Getting to know our neighbors: the Oral Microbes

Action Proposal

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Abstract

Course schedule and mandatory standardized testing has created huge time constraint in the classroom, which has altered how science is taught in most classrooms. Even though inquiry-based learning has been shown to increase student motivation for learning science and improve attitude in science classes, the two factors mentioned earlier has prevented its implementation in most classrooms as teachers race to prepare their students for mandatory tests. My action proposal is to investigate how the inclusion of inquiry-based activities in the cell biology, genetics and biotechnology sections of my IB biology classes will influence my students' motivation and their performance on the individual scientific investigation component of the IB biology course. The effect of these interventions will be assessed using personal meaning mapping, and reflection and survey questionnaire.

Rationale

Incorporating active learning and inquiry-based learning activities in biology courses has been shown to increase motivation, improve attitude and performance (Madhuri and Broussard, 2008), Armbruster et al., 2009), Freeman et al., 2007). Although these studies were based on college-level biology courses, the findings should be applicable to college-level biology taught in high school like IB Biology. The active learning activities include group activities, discussions, clicker and inquiry-based activities. Inquiry-based science has been shown to increase student motivation and attitude towards science in high school (Wang et al., 2015). American Association for the Advancement of Science (AAAS) in its Benchmarks recommended that for students to develop science literacy, the teaching of the nature of science should include the knowledge of scientific inquiry, which is described as scientific investigations that allow students to explore different concepts associated with their content materials. Scientific inquiry-based learning would not only improve motivation in science classes but would help student develop science literacy that is essential for an informed citizenry.

I have been teaching standard level IB biology for two years. The IB Biology course had a curriculum and schedule change from being a two-year course to one-year with a new individual scientific investigation (open inquiry) component added, which is 20% of the final IB biology grade. These changes made the course very fast paced with limited active learning. With the time limitation, students were unable to reach the proficiency level to design and complete an open inquiry investigation. Open inquiry activities that are not preceded by adequate practice to create strong foundational knowledge has been shown to be less effective in helping student gain mastery of content knowledge (Kirschner , et al., 2006). While the overall course pass rate was

relative high in both years (89% and 76%, respectively), the pass rate on the individual scientific investigation was very low with the students exhibiting frustration, some anxiety and lowered motivation. After evaluating the feedback from teachers and students, the school administration changed the course schedule back to a two-year track. This change will eliminate the time constraint of the past two years, and most importantly, provide the opportunity for more active and inquiry-based learning which will help the students acquire the science skills for completing the individual scientific investigation. Secondly, it will hopefully remove the frustration and anxiety of the students over the completion of the individual scientific investigation and increase their motivation for mastering important biological concepts and science skills.

Incorporating inquiry-based learning in my regular biology classes should also have a positive effect on student motivation and achievement. My regular biology students are lower performing and struggling readers compared to the IB biology students with less motivation and commitment to mastering the concepts. The regular biology classes have an end of course (EOC) exam at the end of the school year, which is based on a plethora of standards that places time constraints on implementing more student-centered learning activities. Understanding the nature of science is a significant component of the EOC exam that students struggle to comprehend. Implementing more inquiry-based learning activities will enable my students to practice how science is actually done, understand the process that scientists use in their studies to eventually formulate the content knowledge being taught in the class.

My action proposal is to determine the effect of incorporating inquiry-based activities into my IB and regular Biology on student motivation, attitude and performance. My hypothesis is that these activities will increase student motivation, improve their attitude in class and performance on assessments, as students are able to engage more with the materials as active learners.

Intervention

Inquiry-based activities based on oral microorganisms will be implemented through the school year (see Table 1). Through these activities, students will learning how to use micropipettes, culture microorganisms on nutrient media and identify morphology of different colonies, and prepare wet mount and use microscope. Students enjoy learning about relatable issues like their oral microorganisms. This intervention will allow the introduction of an inquiry-based study of microorganisms into my cell biology curriculum, which was based mainly on lab sessions on plant and animal cells. This study of microorganisms will be a common thread in the curriculum that will be linked into topics in genetics and biotechnology. Although genetics and

biotechnology make up a significant proportion of the curriculum with cutting edge information, I have had no opportunity to introduce my students to important lab skills like using micropipettes, gel electrophoresis, polymerase chain reaction and using DNA sequences generated from students' samples for bioinformatics analyses. Having lab sessions using these techniques will not only make these concepts easier to master but will also make my students relate better to biotechnological information that they come across. Most importantly, these structured and guided inquiry-based activities will enable my students to formulate related research questions that they can investigate to meet the individual scientific investigation requirement of IB biology.

Table 1: Inquiry-Based Activities for 2017/2018 School year IB, AP and Regular Biology Classes

Topic	**Inquiry-based activity**	**Schedule/Course** IB Biology (11th grade) Regular Biology (10th grade)
Biochemistry	Learn pipetting	Fall 2017/IB and Regular Biology
Cell Biology	Culturing oral bacteria on agar plates	Fall 2017/IB and Regular Biology
	Describing morphology of bacterial colonies	Fall 2017/IB and Regular Biology
	Microscopic observation of bacteria colonies	Fall 2017/IB and Regular Biology
Genetics	DNA Extraction from bacteria colony cells	Fall-Spring 2017-18/IB Biology
	DNA amplification using PCR	Fall-Spring 2017-18/IB Biology
	Observation of PCR DNA products with gel electrophoresis and cleaning up PCR product	Fall-Spring 2017-18/IB Biology
	Sequencing oral bacteria DNA	Fall-Spring 2017-18/IB Biology
	Identification of oral bacteria using BLAST	Fall-Spring 2017-18/IB Biology

Data Collection and Analysis

The effectiveness of these interventions will be assessed using personal meaning mapping, reflections and survey questionnaire, performance on the individual scientific investigation component of IB Biology. Implementation of these inquiry-based activities should enable students to be more engaged in the learning process, which should improve their motivation and attitude. With the exposure to new lab techniques and guidance during these guided inquiry-based activities, my IB students should experience less anxiety and frustration completing the open inquiry of the individual scientific investigation.

Words and phrases for Personal Meaning Mapping: Cells, Microorganisms, DNA, DNA technology, Bioinformatics/BLAST
Reflection and survey questionnaire: Attached

Connections to CATALySES summer institute

The oral microorganisms-based inquiry activities are based on the "A Mouthful of Microbes" lab sessions offered during the 2017 CATALySES summer institute. This was a lab protocol that was modified from Strain and Vang (2014). The reuseable equipment required for completing these lab sessions will be obtained on loan from CPET. I would also like to invite CPET staff to assist with the set up of some of these lab sessions.

Literature cited

Armbruster, P., Patel, M., Johnson, E., & Weiss, M. (2009). Active Learning and Student-centered Pedagogy Improve Student Attitudes and Performance in Introductory Biology. *CBE Life Sciences Education*, *8*(3), 203–213. http://doi.org/10.1187/cbe.09-03-0025

CPET, (2017). A Mouthful of Microbes.

Freeman, S., O'Connor, E., Parks, J. W., Cunningham, M., Hurley, D., Haak, D., Dirks, C. and Wenderoth, M. P. (2007). Prescribed Active Learning Increases Performance in Introductory Biology. *CBE— Life Sciences Education*, *6*(2), 132–139. http://doi.org/10.1187/cbe.06-09-0194

Kirschner , P. A, Sweller, J. & Clark, R. E. (2006). Why Minimal Guidance During Instruction Does Not Work: An Analysis of the Failure of Constructivist, Discovery, Problem-Based, Experiential, and Inquiry-Based Teaching, Educational Psychologist, 41:2, 75-86, DOI: 10.1207/s15326985ep4102_1

Madhuri, M., & Broussard, C. (2008). "Do I Need to Know This for the Exam?" Using Popular Media, Inquiry-based Laboratories, and a Community of Scientific Practice to Motivate Students to Learn Developmental Biology. *CBE Life Sciences Education*, *7*(1), 36–44. http://doi.org/10.1187/cbe.07-06-0044

Strain, A.K. and Vang, K.B. 2014. You and Your Oral Microflora: Introducing non-biology majors to their "forgotten organ". *CourseSource.* https://doi.org/10.24918/cs.2014.13

Getting to know our neighbors: The Oral Microbes
Lesson Plan

Title: Getting to know our neighbors: The Oral Microbes

Key Questions:
What are microorganisms (microbes)?
What are the different types of cells?
What are the cell types in microorganisms?
How are microorganisms different?
What are some of the techniques used in biotechnology?
How can DNA information be used to identify microorganisms?

Overall time estimate:
Eight 50-minute class period

Lesson summary:
Students will learn about different types of microbes and cell structures. They will follow a protocol to culture their own oral microbes and analyze the morphology of the colonies. The cultured bacterial colonies will be used for DNA extraction, amplification with PCR, DNA sequencing, protein extraction and proteome identification with mass spectrometry. The DNA sequences will be analyzed using BLAST to identify the different bacterial colonies. The result of the proteomics analysis will be used to determine differences in protein expression of different colonies.

Student Learning Objectives:
The students will be able to:
1. Culture oral bacterial cells on nutrient media.
2. Create wet mount and use microscope to identify prokaryotic and eukaryotic cells.
3. Analyze the morphology of the colonies to identify bacteria.
4. Describe how to extract bacterial DNA and identify its sequencing.
5. Use BLAST to analyze the DNA and protein sequences.

Biology 1 Honors Standards:
SC.912.L.14.1: Describe how continuous investigations and/or new scientific information influenced the development of the cell theory. Recognize the contributions of scientists in the development of the cell theory.
SC.912.L.14.3: Compare and contrast the general structures of prokaryotic and eukaryotic cells
SC.912.L.16.12: Describe how basic DNA technology (restriction digestion by endonucleases, gel electrophoresis, polymerase chain reaction, ligation, and transformation) is used to construct recombinant DNA molecules (DNA cloning).
SC.912.L.16.2: Describe the basic process of DNA replication and how it relates to the transmission and conservation of the genetic information.
SC.912.L.16.5: Explain the basic processes of transcription and translation, and how they result in the expression of genes.
SC.912.L.16.9: Explain how and why the genetic code is universal and is common to almost all organisms.

