

Case Study 1: Withering Syndrome in Red Abalone

Introduction

Abalones (*Haliotis* spp.) have declined world-wide as a result of a variety of factors such as over-fishing, disease and climate change. Although over-fishing has been well documented, disease is an important yet often underappreciated force driving population dynamics, particularly when a pathogen is introduced into naïve populations. Withering syndrome (WS) is a fatal bacterial disease of abalone, which has caused devastating impacts (up to 99% mortality) on wild black abalone, *H. cracherodii*, and cultured white abalone, *H. sorensenii*, in California. This pathogen also infects, with varying degrees, other abalone species in California. WS is caused by infections with “*Candidatus Xenohaliotis californiensis*”, an intracellular rickettsia, in conjunction with elevated seawater temperatures. This bacterium has been transferred to Chile, Israel, Iceland, Ireland, Spain, China, Taiwan and Thailand with cultured abalones.

In the next three laboratory sessions we will use microscopic and molecular techniques (i.e. DNA extraction, PCR, and gel electrophoresis) to diagnose disease in the red abalone (*Haliotis rufescens*). We will be using PCR to test for the causative agent of Withering Syndrome (affecting abalones in California), “*Candidatus Xenohaliotis californiensis*” also called Rickettsia-like prokaryote (RLP) or Rickettsia-like organism (RLO). We will also use QPCR to quantify the number of copies of RLP DNA in each sample.

Part 1

Gross examination

To diagnose a disease or assess the health of an organism, it is important to make a gross (macroscopic) assessment prior to a microscopic or molecular assessment. We will anesthetize the abalone in 3% ethanol in seawater for 10 minutes. Then you will weigh (total weight) and measure (maximum length) your abalone.

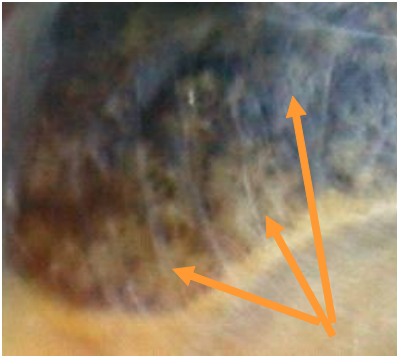
Abalone Accession Number: _____

Total Length: _____ mm Total Weight: _____ grams

After you take the above morphometric data, remove the shell using a metal spatula. Be sure to wear gloves and to put the force on the abalone shell pointing away from you to avoid self injury. Also, remember to record shell weight after it is removed!

Shell Weight: _____ grams

Once you have removed the shell from the adductor muscle and mantle edge, place the abalone into a large weigh boat for dissections as shown on the next page. Before you take your tissue sections, examine the abalone for any abnormalities and record this, along with the morphological data you entered above, in your notebook **AND ON THE CLASS DATA SHEET**. If possible, try to identify the sex of your abalone as well. Males will have a distinctive cream color (sperm) to the digestive gland while females will have a green color (eggs).



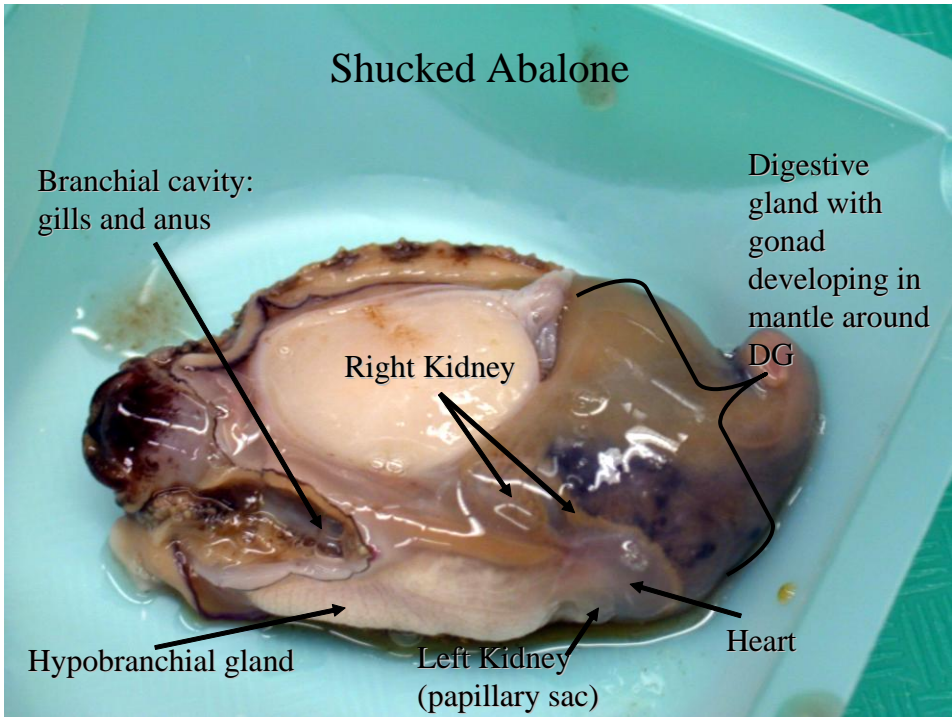
Main abnormalities associated with withering syndrome include an atrophied foot muscle, lack of gonad development and metaplasia (the substitution of one adult tissue type for another). The image to the left illustrates gross signs of metaplasia; arrows point to where the digestive gland is paler than normal indicating a change in tissue from secretory/absorptive to absorptive.

Dissection

The images below show you where to make cuts to excise the proper tissues. We will be sampling 3 tissues types (post-esophagus (PE), digestive gland (DG), and foot muscle) for histology and PCR. An extra section of PE will be taken for fluorescent dye detection.

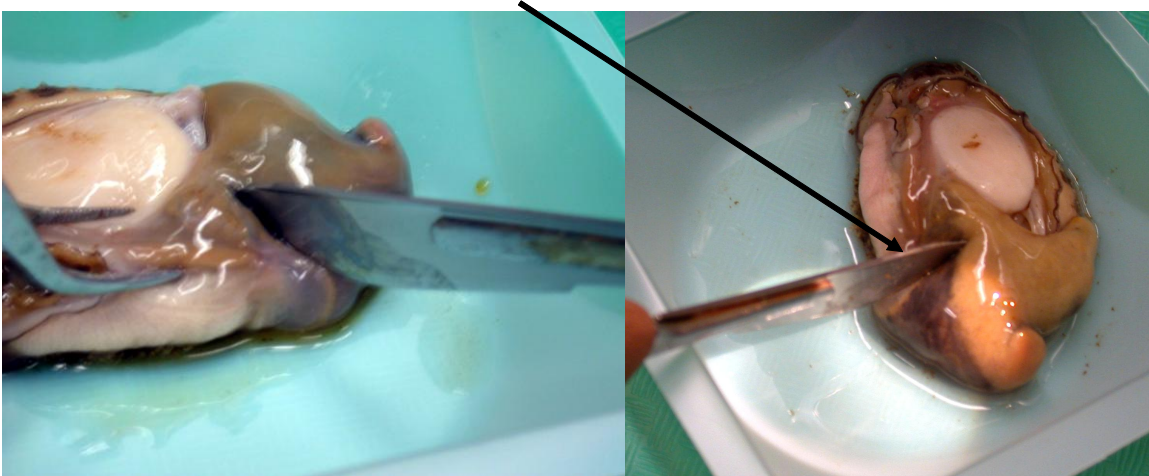
Each time you make a cut you will:

