Week 1

Background: Microscopic observation of anemones

Coral reefs depend on symbiosis with certain species of photosynthetic algae, which are nutritional endosymbionts (i.e., they live inside the cells of their hosts and provide their hosts with nutrients). In this class, we use the anemone *Exaptaisa pallida* as a model system for coral-algal symbiosis because it associates with the same types of algae, can be bleached like coral, and grows quickly in laboratory conditions. In class we will observe anemones in symbiosis with algae as well as aposymbiotic anemones (i.e., anemones that have been bleached and do not contain algae within their cells).

Background: Pipetting exercise

The study of genetics depends heavily on the use of molecular biology techniques. In particular, genetics often requires that we isolate and manipulate DNA from our model organisms. Because the cellular environment is aqueous, many molecular biology techniques ultimately revolve around the manipulation and combination of small volumes of liquids that contain our molecules of interest (e.g., DNA). In this class we will practice pipetting because good pipetting technique is critical to the success of our experiments throughout this semester.

Background: Mutagenesis of *Breviolum minutum*

Breviolum minutum is a species of unicellular dinoflagellate algae and nutritional endosymbiont of corals, anemones like *Exaiptasia pallida* and other cnidarians. They play an active role in establishing and maintaining a symbiotic partnership with their cnidarian hosts, and because of this their genetics are also interesting to scientists studying coral bleaching. In this class we are attempting a **forward genetic screen** in which we cause random mutations with UV exposure and screen for a phenotype (here, resistance to 5-fluoroorotic acid or 5-FOA). This screening is accomplished with the use of selective media (plates containing 5-FOA) which should prove lethal to wildtype algae but is not lethal to *Ura3* null mutants.

Protocol for pipetting exercise:

Warm-up exercise:
What are the ranges of the pipettes at your table?
1)
2)
3)
4)

Select the correct pipette for desired volume:

1) 1.25µl	5) 9.8µl
2)165µl	6) 2.00µl
3) 200µl	7) 12.8µl
4) 20µl	8) 465µl

Exercises 1 & 2

Each student should receive four 1.5 ml tubes. Before pipetting label your tubes A, B, C, D.

Exercise 1: Adding & removing large volumes

1.Add 370µl Blue reagent to tube A.
2.Add 225µl of Red reagent to tube C.
3.Add 495µl of Yellow Reagent to tube B.
4.Add 160µl of Blue reagent to tube D.
5.Remove 155µl from tube A and dispose.
6.Remove 190µl from tube B and add it to tube D (do not mix).

Exercise 2. Adding vol. into vol. & common mixing techniques (Note before and after mixing).

1.Add 285µl of Red reagent to tube A and mix by pipetting up and down.
2.Add 195µl of Blue reagent to B and mix using inversion.
3.Add 275µl of Yellow reagent to tube C and mix using flick/tap method.
4.Add 150µl of Red reagent to tube D and mix by vortexing.

Exercises 3 & 4

Each student should receive four 200ul tubes & label them E, F, G, H.

Exercise 3: Adding small volumes
1) Add 20µl of Blue reagent to tube E.
2) Add 11µl of Red reagent to tube F.
3) Add 2.5µl of Yellow reagent to tube G.
4) Add 1µl of Blue reagent to tube H.

Exercise 4: removing & mixing small volumes
1.Remove 10µl from tube E and dispose of it.
2.Remove 1µl from tube F and dispose of it.
3.Add 7.5µl of Red reagent to tube G and mix by pipetting up and down.
4.Add 9µl of Yellow reagent to tube H and mix by pipetting up and down.

Exercise 5. Making a master mix. (1-1.5 ml tube per person)
Often to save on time and to reduce variation between reactions, it is helpful to make a master mix when doing multiple reactions. Master mixes are made by making reaction mixes that are larger in total volume but contain the same ratios of reactants as 1x. It is often customary to make master mixes larger than what is desired to account for pipetting error.

Example: Desired=5x, Make = 5.5x
Desired =20x, make=22x

1x Reaction	Desired=9x	Make=__x reaction
10µl Yellow reactant.	__Yellow Reactant.	__Yellow Reactant
9µl Blue reactant	__Blue Reactant	__Blue Reactant
1µl Red reactant	__Red Reactant	__Red Reactant
20µl Total	__Total	__Total

Protocol for *Breviolum* mutagenesis

Note to scientists in training:

As a rule, read all protocols from beginning to end before starting. Ensure that you have access to all required materials and equipment. Make sure that you understand both how to perform each step and why you're performing the step. Understanding why you're doing something is important for avoiding mistakes AND critical for troubleshooting. If there is any confusion, resolve it before beginning.

We will be mutagenizing *Breviolum minutum* strain SSB01 using ultraviolet light. Each table will be administering a different dose of UV to randomly induce mutagenesis in a large population of cells. In the following weeks, we will select for mutants which possess the phenotype of interest. This protocol has been carefully designed to eliminate any UV exposure to experimenters

Each table/group is assigned a UV exposure time: 0 min (negative control), 15s, 30s, 1min, 2min, 4min.

