Exploring Native Microbiota Lab Manual

BIO398, Winter 2024

# ***Lab 7 : PCR Cleanup & Pooling for Multiplexed Sequencing***

# **Overview**:

# Last week, we quantified our DNA extracts, diluted them down to 0.5 ng/µL, and mixed 1 µL of this diluted DNA with PCR-clean water & the forward primer. After you left the lab, I added a unique reverse primer to each of your 8 tubes, added the Taq master mix, vortexed, spun down briefly, then the samples went into the thermocycler. The next day, I got the tubes out of the thermocycler and they have been sitting happily in the fridge over the past week (this is fine because DNA is very stable, and the thermocycling should kill any contaminating cells).

# Our goal for this week is to "clean up" our samples, quantify them, then dilute again (this time to 3 ng/µL) so they are ready to pool and send off for sequencing. Why do we need to clean up our samples? It is because we want just the pure amplicon DNA without contaminating salts, enzymes, etc. To accomplish this, we will use the magnetic beads I showed you last week in a simple procedure that is reminiscent of the ethanol rinsing you did of your raw DNA extracts.

A *reminder: cleanliness is still important*. You should wash your hands when you come in, wear gloves when handling your samples, and avoid touching your skin, clothes, phone, etc with your gloved hands. As before, please do not take directly from stock solutions - ask for an aliquot from me (this is to avoid any contamination of the stocks).

That being said, we will actually be reusing pipette tips at a few steps now that the most critical step (PCR) is done, which will save some plastic waste and speed things up. We can do this because a) the PCR products should be of high [DNA], so much higher than anything floating around in the air/reagents and b) the primers we use already have barcodes attached, so there is no concern about sample cross-contamination. However, we still want to be as clean as possible, since any gunk you get into your samples will be shared with everyone else and could cause problems with sequencing.

Protocol source: <https://www.protocols.io/view/fuhrman-lab-515f-926r-16s-and-18s-rrna-gene-sequen-j8nlkpd1g5r7/v2>

**Overall procedure for today's lab:**

1. Add TE and beads to PCR tubes, incubate. ***DNA sticks to beads***.
2. Separate beads on magnet. ***Beads & attached DNA stick to side of tube***.
3. Remove liquid, containing PCR reactants, salts, etc.
4. Add 80% ethanol to rinse. Vortex to mix. ***Ethanol removes any additional contaminating substances.***
5. Incubate on magnet, then discard ethanol.
6. Repeat ethanol rinse, discard immediately. ***Once more for good measure.***
7. Air dry beads (but don't overdry).
8. Once beads are dry, take tubes off magnet, and add 15 µL of low-EDTA TE. Mix up the beads and TE. ***The DNA will now come off the beads and remain in solution***.
9. Transfer this purified DNA extract to a new set of tubes, without transferring any beads. Discard tubes with beads.
10. Quantify, dilute to 3ng/µL, and pool.

# **Safety notes:**

* Physical hazards: we will be using a centrifuge again today, but at much lower speeds. Nonetheless, it is still very important to balance the centrifuge to avoid damage to the machine. Please do not operate it without the instructor.
* The chemicals we are using this time (ethanol, beads) do not present any major safety hazards though please do still wash your hands when you leave, which is good lab practice.

