Exploring Native Microbiota Lab Manual

BIO398, Winter 2024

Lab 6 : PCR Preparation & Thermocycling

Overview:

Last week, we extracted, pelleted, cleaned and resuspended our DNA. This week, we'll be quantifying these DNA extracts, diluting them down to a lower concentration (if necessary), preparing your samples for PCR, and (ideally) starting the thermocycling reaction. In some cases, it may also be necessary to do additional purification of extracts if they contain too much humic acid material, though if the DNA concentration is high enough we will likely attempt PCR since the humic acid will be diluted many-fold before entering the PCR reaction and thus may not cause a problem.

A *reminder: cleanliness is especially important for PCR*. This is because PCR amplifies DNA 2-fold after each cycle, and we will do 30 cycles for a total amplification of 2^30 = up to ~***1 billion-fold***. Therefore, any contaminating templates will be very easily identified in your amplified DNA. 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).

Overall procedure for today's lab:

1. Quantify our DNA extracts from last week to check how successful our extractions were. This will be done with either an A) *absorbance-based assay (spectrophotometric)* or B) *a fluorescence-based assay* depending on the colour of your extract:
    1. If your extract is clear, use A) which is faster and easier, but sensitive to contamination
    2. If your extract looks like black tea (most freshwater soil extracts will be like this), use B) which is less sensitive to contamination of other chromophores (things that absorb light)
2. Based on this information, we will dilute our DNA further in low-EDTA TE to at least 0.5 ng/µL (ng DNA / µL TE) to act as template for our PCR reaction (we will dilute further for humic-y extracts). I will provide a spreadsheet template for these calculations.
3. Next, we will prepare our PCR reaction with 5 basic components:
    1. PCR master mix, containing Taq DNA polymerase, Mg^{2+} enzyme cofactor, buffer, dNTPs
    2. Forward primer (each of you will have a unique numbered forward primer with a unique barcode - this ***must not overlap or your samples will be mixed with the person with the same barcode!***)
    3. Reverse primers 1-8 (each of you will use the same reverse primers 1-8)

4. Template DNA, diluted to 0.5 ng/µL
5. Water to bring up the final volume to 25 µL

4. In addition to your 6 DNA extracts, you will also set up 2 more reactions:
    1. A positive control (A DNA extract that is known to give good amplification)
    2. A negative control (TE buffer)

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 do not present any safety hazards, unless you have to use linear acrylamide to re-purify your DNA.

DNA Quantification and Dilution:

1. Prior to beginning, prepare a PCR strip with your sample IDs and "0.5ng" to indicate these will be your dilutions. Place these into your tube rack. Also, label an 8-strip set of PCR tubes with your name, and place them in a PCR tube rack. UV both for at least 10 minutes to decontaminate (***but whatever you do, don't accidentally UV your DNA!***).
2. Take out your 6 extracts, and set them on the countertop to thaw in a tube rack. Although it is not ideal to freeze-thaw DNA, the TE buffer keeps the DNA stable.
3. Once thawed, give your extracts a brief vortex (or flick to mix) and then spin down briefly in the smaller centrifuge (making sure to balance carefully).
4. Next, proceed to DNA quantification, based on the colour of your extracts:

Quantification Protocol A (spectrophotometric; for clear samples):

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 will pipette 384 µL of low-EDTA TE into each well that is going to be used.
3. Next, you will pipette 1 µL of your DNA extract into the correct well. ***Do not pipette into someone else's well!*** Submerge the pipette tip into the well to make sure the small volume makes it in. Also, use a clean tip each time to avoid cross-contamination.
4. Next, we will follow the Toxopeus lab protocol for running the plate reader.
5. The instructor will demonstrate the calculation of [DNA] and quality ratios.