SC.912.L.18.9: Define a problem based on a specific body of knowledge, for example: biology, chemistry, physics, and earth/space science, and do the following:

1. Pose questions about the natural world, (Articulate the purpose of the investigation and identify the relevant scientific concepts).
2. Conduct systematic observations, (Write procedures that are clear and replicable. Identify observables and examine relationships between test (independent) variable and outcome (dependent) variable. Employ appropriate methods for accurate and consistent observations; conduct and record measurements at appropriate levels of precision. Follow safety guidelines).
3. Examine books and other sources of information to see what is already known,
4. Review what is known in light of empirical evidence, (Examine whether available empirical evidence can be interpreted in terms of existing knowledge and models, and if not, modify or develop new models).
5. Plan investigations, (Design and evaluate a scientific investigation).
6. Use tools to gather, analyze, and interpret data (this includes the use of measurement in metric and other systems, and also the generation and interpretation of graphical representations of data, including data tables and graphs), (Collect data or evidence in an organized way. Properly use instruments, equipment, and materials (e.g., scales, probeware, meter sticks, microscopes, computers) including setup, calibration, technique, maintenance, and storage).
7. Pose answers, explanations, or descriptions of events,
8. Generate explanations that explicate or describe natural phenomena (inferences),
9. Use appropriate evidence and reasoning to justify these explanations to others,
10. Communicate results of scientific investigations, and
11. Evaluate the merits of the explanations produced by others.

IB Biology required skills

- Use of a light microscope to investigate the structure of cells and tissues, with drawing of cells.
- Use of a database to determine differences in the base sequence of a gene in two species.
- Use of databases to identify the locus of a human gene and its polypeptide product.
- Design of experiments to test the effect of temperature, pH and substrate concentration on the activity of enzymes.
- Experimental investigation of a factor affecting enzyme activity.
- Use of Taq DNA polymerase to produce multiple copies of DNA rapidly by the polymerase chain reaction (PCR).
- Deducing the DNA base sequence for the mRNA strand.
- Evaluation of data or media reports on environmental problems caused by biofilms.
- Use of databases to identify the locus of a human gene and its polypeptide product.
- Use of a database to determine differences in the base sequence of a gene in two species.
- Use of software to align two proteins.
- Use of software to construct simple cladograms and phylograms of related organisms using DNA sequences.

Materials: Listed on the protocol for each lab day lesson.

Background information

Microorganisms are indispensable component of any ecosystem however their importance is easily overlooked because they are invisible to the naked eyes. The fact that we have 10 times ore of their cells in our body then our own human cells suggest their importance to our wellbeing as their diversity as been shown to change with our health changes. The advent of polymerase chain reaction, new generation DNA sequencing and bioinformatics has provided a new tool for the identification of different microorganisms.

Advance Preparation

Before the implementation of the lesson,

- Student must have completed the pipetting by design activity and able to use micropipette properly.
- At least 2 days before lesson provide each student with a copy of the protocol to read in preparation for the lab.
- Assemble the materials per 2 students working at a station.

Procedure

1. The day prior to the lesson, the students should have read the protocol. The teacher will discuss the activities for the lab session and answer any questions.

2. Bell work activities and discussion (to ensure students read protocol before the lab): 5 minutes
    a. Lesson 1: Compare and contrast prokaryotic and eukaryotic cells.
       What is the role of the agar medium in culturing bacteria?
    b. Lesson 2: What does each colony represent?
    c. Lesson 3: Why are ribosomes essential to the functioning of a cell?
    d. Lesson 4: How does the polymerase chain reaction work?
    e. Lesson 5: What is the net charge of a DNA molecule?
       How does agarose gel used in gel electrophoresis separate DNA fragments?
    f. Lesson 6: How would sequencing the microbial DNA help identify our microbes?
    g. Lesson 7: What search tool can you use to identify your microbes using DNA sequence?
       How does this search tool work?
3. Teacher must sure that students are following proper lab procedure and completing their lab notebook during each session.

Assessments

1. Each student submit lab notebook for completion and accuracy check.
2. Each student will complete the practical questions (Strain and Vang, 2014).
3. Each student will complete the BLAST assignment (Strain and Vang, 2014).

Resources

CPET CATALySES Summer Institute. 2017. Mouthful of Microbes.

Strain, A.K. and Vang, K.B. 2014. You and Your Oral Microflora: Introducing non-biology majors to their “forgotten organ”. *CourseSource.* https://doi.org/10.24918/cs.2014.13

Getting to know our neighbors: The Oral Microbes

The human body is comprised of approximately 75 trillion eukaryotic cells. As you've already learned, eukaryotic cells contain membrane-bound organelles and have a nucleus containing DNA that is separate from the cytoplasm. In this class you will be learning a lot about how these cells function individually and how they function as complex tissues when grouped together. However, these are not the only cells that are present in and on our bodies. Our bodies are also home to prokaryotic cells, which outnumber our human cells by a factor of 10 [1]. Look up other characteristics of prokaryotic and eukaryotic cells. Answer the questions listed below. The links below are suggested for the review. Do this before coming to lab so you are prepared for the first lab!

Review links: Cell structure: https://www.youtube.com/watch?v=URUJD5NEXC8
History of the cell theory: https://ed.ted.com/on/GmBGQAi2
Shapes of bacteria: https://www.youtube.com/watch?v=e40NrVHygyc

1. Compare and contrast the two types of cell.

	Eukaryotic cells	Prokaryotic cells
Similarities	_____	_____
	_____	_____
	_____	_____
	_____	_____
Differences	_____	_____
	_____	_____
	_____	_____
	_____	_____

2. What do you think are the possible benefits of living together with so many prokaryotic cells?

References

[1] P. J. Turnbaugh, R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight and J. I. Gordon, "The human microbiome project," *Nature*, vol. 449, pp. 804-810, 2007.

Internet resources

Cell structure: https://www.youtube.com/watch?v=URUJD5NEXC8

History of the cell theory: https://ed.ted.com/on/GmBGQAi2

Shapes of bacteria: https://www.youtube.com/watch?v=e40NrVHygyc

Background Information

Microorganisms were first observed under the microscope by Antony van Leeuwenhoek in 1676 [2]. Using samples of dental plaque from his mouth and the mouths of others, he identified what he called "animalcules", which have since been classified as bacteria [rev. in [3]]. Scientists have determined that the human mouth is home to over 1 million bacteria, comprising at least 600 different species of bacteria [4] [5]. Most of these are part of the "normal flora", and serve a protective function, helping to initiate the digestive process, although some of these have been proposed to play a role in systemic diseases such as cardiovascular disease [6]. Likewise, some of the bacteria that have been shown to cause dental plaque may be present in the human mouth without causing disease. Mouth bacteria attach to the surface of a recently cleaned tooth in a particular order. Almost all bacteria live in biofilm, which is an aggregate of microorganisms attached to each other or a surface and enclosed within an extracellular polymeric substance they produced [7]. Bacteria referred to as "early colonizers" will attach first to the tooth surface soon after cleaning (within minutes). The presence of these first "early colonizers" will influence the types of bacteria that attach later (hours to days- "late colonizers") [8].

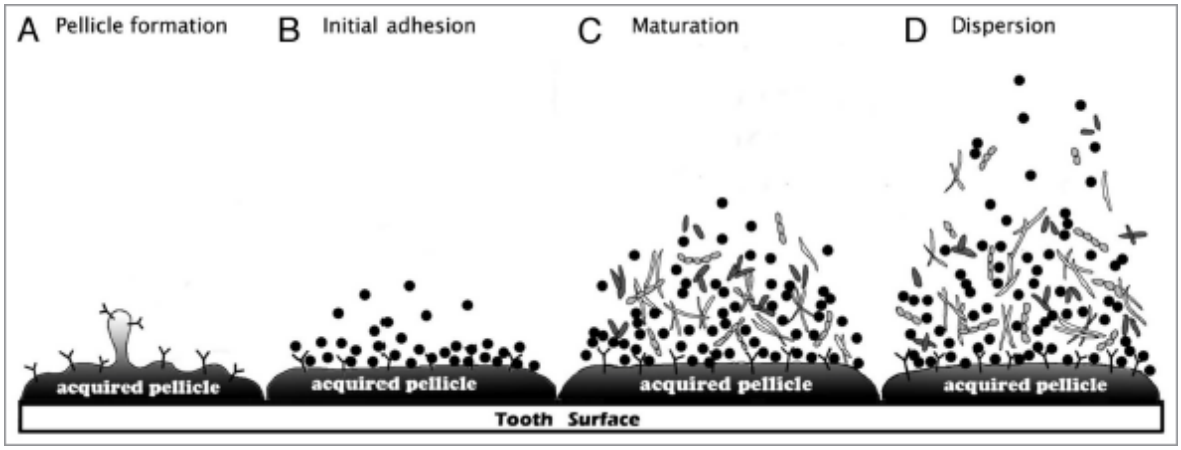

Figure 1: Oral biofilm formation. This diagram represents each step of oral biofilm formation. (A) Pellicle formation. The pellicle is a thin film derived from salivary glycoproteins attached to a clean tooth surface. (B) Initial adhesion. Pioneer bacteria in saliva recognize the binding proteins in acquired pellicle and attach to them. This adhesion is reversible. (C) Maturation. Different bacterial species coaggregate and mature biofilm forms. (D) Dispersion. Bacteria disperse from the biofilm surface and spread to colonize a new site [7].

To more closely study the microorganisms identified in the mouth (and from other sources), culture-based methods were developed soon after Leeuwenhoek first described his oral flora. "Culture-based" means using a growth medium that allows scientists to grow an organism in the laboratory, typically something that uses agar and nutrient sources to support growth of the organisms. In this lab, we are going to sample our oral microflora to identify differences between bacteria that are "culturable", and to begin to appreciate the diversity of microorganisms present on the surfaces of our bodies.

