Library Preparation protocol for use with NEBNext Adapters for Illumina

Notes:

- Write the date opened and the expiration date on top of the box immediately after opening new library preparation kits. Run a test on recently expired reagents prior to use.
- Prepare **800 uL** 80% ethanol, **50 uL** low EDTA-TE, and **90 uL** Speed Beads **per sample** prior to starting.
- If inputting less than 20 ng DNA, the "NEBNext Adapter for Illumina" used in the Adapter Ligation steps should be diluted 10-fold.

Adapter Dilution

Notes:

- Make only as much diluted adapter as will be used in a single day.
- Make more than is strictly required (~double).

Steps:

1. Combine 9 parts 10 mM Tris-HCl (can use low-TE EDTA as substitute) to 1 part NEBNext Adapter for Illumina (e.g. for 20 uL total diluted Adapter, combine 2 uL Adapter with 18 uL Tris-HCl).
2. Thoroughly mix by gently flicking the tube and gently spin down.

DNA Fragmentation (Shearing by Sonication)

Notes:

- The Sonicator's Timer setting has no effect. Manually stop the instrument after it has run for the desired amount of time, otherwise it will continue to cycle.

Preparation:

- Nanodrop the purified DNA to ensure that it is clean enough to amplify successfully. Attempt to amplify the 16S gene if uncertain. Ideally, the 260/280 absorbance ratio will be around 1.8 and the 260/230 ratio between 2 and 2.2 (260 nm = DNA wavelength; 280 nm = proteins & phenols; 230 nm = humic substances).
- Chill the Sonicator to 4 ˚C. Verify that the temperature has reached the desired level prior to use.
- Label one 0.2 mL PCR tube per sample. Use a spreadsheet or notebook to keep track of tube labels.

Steps:

1. In a PCR tube, bring the desired amount of sample DNA to a total volume of **55.5 uL** by combining the appropriate ratio of UltraPure water to sample solution (Volume in = Total input DNA desired / DNA concentration). Unless ug of DNA is available, input no more than 100 ng DNA and preserve the remaining sample. Inputting more than 300 ug could produce undesirable results from shearing.
2. Place PCR tubes in the sample holder assembly. The tubes may need to be gently spun down first to ensure that the sample becomes fully submerged when placed in the cup horn.

3. Place the holder assembly in the sonicator such that the gears line up.
4. Fill the sonicator cup horn with water to exactly the level of the sample liquid.
5. Sonicate at the following settings.
    a. Pulse: 10 sec on 10 sec off
    b. Amplitude: 25%
    c. Time: 30 sec (>90 ng DNA) or 60 sec (< 90 ng DNA)

(optional, as necessary) PCR Quality Check

Notes:

- PCR the 16S rRNA gene using both Bacterial and Archaeal primers to determine whether the purified DNA is clean enough for a successful library preparation.
- Bacterial and Archaeal primers will amplify at different rates, so expect the melt curves to be different.
- Each PCR should include a positive and negative control. The positive control can be anything that had successfully amplified before. The negative control should consist of the supermix, forward, primer, reverse primer, and UltraPure water.

Preparation:

- Thaw "**Universal IT SYBR green supermix**" and **primers** (forward and reverse, bacterial and archaeal).
- If needed, make a batch of archaeal and bacterial primers from primer stock following this guide.

Steps:

1. In a PCR tube, bring **1 ng** DNA to a total volume of **8 uL** by combining the appropriate ratio of UltraPure water to sample solution.
2. To the mixture add **10 uL** "Universal IT SYBR green supermix", **1 uL** "bacterial forward primer" at 10uM (final concentration will be 0.5 uM), and **1 uL** "bacterial reverse primer" at 10uM (final concentration will be 0.5 uM).
3. Repeat steps 1 and 2 using archaeal primers.
4. PCR the mixtures and the positive and negative controls.
5. Verify that the amplification was successful by comparing the results to the controls.