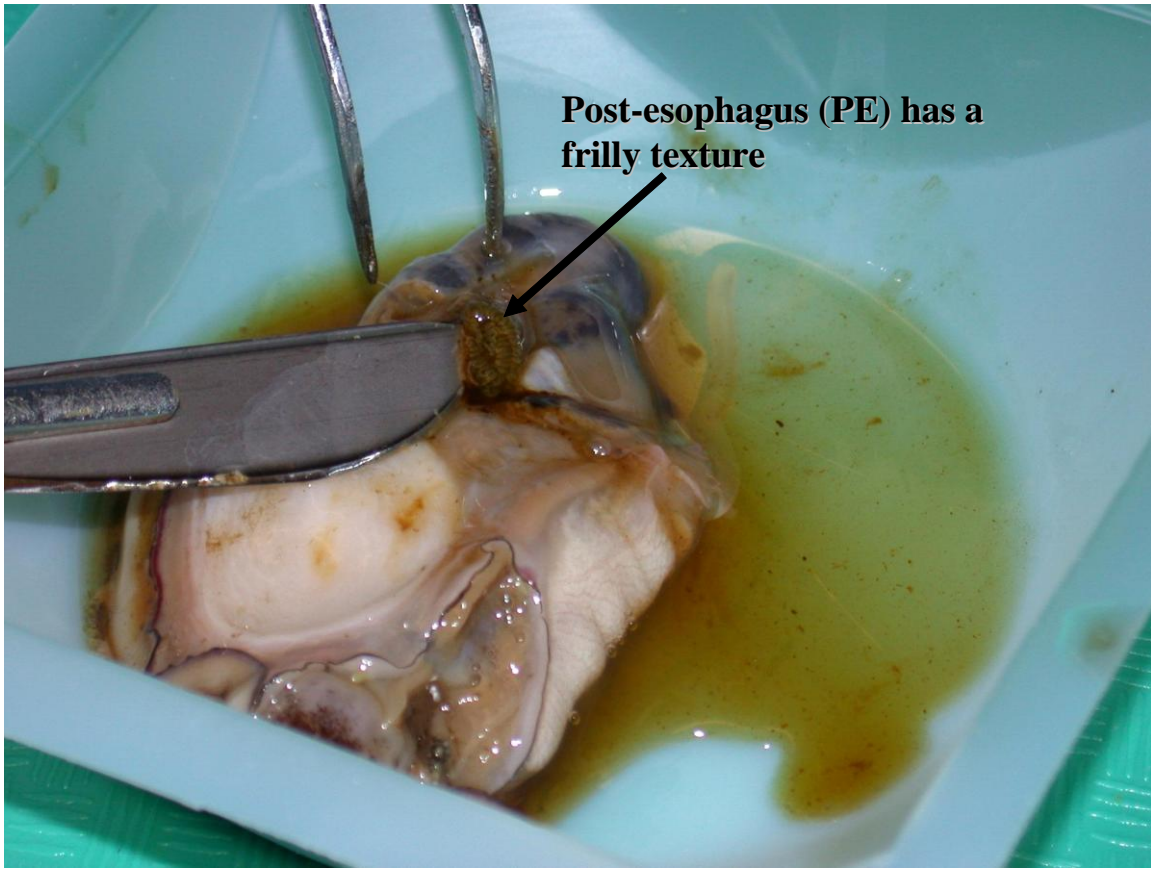
1. Take out a small (~2-3 mm thick) slice of tissue for histology and
 - a. Place it into the appropriate cassette with the side of interest facing down
 - b. Add a sponge and close the lid
 - c. Place cassette into Invertebrate Davidson's fixative for 24hr
 - d. After 24 hr, pour off the fixative and replace with 70% ethanol for storage (TA will do this 😊)
2. Take a second section from PE/DG for PCR (and put into cryovial with 95% non-denatured ethanol).
3. Be sure to take a piece of PE for examination using a fluorescent dye that targets nucleic acid (see pg.6 - Fluorescent Dye Detection of RLOs).



The post-esophagus (PE) lies in the lower portion of the DG just to the right of the heart. Thus, we make our first histology cut at the heart/right kidney (RK) junction with the DG – this is the *optimal* tissue for RLO detection.

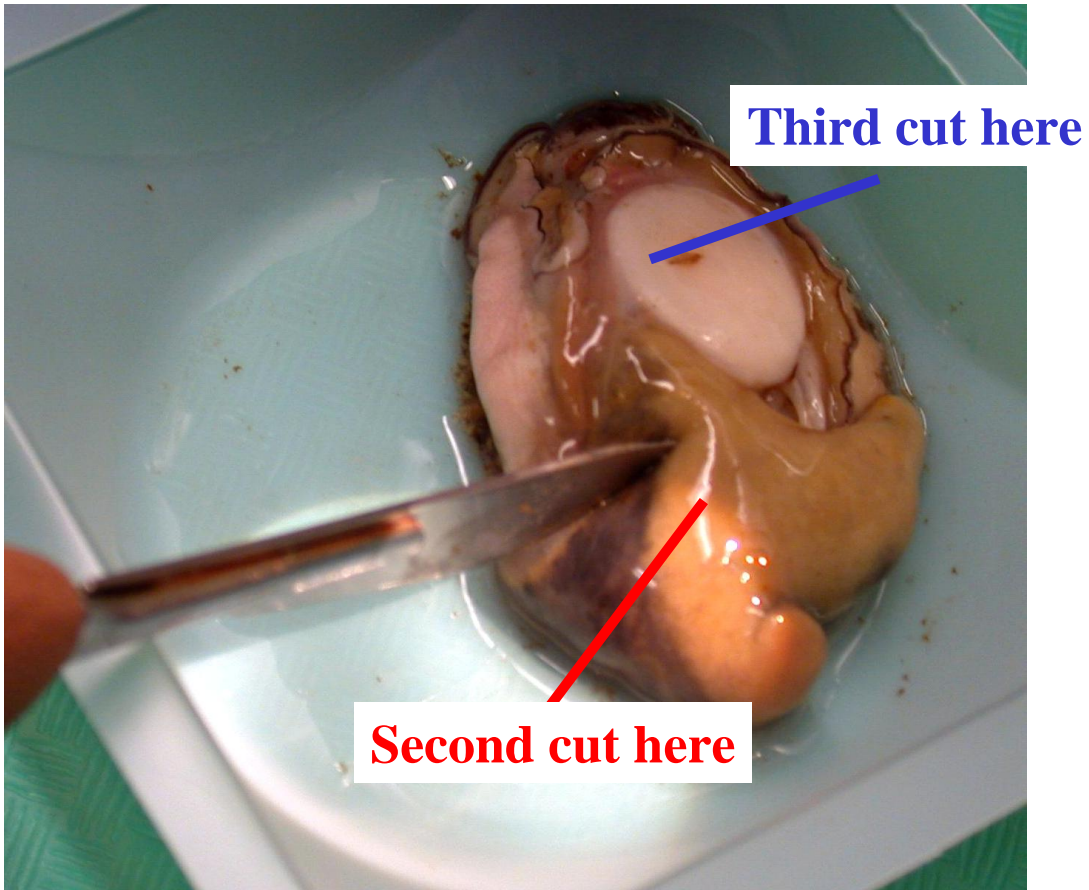
Take a section of PE for histology (try to put the PE facing right side down in cassette), a small piece for PCR, and an additional piece of PE frilly side up on a glass slide (see pg.6 - Fluorescent Dye Detection of RLOs).





