OICR Protocol for 250- 350bp Amplicon Library Preparation

NOTE: Bold texts are deviations from the current recommended PacBio procedure

Pooled PCR amplicons

1. Amplifications may be multiplexed, 1 or 5 amplicons/well, with a total input of 20-30ng/well. The amount of DNA extracted from FFPE's is usually very low, hence less than 5 ng (1-3ng) of material is added into the reaction.
2. 67 amplicons are combined per SMRTbell prep: 12 pools of 5 amplicons, 6 ul/well + 7 individual amplicons, 1.5 ul/well, for a total of 82.5 ul

DNA Concentration

1. **Order of addition: 1x Ampure beads are added to the 1.5ml tubes first,** followed by the addition of pooled PCR products (82.5µl)
2. Samples are mixed thoroughly by pipetting up and down 20x.
3. All steps are done on the bench, not in ice. Lab temperature is 68F.
4. **DNA binding is done on Thermomixer for 15 minutes, 25C, 1400rpm.**
5. **After binding, the tubes are never spun down; this may lead to sticking of the beads to the bottom of the tube**.
6. Tubes are magnetized for 3 minutes.
7. Liquid is aspirated by pipetting leaving some liquid behind.
8. Beads are washed with 200 µl 70% ethanol (up to 3 days old), 200 µl or enough to cover beads. Second wash is the same volume.
9. **Beads are dried at 30C in thermomixer for 5-6 minutes.**
10. Beads are resuspended thoroughly by pipetting (30 µl) elution buffer up and down 20X in the bottom of the tube, only in the area where the beads are located.
11. **Elution buffer / beads are incubated in thermomixer, 5 minutes at 30C.**
12. Tubes are magnetized for 2 minutes.
13. **Sample transfer is done with two pipettes. First aspiration is 28.5 µl, followed by 1.5 µl using a P2. This is 100% recovery of EB added for elution.**

End-Repair

1. **Order of addition is:**
    a. **Water**
    b. **Sample**
    c. **Freshly prepared End Repair master mix containing Template Prep Buffer, ATP, dNTP, and End-Repair Mix**
2. **Mixing is by pipetting up and down, never flicking.**
3. **Incubation at 25 C for 15 minutes in a thermal cycler; do not use a thermomixer.**
4. **Purification procedure is the same procedure used for DNA concentration above. We always use 1x Ampure.**

A-Tailing

Since the A-tailing protocol has a 16-hour ligation, and time is critical, the 500 bp Blunt-end procedure is being used instead of the recommended A-tailing procedure. The amount of adapter dimer contamination is negligible or small, and concatemer formation is not an issue.

Ligation

1. **Prepare two Master Mixes**
    a. **Buffer + ATP**
    b. **Ligase + water (this is made before adding to the reaction)**
2. **Order of addition to sample (in 0.2 ml tubes):**
    a. **Blunt adapter**
    b. **Buffer (a)**
    c. **Fresh buffer (b)**
3. **Mix by pipetting up and down. Take care not to form bubbles. Spin if necessary.**

Exonuclease Treatment

1. Follow PacBio protocol
2. **Mix by pipetting up and down with a large volume pipette. Do not mix by flicking the tube.**
3. In the past, they have stored exo-treated samples at 4C overnight. They haven't noticed any drop in performance. Not a common practice.
4. Purification is done similar to the concentration procedure, recovering the eluted sample
   Two rounds of purification First: 40 µl rxn + 40 µl beads. Elute in 30 µl Second: 30 µl eluted product + 30 µl beads. Elute in 10 µl using two pipettes, with the first pipette set to 8.0 µl and the second set to 2.0 µl.

Primer Annealing

1. Annealing is done at a final concentration of 5nM for 250-350bp libraries.
2. **Order of addition is according to C2 calculator:**
    a. **Water**
    b. **10x Primer Buffer**
    c. **Sample**
    d. **Diluted Primers**

Polymerase Binding

1. **A master mix of dNTP ,DTT and Binding Buffer is made.**
2. **Polymerase is diluted just before binding.**
3. **Order of addition is:**
    a. **Master Mix**
    b. **Annealed library**
    c. **Freshly diluted polymerase**

DNA Quantitation

Accurate DNA quantitation is key to consistent high yield

Conclusions from Qubit vs Nanodrop Study:

- Nanodrop measurements are inflated. Over-quantitation by Nanodrop resulted in low yield
- Using Qubit measurements led to increased yield.