1. Each table will receive a 6-well plate. Each student will label their own well accordingly, with the following information:
   a. Table #
   b. Section #
   c. Experimenter name
   d. Duration of exposure to UV
      e.g. T6, S07, Josh, 32 min
2. Locate the T75 culture flask containing a dense culture of SSB01 on your table and swirl to homogenously suspend cells. Density is approximately 5×10^5 cells/ml.
3. Each student must transfer 1ml of SSB01 culture suspension into one well of the plate (each table will use four wells total). If the culture has settled, swirl before pipetting. Gently swirl plate to distribute culture across bottom of well.
4. When all tables are ready, students can begin carefully putting their plates into the UV chamber under the supervision of their TA. Each table should leave their plate in the chamber with the UV source on for the appropriate time before moving to the next step.
5. As soon as a sample's exposure is complete, add 8 ml of the provided recovery medium. Recovery medium for this particular screen is Daigo's IMK medium supplemented with 1g/L uracil.
6. Incubate at 25°C under a 12:12 light cycle, with 10-50µE/m^2s^1 PAR for 7 days.

TAs: Ensure that the UV source is OFF before leaving.

For your lab notebooks:

As a rule, when you are provided with a protocol, you do **not** need to copy the entire protocol into your lab notebook. It is perfectly fine to write something along the lines of "we performed the mutagenesis according to the protocol we were given in class." However, it **is** important to include any mistakes or deviations from the protocol if any occur, as well as all data and everything listed in the lab manual for the week. You should also include the date and title of that week's lab at the beginning of every entry. Lastly, you should not use a notebook that you are using for another course as your lab notebook, as your TA may need to keep it for a week or more when grading it. For this week, you should include the following information and answer the following questions:

1) Include photographs in your notebook or draw pictures of the symbiotic and aposymbiotic anemones you've observed. Note the differences in pigmentation. Explain what accounts for the differences in appearance you observe.
2) Write in your notebook the answers to the warmup exercises and exercise 5. For exercises 2-4, write the final volumes that each tube should have had. Did your tubes have those volumes when you were finished with the exercise?
3) For this experiment, each table will perform one treatment, and the experiment as a whole will be completed by the class. Because of this, it's important that you write down all data. Record in your notebooks the exposure times that each table used. Which exposure time did your table use? Write your predictions of which tables (if any) will generate mutant algae, and which will generate the most.

Week 2

Background: plating of *Breviolum minutum*

Previously, we mutagenized cultures of the symbiotic algae *B. minutum* (strain SSB01) with ultraviolet radiation. Now, we hope to screen these cultures of randomly mutagenized cells for a specific phenotype: resistance to the compound 5-fluoorooritic acid (5-FOA). 5-FOA is broken down by action of the protein Ura3 into the toxin 5-fluorouracil (5-FU). *Ura3* knockout mutants will not metabolize 5-FOA into 5-FU and as such will be immune to its deleterious effects. We're plating our mutagenized *B. minutum* cells onto media containing 5-FOA to screen for *Ura3*$^{-}$ (i.e., knockout) mutants.

Protocol for plating of *Breviolum minutum*

One week ago, you irradiated your *Breviolum* with ultraviolet light to cause DNA damage and induce random mutations in the cells. We're performing a mutant screen for tolerance to 5-FOA. This week, you will plate these mutagenized cells on agar plates containing 5-FOA at a concentration of 10ug/ml which is lethal to wild type cells. Your plates also contain 1g/l uracil added to the medium. SSB01 cells grow slowly, so it will take several weeks for any mutant colonies to become visible. Because of this long incubation time, cultures are susceptible to mold and bacterial growth, so **it is critically important that you work with good sterile technique**.

1. Retrieve your cells from the incubator.
2. Obtain a 2ml tube and label it with your name.
3. Transfer 2ml of the liquid culture (from your well in your table's 6-well plate) into a 2ml tube labelled with your name.
4. Centrifuge at 10,000g for 1 min.
5. Be mindful of where the pellet is and be sure to not disturb it. **If you disturb the pellet, repeat the centrifugation**. Using a pipette, remove 1.5ml supernatant and dispose of it in the waste cup provided on your table.
6. Add 1.5 ml of the culture to fill up the tube back up to 2ml.
7. Repeat step 4, 5 and 6 until you have no liquid culture remaining and all cells are collected in a pellet in 0.5ml volume in your 2.0ml tube. Resuspend your cells in ~0.5ml volume. The suspension should be dark brown in color because the cells are now densely concentrated
8. Obtain a media plate from your TA and label appropriately. **The label must include your name, your section, your table, and treatment (0, 2, 4, 8, 16, or 32 min)**.
9. Pipette 0.5ml of the concentrated culture on to the center of the plate.
10. Use the sterile plastic spreader to spread the cell suspension evenly.
11. Cover plate and do not move for a few minutes to allow the liquid toabsorb.
12. Cut a 1cm wide strip of Parafilm. Carefully wrap your plate with Parafilm to prevent evaporation and contamination. **Note that if you do not wrap your plate properly, your media will dry and your cells will die.**
13. Turn your plate upside down so that condensation does not drip onto the plate and interfere with the cells.
14. Incubate at 25^{o}C under 12:12 light cycle (~10-40μE m-2s-1PAR).
15. Inspect in 4-6 weeks.

For your lab notebooks

It is not important to copy the entire protocol down into your lab notebook, unless you feel compelled to do so. Instead, you may summarize what you've done in class today. More importantly, state clearly *why* we did this protocol—what do we hope to achieve? If necessary, write down any mistakes or deviations from the protocol, what you expect the outcome of those deviations will be, and what you've done to keep the protocol on track. Finally and most importantly, what do you think will be the outcome of this experiment? Write down in detail what you predict the results will be across the whole experiment (remember, each table is one treatment, so the experiment is all of the groups together). Why do you think this will be the case?