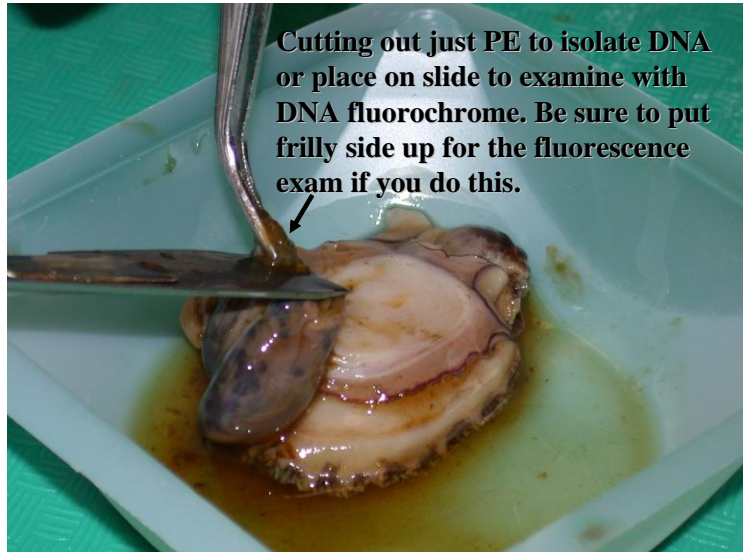
The images above illustrate where to make the initial cuts and how to ensure that you have post-esophagus (PE).

Make a second cut mid-digestive gland (section for histology and small piece for PCR) and a 3rd cut through the foot (section for histology and small piece for PCR).

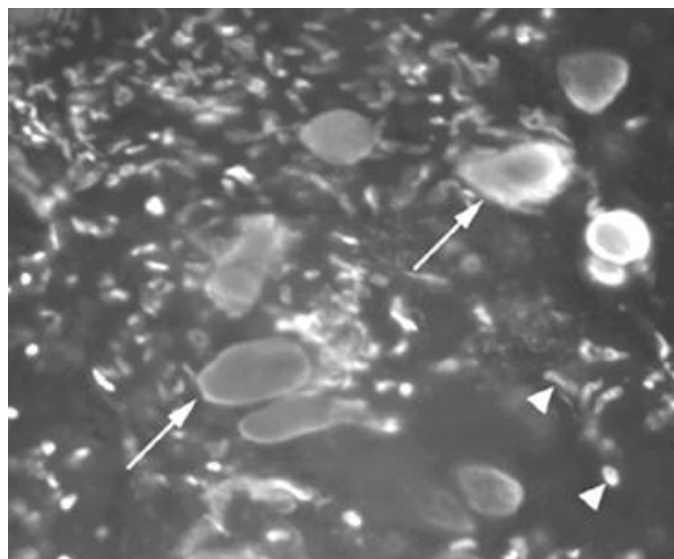


Fluorescent Dye Detection of RLOs

1. After taking a piece of PE for histology and PCR, take an additional piece of PE as shown in the image below and place frilly side up on a glass slide.
2. Tape the slide to the lab bench and heat gently with a hair dryer for ~15-20 min to fix the tissue to the slide and to kill the epithelial cells (the column shaped cells lining the digestive tract, for example) thereby allowing the stain to penetrate into the cells.



3. After drying, circle the tissue with a PAP pen
4. Flood tissue with SYBR green dye (diluted 1:1000 with di water).
5. Incubate for 1 minute at room temperature, add coverslip
6. View with fluorescent microscope at 200x magnification. RLOs (arrows) will be much larger than host nuclei.
7. Quantify the inclusions according to the following scale: WS-RLO infection intensity: **0** = absent, **1** = 1 - 10 inclusions/200 x field of view, **2** = 11 - 100, **3** = > 100 (Friedman et al. 1997). Figure to right: Arrows show RLOs; smaller oval fluorescent bodies are host nuclei.



Part II : DNA extraction

There are many methods to extract DNA, including: salt extractions, organic extractions, chelex extractions, magnetic bead extractions, and silica membrane extractions. In this lab we will use a commercially available kit which relies on the silica membrane extraction, called the Qiagen ® Stool kit. We will extract DNA from the post esophagus/digestive gland, which contains PCR inhibitors. The stool kit has special reagents to remove inhibitors from the tissue.

Materials list:

Tissue samples in 95% ethanol
Qiagen Stool kit
Pipettors, P 20, 100, and P 1000 /tips
Sterilized microcentrifuge tubes
Marking pens
70°C Incubator
Centrifuge
Vortex
Scalpels, blades, and tweezers
Weigh boats
Trays
Bleach bottles
Ethanol jar

Method: DNA Extraction (Qiagen Stool Kit) from Red Abalone

1. Pre-label all the microcentrifuge tubes you will need for this procedure. You will throw away most tubes used in this procedure, so you need to only minimally label these tubes. For every sample, you will need three 2 mL microcentrifuge tubes, two 1.5 mL microcentrifuge tubes, and one spin column. You will also need a final 1.5 mL microcentrifuge tube, please label this with the following information: your sample #, your initials, and the date. Please put the sample # on both the top and the side of the tube. Put this tube aside until the end of the procedure.
2. Remove tissue from tube using sterile forceps and place into a clean weigh boat. With a sterile scalpel blade, cut off a small piece of tissue approximately the size of ½ of a pencil eraser and using the balance record the tissue weight.
Tissue weight _____(g) – Also record on class sampling sheet
3. Cut up the tissue into small pieces and place it into a 2.0 mL microcentrifuge tube. Please take care not to cross-contaminate samples.
4. Add **1.4 mL Buffer ASL** to the sample. Do this by adding **700 µl of Buffer ASL**, vortexing for a minute and then adding another **700 µl of Buffer ASL**. Once all ASL has been added, vortex continuously for 1 min or until the sample is thoroughly homogenized. **Please note:** It is important to vortex the samples thoroughly as it will insure maximum DNA concentration.

