Laboratory 8: Amplification of 16S rRNA Genes from Your Oral Microbes (Thursday Morning)

Background Links for Laboratory 8.1:
2. Human Microbiome Project. www.hmpdacc.org/

Objective of Laboratory 8.1:
Set up ten PCR reactions specific for the 16S gene of a microbial domain or genus using your buccal DNA sample

INTRODUCTION: After reading some of the assigned links for this laboratory, you might be somewhat hesitant to even open your mouth for fear of what you might encounter there. As you have learned, 100 trillion microbes are known to reside in your mouth and several other body niches. Scientists have recently determined that there are more than 600 species of bacteria in the oral cavity alone. The Human Microbiome Project was initiated in 2007 by the National Institutes of Health in an effort to identify and characterize the different microbial species that live both on and in humans. Scientists at TIGR are using a metagenomic sequencing strategy to identify more oral microbial species, which will undoubtedly lead to other studies about the roles these microbes play in health and physiology.

In an effort to determine which oral microbes are associated with different diseases and disorders, scientists plan to compare microbial species from healthy individuals to those suffering from periodontitis, tooth decay, and other diseases. This information will not only identify these microbes but also help elucidate their roles in the disorders. Scientists also hope to determine the composition of oral bacterial biofilms and elucidate the commensal relationships that exist among different microbes and also between microbes and us. Our microbial population (which is estimated to outnumber our own cells by 10 – 20 times!) is believed to play a role in our health because changes in our microbial commensals that lead to absence or presence of beneficial microbes can result in unfavorable changes in metabolism and perhaps even the development of diseases such as inflammatory bowel disease (IBD).
II. EXPERIMENTAL PROCEDURES (You should know the drill by now!):

To investigate the microbial diversity of your oral microbiome, you will set up PCR reactions specific for ten microbes commonly found in the oral cavities of healthy individuals as described next.

A. Setting up PCR Reactions for the 16S rRNA of Oral Microbes: Each person will set up ten PCR reactions specific for the 16S rRNA genes of different oral microbes using the buccal DNA that you isolated last Tuesday.

1. Take an Isotherm and ice to obtain ten 0.65-ml tubes containing the following primer mixtures from the front bench. All oral primers were designed by Dr. Sliski (unpublished results) using PrimerQuest online software that is available from IDT, Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/).

   a. Primers for *Streptococcus mitis* (labeled “Sm1”), a gram positive coccus.

   b. Primers for *Granulicatella elegans* (labeled “Ge1”) a gram positive coccus that lyse red blood cells.

   c. Primers for *Granulicatella adiacens* (labeled “Ga1”) a gram positive coccus that lyse red blood cells.

   d. Primers for *Gemella haemolysans* (labeled “Gh5”) a gram positive coccus.

   e. Primers for *Veillonella parvula* (labeled “Vp1”) a gram negative, anaerobic coccus.

   f. Primers for *Campylobacter concisus* (labeled “Cc1”) a gram negative rod.

   g. Primers for *Haemophilus parainfluenzae* (labeled “Hp1”) a gram negative rod.

   h. Primers for *Megasphaera micronuciformis* (labeled “Mm1”) a gram negative coccus.

   i. Primers for *Abiotrophia defectiva* (labeled “Ad1”) a gram positive coccus.

   j. Primers for *Neisseria subflava* (labeled “Ns1”) a gram-negative diplococcus.

Continue to use ARTips when pipetting. Please **put on fresh gloves** before beginning.

The primer mixtures contain equal parts of forward and reverse primers.

All these bacteria are normal inhabitants of the human oral cavity but may be linked to disease in other tissues.

*S. mitis* grows on the surface of teeth and may contribute to the growth of oral pathogens.

This microbe is the source of the RE HpaI.
2. **Each person** should obtain ten clear 0.5 ml Ready-To-Go PCR Bead Tubes from the front bench.

3. Tap each tube gently on your bench to ensure the bead is at the bottom of the tube before opening that tube.

4. Label the tops and sides of these ten tubes using a fine-tipped black marker with the letters of one of the ten acronyms designating the ten different primers, e.g., “Sm”, “Ge”, etc.

