

## COC Biotechnology Program



### A Fish Taxonomy Investigation Involving Protein

#### Diversity

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- When you buy crab at the market you may or may not be getting *real* crab.
- If it isn't expensive it is probably not genuine crab and will most likely be spelled with a "k" as krab.
- So what are you eating? Don't worry. It is seafood and most people cannot tell the difference.
- Other types of marine fish are substituted as cheap "krab" just as circular pieces of rays and skates have been marketed as scallops.
- How can you tell the difference? One way is to compare protein samples of various fishes with the fake "krab" and real crab meat.
- Although this type of analysis may not be useful in determining evolutionary kinship, it does provide a fairly rapid way to determine similarities and differences between samples that may otherwise appear to be alike.
- In this lab you will use both vertical and horizontal gel rigs to visualize the protein samples.
- By comparison you shall be able to observe the higher resolution afforded by vertical rigs.

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## **I. Objectives**

1. To become familiar with vertical gel electrophoresis rigs and observe the higher resolution afforded over horizontal rigs.
2. To understand why vertical rigs offer the researcher this greater level of resolution.
3. To successfully prepare protein samples of various marine species as well as the fake “krab” sample for use in vertical and unidirectional and bi-directional agarose gel rigs.
4. To determine whether fake “krab” meat is more closely related to fish or real crab using protein electrophoresis.

## **II. Background:**

### ➤ **A Forensic Examination Using Protein Diversity**

High molecular weight proteins, separated by gel electrophoresis, can be used to determine similarities and differences between tissue samples from various organisms. While this type of analysis may not be useful in determining evolutionary kinship, it does provide a fairly rapid way to determine similarities and differences between samples that, visually, may appear to be alike. For example, shirimi, a product produced from processing certain types of marine fish, is sometimes used as a substitute for more costly crab. Circular pieces of rays and skates have been marketed as scallops. This protocol explores differences in the proteins produced by various species of invertebrate and vertebrate animals.

### ➤ **Vertical Gel Rigs**

Vertical gel rigs afford the researcher a greater level of resolution than can be afforded in horizontal rigs. One reason for this is the relative ease and precision with which the gels can be loaded. As a result of gravity, the sample enters the gel in a more uniform manner, and as a result the bands that appear are clearer. In addition to this precision associated with loading, the gels can be prepared in a stratified manner. While stratified gels can be prepared for use in a horizontal gel rig, by pouring and using the gel in the same orientation, associated imperfections can be minimized. Our particular vertical gel has even greater resolution due to the chemical it is prepared from. Agarose gels have a maximum concentration of about 5%. PAGE gels (short for polyacrylamide gel ) can have polymer concentration up to 30%. The reason for this high percentage is that the gel is polymerized in the gel rig (as opposed to agarose, where the preformed polymer is just dissolved.) As a result of this polymerization, greater concentrations can be achieved.

As mentioned, the resolving power of these gels is the fact that they may be stratified. A 5 % gel could be poured atop a 10% gel, which is then atop 20 % gel. As a result, it is like having three gels in one! Many polyacrylamide gels (and the one we are using in this class) are pre-cast. This makes for better gels (as they can be done professionally under very exacting conditions). An additional benefit comes from the fact that the gel, once polymerized, is relatively non-toxic. Unpolymerized acrylamide is a potent neuro-toxin, and hence great care must be used in lab when casting a PAGE gel.

➤ **Gel Electrophoresis Ideas**

After a practice run with the vertical gel rig, three gels will be used (1 set per team). Two gels are 4% agarose. One will have the comb placed near the side of the gel, and the other will have the comb placed near the middle of the gel. A third set of samples will be prepared for the vertical gel rig. See below legend for explanation of these steps.

➤ **Loading buffer, gel rig and electrophoresis buffer**

The first gel will be prepared with the wells at the end of the gel and use SDS loading buffer and SDS Laemmli electrophoresis buffer.

A second agarose gel will be prepared using Triton 100 X loading buffer and Triton 100 X Laemmli electrophoresis buffer. Wells are located in the middle of the gel.

A third gel will use samples prepared with SDS loading buffer. This vertical gel rig will use a Glycine/SDS electrophoresis buffer.

Please ensure that the samples are prepared with the correct loading buffer (and clearly labeled) as seen in sample preparation (part 2) and that the type of loading buffer matches the electrophoresis technique. The following table may be helpful.

