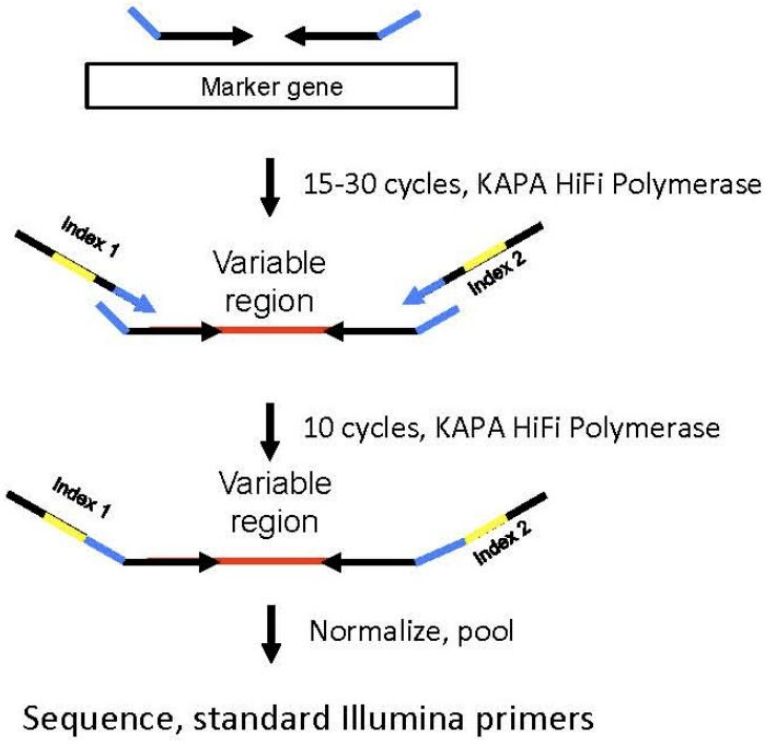
16s V4 rRNA Library prep via dual-indexing
SMack Lab 2019 (modified from Gohl et al, 2016)



Following DNA extraction with the Qiagen's PowerLyzer PowerSoil DNA Isolation kit (Qiagen #12855) as per the manufacturer protocols.

Reagents:

- AMPure XP Beads or suitable alternative (SPRI beads)
- Forward and reverse adapted primers from The Human Microbiome Project (diluted to 10µm concentration):
  515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA)
  806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT)
- NEBNext® Ultra™ II Q5® Master Mix
- Illumina barcoding primers with 10bp index sequence (sequences can be found here)

First PCR: amplification of V4 region

In each PCR well, mix:

1) 12.5 µl Nebnext Ultra II Q5 mastermix
2) 1 µl V4 primer 1 10 µM
3) 1 µl V4 primer 2 10 µM

4) 10.5 µl DNA sample + H2O
   25ng DNA OR
   6µl of DNA samples < 4 ng/µl OR
   1µl of DNA samples > 25ng/µl

Run the following PCR program (in Eppendorf thermocycler with 100ºC heated lid)
5 min @ 95ºC
15 cycles of :
   20s @ 98ºC
   15s @ 62ºC
   60s @ 72ºC
Hold at 4ºC

Cleanup with 2:1 ratio of homemade SPRI beads (50µl beads per sample)

1. Warm the SPRI beads to room temperature and mix thoroughly before use.
2. Add 50µl of beads to each sample, mix thoroughly by pipetting up and down 10x, and incubate for 5 minutes at room temperature.
3. Place the samples on a magnetic separator, when the beads have collected to the wall of the tube and the solution is clear, remove and discard the liquid.
4. Add 200 µl of 70% ethanol. Wash the beads by turning the tube 180° and allowing the beads to re-collect on the side of the tube. Turn the tube 6 times.
5. Remove and discard the ethanol.
6. Repeat steps 4-5 1x.
7. Keeping the tubes on the magnet and the caps open, dry the beads at room temperature. Cracks will be observed in the bead pellet when drying is complete.
8. Add 12 µl of water to the dried beads and pipette up and down to mix thoroughly.
9. Incubate for 2 minute at room temperature.
10. Place the samples on a magnetic separator, when the beads have collected to the wall of the tube and the solution is clear, transfer 10 µl of the liquid to a fresh plate. Label plate FIRST PCR PRODUCT (4µl of this first PCR Product will be used for PCR round two).

Second PCR: indexing PCR
Here we add unique index primer combinations to molecularly barcode each sample.

*Each sample should get a unique i5/i7 primer combination, sequences can be found here.

Each reaction needs the following composition of materials:

1) 6µl of Nebnext Ultra II Q5 mastermix
2) 1 µl n5 primer (e.g., n501, n502,...)
3) 1 µl n7 primer (e.g., n701, n702,...)
4) 4µl of each sample (from 10µl of PCR Product 1 put into a new plate after bead cleaning above).

Run the following PCR program (in Eppendorf thermocycler with 100ºC heated lid):
5 min @ 95ºC
10 cycles of:
    20s @ 98ºC
    15s @ 55ºC
    60s @ 72ºC
Hold @ 4ºC

Cleanup with 2:1 ratio of homemade SPRI beads (24µl beads per sample). Elute in 12µl H2O.

Quantify on Fragment analyzer (or qubit if doing many samples).