Week 3

Background: anemone stress challenge

Coral bleaching is the breakdown of symbiosis between the coral animal and its endosymbiotic algae. The loss of the pigmented algae results in the white coral appearing to have "bleached." Other cnidarians including the anemone *Exaiptasia pallida* and the jellyfish *Cassiopea xamachana* also engage in symbiosis with photosynthetic dinoflagellates and can be

used as models to study coral bleaching. This week, we will begin the second major experiment in this course: the anemone stress challenge. We will be quantifying a phenotype (tolerance to thermal stress or menthol exposure) and then spend the rest of the semester seeing if variations in this phenotype correspond to differences in genotype. Little is known about the mechanisms underlying coral bleaching, so understanding genes involved in this process is of great value to the scientists who study coral bleaching and hope to prevent the loss of coral reefs to climate change.

Every table will have one **genet** of anemones (a clonal colony propagated asexually from a single individual). Each anemone in a genet is called a **ramet**. Students will place one ramet in each of four different treatments: 25^{o}C seawater, 32^{o}C seawater, 34^{o}C seawater, and 25^{o}C seawater with 0.19mM menthol. Menthol is known to bleach anemones, although the mechanism is unknown. These treatments then represent, respectively, a normal temperature they would experience in nature and a **negative control** treatment, mild heat stress, more extreme heat stress, and a **positive control** treatment. Each anemone will be left in the appropriate temperature for one week.

Before going into the incubator, students will measure the color of each anemone using the coral health chart. This is an indirect measure of symbiosis, because more algae in an anemone's cells means more pigment and a darker color. They will then be measured at the end of one week, and loss of pigment (and therefore algae) can be discerned as the difference between color before and after the stress test.

Tolerance to thermal stress of menthol can here be defined as the difference in color of an anemone before and after a stress treatment compared to the difference seen in a control anemone in the same genet. A genet in which a thermally stressed anemones go from very dark to completely bleached is less tolerant to thermal stress than a genet in which thermally stressed anemones go from very dark to only partially bleached (assuming their respective controls behave consistently).

Protocol: anemone stress challenge

Each table will receive one genet (clonal population) of at least 4anemones.

1) Before transferring anemones, score the color of each individual using the coral health chart provided. Take photos of your anemones with reference chart in background.
2) Label your Falcon tubes with genet ID (location), section number, table number, student name and treatment (e.g. ARB31/U02/T3/Josh/Menthol)
3) Each student will transfer ~40ml of seawater into 50ml calibrated Falcon tube. Note: for anemones in the menthol treatment, use 40ml of pre-made mentholated seawater instead.
4) Each student will transfer one anemone using a cut-off transfer pipette to a Falcon tube containing 40ml of seawater or mentholated seawater. Note: the anemones like to adhere to their cups, and sometimes work themselves into corners and are difficult to remove. A firm but delicate touch is best, and if you are afraid you are causing significant damage to your animal, don't hesitate to call your TA over to help. Remember, although they are simple, they are animals and should be treated with respect and care!
5) Loosely cover the top of each tube in plastic wrap and place each tube in the appropriate incubator for seven days.

Notes: if a table has less than four students or a student is absent, each group should still put an anemone in each of the four treatments. Return any remaining anemones to your TA to preserve the lineage. If your cup contains exactly four anemones, your TA may ask you to take additional steps to maintain the genet next week.

For your lab notebooks

Write a brief overview of the whole experiment. Why are we doing what we're doing, and what do we hope to achieve? Record the genet ID of your anemones, and write down the coral health chart score for each individual. How do you predict each of your four anemones will fare? Which treatments will see the most bleaching, and which will see the least? Do you expect to see variation in tolerance among different groups, and if so do you think that there may be a genetic basis for that variation? Why or why not?

Week 4

Background: anemone stress challenge (cont.) and DNA extraction

This week we take our anemones out of their respective incubators and examine the effect that the different treatments had on their pigmentation (which is a proxy for how much algae they have lost, i.e., how much bleaching has occurred) relative to the control group. These data points—along with our pre-treatment data points—will allow us to assess the tolerance of each genet to temperature and menthol stressors and to quantify that tolerance phenotype.

In this experiment, we are interested in seeing if we can correlate those phenotypes to genetic differences. In order to determine the genotype of our anemones and their symbiotic algae, we must first extract DNA from them. This genomic DNA extraction will be used in subsequent labs for amplification of specific regions of DNA and finally sequencing of that DNA. DNA extractions can be performed without a kit, but such techniques may involve harmful chemicals like phenol and chloroform and may require more experience than we have time to provide you with on this one task. Kit extractions are far simpler but follow the same basic principles. Firstly, you must lyse the cells of the organism (or in our case, organisms, as we will be extracting DNA not only from our anemones but from the algae inside their cells). Once the cell and nuclear membranes are sufficiently disrupted, it is possible to access the DNA. At this point your solution will contain your DNA as well as many other types of water soluble molecules. In a traditional DNA extraction, some of these molecules will be removed by centrifugation and phase separation, and the phase containing the DNA is kept and further cleaned by pelleting the DNA in alcohol to remove more alcohol soluble molecules. In a kit extraction, purifying the DNA is achieved using a filter column with selective permeability; the first filter we will use in this extraction allows the DNA to flow through and collects certain large impurities, then the second column binds the DNA and allows us to wash alcohol soluble molecules out of the filter before the final elution step.