IMPORTANT SAFETY INFORMATION BEFORE YOU BEGIN

You will be working with microorganisms in this module. This work should not be harmful to you, but it does require you to use some basic common sense, as well as basic sterile techniques. Some important things to remember:

1) You MUST handle your own specimens at all times! After day 1, you should wear gloves every time you work on this project.

2) Before and after every laboratory session, you should wipe down your benchtop with 10% bleach solution and allow it to air dry. This will remove potential contaminating bacteria from your workspace. Please wear an apron during each lab session.

4) At the end of each session, you will need to:

a) clean up your work space by spraying a 10% bleach solution on your work surface

AND

b) wash your hands well before leaving the lab area.

5) Remember: Ask questions any time you need clarifications and before disposing anything to make sure that you are doing it right.

Very important: Like for all labs, make sure you record all the information from these lab sessions in your lab notebook. The questions and corresponding answers must also be written in your lab notebook.

I. Lab Day One- What's in your mouth?

Have you ever wondered what might be living in your mouth? Have you wondered if there are differences between the different surfaces of your mouth (the tooth enamel versus under the tongue)? Today you will be choosing one or more of the surfaces of your mouth as a source of bacteria to inoculate agar plates. There are two types of agar plates available; the nutrient agar plates are called Luria-Bertani (LB) and are general-purpose medium not designed for bacteria with high nutritional needs; and the chocolate agar, a nutrient rich medium designed to support growth of bacteria that cannot meet their nutritional needs without significant assistance.

Before you begin, here are some questions for you to think about. Write your answers in the space provided:

Questions

1. **How many bacteria do you think may be present on your own entire mouth? How about on a single tooth? How about on a small portion of your check?**

2. **Could you alter the number or types of bacteria present in your mouth? How do you think you would accomplish this?**

General protocol for sampling your oral microflora:

You will be working in pairs throughout this experiment and sharing supplies provided on a tray for two people. Please remember that **you should only handle your own specimens.**

To sample your oral flora, you will need the following per student:

- 1 Nutrient LB agar plate
- 1 Chocolate agar plate
- Sharpie
- 2 sterile swabs
- 4 plastic loops
- 10% bleach solution

Safety precaution:
ALWAYS REMEMBER TO ONLY HANDLE YOUR OWN SPECIMENS!

1. Spray and wipe down your desk with 10% bleach solution before beginning your work. Obtain a tray for each pair of students.

2. Label both plates with your name and date. Write this information on the outside edge of the bottom of the plate (the part that contains the agar) because the lid can easily be moved between plates but the bottom stays with the agar. After labeling the plates, place them lid-side down on the bench in front of you, within easy reach for swabbing. You will be doing much of the work coming up one-handed, so you do not want to have to reach far or fumble around for materials.

3. Obtain four sterile cotton swabs for sampling. These should be individually wrapped, or wrapped in a package of two. Open the "stick" end, the end that does not have the cotton swab on it. If there are two sticks in the package, this allows you to place the unused swab back on the benchtop without losing the sterility of the swab, so long as the swab end stays inside the container. Remember, you'll be putting this swab into your mouth- you don't want it to touch the surface of the lab bench first. Using one swab, sample the area of interest by swiping/rubbing over the surface for at least 15 seconds. Consider the sample site: for hard surfaces you can rub hard, for soft surfaces (such as your tongue or cheeks) you will want to be a bit gentle.

   Sampling site 1: _____

4. Immediately after swabbing, locate the appropriately labeled plate. If you have placed them lid-side down on the lab bench surface, it should be easy to read the writing on the plate and identify your plate. Pick up the bottom of the plate, flip it over to access the agar surface, and inoculate your plate using the three-phase streaking method by rubbing your swab gently over the entire surface of the plate. See Figure 2 for further instructions. Your goal is to try to spread out the bacteria that you swabbed from your mouth so that you will have well-separated colonies on your plate after they have been incubated. This will allow you to analyze individual colonies later, as well as isolate DNA from individual colonies. When finished, flip the plate back over onto the cover that is still sitting on your bench top, and then set this inoculated plate aside. Discard the swab into the biohazard container as directed by your teacher.

Three-phase streaking method: Draw a T on the bottom of your plate with a Sharpie. You should swab your plates in this order. Above the top of the T, rub your swab back and forth across 1/3 of the surface area of the plate. On the right side of the T, using the same fluid motion, drag a new loop once through the area above the T, and then repeat the back and forth swabbing across another 1/3 of the plate. On the left side of the T, use a new loop to repeat the process of dragging once through the area on the right side of the T, and swab back and forth to cover the last portion of the plate. NOTE: Do not cross back over to the other sections more than once after you start covering a new section of the plate!

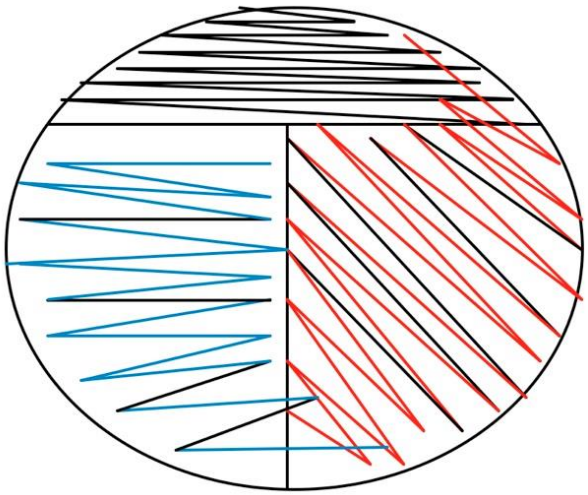

Figure 2: Three-phase Streaking Method
http://fg.cns.utexas.edu/fg/protocol%3A_plate_streaking_and_growth_files/droppedImage.jpg

5. Repeat this procedure with the remaining swabs, loops and plate. Using tape, tape all your plates in one stack and proceed to the next step.

6. Put labeled plates into the bins for your teacher to collect. These plates will be incubated for 48hours at 37^{o}C and then held at 4^{o}C until next lab session.

7. Your gloves should go into the biohazard bag.

8. Please spray and wipe down your benchtop with 10% bleach solution before you leave the workbench. Finally, be sure to wash your hand before you leave the lab.

II. Lab Day Two- Making observations

Your plates were incubated at 37ºC for 24-48 hours in order to allow bacteria to grow. They are then stored at 4ºC in order to keep the bacteria from overgrowing on the plate. It is important to note that only bacteria that can grow at 37ºC in the presence of oxygen, and can utilize LB agar as a nutrient source, will be able to grow on your plates.

Question: Why do you think this might be important to remember?

Bacterial cells "grow" by binary fission. This means that after a single cell has completed the cell cycle, it will divide itself approximately in half, resulting in two (basically) identical cells. On average, bacterial cells can complete a single cell cycle in 20 minutes (some do take longer than this, some less). This means that after 24 hours a single cell has replicated itself 72 times. Of course, with each division, there are additional cells that can now replicate and divide, so after 24 hours a single bacterial cell can result in $2x10^{21}$cells. What you will be looking at on your plates is the result of all of these divisions, for potentially many different types of bacteria. The result of all of these divisions is a pile of cells called a "colony". Each colony arose from a single "colony-forming-unit", indicating that there was a live bacterial cell that was deposited at that spot on your plate, and that the appropriate conditions were provided for it to grow.

There are many different ways to classify and identify bacteria, but one standard technique is to look at the morphology (shape) of the colony that forms when one bacterial cell has divided enough times to be visualized as growth on a plate. Today you will describe your bacteria by answering the following questions:

Use these images in Figure 3 to help you describe your bacterial colonies.

1) **What color are your colonies?**
2) **How high above the surface of the plate are your colonies?**
3) **What is the general shape of the entire colony?**
4) **What does the edge of the colony look like?**
5) **What does the surface of the colony look like?**

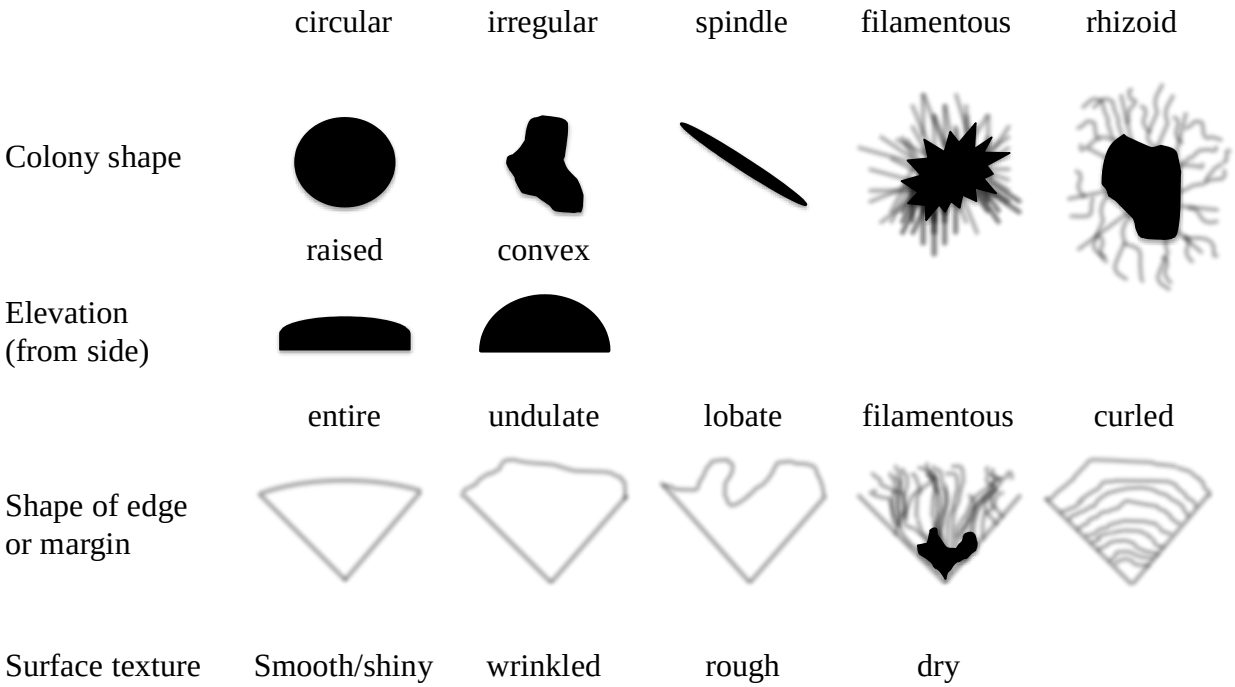

Figure 3. Colony morphology descriptions, adapted from [9].

