**Part 1: DNA Extraction**

1. Use the **Invitrogen PureLink gDNA** extraction kit to extract gDNA from upto ~2E9 cells (usually 1mL overnight culture in LB has this many cells, ~50mL of overnight culture for LTEE isolates)
2. Measure concentration of gDNA using **Invitrogen** **Quant-It Kit**
3. Normalize concentrations of different preps to ~20ng/𝜇L

**Part 2: Nextera Tagmentation Reaction**

1. (for 10𝜇L reactions) Mix 5𝜇L buffer, 2.5𝜇L **TDE1 enzyme,** 2.5𝜇L of gDNA (approximately 50ng) in a PCR tube. Vortex briefly and pulse centrifuge the reaction mixture.
2. Incubate at 55℃ for 10 mins in a thermocycler, keep on ice right after (or in the fridge at 4C if not doing the PCRs instantly)

NOTE: this protocol uses **Nextera TDE1 Tagmentase** enzyme, but in principle, this can be adapted to the **Illumina Library Prep Kit** which uses a bead-linked transpososome.

**Part 3: PCR 1 - adding unique molecular identifiers**

1. Make a list of barcode combinations for each sample (do this beforehand, ensure that the barcodes are color balanced)
2. Aliquot 3𝜇L each of forward and reverse primer in PCR tubes, add all 10𝜇L of the Nextera tagmentation reaction (serves as template)
3. Add 15𝜇L of the Q5 master mix and mix with pipette. Vortex briefly and pulse centrifuge the reaction mixture
4. Run the following PCR program:

|  |  |  |
| --- | --- | --- |
| 1 | 95℃ | 3:00 |
| 2 | 98℃ | 0:30 |
| 3 | 68℃ | 0:30 |
| 4 | 72℃ | 1:00 |
| 5 | Go to 2 | 2X |
| 6 | 72 ℃ | 3:00 |
| 7 | 12℃ | hold |

**Part 4: SPRI Bead Cleanup**

1. Perform magnetic bead cleanup using ~1.2X serapure beads (home-brewed, the exact ratio needs to varied if you’re using AmpPure XP beads)
2. Elute in 15𝜇L dH20

**Part 5: PCR 2 - amplification and addition of Illumina barcodes and adapter sequences**

1. Aliquot 5𝜇L each of forward and reverse primer in PCR tubes, add all 15𝜇L of the cleaned up PCR1 product
2. Add 25𝜇L Q5 master mix and pipette up and down to mix. Vortex briefly and pulse centrifuge the reaction mixture
3. Run the following PCR program:

|  |  |  |
| --- | --- | --- |
| 1 | 95℃ | 3:00 |
| 2 | 98℃ | 0:30 |
| 3 | 68℃ | 0:30 |
| 4 | 72℃ | 1:00 |
| 5 | Go to 2 | 18X |
| 6 | 72 ℃ | 3:00 |
| 7 | 12℃ | hold |

**Part 6: SPRI Bead Cleanup**

1. Perform magnetic bead cleanup using ~1.2X serapure beads (home-brewed, the exact ratio needs to varied if you’re using AmpPure XP beads)
2. Elute in 25𝜇L dH20

**Part 7: Quantifying Sequencing Libraries**

1. Use QuantIT Kit (or an equivalent) to estimate concentration of the libraries after PCR2.
2. Run a few of the libraries on a 2% Agarose Gel, with a 50bp ladder, and get an approximate sense of the average fragment size (NOTE: the exact size doesn’t matter, it just gives us a starting point)
3. Dilute and all libraries to approximately 4nM
4. Pool the libraries together.
5. Perform a qPCR (**KAPA Quantit Kit** or an equivalent) on the pooled libraries. Make sure to run two dilutions (1:10,000, 1:20,000)
6. Calculate the concentration of the pooled library using the calibration curve.
7. Also, run the pooled library on an Agilent BioA high sensitivity DNA chip to get a more accurate sense of fragment size distribution.
8. Ensure that there are no primer dimers. With the current protocol, they’d be at ~125bp if the clean-up isn’t successful at getting rid of them. If you see primer dimers, re-calibrate your beads and ensure they’re size selective.
9. Dilute libraries to 4nM (or the appropriate concentration for the sequencing platform)
10. Send off samples for Illumina sequencing

**Bead Clean-up Protocol:**

If you’re doing a lot of library preps, best to do this in PCR strips, with a compatible magnetic rack and use a multichannel pipette

1. Suspend sample in 1.2x the volume of beads, and mix. Incubate at room temperature for 10 mins
2. Place on magnetic rack, allow beads to pellet and pipette off supernatant
3. Add 100𝜇L of freshly prepared 70% ethanol (volume is not important as long as beads are covered in ethanol)
4. Move the PCR strip to adjacent location (this will move the beads through the ethanol, washing them)
5. Remove the supernatant.
6. Repeat steps 3-5
7. Leave tube open on the bench and let the ethanol evaporate
8. Remove the tube from the magnetic rack and resuspend pellet in an appropriate volume of eluant (e.g. 20 uL molecular grade water)
9. Incubate for 10 mins at room temperature
10. Pellet the beads on the magnetic stand until the eluate is clear. Transfer the supernatant to fresh PCR strips or eppendorfs