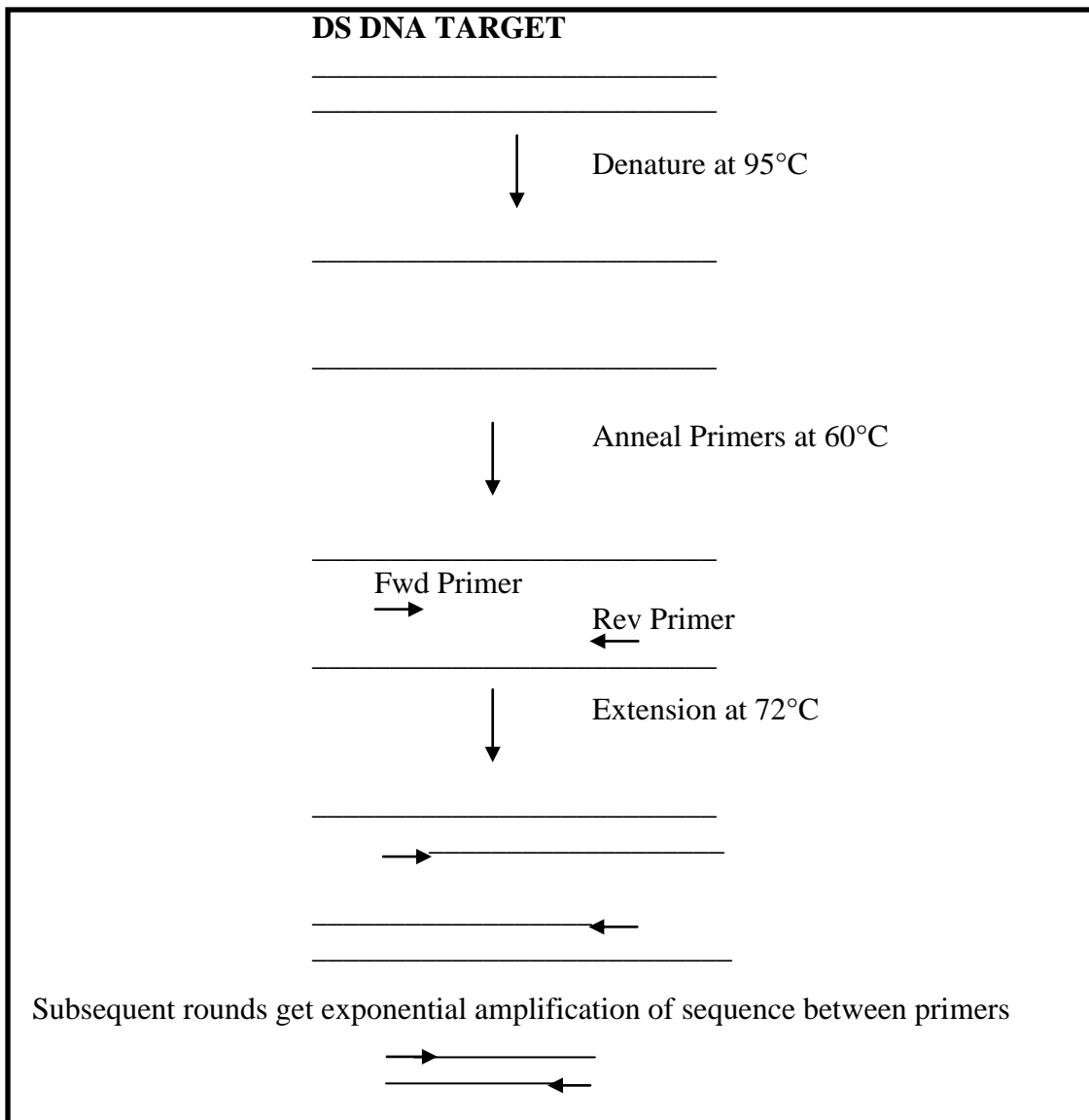
5. Pipet **1.4 mL of the lysate** into a labeled 2 mL microcentrifuge tube.
6. Heat the suspension for **5 min at 70° C**.
7. Vortex for 15 s and centrifuge sample at full speed for 1 min to pellet.
8. Pipet **1.2 mL of the supernatant** into a new 2 mL microcentrifuge tube and discard the pellet.
9. Add **1 InhibitEX tablet** to the sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to absorb the InhibitEx matrix.
10. Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX.
11. Pipet all the supernatant into a new 1.5 mL microcentrifuge tube and discard the pellet. Centrifuge the sample at full speed for 3 min.
12. Pipet **15 ul Proteinase K** into a new 1.5 mL microcentrifuge tube.
13. Pipet **200 ul supernatant** from step 11 into the 1.5 mL microcentrifuge tube containing Proteinase K.
14. Add **200 ul Buffer AL** and vortex for 15s.*DO NOT ADD Proteinase K directly to Buffer AL.*It is essential that the sample and Buffer AL are thoroughly mixed to form a homogenous solution.
15. Incubate at **70 °C for 10 min**.
16. Remove your samples from the water bath and briefly centrifuge. Add **200 ul of 95% ethanol (ETOH)** to the sample and mix by pulse vortexing for 15 seconds. Briefly centrifuge the tubes.
17. Carefully apply the mixture from step 4 to the correctly labeled QIAamp spin column (from my experience this will be approximately 610 ul). If a white precipitate has formed, make sure to add this to the column. Do not wet the rim of the spin column (this can allow cross-contamination of the samples in the centrifuge). Close the cap and centrifuge at 8000 rpm for 1 minute.
18. Place the QIAamp spin column in a clean 2 mL collection tube.
19. Carefully open the QIAamp spin column and add **500 ul of buffer AW1** without wetting the rim. Close and centrifuge at 8000 for 1 minute.
20. Discard the 2mL collection tube containing the buffer and place the spin column into a new collection tube.
21. Open the spin column and add **500 ul of buffer AW2** without wetting the rim. Close the cap and centrifuge at full speed for 3 minutes. *If you still see buffer in the top of the spin column, please re-centrifuge the sample again.*
22. Place the spin column into your final microcentrifuge tube and add **100 ul of buffer AE** and allow to incubate at room temp for 5 minutes.
23. Place the sample into the microcentrifuge and spin at 8000 rpm for 1 minute.
24. Remove the spin column and throw it away. You now have a tube of DNA!

Polymerase Chain Reaction

Introduction

The Polymerase Chain Reaction (PCR), which is the process of *in vitro* targeted DNA replication, combines a master mix and extracted DNA (also known as template DNA) and amplifies (makes a large number of copies) of the template DNA in a thermal cycler (see Figure 1 on next page and attached pages of definitions). Many fish and shellfish disease laboratories are using PCR as a quick less expensive way to measure for presence/absence of a disease. PCR is a diagnostic test for a disease, but not a confirmatory test because it only tests for DNA. Histology is considered the “gold-standard” for disease diagnosis, but can be more time consuming and expensive than PCR.