Protocol: anemone stress challenge (cont.)

Measure the color of your anemones using the coral health chart as you did last week. The genetics lab would like to maintain each genet, so if you do not have any anemones remaining in

your cup from last week, it may be necessary to cut an anemone in half and return one half to your cup, saving the other half for DNA extraction. If necessary, cut the anemone **transversely** (i.e., cut it in half from the mouth to the foot). If a piece of an anemone contains part of the foot, it should be able to regenerate completely. Return one half to your cup and save the other for DNA extraction.

Protocol: DNA extraction

The full instruction manual for the DNA extraction kits we are using can be found here:

https://files.zymoresearch.com/protocols/_d6016_quick-dna_tissue-insect_miniprep_kit.pdf

When performing a DNA extraction from a kit, the specific names of tubes and buffers will vary from manufacturer to manufacturer, but the basic premise remains the same as described above. We will be making some minor deviations to the protocol as listed in the manual, so the instructions below have been modified for clarity and should be followed in class.

1. Add each anemone to a bashing bead lysis tube. This is the tube containing small beads, which will help abrade the cells of the anemone, allowing you to access the DNA. Add 750µl of bashing bead buffer to the tube and cap tightly.

2. Vortex the tubes containing your anemones with buffer for ~1min or until the anemones are completely destroyed. It is important to that there are no visible pieces left after vortexing to maximize our DNA yield. **Note:** you may need to invert your tube several times to trap the anemone under the beads and prevent it from floating above the beads while you vortex.

3. Centrifuge the bashing bead tube in a microcentrifuge at ≥10,000 x g for 1 minute to pellet any large pieces of cellular debris.

4. The supernatant (i.e., the liquid above the beads) contains your DNA at this points, as well as many other water soluble molecules. Transfer up to 400µl supernatant to a III-F filter column (the one with the red ring) in a collection tube and centrifuge at 8,000 x g for 1 minute.

5. At this point, the filter has collected larger cellular debris, and your DNA and other molecules have flowed through the filter into the filtrate. Add 1,200µl of genomic lysis buffer to the filtrate in the collection tube from Step 4. Mix well by pipetting up and down.

6. Transfer 800µl of the mixture from Step 5 to a II-C filter column in a collection tube and centrifuge at 10,000 x g for 1 minute. **Note: DO NOT** discard the remaining 800µl of the mixture from Step 5! You will use it in the next step.

7. Your DNA is now bound to the filter with some other molecules. We can wash it further before causing it to unattach from the filter for collection. Discard the flow through from

the collection tube and repeat Step 6. **Note:** when discarding flow through, you can pour the liquid onto a paper towel and dab the top of the collection tube dry instead of pouring the buffer down the sink. Throw your paper towels away in solid waste at the end of the class.

8. Now all of your DNA is on the filter. Add 200µl DNA pre-wash buffer to the column and centrifuge at 10,000 x g for 1 minute.

9. Add 500µl g-DNA wash buffer to the column and centrifuge at 10,000 x g for 1 minute.

10. Label a clean 1.5ml microcentrifuge tube with your section, table, genet ID and treatment and transfer the column to a clean 1.5ml microcentrifuge tube and add 100µl DNA elution buffer directly to the column matrix. It is important that you put the buffer directly on top of the filter, but do not poke the filter with your pipette. The buffer will cause the DNA to release from the column, as the electrostatic charge of DNA is pH dependent. Centrifuge at 10,000 x g for 1 minute to elute the DNA.

After elution, your DNA will be suspended in the buffer in your labelled microcentrifuge tubes. Return those tubes to your TA so that he or she can freeze them for next week's protocol. When cleaning up, discard all tubes and filter columns that you have used during the extraction, but do NOT throw away any excess buffer, which may be used for later sections and should be disposed of properly by your TA.

For your lab notebooks

Record all coral health chart data from the anemone stress challenge. For easier comprehension, copy your observations from last week and your results from this week into a new table in this week's entry. What did you observe? Are the bleaching results what you expected? Why or why not? How does the tolerance of your genet compare to other genets in the class?

For long protocols like today's DNA extraction, it is not necessary to copy the entire protocol into your notebook (although you may, if you would like it for your own records). Instead, it is sufficient to write "We performed a DNA extraction as directed by the protocol in the lab manual," or something along those lines. It is important, however, to write down a clear statement of purpose for any protocols performed in lab ("We performed a DNA extraction *because…*") and any deviations from the protocol: if you made any errors or received alternate instructions from your TA, that should be noted. Often small errors in procedure can be rectified by other modifications to the protocol, and if you do make a mistake you should seek your TA's advice before proceeding.

Week 5

Background: PCR

Polymerase chain reaction (or PCR) is a method used to amplify specific sequences of DNA found in low abundance in genomic DNA extractions to a workable quantity. Consider the extractions we did last week: we extracted whole genomic DNA from our anemones and the algae that live inside them. Your final samples should contain DNA representing the entire genome of both of these organisms, but those samples will not contain a particularly large amount of *any specific* region of the genome. If you're interested in investigating the role of a specific gene or region of the genome in some process (in our case, coral bleaching) then you must first amplify that region of DNA so you have enough of it to use in downstream applications for further study.

