PCR Protocol

Step 1: PCR

Need:

- Plated colonies
- Eppendorf tubes
- Molecular grade (nuclease free) H2O
- Phusion® High-Fidelity PCR Master Mix with HF Buffer
- 27F and 1492R primers (10 uM) for 16S sequencing
- Ice bucket

Picking colonies

You will receive plates with 6 different bacteria colonies from your TA Laura.

1. Transfer 100 µl water to Eppendorf.
2. Use pipette tip to pick colony and stab into water to make a dilute cell suspension. Mix by pipetting or flicking several times.

PCR

The following guidelines are provided to ensure successful PCR using Phusion® Master Mixes. These guidelines cover routine PCR.

Reaction Setup: We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler. All components should be mixed prior to use. It is important to add Phusion Master Mix last in order to prevent any primer degradation caused by the 3′→ 5′ exonuclease activity.

Component	20 µl Reaction	Final Concentration
Nuclease-free water	7 µl	
10 µM Forward Primer (27F)	1 µl	0.5 µM
10 µM Reverse Primer (1492R)	1 µl	0.5 µM
Dilute cell suspension	1 µl	
2X Phusion Master Mix	10 µl	1X

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Notes: Gently mix the reaction. Also, make two mixtures per sample. One will be used to test the quality of the reactions. The other will be used for Sanger sequencing.

Transfer PCR tubes from ice to a PCR machine and begin thermocycling:

Thermocycling conditions for PCR with 27F and 1492R primers:

STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	3:00 minutes
40 Cycles	98°C 51°C 72°C	10 seconds 30 seconds 45 seconds
Final Extension	72°C	10 minutes
Hold	4°C	

PCR products can be safely stored at 4°C.

Step 2: Assessing quality and submitting for sequencing
Run small volumes of the PCR product through gel electrophoresis to assess quality of amplification and ask TA to send product for Sanger sequencing.

Set up the equipment to cast the gel by fitting the casting tray tightly into the electrophoresis chamber with the orange sides touching the sides of the chamber. Note, this should be tight.

Find the agarose gel (84 mL of TAE + 0.84 g Agarose) in a 250 mL bottle.
Heat in the microwave checking every 5-10 seconds on medium power to melt agarose and keep the agarose from bubbling over. Note, ensure the cap is *loose* so that pressure does not build up inside the bottle. Continue until the mixture is homogeneous. Caution: the glass bottle and cap may become hot during this process. Use heat gloves.

Allow agarose to **cool** to 50C-60C before casting. (This is about when it can be held without discomfort or when small amounts of gel start to harden.) **Pour** gel intro tray and add the 20-well comb so that the 1.5 mm side is in the gel. Let **harden** for 30-45 minutes.

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Carefully **remove** comb from electrophoresis gel. **Remove** the gel cast from the chamber and **rotate 90 degrees** so that the DNA (negatively charged) will flow properly in the chamber. Remember that the black node (left) is negative and the red node (right) is positive.

Submerge the gel beneath 2 to 6 mm of 1x electrophoresis buffer (TAE).

Retrieve one set of the PCR reactions and **put** on ice. **Label** the other set of PCR products for sequencing.
Add 6x loading gel to the PCR reaction in the PCR tubes so that there is a final concentration of 1x loading gel. Note: assume that there is 20 uL of PCR product in each tube.
Combine 10 uL of 1kb ladder DNA plus + 2 uL loading gel.

Carefully **pipette** mix the solutions before putting in wells. Carefully **load** wells with 12 uL of the mixture by putting the end of the pipette tip into the top of or just above the well. It helps to stabilize the pipetting arm with the other hand.

Connect the leads to a **120 V** source. **Turn on** and allow to run until the purple dye is somewhere between the middle and end of the gel (about 1.5 hours).

Move the gel to the ethidium bromide container and turn on the shaker. Note: ethidium bromide is toxic. Wear gloves and a lab coat. Remove contaminated gloves and get fresh gloves. **Stain** for 10-15 minutes.

Place gel on UV light source and image. If possible, ask for the light to be reduced in the room so that it will be easier to see the ladder and PCR reactions. Compare the lengths of the reaction with the ladder using the guide.

Clean up by pouring the TAE in the electrophoresis system down the sink. Place the gel in the used gel container. Use milliQ water to rinse off all electrophoresis components and dry with Kim Wipes.

Ask TA to send samples for sequencing, showing her your samples and supplying 10 uL of the forward and reverse primers labeled as “F” and “R” respectively along with the PCR product.