Schematic of conventional PCR:



Quantitative PCR (QPCR) uses the same general principles as conventional PCR (as described above). In a QPCR reaction, fluorescence is measured at the end of every cycle, and allows us to quantify the amount of pathogen DNA present in the organism (or tissue section). There are two types of QPCR reactions, one is called a SYBR-green assay, and the other is called a TaqMan assay. We will be using a TaqMan assay today. A TaqMan assay uses the same reagents as a conventional PCR assay with the addition of a TaqMan probe. The TaqMan probe is fluorescently labeled with a reporter dye at the 5' end and a quencher dye at the 3' end. Through Forster resonance (FRET) the quencher suppresses the fluorescence emitted through by the reporter. During PCR, the probe “sits” or anneals to its complementary DNA strand. During extension, when Taq polymerase extends the DNA from its upstream primer, the endonuclease activity of Taq polymerase cleaves the reporter from the probe and releases the fluorescence as the reporter and quencher are no longer attached.

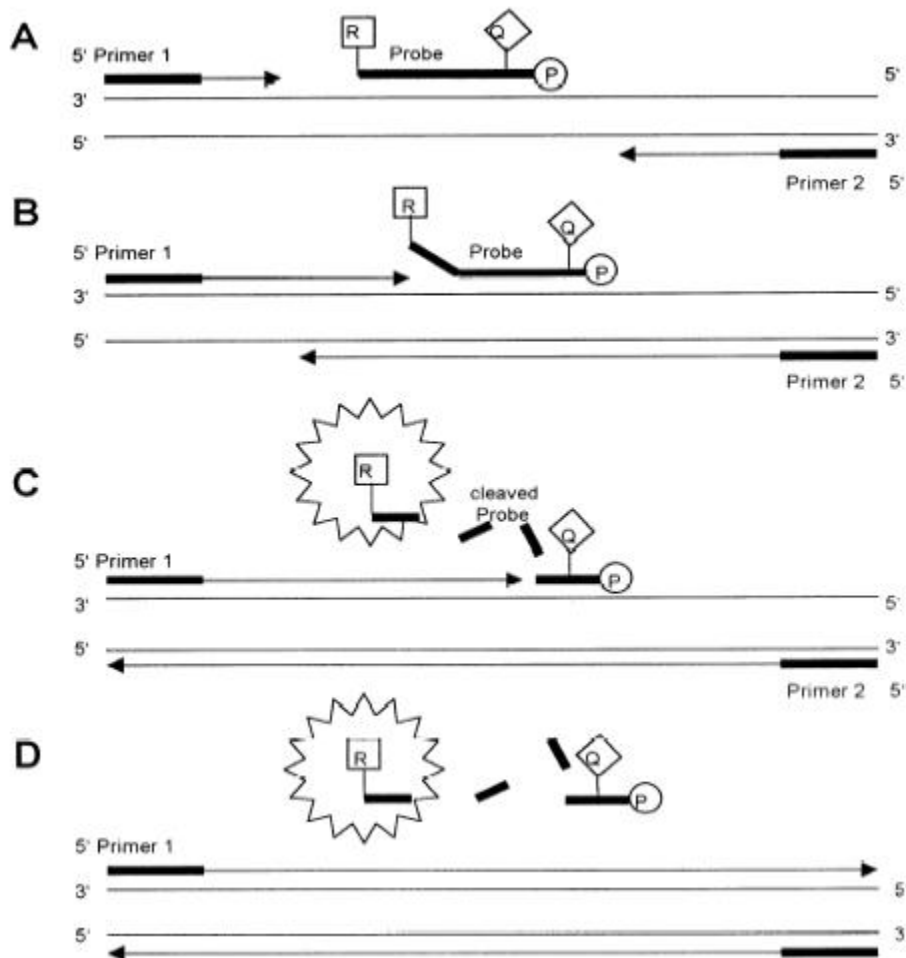


Figure 2. Schematic of TaqMan ® QPCR from :

http://www.fda.gov/ohrms/dockets/ac/02/briefing/3847b1_02_FDA_files/image005.jpg

- A) During annealing, the primer and probe have attached to the top strand (the same would be true on the bottom strand but it's not shown). The Probe is labeled "P" and the Reporter "R" and the Quencher "Q".
- B) and C) During extension, the activity of Taq polymerase cleaves the Reporter "R" from the probe.
- D) The probe is entirely cleaved from the copied strand of DNA.

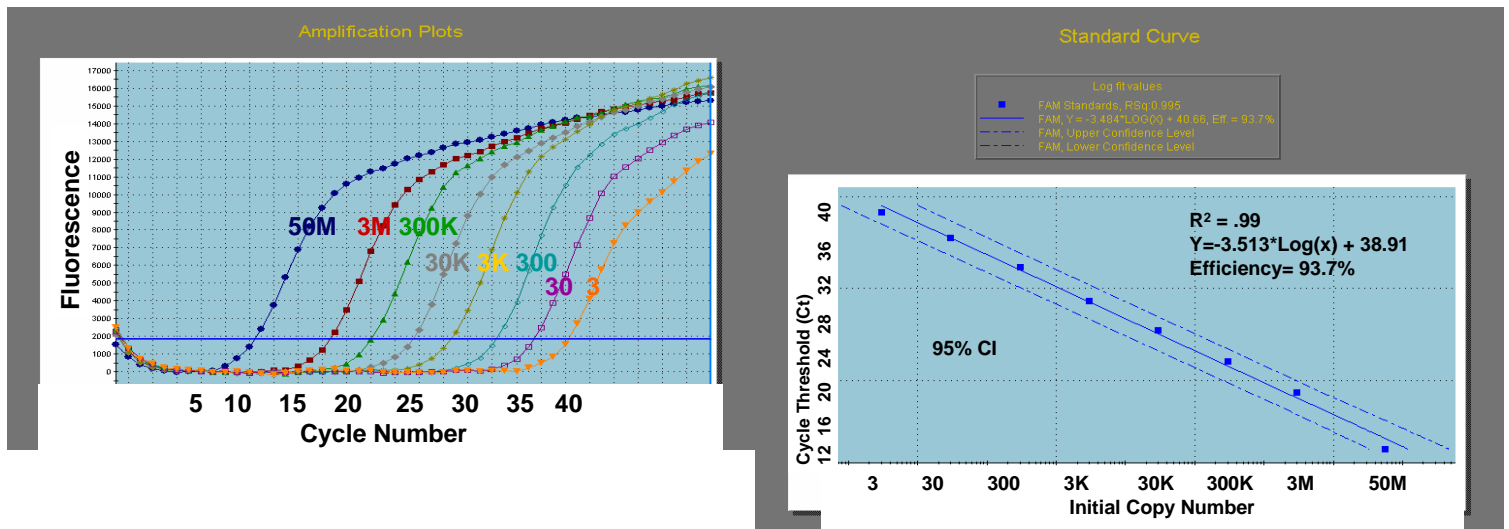


Figure 3. Real-time quantitative PCR (QPCR) Amplification plot (left) showing the cycle number (Ct) when different copy numbers of the target gene amplify above the horizontal threshold. Notice those with 3 copies have a Ct of 40, while 50 million copies have a Ct of 12 in this example. The standard curve (right image) can be used (regression formula) to quantify the number of copies of the target gene in unknown samples.