PCR achieves this goal by approximately doubling the absolute quantity of a specific region of genomic DNA during a three-step cycle. This cycle is then repeated, often 30-35 times, resulting in a DNA sample in which the region of DNA you are interested in is greatly enriched. This is achieved using a reaction mixture containing machinery (an enzyme, primers) that accomplish this goal, as well as the building blocks of DNA, which are necessary for the synthesis of these new DNA strands. Firstly, your reaction mixture must contain a **polymerase**, most commonly Taq polymerase ("Taq" meaning it comes from the thermophilic prokaryote *Thermophilus aquaticus*). Additionally, it must contain single-stranded DNA **primers** which determine the region of DNA that you amplify and which are required for polymerase activity. Your reaction of course must contain DNA (here, from your extractions last week) to serve as a template for the polymerase reaction, and dinucleotide triphosphates or **dNTPs**, the building blocks of DNA.

The three steps of the PCR cycle are **denaturation**, **annealing** and **elongation**. In denaturation, the reactions are heated to a temperature (usually >90^{o}C) at which the hydrogen bonds holding opposite DNA strands will break, resulting in single-stranded DNA and allowing your primers to bind to their appropriate complements in your sample. During the annealing step, the primers will then bind to the newly single-stranded targets in your sample. This usually happens between 50^{o}C and 60^{o}C, although the exact temperature depends on the length of your primers and the ratio of As and Ts to Cs and Gs (which bind by triple rather than double hydrogen bonds and thus require more energy to break). Elongation is the step during which polymerase binds the DNA/primer complex and adds additional bases to the 3' end of the primer, resulting in a new complementary strand attached to each of the single strands that were formed during the denaturation step, resulting in two complete double-stranded DNA molecules from the region of the genome that you specified for each one that existed before. The cycle is then repeated over and over to result in a potentially enormous quantity of your DNA of interest, or **amplicon**.

Today in class we will be amplifying two different sorts of targets. From the anemone, we are amplifying 3 coding regions from putative **TRP channels.** TRP (short for "transient receptor potential") channels are a family of ion channels expressed on the cell membrane and gated by a variety of stimuli including temperature and certain chemicals. Among those chemicals is menthol, which we know from earlier in the semester has a bleaching effect on symbiotic cnidarians. What is the basis for this bleaching effect? The molecular mechanisms are unknown, but we can examine the role TRP receptors play in menthol-induced and other

forms of bleaching by examining the genetic diversity of TRP receptors among genets and seeing if that diversity correlates to differences in bleaching phenotypes. The menthol **ligand-binding domain** for TRPA1 receptors is known and we will be sequencing this domain in three putative TRPA1s in class to determine if there is any variation in that domain across our genets.

The symbiotic algae living inside the cells or your anemones may also contribute to the phenotypes you've observed in your genets. To infer the origin of the algae in your samples, we will amplify the 23S ribosomal region. 23S sequences have been used to "barcode" organisms: simply, a method of identifying species using a short segment of variable DNA. Symbiont type, anemone type and symbiont/anemone interaction all make up components of how your genets will respond to the stress challenge, so it is important to get this information as well!

Protocol: PCR

The TRP PCRs will be performed using the primer pairs TRP1, TRP2 and TRP3 and an otherwise identical mixture. Your reactions for these samples should contain the following proportions:

1) 23ul TRP master mix (premixed and aliquoted by your TA)
2) 1ul of the appropriate forward and reverse primer (again, premixed and aliquoted for you)
3) 1ul of genomic DNA from one of your DNA extraction samples to serve as a template

Because the TRP markers are anemone sequences, it does not matter if the sample that you use for template comes from a bleached or a symbiotic anemone. The sample should, however, come from relatively intact animal, and you should be confident that the extraction of that sample went well.

The 23S PCR mix is slightly different (note that different primers will often require very different reaction conditions based on their specific sequences!) and are as follows:

1) 23ul 23S master mix (**note:** this mix contains $MgCl_2$ and different proportions of Taq mix and water and is **NOT** interchangeable with the SCAR master mix)
2) 1ul 23S F/R primer mix
3) 1ul genomic DNA (**note:**because the 23S template is an algal sequence, it **MUST** come from a sample that was obtained by a symbiotic anemone, as bleached anemones do not contain algae)

Clearly label your reaction tubes with your section and table number and so that you can tell which sample contains which reaction and give them to your TA, who will run the following cycles over the next few hours:

23S PCR conditions		**TRP PCR conditions**
1) 94ºC 1:30		1) 94 ºC 1:30
2) 94 ºC 30s	(denaturation)	2) 94 ºC 30s
3) **56 ºC** 30s	(annealing)	3) **58 ºC** 30s
4) 72 ºC **40s**	(elongation)	4) 72 ºC **1:30**
5) Go to 2 (34x)		5) Go to 2 (34x)
6) 72 ºC 5:00		6) 72 ºC 5:00
7) 4 ºC forever		7) 7) 4 ºC forever

Note the differences between the two cycles; differences in annealing temperatures are determined by the length and GC content of primers. Generally a primer set will work at lower temperatures than the maximum temperature at which it will anneal, but it may bind non-specifically to other sequences in your template, so annealing temperature should be kept as high as possible. Note also the difference in the elongation time; longer amplicons require a longer elongation time for the simple reason that the polymerase must append more dNTPs to the synthesized strand. It takes longer to build a long strand of DNA than it takes to build a short one.

