Cycle Sequencing, Cleaning, and Loading Samples in a Single 96-well Plate

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To save time, money and lab supplies, you can cycle sequence your samples in the plate you are will actually load into the sequencing machine.

To set up your cycle sequence reaction:

- Start with a clean plate (this can be a plate that has been used previously, but should be completely clean and dry before you begin.)
- You will need a silicon mat to cover your samples when you are finished, which should also be clean and dry (these mats can be used over and over).
- Make sure you have all your cleaned PCR samples ready (don't forget to quickly spin these down if they have been in the refrigerator, as there will likely be condensation on the lids).
- Put your 96-well plate on ice (only to prevent evaporation of your DNA template – the cleaned PCR product - once you load them into the wells of your 96-well plate).
- Make up your cycle sequencing master mix minus the Big Dye Terminator (BDT) which you should leave in the freezer until you are ready for it since BDT is light sensitive. You will add the BDT at the very end to the master mix, but after you have your template in the wells.
- Put your master mix minus the BDT on ice.
- Add your DNA template (the cleaned PCR product) to each well of your 96-well plate. Be sure to load the plate from 1A – 1H then to 2A – 2H, 3A – 3H, *etc*. ending on well 12H.
- *Tip* – I usually add the template to the front inside of the tube/well, touching the pipette tip to the inside front side of the tube to insure the droplet of template does not stick to the outside of the pipette tip.
- Once you have your DNA template in each of the wells, get your BDT out of the freezer and add it to your master mix. Be sure to mix well by pipetting up and down several times.
- If you have added your template to the front inside of the wells, then you can add your master mix to the back inside of each well, allowing the pipette tip to touch the inside of the well without chance of contamination.
- After you have added the master mix to each well, cover with the clean, dry silicon mat and quickly spin down your plate in the plate centrifuge to insure that your DNA and master mix are both in the bottom of the wells (be sure you have another mat covered plate to use as a balance).
- After placing in the thermal cycler, be sure to put out a box with aluminum foil to cover your plate, as BDT is light sensitive.
- You can leave your completed cycle sequence reaction in the refrigerator for up to a week before cleaning and sequencing, as long as it remains in the dark.

Cleaning your cycle sequence reaction (EtOH/EDTA precipitation):

- Before you begin, quickly spin down your plate (making sure to balance the centrifuge) as there is likely condensation on the lid/mat. Note orientation of mat on 96-well plate so you can put it back on in the same orientation.
- Make a master mix of the EtOH/EDTA solution in a plastic trough. For a 10µL reaction, you will need to add 30µL 100% EtOH and 2.5µL 125mM EDTA, so for a 96-well plate make up the following:
  - 3300µL 100% EtOH
  - 275µL 125mM EDTA
- Mix solution using pipette.
- Add 32.5µL of the EtOH/EDTA solution to each well of your 96-well plate using a multi-channel pipette (you will only need to use one set of pipette tips if you do not touch the tips to the 96-well plate).
- Seal 96-well plate with silicon mat (paying special attention to place the mat back on the tray in the same orientation as you took it off) and quickly vortex to mix.
- Leave at room temperature for 10-15 minutes in a dark location (*i.e.* in a drawer) or covered in aluminum foil.
- Spin in refrigerated centrifuge at 2500g for 30 minutes at 4°C (program #1 on Eppendorf refrigerated centrifuges). Be sure to balance centrifuge.
- *IMPORTANT*: Proceed to next step immediately (you must be ready and present as soon as the centrifuge stops). If not possible, you must spin the sample again for 10 minutes.
- Remove silicon mat (again noting orientation of mat on 96-well plate) and invert tray onto folded paper towel and place in centrifuge rack (no need to rubberband).
- Place tray inverted into centrifuge and spin 50g for 2-3 minutes (program #2).
- Add 30µL 70% EtOH to each pellet (just eyeball pouring 70% EtOH into trough to use multi-channel pipette to distribute). No need to mix or vortex.
- Seal 96-well plate with silicon mat (again in same orientation as original cycle sequence reaction).
- Centrifuge plate 2000-3000g for 15 minutes at 4°C (program #3).
- *IMPORTANT*: Proceed to next step immediately (you must be ready and present as soon as the centrifuge stops). If not possible, you must spin the sample again for 10 minutes.
- Again, remove silicon mat and invert tray onto folded paper towel and place in centrifuge rack (no need to rubberband).
- Place tray inverted into centrifuge and spin 50g for 2-3 minutes (program #2).
- Place 96-well plate in 65°C oven for 10 minutes to allow to thoroughly dry.
- While waiting, clean gray septa mat cover and make sure it is completely dry.
- If you cannot load samples directly onto sequencing machine at this time, you can cover the 96-well tray with a clean silicon mat, wrap in foil and freeze until you are ready to sequence.

Resuspending samples in HiDi for sequencing

- Add 10µL to each well of the 96-well plate (if you have "blank" lanes without any DNA product, you must still load with HiDi or ddH_2O – there must be HiDi

or ddH_2O in every well so as to not damage the capillary arrays of the sequencing machine).
- If your plate will be the first reaction on the sequencing machine, you must wait 10 minutes before placing your plate on the machine to allow for the pellet to resuspend in the water (if there are other plates ahead of your plate, you can place it directly into the queue).

Special notes:
- You do not need to use barrier/filter tips for cycle sequencing, except when you take aliquots from your stock reagents (like your primers) that are still used in your PCRs.
- For most cycle sequence reactions, there is no need to use more than **0.75µL of BDT per 10µL reaction** (in some cases you may even be able reduce the amount of BDT to 0.50µL per rxn). This will save you a substantial amount of money in the long run.
- If you only need to **sequence a half plate**, you must load your 96-well plate every other row (i.e. 1A – 1H, 3A – 3H, 5A – 5H, *etc*. ending on 11H).
- You can reuse your 96 well plates. Be sure to clean them thoroughly after they come off the sequencing machine, this includes washing with water and UVing when you have this option available. I have reused the same 96-well plates for more than year without any adverse effects.
- **Remember there is no such thing as being too careful in the lab.**