We will be setting up two PCR reactions today. Each student will set-up a reaction using published PCR primers (Andree et al. 2000), and a second reaction using newly designed primers for QPCR.

Supplies:

- Fwd Primer
- Rev Primer
- Your DNA samples
- Positive control
- Sterile water in a microfuge tube
- p200 or p100 + tips
- p10 + tips
- yellow tips
- sterile 1.5 mL microfuge tubes

Methods

1. Calculate the volumes you need for your PCR reactions (see recipes below).
Make at least 10% more than you will need so that you don't run out of reagent.
Make sure to do all calculations in your notebook.
2. Wipe down workbench with 10% bleach.
3. To a 1.5 mL tube add all the reagents **except the DNA**. This is your master mix.
4. Mix gently (by pipetting up and down). Never vortex because it can mechanically disrupt the polymerase protein.
5. Label the PCR tubes with your sample # (e.g. #'s 1-10) and your primers. Label an extra tube for your negative control (-) and one for your positive control (+).
6. For **cPCR** add 18 μL of master mix to each of the PCR tubes.
7. For **QPCR** add 23 μL of master mix to each of the PCR tubes.
8. Add 2 μL of water to your negative controls and close the tubes.
9. Next add 2 μL of template DNA to each tube. Close each tube after adding DNA and take care not to cross contaminate between samples.
10. Finally, add 2 μL of the positive control into the positive control tubes.
11. Briefly flick the tubes.
12. For **cPCR** – add tubes to preheated (95°C) thermal cycler and start program.
13. For **QPCR** – see Lisa.

Recipes

cPCR: using PCR primers from Andree et al. 2000 with optimized protocol of Friedman et al. 2008

Reagent	Stock Concentration	End Concentration	Per Tube (μl)
5 X Buffer	5x	1 X	4
MgCl ₂	25 mM	1.5 mM	1.2
BSA	10 mg/ml	400 ng/ml	0.8
H ₂ O			11.08
dNTP's	10 mM	200 μM	0.4
RA 3-6	100 pmol/ml	0.5 μM	0.1
RA 5-1	100 pmol/ml	0.5 μM	0.1
Taq	5 U/ μl	1.6 U	0.32
Template			2
Total Reaction Volume			20 ml

cPCR Thermal Cycler Program

	Time	Temp (°C)
Step 1	3 min	95
Step 2	1 min	95
Step 3	30 sec	62
Step 4	30 sec	72
Repeat steps 2-4, 40 times		
Step 5	10 min	72

QPCR: using unpublished reaction (Friedman, Wight et al., unpublished)

RLP QPCR recipe with Immomix and RLP-probe	
Immomix 2x	12.5
BSA (10mg/ml)	1.5
WSN1 fwd (10um)	0.8
WSN1 rev (10uM)	0.8
Taqman probe (10uM)	0.5
sH20	6.9
Template	2
Total	25

Thermal profile	Temp	Time
Step1	95	10min
then 45 cycles of		
Step 2	95	15 secs
Step 3	60	1min

Andree, K.B., C.S. Friedman, J. D. Moore, and R. P. Hedrick. 2000. A polymerase chain reaction assay for the detection of genomic DNA of a Rickettsiales-like prokaryote associated with withering syndrome in California abalone. *Journal of Shellfish Research*. 19(1): 213-218.

Part III – Visualization of Histology and electrophoresis

Histology rating:

Deparaffinized (5 μ m) tissue sections will be stained with Harris's hematoxylin and eosin, you will view them by light microscopy and rate by the following scales:

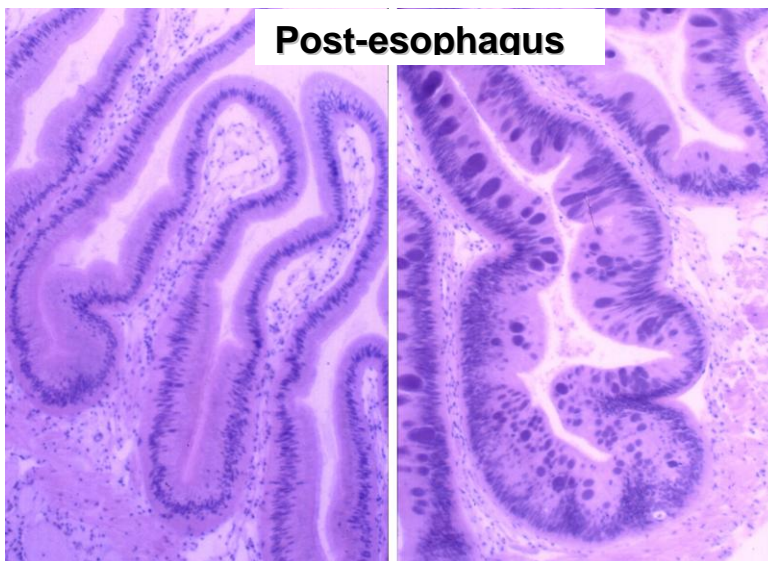
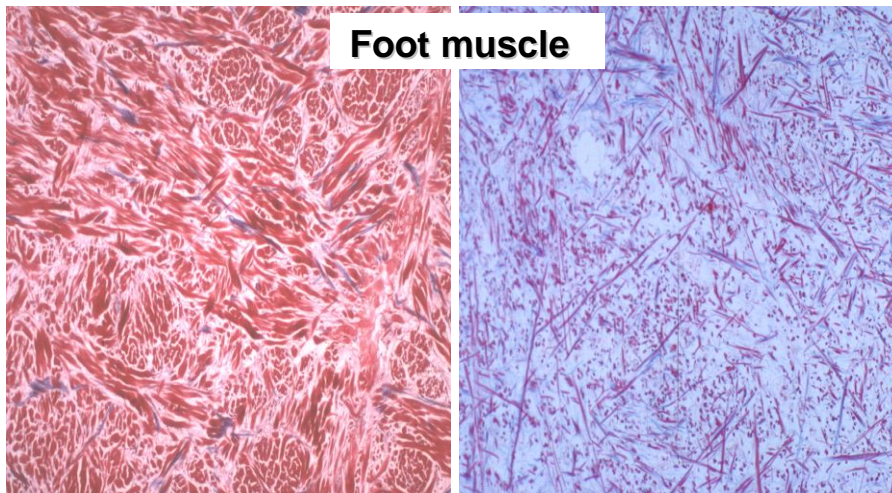
Foot muscle atrophy: **0** = muscle fibers comprise > 90% of tissue present, **1** = 76 – 90%, **2** = 50 – 75%, **3** = < 50%

Digestive gland or PE WS-RLO infection intensity: **0** = absent, **1** = 1 - 10 inclusions / 200 x field of view, **2** = 11 - 100, **3** = > 100

