NEBNext miRNA Sequencing

1. Prepare small RNA libraries from 100 ng of total RNA according to manufacturer's instructions for the NEBNext Multiplex Small RNA Kit (New England Biolabs; Ipswich, MA).
    a. Ligate adaptors to the 3' and 5' ends of the miRNAs as part of total RNA samples. Anneal a complementary primer to 3' adaptor sequences followed by reverse transcriptase (Superscript III; Invitrogen; Carlsbad, CA) to generate a cDNA library of small RNAs.
2. Enrich the library by 15 cycles of PCR employing a common 5' primer and up to 24 unique Index primers (3' adaptor complement; equivalent to Illumina TruSeq Small RNA sequences).
3. Assess PCR products for miRNA products by Agilent Bioanalyzer DNA 1000 (Santa Clara, CA) analysis.
4. Quantify the 130-160bp fraction by region analysis to determine equimolar amounts of libraries to pool.
5. Purify pooled PCR reactions and concentrate using a Qiagen MinElute protocol. Elute in 30□L nuclease-free dH_2O.
6. Fraction small RNA libraries to extract a miRNA-enriched sample via 3% Pippin Prep gel cassettes (Sage Science, Beverly, MA).
    a. Purify the 130-160bp recovered fraction by Qiagen MinElute. Recover in 23□L nuclease-free dH_2O.
7. Assess pooled miRNA fractions by a second Agilent DNA 1000 assay.
    a. A predominant peak at 140-150bp is evidence that the miRNA-modification molecular biology and size selection steps have performed as expected.
8. Determine the final concentration of each library pool by Qubit fluorometry (Invitrogen).
9. Load library pools (12 samples/lane) onto paired end flow cells at concentrations of 8-10 pM to generate cluster densities of 700,000/mm^2 and ~20 million reads per sample following Illumina's standard protocol using the Illumina cBot and cBot Paired end cluster kit version 3.
10. Sequence the flow cells as 51 X 2 paired end reads using the Small RNA Sequencing Primer and an Index read to facilitate demultiplexing of the samples.
11. Sequence libraries on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and HCS v2.0.12 data collection software.
12. Perform base-calling using Illumina's RTA version 1.17.21.3.
13. The raw counts are uploaded to ImmPort for each sample
14. The offset file is the trimmed mean offset using the method of Robinson and Oshlack. The offset can be used in edgeR to run analysis.
    Robinson and Oshlack Genome Biology 2010, 11:R25
    The dispersion file is the per "miRNA dispersion" used in our statistical testing.