**Table 1**

<b>Gel Set Up</b>	<b>Runing/sample Buffer</b>	<b>Electrophoresis Buffer</b>
Agarose w/comb @ end	SDS	SDS Laemmli
Agarose w/comb in middle	Triton 100X	Triton 100X Laemmli
PAGE w/vertical gel rig	SDS	Glycine Buffer

**III.SOP/Lab Activities:**

Important: Always note any addenda that are posted by the instructor.

➤ **Practice run:**

1. Using the information gathered from the demonstration, set up your vertical gel rig. Remember to add a small film of silicone to the rubber seal on the gel rig prior to inserting the gel. **Be sure to remove the comb and the plastic tape/strip at the bottom of the gel rig.** Fill the top chamber (AKA the one in the back) and check for leaks. Once no leaks have been confirmed, fill the bottom well and the top well to the appropriate levels. Have the instructor come by and inspect your rig prior to loading samples.
2. Using the practice dyes, load all of the wells with different samples and with different amounts of dyes (ranging from 5-15  $\mu$ l). *Caution: be sure not to press the pipette tip into the well at all (just rest the tip atop the well, and the fluid will sink into the well) as this will cause separation of the gel plates!* This practice lab will only be mentioned briefly in your lab notebook, so exact sample information is not crucial.

3. Once loaded, run the gel @ 100 volts. As the sample runs, note any anomalies in the pattern (e.g. are all of the lanes running at the same rate?) Run the rig for about 1/2 an hour or until the samples have run out of the bottom of the gel.
4. Turn off the power supply and unplug the chamber. With a transfer pipette, remove and save the buffer from the lower chamber. Dismantle the device (wash thoroughly with soapy water and then rinse with distilled water) and save any remaining buffer. Replace the comb and tape and store gel for later use.

➤ **A Forensic Examination Using Protein Diversity**

**Table 2**

<b>Materials</b>	
<b>Reagents</b>	<b>Equipment</b>
Running Buffers (**)	Sample Buffer
4% Agarose or PAGE Gel	P-20 & P-200 Micropipettes
Coomassie Blue Sample Buffer	Pipette Tips
Crushed Ice	1mL Transfer Pipette
Electrophoresis Buffers (**)	Electrophoresis Chamber
0.1 g of Trout, Tuna, Krab, Shrimp, etc.	70°C Water Bath
Clinical Microcentrifuge	Protein Marker
Floating microfuge rack	1.5mL Microfuge Tubes w/locking caps when noted, very important!
Staining Trays	
White light box	Waterproof Marker

*Note: The following SOP pertains to both the vertical and horizontal gel rigs unless otherwise noted. (\*\*) refers to which running buffer to add depending on which rig you are using as indicated by table 1.*

*## Fish samples may be changed, so see addenda for updates.*

**1. Sample preparation (remember 2 sets in SDS and 1 in Triton 100 X)**

- 1.1 Cut a piece of fish muscle about .25 cm<sup>3</sup> in size.
- 1.2 Into labeled 1.5 ml microfuge tubes, Add 200µl of running buffer (**SDS or Triton**, NOT blue in color) and then the appropriate fish sample (label tubes accordingly) and vortex to mix.
- 1.3 Incubate for 5 minutes at room temperature and clinical centrifuge on high for 3 minutes.
- 1.4 Label appropriate # of locking 1.5 ml tubes with your initials and sample identification.
- 1.5 To each of the above tubes, aliquot 8 µl of sample buffer (made of 25 µl Triton (or) SDS, 65 µl ddH<sub>2</sub>O, 10 µl 10 X reducing agent, the reducing agent is added immediately prior to use). This buffer is either **SDS or Triton and is blue in color so keep them straight and label accordingly** (i.e Salmon, SDS).
- 1.6 Using a fresh pipette for each sample, transfer 7 µl of the liquefied sample into the appropriately labeled locking microfuge tube (from step 1.5). Remember to change pipette to avoid contamination and avoid disturbing the pellet.

**1.7 Repeat steps 1-5 for each sample of tissue. A total of 18-21 samples (12-14 SDS and 6-7 Triton) will be prepared. Make sure to label both sample name and preparation (e.g. Salmon, SDS). Note: This step is a reminder in case you forgot to do this for all samples!**

1.8 Check to see that the microfuge tubes are tightly capped and locked. Place your samples in the 70°C water bath for 3 minutes to denature the proteins. Cool to room temperature and freeze.