For your lab notebooks

State clearly the purpose of performing this PCR today. Where does it fit in the overall context of the anemone stress challenge experiment? Record all of your reaction mixes, the reaction program, which samples you used for which reactions, and any deviations from the protocol as stated.

Week 6

Background: Gel electrophoresis

In the past few weeks, we have collected phenotype data from our genets in the anemone stress challenge, collected DNA from our anemones and algae, and amplified specific gene regions from them in hopes of determining their genotypes. Today we will examine our amplicons from next week using gel electrophoresis. This serves two major purposes: firstly, it allows us to confirm that our PCR reactions from last week worked. Secondly, if there are genotypic differences between our genets that are determined by differences in the *length* of our amplicons, we will be able to see those on our gels.

DNA particles are negatively charged and will move when placed in an electric field. Gel electrophoresis takes advantage of this fact to sort DNA particles by size. The "gel" in the name of the protocol is, in this case, made from agarose and buffer. Agarose is a sugar derived from certain marine algae and is commonly used as an ingredient in certain jelly-like desserts. When placed in buffer, boiled and cooled, it will become semi-solid. On the scale of molecules, however, it will act as a fine sieve: DNA will be able to move through the gel, but the larger a particle of DNA is, the more resistance it will experience in moving through the gel. Because of

this, when an agarose gel is loaded with DNA samples and put in an electric field, smaller particles will migrate further than larger particles in the same amount of time.

The buffer we are using is called TBE buffer, which stands for TRIS, boric acid and EDTA. These ingredients are primarily acting as electrolytes, which are necessary for the conduction of current between electrodes in the gel chamber. TAE is also commonly used, in which acetic acid is substituted for boric acid. These acids are weak and dilute but care should always be taken when handling laboratory chemicals nonetheless.

We will be visualizing our samples using a dye called SYBR safe under near-UV light. Dyes like SYBR safe or the more traditional ethidium bromide operate by binding double stranded DNA and fluorescing under UV or near-UV. This means that as our DNA bands migrate through the gel, dye molecules will stick to them and ultimately show us where our DNA is located. SYBR safe is promoted as a safe alternative to ethidium bromide, and although the risks of ethidium bromide may be overstated, caution should be taken handling any molecule that binds double stranded DNA.

Lastly, it is important that we not only can see our DNA samples, but that we know their size. Use of a **DNA ladder** allows us to compare our bands to a series of bands of known size. In this way we can determine if the bands we observe are the correct size for the product we are trying to amplify. For instance, a common false product is seen in failed reactions is a hazy, low band below 50 basepairs. This is called **primer dimer** and is caused by the partial annealing of our primers to each other when the reaction doesn't amplify (primers may bind to less optimal partners if there are no target sequences to be found). Use of a DNA ladder can confirm that your bands are the size they are supposed to be and rule out primer dimers or other false products.

A word about genotyping: many alleles may have different sequences but be the same length. Gel electrophoresis alone will not allow us to distinguish among these. However, alleles that differ from one another by larger insertions or deletions can be detected by their differences in length on a gel. These differences in PCR product size are known as **amplicon length polymorphisms**, and while we are not necessarily expecting to find any, many of our TRP sequences contain introns, which are more likely to vary in sequence length. For this reason, we should consider that modest deviations from our expected band size may actually be the correct amplicons.

Protocol: Gel electrophoresis

1) There are three gel rigs available for each section. Thus, two groups will share each gel by each using one comb per gel. Use a rubber seal or masking tape to make the mold watertight and prevent the gel from leaking.

2) Weigh 1.25g of agarose powder on a weighing boat using the scale. Transfer it into an Erlenmeyer flask and add 100ml of TBE buffer. Microwave ~1 minute, then check to see if the agarose is fully dissolved and the liquid is clear. If not, put the beaker back and microwave for short intervals at your TA's discretion. Swirl the gel, cooling it under the faucet until it has stopped steaming. Add 10ul of SYBR safe. **If SYBR safe is not added you will not be able to view your results.** Carefully pour your gel into the mold, taking care to avoid creating any bubbles. Bubbles may be removed with a pipette tip or

by spraying ethanol. Add your combs so as to divide the gel into two equal parts and leave the gel to solidify.

3) When the gel completely solidifies, carefully remove the masking tape (if present) and place it in the rig. (Note the appropriate orientation). Pour running buffer in the gel rig until it completely covers the surface of the gel.

4) Obtain DNA ladder. TAs should demonstrate how to load DNA using the ladder. >Add 5ul of ladder to the first well of each row for reference. Add 8ul of each PCR to the next four wells in the following order: TRP1, TRP2, TRP3, 23S. It is okay that there are empty wells.

5) Run the gels for 35 minutes at 130 volts. Ensure that the chamber is working (bubbles should come up from the electrodes) and that the samples are migrating in the correct direction (you will be able to see the dye moving through the gel in the direction of the positive electrode). TAs may use this time to explain expected results. Remove the gel and view using the transilluminator). Take a picture of your gel for your lab notebooks so you can interpret results

The approximate sizes for each of your bands are as follows:

TRP1: 2142bp
TRP2: 1323bp
TRP3: 1925bp
23S: ~500bp

For your lab notebooks

What purpose does this electrophoresis serve in the broader context of the anemone stress challenge experiment? What do you make of your results? Did all of your PCRs work as planned? If they failed, why do you suppose that is? Did you experience issues with your DNA extraction or PCR? Did you simply misload your sample into the gel? Did you see any ALPs? Please include a picture of your gel in your notebook and **clearly label which sample is which** so that your TA can understand your interpretation of your results.

