**Library prep/Pooling strategy for metabarcoding samples (16S and 18S rRNA)**

*Pre-pooling steps*

1. With respect to DNA concentration, make sure you have a good understanding of your samples, i.e., do they have similar (i.e., same order of magnitude) Qubit values? Is there an agreement between Qubit values and band intensity from your agarose gels (i.e., bright bands have higher DNA concentration, something close to your positive controls)? This information should guide your final decision on how you will pool your samples.
2. After reviewing the DNA concentration of your samples and deciding what strategy to use for pooling (e.g., based on DNA concentration or based on band intensity), organize your samples in a way/order that facilitates your work at the bench (e.g., you might organize samples according to the volume to be added, or just following the sample ID order). Make sure you have a printed list of the sample order with their respective volume to be added during the pooling.
3. Make sure to clean up your bench space (a large area) with EtOH 70% prior to pooling. Depending on the number of samples you have, you might decide to take them out from the refrigerator/freezer in batches.
4. Make sure have the pipettes (based on the volumes you will be adding) and the respective tips on the bench space you cleaned for the pooling process.

*Pooling, cleaning, and Qubit steps*

1. Depending on the final volume you might reach after pooling all samples, you can choose between tubes of 1.5 ml or 5 ml (mostly likely 5 ml). UV/Sterilize the tube (5-10 min) prior to pooling any of the samples.
2. Transfer the samples to the tube (1.5 ml or 5 ml) following the list you generated. Make sure you are adding the correct volume for each sample and changing the pipette tips accordingly. To avoid any confusion, you can always mark/check on your list those samples already added into the tube.
3. After completing the pooling, samples need to be cleaned using our bead cleaning protocol (basically an extra bead cleaning step). You will have split the volume of your final library into 1.5 ml tubes. For example, if you used the 5 ml tube for the pooling and ended up with about 2000 ul, you can split this volume into 8 tubes (i.e. 250 ul per tube). Make sure to vortex the library prior to split it into separate 1.5 ml tubes. Using this strategy, you will have enough space to add the bead solution and the PEG.
4. Assuming you had 250 ul of your pooled sample in each 1.5 ml tube, add 500 ul of beads and 500 ul of PEG (i.e. 2X your sample volume). Samples should be incubated for 10-15 min in the mixer (you might have to change the rack for 1.5 ml tubes). Alternatively, you can just incubate the samples in a regular plastic rack for the same period.
5. Place/Incubate your samples in the magnetic rack for 10 min.
6. Aspirate the solution/liquid (i.e. beads and PEG), then transfer your samples to a plastic rack and add 1000 ul of EtOH 80%.
7. Place/Incubate your samples in the magnetic rack for 10 min.
8. Repeat steps 6-7 an additional time, so you have two EtOH 80% washes. Before eluting samples, you might let the samples air dry for 1 min (so any excess of EtOH 80% evaporates). Do not let the samples air dry for too long.
9. Elute samples in 105 ul using Molecular Grade H2O.
10. Place/Incubate your samples in the magnetic rack for 10 min.
11. Transfer 100 ul from each sample/tube to a final collector tube (mostly likely a 1.5 ml).
12. Before submitting samples for a MiSeq run, the final concentration of your library must be determined. Prepare Qubit reactions for Standards 1 and 2 and your library(s) following our Qubit protocol. You might decide to Qubit your samples using 2 ul instead of 1 ul. If so, make sure to change/adjust the Qubit settings.
13. We usually ship about 500 ul of a library for MiSeq sequencing. So, you have a larger volume, you might want to transfer only 500 ul into a new 1.5 ml tube. Make sure to properly label your tube with the library info (e.g., date, gene, library name/code).
14. Finally, properly seal your tube(s) with parafilm.