1.9 Each team should prepare 10 µl of a protein standard by adding 8 µl of SDS sample buffer to 2 µl of “Kaliedoscope” Protein Standard.



## **2. Casting the agarose gels**

2.1 Prepare two gel trays for casting by removing them from the electrophoresis chamber and pushing the gates into the *up* position. The screws holding the gates to the tray should be snug *but not tight*. If screws are too tight, the gates will leak.

2.2 Position the 8-tooth comb into the slots nearest the end of the tray or in the middle(\*\*). Make certain the comb is seated on both sides. Set the tray flat on the table.

2.3 Melt the 4 % agarose in a microwave. Set on high for 2 minutes and then heat at 30-second intervals. Watch for boil over and add 35 mls to one rig (with comb near side) and 35 mls to the other rig (with comb in middle.)

2.4 Pour the agarose into the tray without creating any bubbles. If there are a few bubbles that have formed, try to remove the bubbles with a *clean* pipette tip. Bubbles and other inconsistencies in the gel can disrupt the flow of electrical current through the gel. Allow about 20 minutes for the gel to solidify. While the gels are solidifying, start work on the vertical gel rig (step 3).

## **3. Loading samples for electrophoresis**

### **Vertical Gel Rig Loading**

3.1. Attach vertical gel to box (remember to add a small film of silicone to the rubber seal on the gel rig prior to inserting the gel), fill with glycine buffer and remove comb and tape.

3.2 Rinse all of the wells three times with 150 µls of SDS running buffer using a micropipette (it is not necessary to change the tip between samples).

3.3 For continuity's sake, add the samples in the same order that the agarose gel samples will be added. (See legend provided by faculty on dry erase board).

3.4 Load samples (15 µl per lane) and the 10 ul of protein standard. Fill any unfilled lanes with 15 µl of sample buffer. Run the gel @ 80 volts for 5 minutes and then 125 volts for 50 minutes, or until the dye is 1 cm from the bottom of the gel.

While gel is running go to next page: **agarose gel rig loading**.

3.5 Shut off the power and remove the electrophoresis buffer from the bottom chamber using the aforementioned technique.

3.6 Pour the buffer from the top chamber (using a funnel) directly into the container with the electrophoresis buffer. Once most of the buffer has been removed, remove the gel from the rig.

3.7 Clean the rig using soapy water first and then distilled water, and invert it to allow it to dry.

3.8 With the instructors help, remove the gel from the casing and nick the lower right corner (to help identify the gel lane's orientation.) Label a piece of tape with your group names, gel information and date and affix to the bottom of a gel staining tray. To the tray, add 50 mls of Coomassie blue stain. Place lid on the tray and put tray onto oscillating tray as directed by instructor. The instructor will then stain and de-stain the samples.

#### 4. Agarose Gel Rig Loading

- 4.1 After the agarose gel has solidified, push the gates into the *down* position and carefully place it into the electrophoresis chamber so that the comb-end is nearest the negative (black) electrode (for SDS sample). For Triton 100 X sample, center gel comb between both electrodes as evenly as possible.
- 4.2 Using both hands, carefully pull the comb straight out of the gel being careful to avoid tearing the sides or bottoms of the wells.
- 4.3 For the gel with wells located near one end, use SDS Laemmli buffer, and for the gel with the wells located in the middle, use the Triton X 100 Laemmli buffer. Fill the chamber with buffer (\*\*\*) until the level is about 1-2 mm *above* the surface of the gel. Check the buffer level to be certain it is still covering the gel. *There should be no observable "dimples" above the wells.* Add additional buffer if necessary.
- 4.4 Place a new tip on a P-20 micropipette and adjust the pipette to 15 $\mu$ L. Depress the plunger to the *first stop* and aspirate up 15 $\mu$ L of the protein. Place above well on gel and slowly depress the plunger to expel the sample. Note lane position of the sample in your notebook, and repeat with remaining samples and other gel (be consistent with all three gels with respect to sample lane loading). Load any empty lanes with 15 ul of samples buffer (Triton or SDS as needed).
- 4.5 Close the cover tightly over the electrophoresis chamber. Connect the electrical leads to the power supply. Be certain that both leads are connected properly with the cathode (-) to cathode (black to black) and anode (+) to anode (red to red).
- 4.6 Before turning the power supply "on," rotate the voltage adjustment *counterclockwise* to set the voltage to zero. Turn on the power supply. Set the voltage to 100v.
- 4.7 When the blue tracking dye has moved to within 1-2 cm of the far edge (+ end) of the gel, set the voltage back to zero, and turn the power supply off. The box will be foggy, so to see the blue dye, look through the side of the gel box. The dye and gel will be visible as you look through the buffer solution. The Triton gel will be done on roughly half of the time of the SDS gel. Unplug the electrical leads *from the power supply* by grasping the plugs, not the cords. Remove the cover of the electrophoresis chamber.
- 4.8 Carefully remove the gel and rig. Slide the gel off the rig and place your gel into the plastic tray designated for staining. Label with tape as done for the vertical gel. Take the gel and tray to your instructor for staining.
- 4.9 Stain the gels for 4 hours in Coomassie blue.