**DNA Quantification and Dilution:**

1. Prior to beginning, label two PCR strips (pre-UV'd by instructor) with your sample IDs and "CLN", and "3 ng" to indicate one will be your clean PCR product, and the other your dilutions for final pooling.
2. Take PCR reactions out of fridge, allow to warm to room temperature. Spin down briefly.
3. To each of your 25 µL reactions, add 15 µL low-EDTA TE (provided as aliquot).
4. To each of your tubes, add 32 µL of beads (provided as aliquots).
5. Resuspend the beads fully by vortexing or flicking, making sure to not lose your liquid accidentally. Spin briefly to bring any drops down.
6. Incubate for at least 5 minutes (longer is OK). This allows the DNA to bind to the beads.
7. Once the 5 minutes is up, pass the tube to the instructor or place on the magnetic plate yourself.
8. Allow to separate for at least 3 minutes (longer is OK). The beads will form a ring around the side of the tube. Your DNA is with the beads, attached to the side.
9. The instructor will discard the clear buffer for you (this is tricky, and I want to make sure it works for everyone).
10. Once the buffer has been discarded, use a P1000 to add 200 µL (0.2 mL) of 80% ethanol, prepared by the instructor (provided as aliquots). The P1000 tip can be reused, so long as you don't touch the tip to anything. Keep on top of the P1000 box when not using. (Note: *While you won't need to worry about this, this is a step where things can go wrong if you accidentally prepared a low concentration of ethanol (e.g. 30% instead of 70%). This is because at concentrations less than about 70%, DNA will come off the beads and be lost. We use 80% just to make sure we don't accidentally have less than 70% if the ethanol has absorbed some water from the atmosphere.*)
11. Mix your samples by flicking or vortexing. This is to get the ethanol to come into contact with the beads and wash them. The beads probably won't go into solution, which is OK. Incubate for 3 minutes off the magnet.
12. Put back on the magnet, and wait at least 3 minutes.
13. The instructor will then discard the ethanol for you using a multichannel pipettor (this is tricky, and I want to make sure it works for everyone).
14. Get your clean tubes from the instructor. Use a P1000 to add another 200 µL (0.2 mL) of 80% ethanol, prepared by the instructor (remember, the tip can be reused).
15. Put the tube back on the magnet. Wait until the beads are fully separated.
16. Get the instructor to remove the ethanol, as before.
17. Now, spin your tubes briefly to collect all the ethanol at the bottom, *put back on the magnet,* and use a P200 tip to try and remove as much ethanol as possible without disrupting the beads, *checking your pipette tip to make sure you minimize bead carryover*. You can use the same tip for all 8 of your samples to conserve plasticware.
18. Once you have removed all the ethanol, allow your beads to air dry in the tubes with *caps off* for 5 minutes. ***Don't exceed 5 minutes. Show to the instructor once the beads are dried to make sure it looks good before proceeding.***
19. Next, add 15 µL of low-EDTA TE to each tube with a P10 by pipetting 7.5 µL twice (can use the same tip so long as you don't touch the tubes). ***Resuspend the beads in the TE. Do so by flicking, vortexing, etc.***
20. Incubate for 5 minutes *off the magnet* to allow the DNA to come off the beads.
21. Incubate for 3 minutes *on the magnet* to allow the beads to go to the side of the tube.
22. Get the instructor to transfer 14 µL of your clean PCR product into your "CLN" tubes. These samples will now be quantified in the following protocol.

**Quantification Protocol A (spectrophotometric; for all samples which should all be clear):**

1. Everyone using this protocol will be putting their samples on the same plate. So the first step is to allocate locations on the plate (see image on Toxopeus lab protocol; several copies will be provided).
2. The instructor (or a willing student) will pipette 380 µL of low-EDTA TE into each well that is going to be used.
3. 385 µL of low-EDTA TE will also be pipetted into two wells as a blank.
4. Next, you will pipette ***5 µL*** of your DNA extract into the correct well using a P10. ***Do not pipette into someone else's well!*** Submerge the pipette tip into the well to make sure the small volume makes it in. Use a clean tip each time to avoid cross-contamination. ***Double-check the volumes here - it's critical to be accurate!***
5. Next, we will follow the Toxopeus lab protocol for running the plate reader.
6. The instructor will demonstrate the calculation of [DNA].
7. Continue with the protocol below to calculate how to dilute your samples to 3 ng/µL.

**Dilutions to 3 ng/µL (for all samples):**

*Note:* ***this step must be done accurately or everyone's pool concentrations will be off****! Please make sure you can accurately pipette small volumes. Please ask the instructor if you're not sure.*

1. The instructor will do the calculations in a spreadsheet and provide you with printed values, which indicate a volume of TE to put into your 8-strip tubes (like last week).
2. Being careful not to mix up your samples, pipette in the correct amount of TE in each of your 8 "3 ng" tubes.
3. Now, transfer 1 µL of your clean PCR product into the corresponding tube. For samples with [DNA] below 3 ng/µL (e.g. your negative control or poorly-amplified samples), please transfer 5 µL into the "3 ng" tubes.
4. Mix by vortexing or flicking, spin down, and ***provide to the instructor for pooling / sequencing\****. To pool I am just going to take a few µL of your samples and add them to a single tube. Probably what I'll do is add 3 µL from each of your samples for a total of 192 µL (i.e. 64 samples \* 3 µL).
5. Put the "CLN" tubes in the -80°C for long-term storage.

\*This will then be sent to Dalhousie's Integrated Microbiome Resource sequencing center (<http://imr.bio/about.html>). We will do a nano MiSeq 2x250 sequencing run that will give us about a million sequences total, shared among all our samples (more on that later!).