(optional, if desired) Division of Sheared DNA into Large and Small Libraries (small library will have range of 500-800 bps)

Large side selection:

1. Bring speed beads to room temperature and vortex to mix.
2. To 55.5 uL of sheared sample add 0.55x (30.25 uL) beads.
3. Incubate for 3 minutes.
4. Pellet on magnetic stand for 3 minutes.
5. Remove supernatant to fresh tube and set aside. **DO NOT DISCARD SUPERNATANT**. Supernatant contains small fragments and beads contain large fragments.
6. Leaving tube with large fragments on magnetic stand, add **200 uL** of 80% EtOH.
7. Wait 2 min
8. Remove EtOH

9. Repeat wash steps
10. Air dry beads 3-5 min on magnetic stand (be careful not to let beads overdry and crack)
11. Remove tube from stand and elute beads with 55.5uL low EDTA-TE and proceed to library prep.

Small side selection:

1. To tube with small fragments, add 1.8x speed beads.
2. Incubate for 3 minutes.
3. Pellet on magnetic stand for 3 minutes.
4. Discard supernatant.
5. Leaving tube on magnetic stand, add **200 uL** of 80% EtOH.
6. Wait 2 min.
7. Remove EtOH.
8. Repeat wash step.
9. Air dry beads 3-5 min on magnetic stand (careful not to let beads overdry and crack).
10. Remove tube from stand and elute beads with **55.5 uL** low EDTA-TE and proceed to library prep.

DNA End Repair

Notes:

- The purpose of this section is to repair the ends of double-stranded DNA that may have become staggered during shearing.

Preparation:

- Thaw "**End Prep Enzyme Mix**" and "**End Repair Reaction Buffer**" at room temperature. Enzymes have a tendency to precipitate out of the solution after freezing. Prior to use, gently finger flick to mix and then quickly spin down the tubes. **Do not** vortex reagents used in library preparation.

Steps:

1. Mix **55.5 uL** sheared DNA with **3.0 uL** "**End Prep Enzyme Mix**" and **6.5 uL** "**End Repair Reaction Buffer**" in a PCR tube. You should have a total of **65 uL**.
2. Thoroughly mix by flicking, then gently spin to gather liquid.
3. Place in the PCR machine and use protocol "**Library Prep 1**" (30 minutes at 20 C, 30 minutes at 65 C, hold at 4 C). Select "SYBR Only" in the "Express Load" dropdown under "Plate" settings.

Adapter Ligation

Notes:

- Do not allow the samples to sit for long in the PCR machine after each PCR step.

Preparation:

- Thaw "**Blunt/TA Ligase Master Mix**", "**Ligation Enhancer**", and "**Adapter for Illumina**".

Steps:

1. To the end-repaired DNA solution (**65 uL**), add **15 uL** of "**Blunt/TA Ligase Master Mix**", **2.5 uL** of "**NEBNext Adapter for Illumina**", and **1 uL** of "**Ligation Enhancer**" for a total volume of 83.5 uL.
2. Mix by flicking, then gently spin to gather liquid.
3. PCR using protocol "**Library Prep 2**" (15 minutes at 20 C). Ignore error: "The selected protocol does not contain plate reads". Thaw "**USER Enzyme**" after having let the PCR run for 10 minutes.
4. Add **3 uL** of "**USER Enzyme**" to the mixture of **83.5 uL**. The total volume should be 86.5 uL.
5. Mix well and place back in the PCR machine. Select protocol "**Library Prep 3**" (15 minutes at 37 C). Ignore error: "The selected protocol does not contain plate reads". Can move on to the preparation steps in next section during the wait.

Size Selection (for an average insert size of 500-700 bp)

Notes:

- Skip this section if size selection was already performed above
- If greater than one month old, test speed beads against a ladder prior to use.

Preparation:

- Label one PCR tube and two 1.7 mL or 2.0 mL microtubes per sample.
- Bring Speed Beads to room temperature.
- Add **13.5 uL** of UltraPure H2O to one 1.7 mL or 2.0 mL tube per sample.