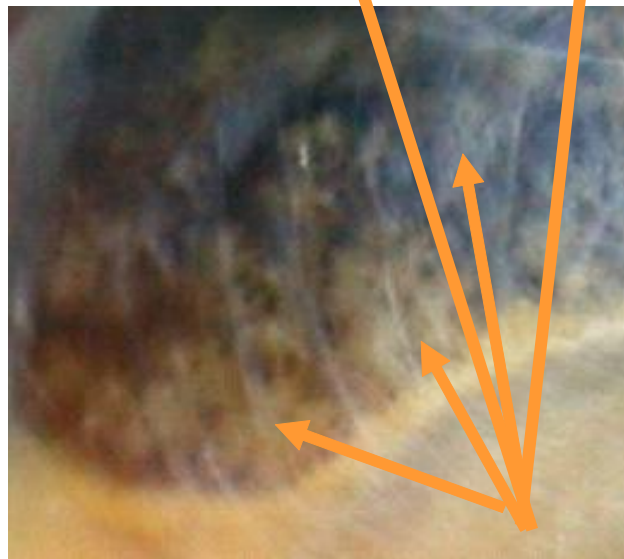
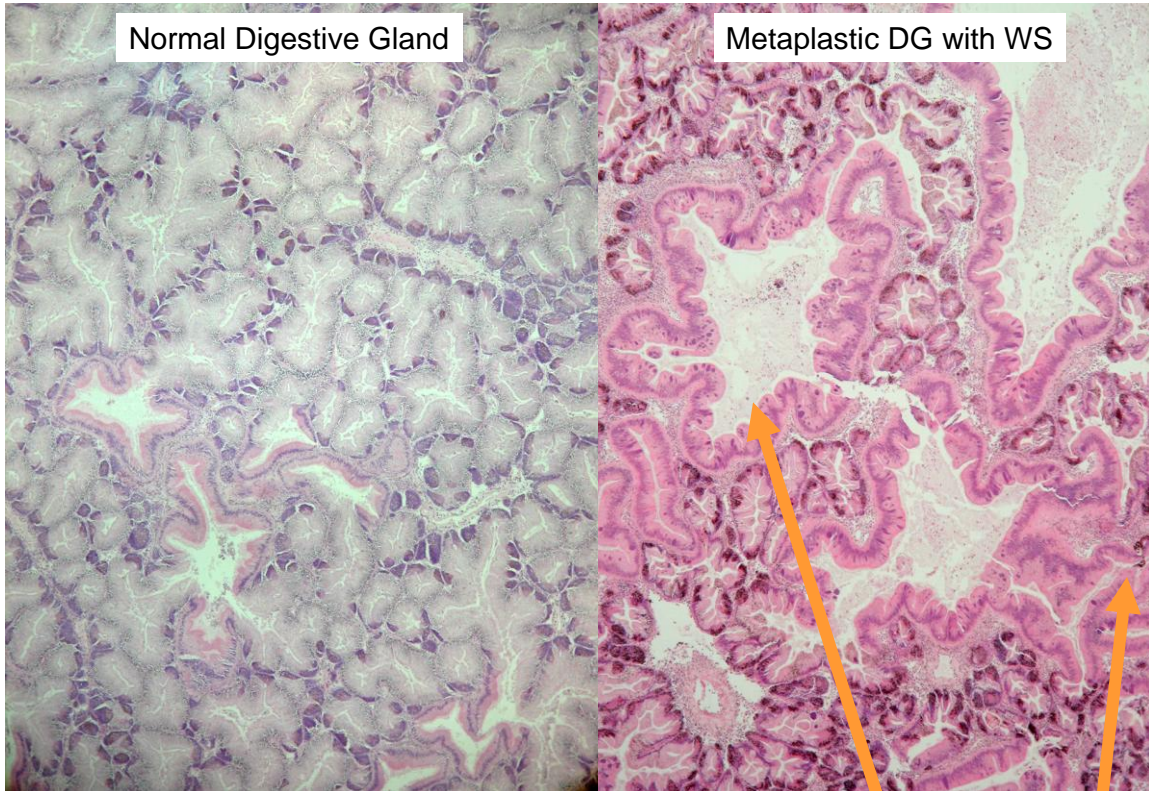
Digestive gland metaplasia: **0** = 0-4%, **1** = 5-10%, **2** = 11-25%, **3** = > 25%

(Friedman *et al.*, 1997; Moore *et al.*, 2000).

The left images are rated as 0, while those on the right represent the rating of 3.



The arrows highlight metaplasia in the digestive gland (DG). Metaplasia is the substitution of one adult tissue type for another. In this case the terminal tubules that secrete digestive enzymes and play a role in absorption are substituted for those that resemble the PE and can only play an absorptive role. Thus DG metaplasia results in a loss of function of this gland and also provides more target tissue for the WS-RLO.

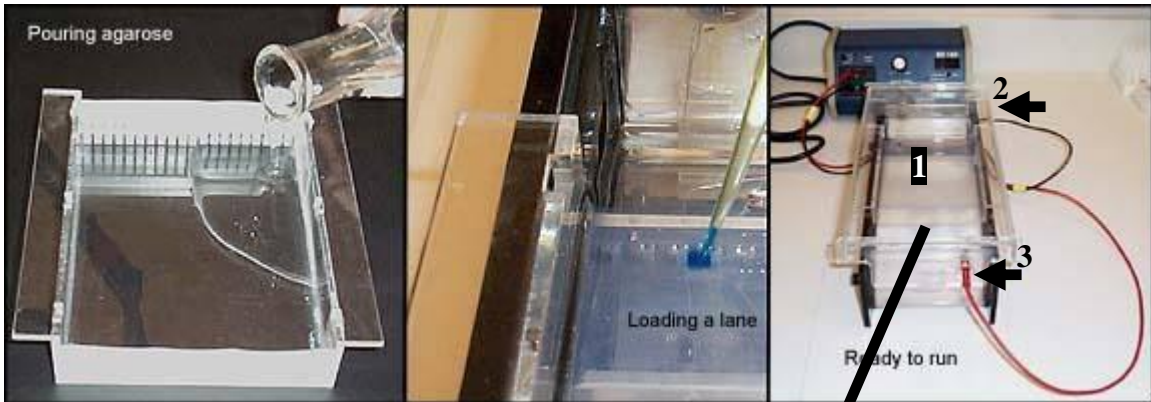


Gel electrophoresis

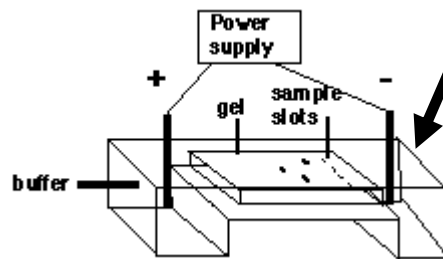
Introduction

In our lab we are using molecular (PCR) and traditional (histology) techniques to test for the causative agent of Withering Syndrome in red abalone. We first necropsied 10 red abalone and sampled PE, DG, and foot muscle tissues for PCR and histology. Next, we extracted DNA from the digestive gland of each of the abalones we sampled. Using this extracted DNA as template, we set up a Polymerase Chain Reaction (PCR) test using RLO specific primers. We used two tests to compare RLO detection. One was a conventional PCR (cPCR) test and the other was a quantitative PCR (QPCR) test. We used two different sets of primers; for the cPCR test, we used primers RA 3-6 and RA 5-1, and for the QPCR test we used primers WSN1 and WSN2. The RA primers amplify a ~160 bp fragment of the 16S RLO genome and the WSN primers to amplify a ~148 bp fragment of the 16S RLO genome.

