**Protein Evolution: Fingerprinting with Agarose Gel Electrophoresis**

**Introduction:** Mutations in an organism’s DNA can change the phenotypic characteristics of that organism. These changes may be beneficial, helping the organism survive and reproduce better than other phenotypes in the population. Alternatively, these changes may have a negative impact on the organism’s ability to survive and reproduce, or they could have no impact on the organism. Over many generations, organisms can accumulate so many mutations and phenotypic differences that we identify the organisms as separate species. For example, cows and goats we see today are descendent from the same common ancestor many generations ago, similar to two humans sharing the same great-great-great-great grandparents. Over time, descendants of that ancestral population accumulated different mutations and different phenotypes that eventually led to two different species today – cows and goats that can no longer reproduce with each other successfully.

Although some of the phenotypic characteristics of cows and goats became different, other characteristics stayed the same as those found in their common ancestor. Therefore, if you were to compare the DNA of cows and goats, you would find that some of the DNA sequence would be the same and some would be different. The same can be said for the proteins produced by each species – some proteins would be the same and others would be different.

Picture a cow and a goat. What characteristics do they have in common?

What characteristics differ between cows and goats?

In this lab investigation, you will isolate and then separate the proteins from muscle tissue of different organisms using gel electrophoresis. The result will be a “**protein fingerprint**” for each organism that you will use to determine how closely related these organisms are to each other.

**Constructing a Phylogenetic Tree:** Before beginning the lab activity, think about the physical characteristics of the organisms you will be investigating and how the organisms might be related to each other. This information can be displayed as a **phylogenetic tree**, which shows the ancestral descent of different species, similar to how a family tree shows relationships among family members and their ancestors. The figure on the right is an example of a phylogenetic tree with five species: worms, fruit flies, frogs, turkeys, and horses. These organisms have some characteristics in common (e.g., they all are multicellular, they all have mouths), and some characteristics that are different (e.g., some have hair, some have feathers, some have eyes). The more closely related two organisms are, the closer they will be on the phylogenetic tree (e.g., fruit flies are more closely related to worms than they are to frogs, turkeys, or horses).

You will be examining the protein fingerprint, which is a phenotypic trait seen at the molecular level, of four species: **cow**, **fish**, **chicken**, and **squid**. Use what you know about each organism to infer how they are related to each other and diagram this relationship by drawing a phylogenetic tree in the space provided below. Later, you will compare your protein fingerprinting results to this tree.

**Equipment and Materials:**

*For the class:*

Hot water bath (70°C) or microwave

Hot water bath or heat block (95°C)

3% agarose in 1X Tris-glycine buffer

1X Tris-glycine-SDS buffer

Coomassie blue gel stain

Tissue samples (cow, fish, chicken, squid)

*For each group:*

Horizontal gel electrophoresis apparatus, electrodes, and power supply

P200 or 50 µL fixed volume micropipette and 3 micropipette tips per sample

4 microcentrifuge tubes with 500 µL of sample buffer

4 empty 1.7 mL microcentrifuge tubes

Gel staining tray

**Pouring an Agarose Gel:**

1. Melt the bottle of 3% agarose in 1X Tris-glycine buffer by heating the bottle in the microwave until the solution is completely clear (i.e., no solid agarose remains). Alternatively, your teacher may have pre-melted the agarose, in which case, it will be kept melted in a 70°C water bath.
2. Seal both ends of the gel caster with stoppers or masking tape.
3. Slowly pour the melted agarose into the sealed caster – pour a thicker gel than you would for DNA gel electrophoresis because you will need to load more sample than normal.
4. Insert the comb near one end of the caster (the black/negative electrode if pouring inside the gel box).
5. Allow the gel to cool and solidify (10-30 minutes depending on gel size and thickness). In the meantime, prepare your samples.

**Sample Preparation:**

1. Label one sample buffer tube for each of the four tissues your group will be using.
2. Cut a small sample of each tissue (approximately the size of a pencil eraser or one of your fingernails) and put it into the corresponding sample buffer tube.
3. Gently shake the sample tubes and let them sit at room temperature for 5 minutes – make sure the tissue samples are submerged in the buffer before starting your timer (you may need to use a pipette tip to force the sample down into the buffer; you can also use the tip to try and macerate the sample a little).
4. Label four new 1.7 mL tubes – one for each tissue sample.
5. Pipette as much of the liquid as you can (not the tissue!) from your sample buffer tube to the corresponding new tube.
6. Incubate the new sample tubes at 95°C for 5 minutes in order to denature the extracted proteins before loading them on your gel.