Week 7

Background: PCR cleanup

Last week, we confirmed that our PCR reactions worked using gel electrophoresis. This week, we will clean up those PCR products and begin Sanger sequencing. This is the final step towards getting full genotype information from our samples down to the sequence level; that is to say, when we are done with Sanger sequencing, we will receive an output file detailing the exact nucleotide sequence of each of your samples.

Before we can sequence our PCR amplicons, we must first clean them up. There are still a number of chemicals in your PCR reactions that can interfere with downstream applications such as Sanger sequencing. These impurities can be removed by two main strategies: physical means (for example, a column purification similar to the one we performed in DNA extraction) or chemical means. We will be cleaning up our samples chemically using a product called ExoSAP. The chief advantage of chemical cleanup is that you can recover 100% of your sample, whereas cleaning your sample on a column typically results in some amount of sample loss.

ExoSAP is so named because of its two chief components: **exonuclease I** (exoI) and **shrimp alkaline phosphatase** (SAP)**.** ExoI is an enzyme that chews up single-stranded DNA molecules. This is useful for removing excess primer molecules from our reactions, which can interfere with downstream applications. Note that while primers are incorporated into new DNA strands by Taq polymerase, it is likely that there are still some left in your reactions as PCR is not always perfectly efficient. SAP removes phosphates from dNTPs, the monomers of DNA may also persist in your reaction. dNTPs are necessary in PCR because they are the building block of new DNA strands, but for Sanger sequencing we will want to build new strands with a new mixture of subunits so these must be removed for tighter control of the sequencing reaction. Phosphates are required for the dehydration synthesis reaction that links nucleotide monomers, so removal of phosphates by SAP prevents dNTPs from being incorporated into new DNA polymers later.

Background: Sanger sequencing

Sanger or "cycle" sequencing was developed by Frederick Sanger in 1977 and is still one of the most widely used DNA sequencing technologies due to its low price point and simplicity. Sanger sequencing relies upon a reaction that is in many ways similar to PCR, with a couple of important differences. Firstly, Sanger sequencing uses only one primer, which causes synthesis of new strands to only occur in one direction. Secondly, instead of only containing dNTPs as building blocks for new strands, Sanger reactions utilize a mixture of dNTPs and radio- or fluorescently labelled **dideoxynucleotides** (ddNTPs). The 3' hydroxy (-OH) group of a dNTP is necessary for polymerase to form a bond to the next nucleotide in the chain, so the removal of this group from dNTPs terminates elongation.

These two differences from PCR result in a final reaction product that is a mixture of complementary strands starting at one primer and terminating after a random number of bases. Additionally, with each nucleotide letter—A, T, C, and G—being coded with a different fluorescent tag, the last base of each molecule can be read by a machine. After your reactions are done, your TA will bring your samples to FIU's sequencing facility. The molecules in your reaction—all of different lengths and each labelled by color according to the last molecule—can then be separated by size using capillary electrophoresis (a process fundamentally similar to the gel electrophoresis you ran last week). This allows a computer to literally read the nucleotide sequence of your sample based on the order of the fluorescent signals it receives! (Note that back in the 1970s this part was painstaking and accomplished on a gel, as sufficiently advanced machines were not feasible.)

Protocol: PCR cleanup and Sanger sequencing

PCR clean-up protocol with ExoSAP-IT

(Your TA will complete Step 1.)

1) Make a 1:10 dilution of ExoSAP-IT reagent. 2µlof the 1:10 working solution is required for each reaction. For each section pipette 54µl of molecular biology grade water into a microcentrifuge tube. Label this tube ExoSAP 1/10. Remove stock of ExoSAP-IT reagent from –20°Cfreezer and place it on ice. ExoSAP-IT is in a glycerol solution and does not freeze at -20°C. Briefly spin down the ExoSAP-IT stock in a microfuge (~3 sec) and return it to ice. Pipette 6µl ExoSAP-IT stock into the tube you labelled ExoSAP 1/10. Close the tube, briefly flick to mix, and briefly spin down in microfuge. Immediately return the ExoSAP-IT stock (NOT your dilution) to the -20°C freezer. Keep your dilution (the tube labelled ExoSAP 1/10) on ice. This dilution can only be used today; any leftovers should be discarded.

2) Students will collect their PCR products from the TA.

3) Each student must label a new PCR tube. It may be easiest for the TA to assign numbers 1-24 to each student. Each table can keep track of the numbers used for its four samples; you need to be able to identify your own tubes. You may want to keep your table's four tubes attached for ease of handling by your group and your TA.

4) Briefly spin down PCR products to collect at bottom of each tube. Transfer 5µl of each PCR product into the new labeled PCR tubes. If any of your products did not show up during electrophoresis or you have no more PCR products due to pipetting mistakes, you may want to use one product for more than one sequencing reaction (you can sequence the same product from two directions to provide a more complete dataset, but note that you will have to use an appropriate primer later on).

5) Pipette 2µl of 1:10 ExoSAP dilution into the labelled PCR tube containing 5µl of your PCR product.

6) Close the PCR tubes, flick to mix, and spin down in a balanced microfuge (under the supervision of your TA).