General protocol for analyzing your plates and preparing wet mount slides:

What you will need:

Your plates from last time
Colony morphology worksheet (Figure 3)
Glass microscope slides
Coverslips
Plastic loops
Gloves
Water
Water dropper or 20uL pipette with tip
Microscopes

1. Spray and wipe down your benchtop with 10% bleach solution before beginning your work. Procure a tray with the materials needed teams of two. REMINDER: YOU ARE WORKING ONLY WITH YOUR OWN SPECIMENS! Wear gloves at all times.

2. Find your plates from the bin from last lab.

3. Analyze your plates from last lab.

   a. **Approximately how many total bacterial colonies do you have on each plate?**

   b. **Different bacteria form different types of colonies when grown on LB. They are judged based on their colony color, the shape of the colony, the height of the colony on the plate, if they look wet or dry, if they look like they are embedded into the plate, etc. Using the colony morphology descriptions above, classify the number of different types of colony morphologies observed on your oral swab plates.**

      i. **How many colonies (approximately) do you have on each plate?**

      ii. **How many different kinds of bacterial colonies do you have on each plate?**

      iii. **Does one colony morphology seem to predominate?**

      iv. **Take pictures of your plates to keep for your records. This data gives you some preliminary information about your isolated organisms, and provides you with some of the details that would be used to identify and classify which organisms are present within your mouth.**

      v. **What other technique could be use to identify and classify which organisms are present within your mouth.**

4. Make a wet mount of some of your colonies so that you can look at them under the microscope to compare them to the eukaryotic cells you will also be looking at this week. To make a wet mount side, follow the directions below.

REMINDER: Wear gloves, work only on the bench paper, work with your OWN plates.

5. To make a wet mount, find the plastic loop, a glass microscope slide, a cover slip, a pipette and pipette tips, and a pair of gloves. Put on your gloves! Look at your plates and decide which colony you want to pick cells from. It might be useful to draw a circle on the bottom of the plate around the colony chosen using a Sharpie, so that you know which colony you sampled from. You may want to use this same colony later for the DNA analysis. Prepare a wet mount by placing a drop of water (no more than 20 microliters) on the microscope slide. Dispose of the pipette tip in the tip bucket. Pick up a sterile toothpick (touching only one end) with one hand, and carefully lift the lid off one of your plates with the other. Using the plastic loop, pick up a small portion of one well-isolated colony with the tip of your toothpick and spread it around in the drop of water. Replace the lid of your plate. Dispose of the used plastic loop in the beaker with the biohazard bag, as directed by your teacher. Cover your sample on the microscope slide with a cover slip, being careful not to get any bubbles trapped under the cover slip.

6. Observe your cells under the compound microscope. Keep in mind that prokaryotic cells range in size from 0.2-5 microns, while eukaryotic cells can be 10-100 micrometers in size. If you need help finding bacterial cells, don't hesitate to ask for help.
    a. **Draw what you see in your lab notebook.**

7. When done, return your plates to the bins for your teacher to put back into storage until next week. Be sure to dispose of your slide(s) in the appropriate waste container for glass waste. Throw your gloves and the blue drape into the biohazard bag for disposal, and spray down your bench top with the 10% bleach solution.

8. Save your colony morphology data in your lab notebook, and wash your hands before you leave the lab area.

Colony Morphology Worksheet

(fill in as many as you can)

	Shape of entire colony	Elevation (seen from side)	Shape of edge or colony margin	Surface texture
Colony type 1				
Colony type 2				
Colony type 3				
Colony type 4				
Colony type 5				
Colony type 6				
Colony type 7				
Colony type 8				
Colony type 9				
Colony type10				
Colony type11				

Overall Number of colonies:

Plate 1 _____

Plate 2 _____

Overall number of different colony types:

Plate 1 _____

Plate 2 _____

III. Lab Day Three - Extracting DNA

Now that you have determined the number and kinds of colony morphologies formed by bacteria that can be cultured from your mouth, we are going to attempt to identify some of these microbes. There are many different ways that microbiologists have used in the past to identify and classify microbes, including biochemical, molecular and physical tests. These typically require additional time for growth, as well as special media and reagents. The molecular technique that we are going to use to classify our oral microbes also requires special reagents but does not rely on growth. Instead, it relies on the ability to obtain an adequate source of DNA. Since you've isolated single colonies, you should have an ample source of genetic material to utilize! The problem will be deciding what part of that genetic material to analyze.

Bacterial genomes can be as small as 500,000 base pairs or as large as 5 million base pairs, which is a lot of genomic material to cover. What is needed in order to classify microbes is a gene that is found in all bacterial cells and that has a conserved function so that it will not change (evolve) quickly. One gene that serves this purpose quite nicely for bacteria is the sequence that codes for the 16S ribosomal RNA (16S rRNA; the gene sequence encoding this molecule is referred to as the 16S rDNA). This molecule is part of the 30S ribosomal molecule in prokaryotes, and is required for the production of proteins.

Because the ribosome serves an essential role in the cell, its structure and function must be maintained. Any mutation that significantly alters the ability of the ribosome to bind to the mRNA or to the incoming tRNA could be lethal. Further, the 16S rRNA has conserved and variable regions, based on the portion of the molecule that is important for function. When the 16S rRNA molecule folds and becomes a part of the ribosome (RNA plus protein), the conserved regions play a role in maintaining functionality (ribosomal structure and translation initiation), while the variable regions are not as important for these functions. The variable regions are useful for classifying, because they generally differ between species. You will be using a portion of the 16S rDNA sequence within the V5-V6 hypervariable region to classify your oral microflora by amplifying this region using the polymerase chain reaction (PCR). This procedure will be described in more detail later; for now, you need to obtain the genetic material that will be used in this analysis using the Chelex protocol described below.

Remember this: The 30S ribosomal subunit of prokaryotes is made up of the 16S rRNA and proteins. The 16S rRNA gene has both conserved and variable regions, which can be useful for identifying and classifying prokaryotes

REMINDER: Even though you are sharing a bench space with your benchmate, you must continue to work with YOUR OWN bacterial plates.

General Protocol for the isolation of bacterial DNA, Chelex procedure [adapted from [10]]
Materials needed:
Chelator beads solution (Instagene matrix)
Pipettes (P1000, P200, P20, P10)
Pipette tips for the above pipettes
Sterile water (one bottle for each of group of 2 students)
Screw-cap Microcentrifuge tubes
Plastic loops
Microcentrifuge tube rack
Gloves
Ice bucket
Heat block with water in each well
Sharpies (extra-fine or Ultrafine tips, for writing on caps)

1. Spray 10% bleach on the benchtop surface. Put on your gloves. Place your plates from Day 1 onto the lab bench, along with the other materials you need for steps 2-5.
2. Choose two different colony morphologies (total) from your two agar plates. You will be taking a small (toothpick-tip sized) sample of each colony to analyze by PCR. Label two sterile microcentrifuge tubes. Also indicate somehow what the sample is that you will be placing in each tube. Keep a record of which colonies you are sampling! Again, it may help to circle the colony on the bottom of the plate in order to be able to visualize which colony you're working with. After choosing your colonies, place the plates back on your lab bench, lid side down. Record this information here or in your laboratory notebook.

a. Colony 1: (agar type, morphology)
b. Colony 2: (agar type, morphology)

3. Using the p1000 micropipettor set at 1 ml (1000), with a 1ml size tip, transfer 1 ml of sterile water into each sterile screw-cap microcentrifuge tube. Close the caps securely and set these tubes aside in a tube rack.

4. Obtain a plastic loop with one hand, being careful not to touch the end of the loop that will be going into your colony. Pick up your plate from the bottom, so that you can flip it over and pick up a small portion of your first selected colony. Carefully pick up a small amount of a selected colony from your plate (enough to cover the end of the loop). After getting your cells, return your plate to its lid, which should still be sitting on the benchtop.
5. Open the appropriately labeled screw-cap microcentrifuge tube with your free hand. Insert the inoculated loop into the tube. Swish the loop around until the water looks cloudy. Close the tube, dispose of the loop in the appropriate waste container indicated by your teacher, and set the tube into a tube rack on your bench while you inoculate the remaining sample.
6. Repeat steps 4-5 for the remaining tube and chosen colony, using a fresh loop each time. Be sure to put the appropriate colony sample into the appropriate tube.
7. Place your tubes in the microcentrifuge on your bench, making sure that your tubes are balanced against other tubes in the opposite positions in the rotor. Centrifuge at 10,000 RCF for1 minute to pellet the cells to the bottom of the tubes. Wait 30 seconds before opening the microcentrifuge to allow any microaerosols to settle before you open the lid.
8. Gently pour off the liquid into a plastic beaker (or paper cup). Be careful not to disturb the cell pellet at the bottom. Do not shake the tubes. A small amount of liquid will remain in the tube.
9. You will use the p1000 micropipettor (set at 200) and an appropriate tip to transfer 200 ul of chelator beads solution to the cell pellet. Chelator beads will grab up any metal ions in a solution that might interfere with the PCR reaction you will be performing in the next lab period. To use the chelator beads, shake the beads solution to suspend the chelator beads – they settle to the bottom very quickly! Before the beads have had a chance to settle, transfer 200 ul to the tube containing your cell pellet. Look at the tip to make sure you have gotten at least a few chelator beads before transferring to your tube. Use the pipet tip to break up the cell pellet. Dispose of the pipet tip after a single use, into the biohazard bag.
10. Repeat step 9 for the remaining tube.
11. Mix the cells with the chelator beads by vortexing at high speed for 10 seconds, or by flicking the end of the tube roughly with your finger (your teacher will show you how to do this).