Steps:

1. Combine sample mixture with the UltraPure water, for a total volume of **100 uL**.
2. Vortex Speed Beads to resuspend.
3. Add **55 uL** Speed Beads to the samples. Thoroughly mix by flicking.
4. Incubate for 5 minutes at room temperature. Periodically mix using gentle flicking of the tube to prevent beads from settling.
5. Gently spin to make sure there are no liquid droplets or beads on the sides and place on a magnetic stand until the solution is clear (~5 minutes).
6. Transfer SUPERNATANT containing DNA to a new 1.7 mL tube. Discard the beads. Larger DNA fragments will have preferentially bound the beads and will be removed with the discarded beads.
7. Add **15.5 uL** Speed Beads to supernatant. Thoroughly mix by flicking.
8. Let incubate for 5 minutes. Periodically mix using gentle flicking of the tube to prevent beads from settling.
9. Spin gently and place back on magnetic stand.
10. After solution is clear, discard supernatant. Be careful not to disturb beads. Once the large fragments have been removed, the medium size fragments will preferentially bind the beads. Discarding the supernatant will remove small DNA fragments.
11. Leaving the tube on the magnetic stand, rinse the beads with **200 ul** of 80% ethanol. Wait until the solution is clear, then discard the supernatant.
12. Repeat the previous wash step, removing supernatant when done.

13. Let the tube sit on the magnetic stand (with the lid open) until dry (~ 3 minutes). Be careful not to let the beads become overdry (beads will begin to crack if too dry).
14. Remove the tube from the magnetic stand. Elute the beads in **17 uL** of clean low EDTA-TE. Mix well, getting all beads off the side of the tube.
15. Let incubate for 2-3 minutes. Gently flick the bottom of tube so that beads do not settle.
16. Gently spin the tube and place back on the magnetic stand. After the solution is clear, carefully transfer **15 uL** of the supernatant to a new PCR tube.

DNA Amplification

Preparation:

- Select the Index primers that will be used for the current batch of samples and log them in a notebook.
- Thaw index primers, "Universal PCR Primer", and "**Q5 Hot Start HiFi PCR Master Mix**" at room temperature. Invert the master mix to dissolve precipitates.

Steps:

1. Add **25 uL** of "**NEBNext Q5 Hot Start HiFi PCR Master Mix**", **5 uL** of "**Index Primer**", and **5 uL** of "**Universal PCR Primer**" to the adaptor-ligated DNA fragments from the previous section, for a total volume of **50 uL**. MAKE SURE to take note of what barcode encodes what sample.
2. Finger flick to mix and then gently spin down tube.
3. PCR using protocol **Library Prep 4**. Select "SYBR Only" in the "Express Load" dropdown under "Plate" settings.

Standard Cleanup (Primer, Nucleotide, and Enzyme Removal)

Preparation:

- Label one 1.5 mL (with original ID) and one 1.7 mL eppendorf tube per sample.
- Bring Speed Beads to room temperature, if not already done.

Steps:

1. Transfer amplified product to a clean 1.7 mL eppendorf tube.
2. Vortex Speed Beads to resuspend.
3. Add **45 uL** Speed Beads to the PCR reactions. Mix well by flicking.
4. Let incubate for 5 minutes. Periodically mix using gentle flicking of the tube to prevent beads from settling.
5. Quickly spin the tube and place on the magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes) remove and discard the supernatant (DNA should be attached to the beads).
6. Leaving the tube on the magnetic stand, rinse the beads with **200 uL** of 80% ethanol. Incubate for 30-60 seconds to allow the beads to re-bind DNA, then remove and discard the supernatant (DNA should still be attached to beads).
7. Repeat previous wash step once.
8. Air dry the beads on the magnetic stand.
9. Elute the beads in **33 uL** low EDTA-TE. Mix well. Be sure that all beads from the side of the tube are suspended in the solution.

10. Quickly spin down the tube and let sit at room temperature for 2-3 minutes. Gently flick the bottom of tube so that beads do not settle.
11. Place the tube on the magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant to a clean 1.5 mL eppendorf tube. Discard the beads.
12. Qubit **1 uL** of the final product and record the values (2-10 ng/uL = PASS, <1 ng/uL = FAIL, 1-2 ng/uL = maybe, redo if possible).
13. Store final product at -4 ˚C if it will be used immediately, -20 ˚C otherwise.