6. Continue with the protocol below to calculate how to dilute your samples to 0.5 ng/µL.

Quantification Protocol B (fluorometric; for coloured samples):

1. First, work with the instructor to figure out how many samples total will be measured. Then, prepare enough of the qBit fluorometric solution for those samples + 4 more samples (2 standards plus extra to account for pipetting error). The fluorometric solution is a 1/200-fold dilution of the fluorescent orange stock solution in the buffer provided in the kit (i.e. 1 µL orange stuff + 199 µL buffer per each sample). Keep this out of light until use (wrap in tinfoil).
2. Next, get tubes from the instructor for the number of samples you will be measuring. Label tubes ***only on the top!*** This is because the assay uses light that passes through the bottom to measure [DNA], and if you write on the bottom you may block the light and get an inaccurate reading.
3. To each of these tubes, add 199 µL of the solution mixture prepared in the previous step. Keep under tinfoil when not using to protect from light.
4. Then to each of these tubes, add 1 µL of your sample. Once you have added all of your samples, flick them briefly to mix. Make sure there are no bubbles at the bottom. Wipe the sides with a small Kimwipe.
5. Incubate for at least 2 minutes in the dark.
6. Once you are ready to measure, go to the qBit and measure standard 1 and standard 2, prepared by the instructor (after wiping with a Kimwipe), then proceed to measuring your samples. Carefully note down the concentration of DNA in ng/µL. Double-check your numbers to make sure they match the screen.
7. Continue with the protocol below to calculate how to dilute your samples to 0.5 ng/µL.

Calculating dilutions to 0.5 ng/µL (for clear extracts only):

1. Download the spreadsheet provided by the instructor from Moodle.
2. Input your data, double-checking to make sure you haven't made a data input error.
3. Using the information from the spreadsheet, add appropriate volumes of TE to each tube (this should be done with filter tips, but you can use the same tip so long as you keep it clean). In some cases, you may have to use a microcentrifuge tube if you have ***lots*** of DNA.
4. Add 1 µL of your DNA template to each tube. Submerge the tip to make sure the volume gets in. ***Make sure to use new tips each time to avoid cross-contamination!***
5. Flick to mix / vortex briefly, and spin down. This is now the template for your PCR reaction.

Calculating dilutions for humic-rich samples:

1. Download the spreadsheet provided by the instructor from Moodle.

2. Input your data, double-checking to make sure you haven't made a data input error.
3. Choose a final dilution that is at least 1/1000 (up to 1/10.000) and consistent for all humic-y samples, if possible (the instructor will assist with this).
4. Add appropriate volumes of TE to each tube (this should be done with filter tips, but you can use the same tip so long as you keep it clean). For these samples, you will likely have to use a microcentrifuge tube since we will be diluting a lot.
5. Add 1 µL of your DNA template to each tube. Submerge the tip to make sure the volume gets in. Make sure to use new tips each time to avoid cross-contamination!
6. Flick to mix / vortex briefly, and spin down. This is now the template for your PCR reaction.

PCR Preparation and Thermocycling:

Important note: Small volumes can evaporate quickly or get caught on the sides of the tubes. To avoid this, only keep the caps open when you're actively working with the samples and pipette small volumes (i.e. 1 µL) into the liquid at the bottom of the tube.

1. To your 8-strip set of PCR tubes, add the following items *to each of 8 tubes* (in this order, using ***filter tips***):
    1. 9.5 µL of nuclease-free water, provided as aliquots to each pair.
    2. 1 µL of the forward primer (working concentration = 7.5 µM; ***make sure you're not using the same primer number as someone else!)***. This will also be provided as an aliquot.
    3. 1 µL of your template. Make sure to pipette into the correct well. I suggest moving your tube or using the pipette tip box arrangement as a memory aid. Make sure to also do a negative control (low EDTA TE buffer) and a positive control (DNA will be provided).
    4. 1 µL of the reverse primer (working concentration = 7.5 µM; this will be done by the instructor once I verify the well volumes look good).
    5. 12.5 µL of GoTaq master mix (2x master mix Promega M5132 or M5133; this will also be done by the instructor).
    6. Close caps tightly, vortex briefly, and spin in the centrifuge to bring liquid down. Make sure to balance the centrifuge with someone else's tube rack.
    7. Your samples are now ready for thermocycling. They can be safely left on the counter until thermocycling begins since this is a "Hot Start" enzyme which needs to be cooked at 95°C before beginning.
    8. Place in thermocycler and next week we'll clean up the PCR reaction and quantify the [DNA] to see how the PCR worked! Note: PCR program = 95°C for 120s (hotstart), followed by 30 cycles of 95°C (45s; denaturation), 50°C (45s; annealing), 68°C (90s; elongation), followed by a final 300s elongation step @ 68°C, and hold at 4°C (forever).