12. Place the tubes in the 100°C heat block for 8 minutes. This will burst open the cells, releasing the DNA.
13. Mix the cells with the chelator beads by vortexing at high speed for 10 seconds, or by flicking the end of the tube roughly with your finger (your teacher will show you how to do this).
14. Place the tubes in a balanced configuration in the microcentrifuge rotor, and centrifuge at 10,000 RCF for 2 minutes to pellet the chelator beads at the bottom of the tube. Wait 30 seconds before opening the microcentrifuge lid to allow any microaerosols to settle. After centrifuging, the supernatant will contain your DNA. DNA is very light, so the cell debris and chelator beads will fall to the bottom and the DNA will remain suspended in the liquid.
15. Obtain an additional 2 sterile microcentrifuge tubes and label them as before. Place them into the tube rack. You will be transferring the DNA from step 14 into these new tubes for storage until next time. Be sure not to mix your DNA samples together, and make sure you transfer the DNA from step 14 into a new tube labeled with the same information. You've worked hard to get your DNA; you don't want to mix up your samples at this stage! Use the p200 micropipettor (set at 100) and a sterile tip to transfer 100 ul of your DNA from step 14 into your new sterile microcentrifuge tube. Be careful not to transfer the chelator beads or any of the cell debris from the bottom of the tube. Dispose of the used tip in the used tip beaker, as indicated by your teacher. Place the labeled tubes containing your transferred samples into the rack indicated by your teacher for storage at -20°C until next time.
16. Dispose of your used microcentrifuge tubes into the biohazard bags as directed by your teacher, along with your used gloves. Spray down your benchtop with 10% bleach solution.

Be sure to wash your hands before you leave the lab area today.

IV. Lab Day Four- Amplifying your DNA

During the previous lab period you extracted DNA from two different colonies, which could be from four different species of bacteria, if they each had distinct colony morphologies. The extraction protocol used was fairly simplified, but has proven to be very effective at obtaining enough genetic material to amplify a selected region of the bacterial genome. In order to increase the chance we'll be able to identify the organism present in your sample, we will first perform a polymerase chain reaction (PCR) to selectively amplify the piece of DNA that we are interested in (the 16S rDNA).

The polymerase chain reaction (PCR) was developed by Kary Mullis in the 1980s, for which he won the 1993 Nobel Prize in Chemistry [11]. The basic process is similar to the process used by cells to replicate DNA, although there are a few key differences. PCR requires the user to supply all of the necessary reagents, which are found within the cell. These include the template DNA, two primers, deoxynucleotides (dATP, dCTP, dGTP, dTTP), a thermostable polymerase, water and some buffer (to keep everything working).

DNA replication begins with the separation of the DNA template strands, which is done by enzymes within the cell, while PCR uses a high heat step to break the hydrogen bonds that hold the two strands together. All of the steps for PCR happen inside a machine called a thermocycler, which will go through multiple heating/cooling steps in order to amplify a given template.

During cellular DNA replication, enzymes are used to produce an RNA primer to provide the free 3'OH group to initiate replication at the origin of replication. In PCR, the user provides two DNA primers that are complementary to either end of the region of the template that they wish to copy. This is an important difference, as only the region between where the primers are complementary will be replicated during a PCR reaction, while in a cell DNA replication results in the complete replication of the entire genome.

For our reactions, we will be using primers that should allow us to make millions of copies of approximately 280 base pairs of the V5/V6 region of the 16S rDNA. One of the primers is complementary to nucleotides 785 to 805 on one strand of the 16S rDNA template, while the other primer is complementary to nucleotides 1041-1061 on the opposite strand of the 16S rDNA template (remember that the two strands of DNA are paired together by complementary base pairing). When the two primers are used together in a PCR reaction, only the region between those

two primers will be copied, resulting in millions of copies of a piece of DNA that is ~280 base pairs in length.

After amplification, it is easy to determine whether your PCR reaction worked by running your samples on a SyBrSafe-stained gel. After separating the DNA by size and charge through an agarose gel, a positive PCR reaction should result in a single band in the gel, indicating that only the portion of DNA that you wanted to copy was actually copied. Review the special emphasis paragraph below for a general outline of how PCR can selectively copy a single region of DNA.

Special emphasis. PCR consists of three basic steps:

1) Denaturation (of the hydrogen bonds holding the template strands together) by heating to >95ºC.

2) Annealing of the primer to the template by cooling to the "melting temperature" of the primer set, which allows the primers to "sit down" on the template, also bringing in the thermostable polymerase to begin replicating the DNA.

3) Extension, which allows the DNA polymerase to add on nucleotides to the initial primer, resulting in two copies of the initial piece of DNA.

This process is then repeated 25-35 times, resulting in exponential copying of the selected portion of DNA.

General protocol for PCR amplification of the 16S rDNA sequence

Materials you will need:
4 tubes containing colony DNA, isolated during the previous lab meeting
1 sterile microcentrifuge tube
5 PCR tubes
Sterile water (one bottle per group of 2 students)
Pipet tips (P200, P20)
Pipettes (P200, P20, P10)
PCR tube rack
Ice bucket
iProof PCR Master Mix (2X)
Primer mix; Primers 1 and 2 equally pre-mixed, based on 16S rDNA sequence from [4]
Gloves
Microcentrifuge tube rack
Ultra-fine point sharpie

1. Spray your benchtop with 70% ethanol before beginning your work. Obtain the reagents listed above. Put on your gloves; these are being worn today in order to keep "you" from getting into your PCR tubes. Label your PCR tubes with your identifier code and some identifying mark that will let you know which tube contains the DNA from which of your original DNA samples. For example, C1 for "Colony 1". Place these tubes into your PCR tube rack, and put the rack into the ice bucket. Put your tubes containing your DNA from last time into your ice bucket.
2. Label your empty microcentrifuge tube as "MasterMix"(this will have 5 total reactions, to allow for fluctuations in pipettors) and place into your ice bucket. Add the following items to this tube, using a fresh tip each time (dispose of each used tip in the appropriate tip container):

   a. 125 ul of the 2X iProof Master Mix (this contains buffer, dNTPs and the polymerase)
   b. 15 ul of sterile water (keep this sterile- you never know when you might need it again!)
   c. 10 ul of the primer mix (this contains a mix of the forward and reverse primers).

3. Mix these together well by briefly vortexing or flicking the bottom of the tube. Place the tube back into the ice bucket.
4. Reset the P200 tip to 30 ul. Carefully open each of the PCR tubes in your rack and add 30 ul of your "Master Mix" to the bottom of each tube. You may use the same tip each time for this process, so long as you do not touch any outside surfaces. After adding your "Master Mix" to each of the four tubes, discard the used pipette tip. Save your Master Mix tube!
5. Your PCR tubes should now contain everything needed for the PCR reaction except for the DNA, all in 30 ul. They should also be in a PCR rack in an ice bucket. To finish prepping your tubes for PCR, you need to add your template DNA. You will add 20 ul of template DNA to each tube. Set your P200 pipette to 20 ul. Gently vortex one of your DNA tubes prepared last session. Obtain a pipette tip and carefully draw up 20 ul of template DNA. Check the end of your tip to be sure that you have 20 ul of liquid in the end of your tip. Carefully dispense these 20 ul into the bottom of the appropriately labeled PCR tube, trying not to get any bubbles in the tube. There should now be 50 ul of liquid in your PCR tube. Discard the pipette tip.
6. Repeat step 3 for the remaining three PCR tubes, disposing of the pipette tip between each sample.

7. In a fifth PCR tube, combine the remaining Master Mix with 20 ul of sterile water. This is your negative control for PCR.
8. Once you have all four tubes prepared, bring your sample (in your ice bucket) to the thermocycler. Place your PCR tubes into the thermocycler.

IMPORTANT: Be sure to note in your notebook and MOST IMPORTANTLY on the 48-well grid sheet provided the location of your PCR tubes so that you can get your samples back next week.

Even though you've labeled your tubes, that labeling may not stand up to the rigors of the thermocycler, so we need to have an additional means of identifying your samples. The grid sheet is laid out identical to the PCR rack, with letters going down the side of the rack from A-H and numbers across the top from 1-12. Your samples will be in (for example) A1, A2, A3, and you should write this information down on the grid. Your samples will be run using the program listed below.

a. Thermocycler program name: ORALBUGS

i. First denaturing step: 98ºC for 2 minutes

ii. Denaturing step: 98ºC for 10 sec

iii. Annealing step: 59ºC for 30 sec

iv. Extension step: 72ºC for 15 sec

v. Repeat steps ii-iv 30times

vi. Final extension: 72ºC for 5 minutes

vii. Hold: 16ºC indefinitely

b. The expected product should be about 280 bp

9. When finished setting up your tubes, dispose of your gloves in the biohazard bag. Place your DNA tubes back into the tube rack for your teacher to store.

Please record in your laboratory notebook:

	Label of sample	Location of sample in 48-well grid
1.	_____	_____
2.	_____	_____

V. Lab Day Five- Looking at your DNA

The PCR reactions run last time should have resulted in numerous copies of the 16S rDNA gene from your bacterial cells. To verify that everything worked as expected to, before you send samples out for sequencing, you will be running your samples on an agarose gel to separate out the bands of DNA based on size and charge. The agarose gel is run in a liquid buffer that has an electrical current running through it. The DNA will travel through the gel from the negatively charged anode to the positively charged cathode, because DNA carries a net negative charge. The agarose in the gel functions as a sieve to additionally sort the fragments based on size. We are looking for a single band on the gel, indicating that the primers were specific for only one region of DNA and that they made copies of the appropriate piece of DNA.