- 4.10 Destain gels in destain solution 1 for 1 hour and destain solution 2 for 24 or more hours.
- 4.11 Change destain solution 2 at least once during the first 4-6 hours. Note: steps 4.10 and 4.11 will be carried out on both agarose and PAGE gels by the instructor/lab tech.
- 4.12 Once the destaining is complete, place PAGE gel into tray and cover with about 50 ml of gel-drying solution (45% ethanol, 5% glycerol).
- 4.13 Wet two cellophane sheets in the above pre-drying solution. Set one of the sheets on the drying rig and carefully place the PAGE gel atop this sheet. Try to center the gel on the drying rig and BE CAREFULL as gel is quite fragile. Place the second cellophane sheet atop the gel. "Burp" the air bubbles from between the sheets. Place the other half of the rig onto the sheets and secure with clamps.
- 4.14 Label rig with your group name, and place in the dry shaker bath (set at 37 °C) overnight to dry.
- 4.15 Take the dried PAGE gel and both agarose gels to the gel imaging system. By adjusting the contrast, a reasonable quality gel image can be produced. Make enough copies for all of the members in your group. Put the gel images into you lab notebook and completely label the gel image.

**IV. Post-Lab Questions/Activities:** The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) before making any lab-notebook entries.

1. Which gel, horizontal or PAGE, gave you the best resolution? Why?
2. Why is it important to keep your samples on ice during preparation?
3. In a table format, discuss the three gels created and the rationale for the different buffers used. A 3 by 3 array will be sufficient to discuss the 3 gels and 3 buffers used. Get Fotodyne copies of all three gels and label fully (type, running time, voltage and lane contents).
4. Why was the reducing agent added to the sample buffer? What does the reducing agent do? What specific chemical reaction is occurring and how does this alter the protein's movement in the gel?
5. Decide which samples (fish or shrimp) are more closely related to the "Krab". Cite one piece of evidence from each agarose gel and three pieces of evidence from the PAGE gel.
6. Pick a high quality lane from the PAGE gel. Using graph paper and the colored protein standards (mass available from the instructor) make a standard curve of protein mass verse distance migrated. Using interpolation, determine the molecular weight of the proteins in the lane you have chosen to analyze. Referencing the anatomy/physiology texts in class, try to identify some of the proteins by name. Lastly, a graph of distance migrated verses molecular mass for protein is often less accurate than a similar graph created for DNA. Name three factors that may affect the distance migrated of a protein that one would not expect to be a factor influencing DNA. (Hint, DNA has a stable charge to mass ratio, i.e. a piece of DNA twice as big will have twice the charge, and it is always negative).

**V. Notebook Entries:** Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. Section V will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities.

**Your intro should:**

- Role of detergent/gels in protein analysis.
- Muscle tissues as a model tissue (only a few proteins)
- Comparison of muscle tissue types in various fish.
- Gel resolution, fish relationships and fragment mass should also be addressed.

**Results should be:**

- Three gel images completely labeled.

**Discussion should consider the following:**

- What are the strengths and weaknesses of each gel image?
- On PAGE gel comment on any relationships of fishes, type of fish for Krab and possible proteins seen in each lane.

The previous lab protocol can be reproduced for educational purposes only. It has been developed by Jim Wolf, and/or those individuals or agencies mentioned in the references.

References:

Professor Martin Ikkanda, Pierce Community College, Woodland Hills, CA.