Deep-amplicon sequencing protocol for fecal nematode identification

SMack Lab 2019 (adapted from Avramenko et al. 2015)

Following extraction with the MoBio DNEasy Blood and Tissue Kit (larval DNA) or Powerlyser Powersoil kit (fecal DNA) as per the manufacturer protocols.

Reagents:

- AMPure XP Beads or suitable alternative
- Forward and reverse adapted primers (Pafco et al., 2018): Strongyl_ITS-2_F (acg tct ggt tca ggg ttg) and Strongyl_ITS-2_R (atg ctt aag ttc agc ggg ta) (diluted to 10um concentration).
- NEBNext® Ultra™ II Q5® Master Mix
- Illumina barcoding primers
    - Illumina P5 sequence with NEBNext adaptor site and 10 bp i5 index
    - Illumina P7 sequence with NEBNext adaptor site and 10 bp i7 index

PCR Round One. This phase puts primers on for our target region (ITS-2).

- Make a master mix as follows (19µL per reaction)
    - 10µL NEBnext
    - 1µL Strongyl_ITS-2_F adaptor pool (10µM)
    - 1µL Strongyl_ITS-2_R adaptor pool (10µM)
    - 7µL nuclease-free H20.
- Add 19µL of the master mix into each well.
- Add 1µL of sample DNA per well.
- Mix and spin
- PCR I protocol: 95°C for 3 minutes (1x), 98°C for 20 seconds, 62°C for 15 seconds, and 72°C for 15 seconds (25x), and then 72°C for 2 minutes.
- Perform a 1x bead-clean with room temperature ampure beads.
    - Add 20µL of beads to each sample, mix thoroughly
    - Incubate at room temperature for 5 minutes
    - Place on magnetic separator, remove and discard liquid
    - Add 200µL of 70% ethanol, mix to wash beads
    - Place on magnetic separator, remove and discard liquid
    - Repeat ethanol wash as above
    - Dry beads at room temperature on magnet

    - Elute in 12μL of water, mix thoroughly
    - Incubate at room temperature for 2 minutes
    - Place on magnetic separator, transfer 10μL of liquid to a new plate.

PCR Round Two: This phase adds Illumina adaptors and barcodes.

- Make a master mix as follows (15.5μL per reaction)
    - 10μL Nebnext buffer
    - 5.5μL H20
- Add 15.5μL of master mix into each well.
- Add unique combination of Illumina P7 (1μL) + P5 (1μL) primers into each well (keep close track of unique combinations).
- Add 2μL of sample DNA per well.
- Mix and spin.
- PCR II protocol: 98°C for 45 seconds (1x), followed by 98°C for 20s, 63°C for 20s (7x), 72°C for two minutes (1x).
- Perform a 1x bead-clean with room temperature ampure beads.
    - Add 20μL of beads to each sample, mix thoroughly
    - Incubate at room temperature for 5 minutes
    - Place on magnetic separator, remove and discard liquid
    - Add 200ul of 70% ethanol, mix to wash beads
    - Place on magnetic separator, remove and discard liquid
    - Repeat ethanol wash as above
    - Dry beads at room temperature on magnet
    - Elute in 22μL of water, mix thoroughly
    - Incubate at room temperature for 2 minutes
    - Place on magnetic separator, transfer 20μL of liquid to a new plate.