Today we will use gel electrophoresis to visualize the results of your cPCR (often called “PCR products”). Your PCR products (plus a dye which acts to “hold” the DNA in the gel so it doesn’t float away) will be added to a 1.5% agarose gel (1.5 grams agarose per 100 mL) with 0.1 µg/mL Ethidium Bromide (EtBr) submerged in 1X TBE which is made up of Tris-HCl, Boric Acid, and EDTA. EtBr is a fluorescent dye (visualized under UV light) which intercalates (or wedges) between DNA bases which allows us to visualize our DNA.

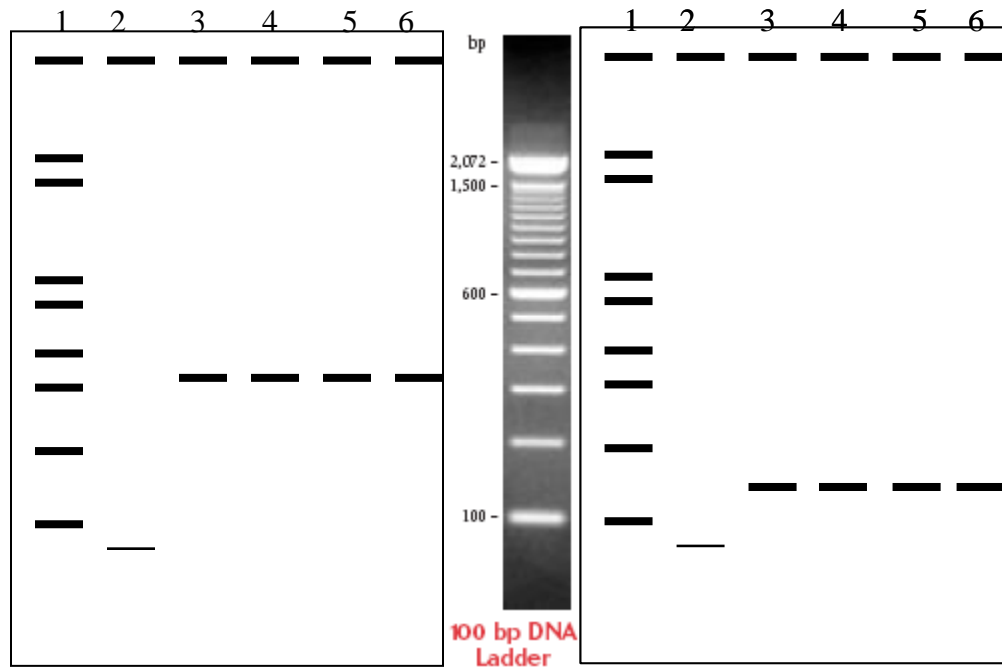


<http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agarad>



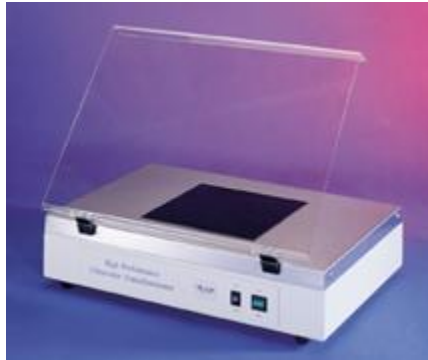
<http://opbs.okstate.edu/~melcher/MG/MGW4/MG422.htmlna.html>

Your gel will be submerged in 1X TBE Buffer in a “gel box” (1). Your gel box is hooked to an electrical current with a negative electrode (2) and a positive electrode (3). DNA is negatively charged and when placed in an electrical field will migrate to the positively charged electrode. Pieces of DNA of varying sizes migrate at different speeds so that larger pieces migrate more slowly. A molecular weight ladder (DNA cut into known sizes with restriction enzymes) is used as a marker to estimate the size of your amplified DNA (also called an amplicon). Below are three pictures, the picture on the left is a depiction of a gel with a ladder in lane 1 (the same ladder as the picture in the middle minus a few of the rungs), a negative control in lane 2 (what’s the slight band on the bottom?), and samples in lanes 3-6. The picture in the middle is an example of a 100 bp ladder (which depicts how DNA migrates). The picture on the right is another gel, with a ladder in the first lane, a negative control in the second lane (what’s the slight band on the bottom?), and samples in lanes 3-6. Approximately what sizes are the amplicons in each of the gels?



Answers- Left Picture: The slight band in lane 1 is called primer dimer and not an actual band. The amplicons are ~ 300 bp. Right Picture: The slight band in lane 1 is called primer dimer and not an actual band. The amplicons are ~150 bp.

We will visualize our gel with a UV transilluminator similar to one shown in this picture.



Materials list:

Pipettors, P 20 and tips
Sterilized microcentrifuge tubes
100 bp Ladder
Loading dye
Agarose
1X TBE
Gel Box
Power supply
Ethidium bromide
Lab Mat
PCR Water

Methods

1. Weigh 1.5 g agarose and add to 200 ml flask
2. Add 100 mL 1 X TBE
3. Boil in a microwave for 3 minutes.
4. Add 10 μ L of ethidium bromide (A POTENT MUTAGEN!!), swirl gently and pour into the gel mold
5. Place in combs and allow to set for ~ 15min
6. Add 1X TBE to the gel box (~ 1/2" over the top of the gel)
7. Pipet 7 μ l of 100 bp molecular weight ladder in the far left well

8. Add 5 μ l of loading dye to your PCR products and LEAVE IN THE TUBES. Change tips every sample to avoid contamination.
9. Pipet 7 μ l of your sample + loading dye into each well.
10. Run at 115V for 45 mins or until dye is $\frac{3}{4}$ way down the gel.
11. Carefully remove gel and examine under UV light (wearing nitrile gloves and lab coat).
12. Photograph your gel.



For Your Lab Report:

Results: Please include a table with the results of your gross exam, fluorescent dye detection, histology, and the two PCR tests. Include presence/absence of RLO for each test and the severity of disease (i.e. copy # and the severity of infection based on histology scale). Remember to include legends with your tables and figures and to properly cite all outside references including histology scales.

Discussion: Your discussion should address the following questions

- **How do the PCR and histology results compare (both for detection and the copy # in QPCR)?** (this is a good place to look in the literature at copy # and progression of disease, do we see a difference in disease progression (i.e. histological signs and copy #)? If we had individuals in a later stage of disease (i.e. withered foot and/or further disease progression based on histology), do you think this would change?
- **Are your histology and PCR results what you would expect?**
- **Which test should be more sensitive?** (this is a good place to look at the literature to compare histology and PCR)?
- **PCR is a quick, inexpensive diagnostic test, but do you think it might require more training or practice than what you had in class?**

Please use peer reviewed references in your discussion. Some good starting references have been provided in this handout.