5. Using your P200 set to “0-2-2.5” and a fresh 200 µl ARTip, add 22.5 µl of each of the primer mixtures to one of the ten appropriately labeled Ready-To-Go Bead Tubes as you did in the D1S80 lab. **Use a clean tip for each primer mixture.**

6. Flick all ten Bead Tubes gently but well with your fingers until the PCR beads are thoroughly dissolved.

7. Using your P20 with a fresh 20 µl ARTip, transfer 2.5 µl of your buccal DNA to each of the ten Ready-To-Go PCR Bead tubes you just prepared. **Be sure to use a fresh tip for each transfer.**

8. Flick these tubes gently but thoroughly to mix.

9. Spin all ten tubes 10 sec in the microfuge. **This brings all droplets to the bottom of each tube.**

10. Obtain one strip of eight 0.2-ml PCR tubes and a PCR rack from the front bench. The other two samples will remain in the 0.5-ml Bead tubes. **Be sure to wear gloves when obtaining these tubes.**

11. Use a black marker to label each PCR tube with your photo ID number and the letters of one of the first eight acronyms denoting the different primer pairs for oral microbes. **Label the side and hinge of each tube if possible. Use the strip of 8 tubes for reactions a – h.**

12. Set your P200 to “0-3-5” (the bead adds volume) and use a new tip to transfer the entire contents of the first 8 Ready-To-Go Bead tubes into one of the strip of PCR tubes with the matching label. **Transfer reactions a – h described above and on the previous page into the appropriately labeled strip tube.**

13. Leave reactions “i” and “j” (“Ad1” and “Ns1”) in the 0.5-ml Bead tubes.

14. Return the tube containing the buccal DNA you isolated to the front lab bench for storage. **Make sure your initials or photo ID number is still visible.**
15. Bring your strip of eight PCR tubes to the front of the lab and put them into the PCR rack.

16. Bring your remaining two tubes labeled “Ad1” (i) and “Ns1” (j) to the hallway and load them into the Techne cycler.

Your oral microbe PCR reactions will be amplified using the conditions described below.

95° C 10 min

30 cycles:

- *Denaturing time and temperature:* 15 sec at 95° C
- *Annealing time and temperature:* 15 sec at 65° C
- *Extending time and temperature:* 30 sec at 72° C

72° C 10 min
Laboratory 8.2: Analysis of the PCR Products from Your Oral Microbes using E-Gel Electrophoresis (Thursday Afternoon)

Objective of Laboratory 8.2: Analyze your oral microbe PCR products using e-gel electrophoresis

I. EXPERIMENTAL PROCEDURES: Although you should almost be able to load and run an e-gel in your sleep by now, instructions for this process are provided (yet again) below.

A. Preparing Your PCR Products for Electrophoresis: Each person should obtain an Isotherm, ice and her/his ten PCR products from the front bench.

1. Add 2.0 µl loading dye (clear tube, purple dot) to each of your ten PCR tubes. > Do not discard this tube of loading dye.

2. Flick the tubes with your fingers to mix in the dye and spin these tubes in the microfuge for a few seconds using black adaptors. > This pulse spin will bring the samples to the bottom of the tubes.

B. Preparing your E-Gel for Electrophoresis: Each person will use a 2% e-gel today to estimate the diversity of your oral microbes. Use scissors to open the e-gel package and prepare this gel as you have previously as described in the Appendix. Each person should prepare an e-gel and follow the directions below.

1. Obtain two tubes of the 100 Base Pair Ladder (clear tube, orange cap, black line) from the front bench. > Remember to always wear gloves when working with e-gels because they contain ethidium bormide.

2. Load 20 µl of the 100 bp Ladder (clear tube, orange cap, black line) into lane 1 and lane 12 of your e-gel.

3. Load 20 µl of each oral PCR product sample into one well in the order in which the primers are listed on p. 88. > Be sure to record the order in which your samples are loaded.

4. As usual, be careful not to introduce bubbles while loading, as they will cause bands to distort. > You can avoid introducing bubbles by setting your Pipetman to 20 µl, which is the exact volume you want to load.

5. Add 20 µl of water to any empty wells. > Add 20 µl of this mixture to any empty wells.
6. Press the 30 min button to begin the run.

7. After the electrophoresis ends, put on gloves and remove the gel cassette from the apparatus.

8. Place the cassette on top of the UV transilluminator and take four photographs of your gel, one for you and three for Drs. Sliski and Thieringer and the lab staff. Thank you!

> Check that the dye is moving out of the well.

> Remember to use the red filter with the camera. The camera should be set to 4.5 (f stop) and 2 (an exposure time of 1/2 sec). If a lighter exposure is needed, set the camera to 4.5 and 1 (exposure time of 1 sec).