Materials you will need:
Your PCR reactions from last time
Pipette tips for P20 pipette
Pipette tips for P200 pipette
Pipette tips for P1000 pipette
P20 pipette
P200 pipette
P1000 pipette
1.2% Agarose E-gel
1kb ladder
1X E-gel Loading dye
Parafilm square
Nuclease-free or sterile water
Buffer PB
Buffer PE (with ethanol added)
4 each, Qiaquick spin columns
8 each, 2.0 mL collection tubes
8 each, 1.5 mL microcentrifuge tubes

1. Spray your benchtop with 10% bleach before beginning your work. Prepare the gel: Remove gel cassette from package and insert the gel (with comb in place) into the base, right edge first. The Invitrogen logo should be located at the bottom of the base. Press firmly at the top and bottom to seat the gel cassette in the PowerBase™. A steady, red light will illuminate if the gel cassette is correctly inserted. Pre- run the gel (with comb in place) by pressing and holding either the 15 minute or 30 minute button until the red light turns to

a flashing green light indicating the start of the 2 minute prerun. Release the button. At the end of the pre- run, the current will automatically shut off and the power base will display a flashing red light and beep rapidly. Press either button to stop the beeping.

2. Find the following items: your PCR reactions in a PCR tube rack, the P20 pipette and pipette tips, loading dye, sterile water and a piece of parafilm. Take these items over to one of the gel loading areas. Place the piece of parafilm down in front of the gel box and pipet 2 ul of loading dye onto one spot on the parafilm. Make three more 2 ul spots of loading dye on the parafilm. Discard this used tip. Pipet 10 ul of sterile water into each of these spots (no need to mix yet).
3. Gently mix your PCR reactions either by flicking the tubes or vortexing. Set your pipet to 8 ul, obtain a new tip, and draw up 8 ul of one of your PCR reactions. Mix this into one of the spots of loading dye on the parafilm by pipetting up and down several times. Carefully pull up all of the liquid from this one spot (20 ul) and load it into the second lane from the left on the agarose gel. Carefully note which gel and which lane you have loaded your sample into.
4. Repeat this process (Step 2) for your remaining three samples and negative control, loading each into the next lane to the right. Keep your PCR reactions at your bench. We will run these gels now to determine which samples will be used for sequencing.
5. Each gel should have a ladder run on it (~8 ul) to determine the size of the fragments that are amplified. In addition, at least one gel will have the following control samples run, to ensure that the PCR reactions worked appropriately:
    a. E. coli DNA
    b. Saccharomyces cerevisiae DNA
6. If there are any remaining lanes in the gel, fill each of them with 20 ul of sterile water. In the space provided answer the following question: When using the primers for 16S rDNA, one of these samples should produce a specific band, while the other should not. Which one should not produce a band, and why not?
7. Press and release the 30 minute button on the E- Gel® PowerBase™ to begin electrophoresis. At the end of the run, the current will automatically shut off and the power base will display a flashing red light and beep rapidly. Press either button to stop the beeping. Remove the gel cassette and analyze your results by viewing on one of the transilluminators. The instructor may

take a picture of your gel as well, to facilitate quantifying the amount of DNA present in your samples. This is important when we set up the sequencing reactions.

8. While the gel is running, clean up the reactions using the QiaQuick kit from Qiagen. Label a new 1.5 mL tube for each PCR reaction, then use a P200 pipettor to place 200 ul of Buffer PB into each 1.5 mL tube. Next, transfer 40 ul of each of your PCR samples into the corresponding 1.5 mL tubes and mix. Check that the color of the mixtures is yellow. If they are yellow, proceed to the next step. If not, see your instructor.
9. Place a QIAquick spin column in a 2 ml collection tube. For your first PCR sample (now mixed with Buffer PB), transfer the entire sample to the QIAquick column and centrifuge at 10,000 RCF for 60 seconds. Discard the flow-through (liquid at the bottom of the tube) by dumping in a waste beaker. Place the QIAquick column back into the same tube. For this same sample, use a P1000 pipettor to transfer 750 ul Buffer PE to the QIAquick column and centrifuge at 10,000 RCF for 60 seconds. Discard the flow-through again, and place the QIAquick column back in the same tube. Centrifuge the column for 1 minute at 10,000 RCF (you should not add liquid to the top of the column this time). After centrifuging, place the QIAquick column in a clean 1.5 ml microcentrifuge tube – make sure this tube is properly labeled; you don't want to mix up your samples now! For the final step, add 30 ul nuclease-free water to the center of the QIAquick membrane, let the column incubate at room temperature for 1 minute, and then centrifuge at 10,000 RCF for 60 seconds.
10. Repeat the cleanup for the remaining three samples. Now, the PCR products can be sent for sequencing. If you are preparing for sequencing today, proceed to the next section. If you are preparing for sequencing in the next lab session, please place your "clean" DNA into the community microcentrifuge tube rack, again indicating where your samples are in the new grid. This will ensure you get your clean samples back next time.

VI. Lab Day Six- Sequencing your DNA

The bands that you saw on the gel at the end of day five were the result of multiple rounds of replication of a single portion of the DNA template that was present in the original sample. The presence or absence of a band can provide you with some information about your sample. If a single band of the appropriate size is present in the gel, this suggests that there was an appropriate DNA template present in your sample, one that contained sequences that were complementary to the E.coli-based 16S rDNA primers that were used in the PCR reaction. This would suggest that perhaps you amplified bacterial 16S rDNA, although it doesn't guarantee that is what was amplified. If you ran a sample of known E. coli 16S rDNA in the PCR reaction and then on the gel, this would provide further evidence that you amplified the appropriate DNA fragment. If you also ran a PCR reaction using those primers and S. aureus DNA and obtained a band it would suggest that the primers work on more than just one type of bacteria, which could be helpful if you didn't know exactly what type of bacteria you were working with. Finally, if you used a sample containing eukaryotic DNA and the 16S rDNA primers and didn't see a band, you could feel fairly confident that the primers were specific for prokaryotic DNA and not eukaryotic DNA. All of this information still doesn't tell you exactly what type of DNA you've amplified. For this, you will need to determine the sequence of the DNA that you amplified, to see

1) if your primers amplified the 16S rDNA gene and if so,

2) what species of bacteria was the sequence amplified from?

Reading the sequence of 16S rDNA can help in identifying an unknown sample because there are enough 16S rDNA sequences available in the database to allow for comparison across thousands of species to see how closely related different sequences are.

In the early days of DNA sequencing the samples you obtained last time would have been run on a very large sequencing gel using radioactive di-deoxynucleotides to determine the sequence of the DNA we had amplified. Luckily, we no longer need to run these reactions ourselves but can simply supply our template DNA, one primer and a little water in tube and send the samples out to be sequenced for us. This process again is similar to the process that occurs during DNA replication in the cell. This time, however, in order to identify the nucleotide sequence special nucleotide analogs called di-deoxynucleotides are used that will terminate replication after they are incorporated into the sequence. This is because they do not have a free 3' hydroxyl group to allow

the addition of the next nucleotide. In addition, they are labeled with a fluorescent dye that can be detected and read by the sequencer. Each di-deoxynucleotide (ddATP, ddCTP, ddGTP, ddTTP) has its own color, so they can be readily distinguished from one another. The results that are sent back from sequencing facilities will include the raw data obtained from the sequencing read and the sequence itself. You will use these results to identify at least one of the bacteria present in your mouth.

Materials you will need:
Your cleaned-up PCR reactions from last time
P20 pipette and pipette tips
Primer 1
Two clean PCR tubes

1. Spray your benchtop with 10% bleach before beginning your work. Obtain four PCR tubes, and label with your identifier code and your sample number.
2. Use all of your samples that gave a nice band on the gels run last time. Using the P20 pipette set to 8 ul, transfer 8 ul of your first PCR sample to the corresponding PCR tube. Repeat for the remaining three PCR samples.
3. Gently mix the tube containing Primer 1. Set the P20 pipette to 4 ul. Obtain a new tip and transfer 4 ul of Primer 1 to your first sequencing tube marked “P1”. Discard the used tip. There should now be 12 ul of liquid in your sequencing tube. Repeat this for the remaining three PCR samples.
4. Take your labeled sequencing PCR tubes to the community PCR rack and ice bucket. Place your tubes into the PCR rack, and indicate in the 96-well gridsheet where your sample is located in the rack. This is again going to be important for identifying which sequence belongs to you when it comes back from Eurofins. The instructors will transfer a portion of your sample into a 96-well plate format for sequencing, as this is less expensive than running individual tubes. The results should be available within ~48 hours of submitting the sequences.

VII. Lab Day Seven- Identifying your bacteria

At last, we reach the culmination of all of our work of swabbing cheeks, plating, waiting for growth, analyzing colonies, extracting DNA, running PCR, cleaning up reactions and sending the samples out for sequencing. We should have received all sequencing results via the Eurofins site by now, and you should be ready to take the next step to determine the possible identity of at least one member of your oral microflora.

The typical process for this step would be to analyze your sequence traces, to verify that the sequences that the computer automatically "called" for you were actually what they should be. An example of a sequence trace is shown below, using a program called "Geneious". Note that the first 30 or so peaks on the chromatogram are "junky", and often overlap. These peaks correspond to the nucleotides that were incorporated into the growing DNA chain during the sequencing reaction. However, even though the machine suggests that it could distinguish which nucleotide was added to the growing chain, it is clear from the chromatogram that some of the "calls" that the computer made could not possibly be accurate (look at the first 15 peaks in particular). Because it takes a bit of time and experience to be able to read a chromatogram, you are not going to do that step of the analysis. The results that were sent to you were the text files indicating the bases that the computer determined for your sequences, with the "junky" ends trimmed off. This information is sufficient to move on to the next phase in the analysis, which is to perform a BLAST search.