**Gel Electrophoresis:**

1. When your gel has solidified, remove the stoppers/tape from the caster. Carefully remove the comb from the gel by pulling it straight up (do not pull at an angle as this will break the wells).
2. Add enough 1X Tris-glycine-SDS buffer to the gel box so that it covers the top of the gel, filling the wells without overflowing the gel box. Position the gel box in a suitable spot before loading your samples because once you start loading, you cannot move the gel box.
3. Use a micropipette to load as much of the first sample as you can into the well without overflowing the well (40-50 µL would be best). Repeat the process for each of your samples. Include a diagram of your gel below, labelling where you loaded each sample on the gel.
4. Connect the red/positive and black/negative wires to the appropriate electrodes of your gel box (black-to-black and red-to-red) and to their corresponding location on the power supply.
5. Set the power supply to 125 volts and run your gel for 20-30 minutes. The dye should travel 6-8 cm from the wells during this time.
6. Turn off the power supply and unplug the electrodes from the gel box.
7. Carefully remove the gel from its caster and place the gel in a staining tray.
8. Pour enough Coomassie blue stain into the tray to cover the gel completely. Be careful with the gel stain as it will also stain your skin, clothes, etc.
9. Cover your gel and stain it for at least 30 minutes; if you cannot see any protein bands after 30 minutes, continue to stain your gel until you do. You can also leave your gel in the stain overnight.
10. After staining, your protein bands may be robust enough to view on a white light box for analysis without needing to destain the gel (if so, proceed to the next step). However, you can increase the contrast between the protein bands and the surrounding gel for easier analysis by destaining the gel in water. To do so, use gloved hands to prevent your gel from coming out of the staining tray while pouring off the Coomassie blue stain (the stain is drain safe). Rinse the gel and the tray with water and pour off the rinse. Add enough water to the staining tray to cover your gel completely (the more water, the faster the gel will destain, but be careful not to overfill the tray, especially if you will be using an orbital shaker to make the destaining process more efficient). Your gel should be sufficiently destained overnight, but you can leave it in the water for several days without affecting the protein bands.
11. Place your gel on a white light box (or white paper) for easy viewing. Draw a picture of your gel bands below and label which sample was loaded into which well:



**Analysis:** Agarose gel electrophoresis separates proteins based on size. Each band (line) in the fingerprint represents a protein of a given size. There may actually be several different proteins of similar enough size that they are found in what appears to be one band on the gel; however, without further analysis with more advanced separation techniques (e.g., that use both size and electrical charge), we cannot tell how many proteins are really found in one band. For this activity, we will assume that each band represents a single protein.

1. Compare the protein fingerprints from the different samples. Are there any bands in the fingerprints that look the same for all, or almost all, of the organisms? Why might some proteins be made by many different organisms?
2. Are there any bands that you observe in only one or two organisms? Why might some proteins not be made by all organisms?
3. Which organisms have the most similar protein fingerprints?
4. Which organisms have the most different protein fingerprints?
5. Based on protein fingerprint similarities, which organisms do you think are most closely related?
6. Based on protein fingerprint differences, which organisms do you think are least related?
7. Keeping in mind your observations from the protein fingerprints, draw a new phylogenetic tree of the organisms. If this tree is different from your initial tree, please explain your reasoning.

**Biological Classification:** Before molecular evidence like protein fingerprinting and DNA sequencing, scientists only used easily observable phenotypic characteristics (e.g., legs, feathers, skull shape) to “classify” relationships among living organisms and fossilized ancestors. The classification system included Domain > Kingdom > Phylum > Class > Order > Family > Genus > Species. Organisms classified in the same kingdom are more closely related to each other than to organisms in different kingdoms. Organisms in the same phylum are more closely related to each other than to those in different phyla (the plural of phylum), and so on. Conduct textbook or online searches to determine the kingdoms, phyla, and classes of the organisms you fingerprinted and include this information in the table below.

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| --- | --- | --- | --- |
| **Organism** | **Kingdom** | **Phylum** | **Class** |
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1. According to their biological classification, which organisms are most closely related? Please explain.
2. According to their biological classification, which organisms are least related? Please explain.
3. Does the protein fingerprinting evidence support or contradict these biological classifications? In other words, are the organisms that are more closely related according to the fingerprints also the organisms that are more closely related according to biological classification? Please explain.
4. What do you think is a more reasonable way to examine relationships between different organisms – their phenotypes, genotypes, or both? Why? Phenotypes can be easily observable ones like number of legs and presence/absence of feathers, or they can be visible at the molecular level like protein fingerprints. Genotypes are determined from methods like DNA fingerprinting or DNA sequencing.