to enhance viewing. Common stains include **hematoxylin**, which stains DNA and RNA blue, and **eosin**, which stains proteins pink. Digital techniques can also be used to enhance light microscopy pictures.

7.2 Electron Microscopy

Electron microscopy has the benefit of being able to resolve smaller structures. This is because instead of sending light through specimens, a beam of electrons are used. Since electrons have a smaller wavelength than photons, they lead to higher resolution pictures. There are two major types of electron microscopy, transmission electron microscopy and scanning electron microscopy.

Transmission electron microscopy (TEM) involves passing electrons through a specimen to see what it looks like, using magnets instead of lenses to focus the beam. Before viewing, the specimen is stained with heavy metals like lead, making certain areas of it more electron-dense than others. When the beam is cast on the specimen, more electrons pass through areas of the specimen that are less electron-dense. These electrons that originated from the beam pass through onto a screen, where their pattern can be viewed to gain information about the specimen.

Scanning electron microscopy (SEM) is cheaper and easier to use than TEM, but has lower resolution. In SEM, electrons are passed over a specimen and the scattering of the electrons off of the specimen gives information about what it looks like. A disadvantage is that SEM can only give information about the surface of a specimen, as opposed to TEM, which usually involves thin slices through tissues.

Cryogenic electron microscopy (cryo-EM) is a modification on traditional electron microscopy, quickly freezing a specimen instead of using fixation and staining techniques that may introduce artifacts. It is often used on membrane proteins.

8 Conclusion

Biotechnology allows biologists to manipulate cells and tissues without physically being able to. Currently, the impacts and uses of biotechnology are increasing, with the global biotechnology market having an estimated compound annual growth rate of 13.96% from 2023 to 2030. Now, we can see the impact it has on the common person's life, from DNA test kits to flu tests. With so much of biology still unknown, biotechnology is our ally to understanding more of the world.