What is a BLAST search? BLAST stands for Basic Local Alignment Search Tool, and it is one of the most-used tools in bioinformatics. It can be found on the National Center for Biotechnology Information website at http://blast.ncbi.nlm.nih.gov/. The BLAST search tool has been around since 1990 [12], as a way to search through, organize and compare the millions of sequence records (DNA and protein) that have accumulated (and continue to accumulate) at an exponential rate. The BLAST tool allows you to enter your query sequence on the database server and it then uses an algorithm to compare it to all the sequences present in the database, depending on your search parameters. The final output is then an alignment of your sequence with all the sequences that the search program decided that it is similar to, along with some numbers to help you determine how well those sequences match one another. How to perform a BLAST search may have already been discussed in class, so we won't go into detail here, other than to explain what to do with your sequence results.

General protocol for performing a BLAST search.

Materials you need: your sequence text files, access to a computer with internet access

The assignment: identify the top hit for your sequence using the BLAST algorithm, which will provide you with the identity of one of the organisms found in your mouth. After identifying this organism, find out a bit more information about it, such as whether it is a gram positive or gram negative microorganism, is it something that would normally be found in a human mouth, is there anything else interesting about this microbe? We will also use the sequence information to construct a phylogenetic tree of the bacteria throughout our small "community", with a combination of Clustal Omega, ClustalW2 and the ETE Toolkit, in the next lesson focused on Phylogenetics (to be distributed separately).

1. Go to the NCBI BLAST webpage at http://blast.ncbi.nlm.nih.gov/.
2. Click on the link for "nucleotide blast"
3. In the box that says "Enter Query Sequence", do the following. On the first line, it is helpful to include some information about your sequence. This should be entered exactly as shown below, replacing the phrase "My tooth sequence" with your name and however you identified your specimen throughout this experiment.

   > My tooth sequence
4. After entering that information, you need to add a carriage return by pushing the "enter" button to move the cursor to the next line. On this next line, you should copy and paste the text for one of your DNA sequence files. Only enter one of the sequences at this time.
5. Under the "Choose Search Set" section, be sure to check the "Others (nr etc)" choice instead of the default "Human genomic+transcript". If you leave it on the default, it will only be searching for similarities among human sequences, and hopefully you have amplified prokaryotic sequences. "nr" stands for non-redundant, which means that it will not search through sequences that are incomplete or partial reads.
6. You may leave all the other parameters on the default settings initially. After making the indicated changes above, feel free to click the "BLAST" button.
7. After a period of time, you will be taken to your results page. It should look something like the screen shots below. Reading the color coded heat map will give you some pretty good information as to how well your sequence aligned to the sequences in the database. Each of

colored lines corresponds to a single alignment between your sequence and a sequence in the database. You can point your mouse over the top of each one to see what each sequence is, or you can scroll further down the page to see those alignments for yourself.

8. The next screen shot shows additional details about your sequence alignment, providing you with the hard numbers such as "Max score", "E-value", "Query coverage" and "Max. Ident."
    a. The information that is most useful is E-value, which tells you the likelihood that this match happened by chance. In general, the lower the e-value (the closer it is to zero), the better (although this is not always true- read on).
    b. The "Query coverage" statistic tells you how much of the length of your sequence matches the sequence in the database. This is important, because you can have a very low E-value, but if only 10 nucleotides out of 200 nucleotides in your original query are all that is included in that match, then the E-value doesn't really matter here and you don't have a good match.
    c. Another important statistic is the "Max. Ident.", which stands for maximum identity. This tells you how many of the nucleotides in your sequence are identical to the given sequence from the database.

9. Finally, if you click on the hyperlink listed under "Max score", it will take you to the actual sequence alignment of your sequence with the sequence from the database. This will show you exactly how well the two sequences match. Use this information to complete the homework assignment listed above.

What happens next?

After all of this work, you are perhaps wondering if this is really how microbiologists identify the bacteria living in a given environment. This is the way that well-known microbiologists such as Robert Koch and Louis Pasteur first identified various microorganisms, especially those that are involved in disease in humans and animals. Robert Koch, a German scientist, developed a set of postulates (rules) that needed to be observed in order to link a particular microorganism to a particular disease. His work proved that specific bacteria cause anthrax, tuberculosis and cholera, diseases that have played important roles in human health. In order to apply Koch's postulates to an organism, however, microbiologists must be able to do one very important thing. Think back to day

one of this experiment, when you swabbed your cheek and used that swab to inoculate an agar plate. What would have happened if the bacteria in your mouth were unable to grow on the plate? What if the nutrients were not available in the media for the bacteria to grow? What if oxygen was actually deadly to the bacterial cells that you swabbed on your plate? Nothing would grow on your plate! Does that mean that there are no bacteria present? No, it does not.

With the advent of DNA sequencing technology and techniques for obtaining DNA from many different types of environments, it has become clear that less than 1% of the microbes present in a given environment are able to grow in the lab and that many more species are present than were originally thought [1]. To identify the missing 99%, microbiologists have begun to sequence the 16S rDNA gene from non-cultured samples to identify all of the microbes present in a given sample site. This field of science, called "metagenomics", allows scientists to study all of the DNA present in a given site, whether it's bacterial, viral, or eukaryotic.

Metagenomics is a fast-growing field and is being used to answer questions about the populations of microbes present in environments as diverse as the human mouth, the human gut, deep-sea thermal vents, and hyena scent glands. The most important thing required, after a source of DNA, is some sequence within all those many DNA sources that will be able to represent all of a given type of organisms present in a sample, whether bacterial or fungal, for example, and that can differentiate between the types of organisms present. As we talked about on day 4, this is why bacteriologists choose to use the 16S rDNA sequence to identify the types of bacteria present in a sample. Metagenomics allows microbiologists to study and identify microbes that can't be grown in the laboratory, because the conditions needed for growth just aren't known. Metagenomics comes with its own downsides, as sequencing a metagenome results in tens of thousands (or more) sequences that need to be analyzed from a single sampling site, and this sequencing is not cheap. However, it does open up entirely new areas for research, and is allowing scientists to ask questions about how different microbes and microbial populations may play a role in health and disease.

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You and your oral microflora BLAST assignment

Follow the instructions in your Oral microflora handout to complete this assignment.

Please provide the following information about your sequence:

1. The name of your sequence (e.g. SC): _____
2. The amount of DNA you used when you set up your sequencing reaction: _____ (i.e. which recipe did you follow?)
3. The length of the sequence you pasted into the BLAST algorithm: _____
4. Identify the top hit for your sequence using the BLAST algorithm (note, if your top hit is an "uncultured bacterium, indicate that here, and refer to the "Uncultured bacterium instructions" below to answer question 5).
    a. Accession number: _____
    b. Description: _____
    c. E-value:_____
    d. Query coverage: _____
    e. Max identity: _____

    The following information can be found by clicking on the hyperlink for your top hit's "Max Score", which will take you to the actual alignment of your "query" sequence to the "subject" sequence that it matched in the database.
    f. Identities: _____
    g. Gaps: _____
    h. Were all of the nucleotides that you submitted used to find a match in the database? In other words, if your query sequence was 245 nucleotides, were all 245 nucleotides accounted for in the identities equation, or for example, were only 150 nucleotides used? _____
    i. If only a portion of your query sequence was used, what could that mean about the strength of your match? _____
    _____
5. Now that you know the identity of one of the organisms in your mouth, you need to find a little bit more information about this organism. Use the websites listed below to help

you find information about your newly identified oral microflora. (NOTE: if your top hits are "uncultured bacterium", see number 6 below)

a. Is it gram positive or gram negative? _____

b. Is it typically found in the human mouth? (If not, please indicate where it is normally found, and why you think you might have found it in your sample).

_____

c. Can it be found elsewhere on the human body? If so, where else can it be found?

_____

d. Find one other interesting fact about this organism, and indicate where you found this information.

_____

_____

_____

_____

6. **"Uncultured bacterium" instructions.** If your top hit is an uncultured bacterium, repeat your BLAST search with the following changes: In the "Choose Search Set" section, be sure that you have checked the box marked "Uncultured/environmental sample sequences" next to "Exclude". This will exclude unknown species from the database search and should provide you with the information to answer question 5 above.

Some useful websites for finding information about microorganisms.

NCBI PubMed, a resource for scientific literature (experimental research and scientific reviews): http://www.ncbi.nlm.nih.gov/pubmed/

NCBI Bookshelf, a resource of scientific textbooks: http://www.ncbi.nlm.nih.gov/books

Microbeworld, a website sponsored by the American Society for Microbiology (www.asm.org) to enhance the understanding of microbiology, and to provide access to the latest in microbial research: http://www.microbeworld.org/

Centers for Disease Control and Prevention, a division of the federal Department of Health and Human Services: www.cdc.gov

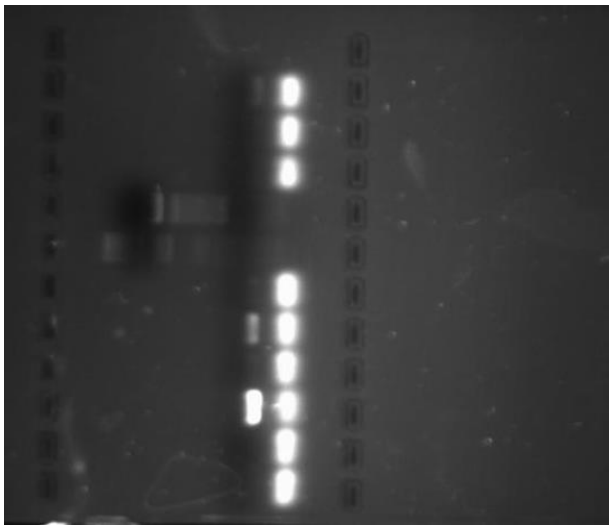
Practical Question

1. (1 point) A sampling of the cells in the human mouth would show that
    a. There are more bacterial cells than human cells.
    b. There are more human cells than bacterial cells.
    c. There are an equal number of bacterial and human cells.
    d. There are no bacterial cells in the human mouth.

2. (1 point) One difference between replicating DNA by PCR and cellular DNA replication is
    a. PCR uses enzymes to separate DNA strands.
    b. PCR makes many copies of all the DNA in the test tube.
    c. PCR uses a polymerase to copy DNA
    d. PCR makes many copies of only a targeted portion of the DNA in the test tube.