7) Using the thermal cycler, incubate at 37°C for 30 minutes to degrade remaining primers and nucleotides, and then for at 80°C for 15 minutes to inactivate the enzymes in ExoSAP-IT. This should be automatically accomplished using a program called EXOSAP; check the program before running it.

8) Keep on ice while you prepare for the next step.

Cycle sequencing protocol with BigDye Terminator kit

Before beginning, you need to dilute the primers you're using for sequencing. First, be sure that you're using the appropriate primer for your PCR product. For instance, if the PCR product you are sequencing is TRP1, you should be using the TRP1 FORWARD primer. If, for instance, TRP2 did not work and you are sequencing TRP1 twice, use the TRP1 REVERSE primer for the second reaction. Attention to detail is critical: note that if you have a purified TRP1 PCR product but use a TRP2 primer for sequencing, **your cycle sequencing reaction will fail**. We want each sequencing reaction to contain 0.935pmol of one (and only one) primer. A typical concentration used for primer working stocks is 10μM; this is equivalent to 10pmol/μl. If you were to use this directly, that's far too small of a volume to accurately pipette. The solution is to dilute your 10μM stock to 0.468μM. You can then add 2μl of this 0.4675μM dilution to your reaction to add 0.935pmol with reasonable accuracy.

1) In order to make this dilution, first pipette 95.33μl of molecular biology grade water into a microcentrifuge tube.

2) Acquire the appropriate primer stock for the PCR product you are sequencing.

3) Label the tube to which you have added 95.33μl of water with the name of your primer (e.g. "23S-FORWARD") and "0.4675 μM." Add 4.67μl of the 10μM stock primer to tube you just labelled. Close tube and flick to mix. Spin down tube briefly to collect contents at bottom.

4) Setting Up the Cycle Sequencing Reaction Mix: Uniquely label a PCR tube as before. This full information need not appear on the tube, but each student must capture the following information in their lab notebook:

    a. Unique genet ID (e.g. ARB-42)
    b. Amplicon (e.g. 23S, TRP1, TRP2 or TRP3)
    c. Primer used for sequencing (e.g. 23S FORWARD, TRP1 REVERSE, etc.); Students: this **must** be recorded in your lab notebooks in order for any data to be useful. **You are responsible for this information and will be asked for it in coming weeks.**

5) Add the following to the labelled PCR tube:
    a. 3μl molecular biology grade water
    b. 2μl 5x buffer
    c. 2μl purified PCR product
    d. 2μl primer (0.4675μM)
    e. 1μl BigDye Terminator
    10μl total

Double check that you're using the appropriate primer for each PCR product!

6) Close PCR tubes, flick to mix, briefly spin down, and keep on ice until the thermal cycler is ready to cycle your reaction.

Cycle Sequencing Program:

1) 95℃ for 15s
2) 50℃ for 10s
3) 60℃ for 4min
4) Go to 1 34x
5) 4℃ forever

Note: This program requires approximately 3 hours to complete. Store products at 4℃ until ready for capillary analysis at the DNA Core.

For your lab notebooks

Clearly describe which samples you used and why (bad gel, no more product, etc.). Record which numbered reaction contains which amplicon/primer set. Make note of any issues deviations from the protocol and what steps were taken to salvage it. What is the purpose of these steps in the context of the anemone stress challenge experiment?

Week 8

Background: Scoring the UV mutagenesis of *Breviolum minutum*

Note: This is just a brief refresher as most of the relevant material has been previously covered in weeks 1 and 2. Please refer to those sections of the lab manual for more detailed explanations of the concepts underpinning this mutagenesis screen.

Previously, we exposed the algae *Breviolum minutum*—an intracellular symbiont of cnidarians—to ultraviolet light for different lengths of time with the goal of creating random mutations. We then plated those cultures on 5-FOA+ plates to screen for mutants with a 5-FOA resistance phenotype. We also plated cells on 5-FOA- plates to see how much growth we would observe in the absence of selection.

Protocol: Scoring the UV mutagenesis of *Breviolum minutum*

Note: in the event of extensive contamination of plates, the TAs may elect to combine and count data across sections. In this event, a representative sample of plates may be left out for your section to examine.

1) Examine each plate for signs of algal growth. *B. minutum* colonies should be visible to the naked eye and will appear as very fine, brown spots. Dead cells may also be visible but will appear grayer in color and should not be counted towards our analysis, as cells

that survived the mutagenesis but died on the plate probably did not acquire resistance to 5-FOA.

2) If the number of colonies is few or zero, record that number for all plates that apply. If the number of colonies on a plate is too large to easily count, use the following technique outlined in step 3.
3) To estimate the number of colonies on densely populated plate, use a ruler and fine-tipped sharpie to draw a 1cm square over a representative portion of the plate (i.e., the square should have roughly the same density of algae as the part of the plate that contains algae). Count the algae within this region under the dissecting scope. Then, estimate the average diameter of the region covered in algae using a ruler. These numbers will allow you to estimate the total number of algae using the following formulas:

    - Area = π x (diameter/2)^2
    - Area in cm^2 x cells/cm^2 = total cell count estimate

4) Share these figures with your TA. **Each table should record all data from your section.**

For your notebooks:

Describe the appearance of the positive and negative plates. What are the two types of controls we used in this experiment and why are they important? Which plates (if any) do you think are likely to contain 5-FOA resistant mutants? Why? Please specify time point **AND** 5-FOA+/- condition. Lastly, how might you use this type of experimental design to discover algae genes involved in cnidarian symbiosis?