This protocol is for 6 samples

Ligate 3' adaptor

1. Thaw on ice: RA3, ligation buffer, stop solution
2. Prepare a strip tube with labels; set on ice
3. Preheat cycler to 70 degrees
4. Add 1 ul of RA3 to each tube
5. Add 5 ul sample to each tube (50ng), mix by pipetting, spin down briefly
6. Set in thermocycler for 2' at 70 degrees to denature samples
7. Remove and set on ice
8. Preheat cycler to 28 degrees
9. Combine in a 1.5 ml tube
    a. HML – 13.2 uls
    b. RNase inhibitor – 6.6 ul
    c. T4 RNA Ligase 2, Deletion mutant – 6.6ul
10. Mix by pipetting, spin down briefly
11. Add 4 ul of HML mix to each sample, mix by pipetting. Total volume: 10ul
12. Set in thermocycler for 1 hour exactly at 28 degrees
13. Thaw on ice: ATP
14. Add 1 ul of Stop solution to each sample, mix by pipetting, spin down briefly
15. Set back in thermocycler for 15'
16. Remove samples from thermocycler, keep on ice

Ligate 5' adapter

17. While samples are on thermocycler, preheat other cycler to 70 degrees
18. Add 6.6 ul of RA5 to new strip tube
19. Set in cycler at 70 degrees for 2' to denature adapter
20. Remove from thermocycler, keep on ice
21. Add 6.6 ul 10mM ATP to the tube
22. Add 6.6 ul of T4 RNA Ligase (provided in kit) to tube
23. Mix by pipetting, spin down and keep on ice
24. Add 3 ul of RA5 mix to each sample, mix by pipetting, spin down briefly. Total volume: 14 ul
25. Set in thermocycler for 1 hour at 28 degrees

26. Remove samples from thermocycler, keep on ice

Reverse transcription

27. Thaw on ice:
    a. 1st strand buffer
    b. 100 mM DTT
    c. 25 mM dNTP mix
    d. RT primer
28. Preheat cycler to 70 degrees
29. Dilute 25 mM dNTP mix (3.3 ul of 25 mM dNTP mix + 3.3 ul of RNase-free water) to create a 12.5 mM dNTP mix. Set aside
30. Prepare master mix for reverse transcription. Combine the following in a 1.5 ml tube:
    a. 1st strand buffer – 13.2 ul
    b. 12.5 dNTP mix – 3.3 ul
    c. 100 mM DTT mix – 6.6 ul
    d. RNase –inhibitor – 6.6 ul
31. Prepare a new strip tube
32. Add 1 ul of RT primer to each well on ice
33. Add 6 ul of each sample to new strip tube containing RT primer
34. Set samples on cycler at 70 degrees for 2'
35. Remove and set on ice
36. Preheat cycler to 50 degrees
37. Add 6.6ul Superscript II to the 1.5 ml master mix above
38. Gently flick or mix tube by pipetting, spin down
39. Add 5.5 ul of mix to samples. Total volume=12.5 ul
40. Place on cycler at 50 degrees for 1 hour
41. Remove samples and set on ice

Amplify libraries

42. Thaw on ice: Ultra pure water, PCR Mix (PML), RP1, and all the indices you will be using
43. Combine on ice:
    a. H2O – 56.1 ul

b. PML – 165 ul
c. RP1 – 13.2 ul

44. Add 2 uls of corresponding adapter to each sample

45. Add 35.5 uls of Master mix above to each sample
46. Mix by pipetting, spin down briefly and set in thermocycler
47. Run program 'Illumina pcr'
    a. 98 – 30s
    b. Repeat the following 12 x's:
        i. 98 – 10s
        ii. 60 – 30s
        iii. 72 – 15s
    c. 72 – 10'
    d. 4 – infinity
48. Run DNA HS chip on samples to visualize desired peak
49. Good stopping point – freeze at -20

Gel Extraction & Purification

1. Make 1xTBE
2. Prepare gels. Pipette TBE into wells of gel and shake out. Be sure to remove white strip. Place in rig
3. Fill rig with TBE until wells are covered
4. Thaw on ice: samples, Custom ladder (CRL), High resolution ladder (HRL) and load dye
5. Make 2x load dye (1.1 ul 6x dye + 2.2 ul H2O)
6. Combine 2 ul CRL + 2 ul 1x load dye per gel
7. Combine 1 ul HRL + 1 ul 1x load dye per gel
8. Fill gels with ladder as desired. Use HRL to orient gel wells. Use CRL or empty wells between samples. Samples will take 2 lanes each
9. Combine 9 uls 6x load dye + sample
10. Fill sample wells using p20 set at 13.5uls. Do not exceed ~ 27 uls per well

11. Run gel at ~140 volts for ~55 min or until lower marker is practically running off gel
12. Prepare 1.5 ml tubes with gel breaker tube inserts
13. Combine 10 ml milli-q water with 5 ul ultra-pure EtBr (screw cap, not dropper). Vortex to mix
14. Excise gels from their cassettes. Push tool between the 2 pieces of cassette until you hear it crack. Do this on all three sides. Then push tool into crack at bottom of gel to release gel. Pour a little buffer on it to untangle it and empty buffer into sink
15. Apply EtBr mix to gel and incubate 10' (use hood)
16. If desired, rinse EtBr off gels using a little TBE
17. Using transilluminator and a clean razor blade, excise the portion of the gel that lies between the 2 custom ladder bands. Place in gel breaker tube.

147 nt band: mature miRNA generated from ~22 nt small RNA fragments.

157 nt band: piwi-interacting RNAs, some miRNAs, and other regulatory small RNA molecules.

CRL: 3 dsDNA fragments: 145 bp, 160 bp, and 500bp.

18. Spin at 20,000 xg for 2 minutes.
19. Remove gel breaker tube from 1.5 ml tube.
20. Add 300 ul ultra-pure water to sample.
21. Gently shake for at least 2 hours. I use 600 rpm
22. Adjust centrifuge temperature to 4 degree.
23. Prepare p1000 tips by cutting the tip with a clean razor blade.
24. Thaw on ice: glycogen, pellet paint, 3M Sodium acetate(NaOAc).
25. Prepare new 1.5 ml sample tubes for storage of libraries.
26. Remove gel/water from tube using prepared p1000 tip and place in 5 um filter tube.
27. Spin tubes at 600 xg for 10 seconds.
28. Remove, remove filter.
29. Prepare a 2% glycogen solution
30. Prepare a .1% pellet paint solution (1.3 ul 1x solution + 11.7 ul H20)
31. Add 2 ul of 2% glycogen to tube
32. Add 30 ul of 3M NaOAc
33. Add 2 ul of .1% pellet paint
34. Add 975 ul of 100% cold EtOH
35. Gently mix by pipetting and transfer to prepared 1.5 ml storage tube.
36. Spin tube at 20,000 xg for 20' at 4 degree.

37. Aspirate solution. If the pellet becomes loose, spin again
38. Add 500 uls 70% EtOH
39. Spin 2’ at 20,000 xg
40. Repeat EtOH wash
41. Set pellet in bead bath at 37 degrees for 7’ to dry the pellet
42. Resuspend pellet in 11 ul of 10mM Tris-HCL, pH 8.5
43. Run DNA 1000 Bioanalyzer chip. Identify desired fragment around ~140-150 bp. Check for any contamination from other sized fragments. Calculate normality using all fragments.
44. Store.