3. (1 point) Your friend learned in class recently that some antibiotics work because they target the ribosomal subunits of prokaryotes but don't affect the ribosomes of eukaryotes. Your friend isn't sure how this can be true, since both eukaryotes and prokaryotes use ribosomes to make proteins. You explain to your friend that this is because the ribosomal genes are different in prokaryotes and eukaryotes and you can prove it by performing a PCR reaction for a ribosomal gene. You use the primers for the 16S rRNA gene, DNA from a prokaryote and DNA from a eukaryote, and run a gel to show what the PCR product looks like. What do you expect to see on the gel?
    a. A band of the appropriate size in the lane with eukaryotic DNA, no band in the lane with prokaryotic DNA
    b. A band of the appropriate size in the lane with prokaryotic DNA, no band in the lane with eukaryotic DNA
    c. A band of the appropriate size in both the prokaryotic and eukaryotic DNA lanes.

4. (5 points) Look at the gel shown below. Based on what you know about the charge on DNA and how DNA runs through a gel, indicate on the gel 1) which is the positive end of the gel, 2) which is the negative end of the gel, 3) which set of wells the samples were loaded into, 4) which direction the DNA ran in the gel, and 5) which bands correspond with the smallest pieces of DNA.



5. Your little sister had a secret stash of "study foods" in her apartment that she shares with three other students. She noticed that her secret stash was disappearing and she thought perhaps one of her roommates was sneaking in and stealing some of her food. Her roommates said it wasn't any of them stealing the food, but that maybe there were some mice or stray cats getting into the stash and eating it (your sister tends to leave her window open, and there is a balcony outside her window). Your sister ordered a do-it-yourself CSI kit from the internet to discover the culprit. She found a sample of DNA on one of her pilfered snacks, did a PCR reaction to amplify a portion of the genetic material and then did a BLAST search to try to figure out what it was. She isn't quite sure what the results mean, so she's asked you to help her figure out which of the following "hits" is most likely to be the food thief. Looking at the results below, tell your sister which sequence from the database best matches the organism that is stealing her food (circle your choice). Then answer this question: Does she need new roommates, or does she need to invest in some better screens for her windows?

Hit #1

Select All Get selected sequences Distance tree of results

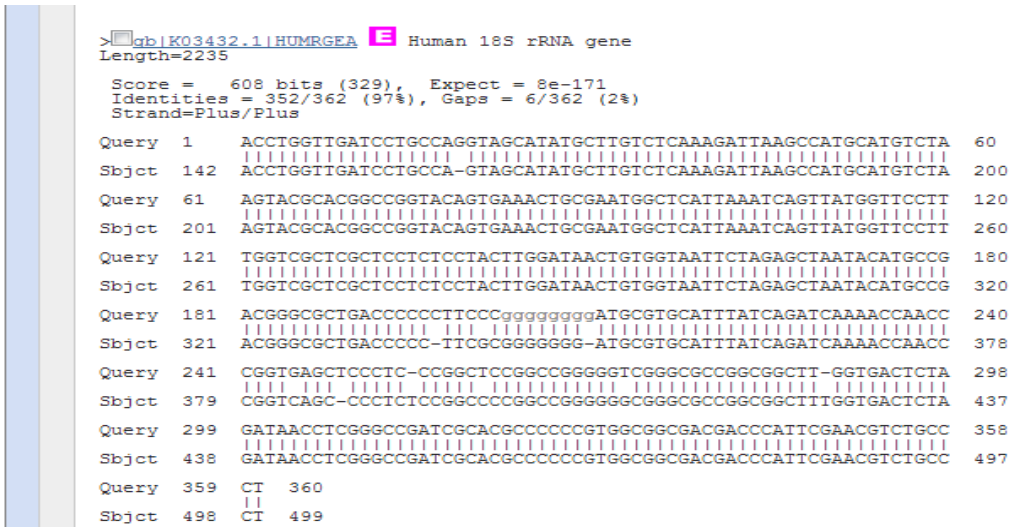
>ref|NR_046233.1| Mus musculus 45S pre-ribosomal RNA (Rn45s), ribosomal RNA
Length=13404

GENE ID: 100861531 Rn45s | 45S pre-ribosomal RNA [Mus musculus]
(10 or fewer PubMed links)

Score = 665 bits (360), Expect = 0.0
Identities = 360/360 (100%), Gaps = 0/360 (0%)
Strand=Plus/Plus

```
Query  1     ACCTGGTTGATCCTGCCAGGTAGCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4008  ACCTGGTTGATCCTGCCAGGTAGCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA  4067

Query  61    AGTACGCACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4068  AGTACGCACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTT  4127

Query  121   TGGTCGCTCGCTCCTCTCCTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCCG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4128  TGGTCGCTCGCTCCTCTCCTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCCG  4187

Query  181   ACGGGCGCTGACCCCCCTTCCCgggggggATGCGTGCATTTATCAGATCAAAACCAACC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4188  ACGGGCGCTGACCCCCCTTCCCGGGGGGGGATGCGTGCATTTATCAGATCAAAACCAACC  4247

Query  241   CGGTGAGCTCCCTCCCGGCTCCGGCCGGGGGTCGGGCGCCGGCGGCTTGGTGACTCTAGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4248  CGGTGAGCTCCCTCCCGGCTCCGGCCGGGGGTCGGGCGCCGGCGGCTTGGTGACTCTAGA  4307

Query  301   TAACCTCGGGCCGATCGCACGCCCCCCGTGGCGGCGACGACCCATTCGAACGTCTGCCCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4308  TAACCTCGGGCCGATCGCACGCCCCCCGTGGCGGCGACGACCCATTCGAACGTCTGCCCT  4367
```

Hit #2



>gb|K03432.1|HUMRGEA Human 18S rRNA gene
Length=2235

Score = 608 bits (329), Expect = 8e-171
Identities = 352/362 (97%), Gaps = 6/362 (2%)
Strand=Plus/Plus

```
Query  1     ACCTGGTTGATCCTGCCAGGTAGCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA  60
             ||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  142   ACCTGGTTGATCCTGCCA-GTAGCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA  200

Query  61    AGTACGCACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201   AGTACGCACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTT  260

Query  121   TGGTCGCTCGCTCCTCTCCTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCCG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261   TGGTCGCTCGCTCCTCTCCTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCCG  320

Query  181   ACGGGCGCTGACCCCCCTTCCCgggggggATGCGTGCATTTATCAGATCAAAACCAACC  240
             ||||||||||||||| ||| |||||||| ||||||||||||||||||||||||||||||
Sbjct  321   ACGGGCGCTGACCCCC-TTCGCGGGGGGG-ATGCGTGCATTTATCAGATCAAAACCAACC  378

Query  241   CGGTGAGCTCCCTC-CCGGCTCCGGCCGGGGGTCGGGCGCCGGCGGCTT-GGTGACTCTA  298
             |||| ||| ||||| ||||| |||||||||| ||||||||||||||| |||||||||||
Sbjct  379   CGGTCAGC-CCCTCTCCGGCCCCGGCCGGGGGGCGGGCGCCGGCGGCTTTGGTGACTCTA  437

Query  299   GATAACCTCGGGCCGATCGCACGCCCCCCGTGGCGGCGACGACCCATTCGAACGTCTGCC  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438   GATAACCTCGGGCCGATCGCACGCCCCCCGTGGCGGCGACGACCCATTCGAACGTCTGCC  497

Query  359   CT  360
             ||
Sbjct  498   CT  499
```

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Survey Questionnaire

The following questionnaire is anonymous. *Your feedback is very important in helping me understand what aspects of this course need to be changed.* Thank you for your candid comments! Please indicate:

Subject/Period:
Date:

1A. Please rank your *interest* in the topics covered in this class **BEFORE** taking the course (1= no interest at all, 5 = very interested).

1 2 3 4 5

1B. Please rank your *interest* in the topics covered in this class **AFTER** taking the course (1= no interest at all, 5 = very interested).

1 2 3 4 5

1C. If your interest in the material has changed (either increased or decreased) please explain why.

1D. Which concepts in the course, if any, were easier to understand when you had the opportunity to work on them in a lab setting?

2A. Please rate the extent to which you feel the material covered in this course is relevant to your short and long term goals: (**1= not relevant, 5= highly relevant**)

1 2 3 4 5

2B. Please rate the extent to which you feel the material covered in this course has influenced your motivation in this course: (**1= not relevant, 5= highly relevant**)

1 2 3 4 5

2C. Please explain your answer to 2A and 2B above.

Modified from Freeman et al., (2007) and Madhuri & Broussard (2008).

3A. Please rank each of the following elements of the lab portion of this course according to how helpful you found them to be in terms of your learning. **(1 = not helpful, 5 = extremely helpful)**

Pipetting	1	2	3	4	5
Culturing oral microbes	1	2	3	4	5
Using microscope	1	2	3	4	5
DNA extraction	1	2	3	4	5
Gel Electrophoresis	1	2	3	4	5
DNA sequence	1	2	3	4	5
Analyzing DNA sequence with BLAST	1	2	3	4	5
Proteomics	1	2	3	4	5
Bacterial transformation	1	2	3	4	5
Weekly quizzes	1	2	3	4	5

3B. How did designing of your own scientific investigation affect your lab experience? (**1= not at all, 5 = extremely well**)

Learned more about experimental techniques	1	2	3	4	5
Made lab more interesting	1	2	3	4	5
More trial and error, more stress	1	2	3	4	5

4A. How well do you feel the lab portion of this course prepared you for the tests? (**1= not at all, 5 = extremely well**)

1 2 3 4 5

4B. How well do you feel the lab portion of this course prepared you for planning your individual scientific investigation/open inquiry? (**1= not at all, 5 = extremely well**)

1 2 3 4 5

4C. How well do you feel your study strategy prepared you for tests? (**1= not at all, 5 = extremely well**)

1 2 3 4 5

4C. Please provide more information on your study strategy.

4D. Do you have any comments or clarifications for your answers to the above questions about exams (4A-4C)? Do you have anything to add about examinations?