Purchases

Beckman Coulter
RNAClean XP beads
A63987

AMPure XP Beads
A63880

Life Technologies
Arrayscript (AM2048 or from MessageAmpII kit – AM1751)

NEB
Phusion® High-Fidelity PCR Master Mix with HF Buffer – M0531

Primer manufacturer
randomhexRT primer – GCCTTGGCACCCGAGAATTCCANNNNNN – used at 100µM
small RNA PCR primers - PCR primers used at 10µM.
RP1: 5´-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCG-s-A-3´
RPI1: 5'-CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI2: 5'-CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI3: 5'-CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI4: 5'-CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI5: 5'-CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI6: 5'-CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI7: 5'-CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI8: 5'-CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI9: 5'-CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI10: 5'-CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI11: 5'-CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'
RPI12: 5'-CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCCTTGGCACCCGAGAATTCC-s-A-3'

aRNA Clean up and fragmentation

Clean up

1) Pool 5µl of each of the harvested aRNA libraries (~480µl total)
2) Split into four 0.5-ml tubes (~120µl each)
3) Add 1.5× volumes (180µl) of RNAClean XP beads to each. Mix well until the liquid appears homogeneous. Incubate for 5 min at room temperature.
4) Put the tubes on a magnetic stand for bead separation for 5 min.
5) Aspirate and discard the supernatant.
6) Wash 3 times with 70% ethanol.
7) Remove the ethanol and let dry for 10 min or until completely dry.
8) Elute the aRNA with 20 µlTE or RNase-free DDW. Combine eluates and mix until homogeneous.
9) Place the tube on a magnetic stand for 5 min
10) Transfer the eluted aRNA to a new Eppendorf

Fragmentation

To 20µl aRNA add 5µl of fragmentation buffer (200mM Tris-acetate, pH 8.1, 500 mMKOAc, 150 mMMgOAc)
Incubate for 3 min at 94^{o}C
Move to ice and add 2.5µl stop solution (0.5 M EDTA pH8)

Repeat the clean up as above, but add 1× beads (27.5µl). Elute in 20µl.
Check aRNA amount and quality on 2100 Bioanalyzer

Library preparation

Protocol designed for 5-10ng amplified RNA, although as little as 1-2ng can be used, but then additional PCR cycles are required. Sample volume should be adjusted to 5µl, either by adding water or drying down in a speedvac, depending on RNA concentration.

RT reaction (Ambion kit)

To 5 µl aRNA add 1 µl randomhexRT primer.
Incubate 2min at 70^{o}C, quick chill on ice.
Add 4µl of the following mix to each reaction:

First Strand buffer	1µl
dNTP (2.5mM)	2µl
RNase Inhibitor	0.5µl
ArrayScript	0.5µl

Incubate 10 min at 25^{o}C.
Incubate 2hr at 42^{o}C (in hybridization oven, or thermal cycler with lid at 50^{o}C)

PCR amplification:

To each reverse transcription reaction add 38µl of the following mix:

- Ultra Pure Water 11µL
- Phusion PCR mix 25µL
- RNA PCR Primer (RP1, from Illumina small RNA kit) 2µL

To each reaction add 2µl of a uniquely indexed RNA PCR Primer (RPIX, from Illumina small RNA kit)

Amplify the tube in the thermal cycler using the following PCR cycling conditions:
30 seconds at 98°C
12 cycles of:
10 seconds at 98°C
30 seconds at 60°C
30 seconds at 72°C
10 minutes at 72°C
Hold at 4°C
Can go up to 15 cycles if necessary, or down to 11 if starting with the full 10ng.

Bead Cleanup of PCR products – Repeat 1:

- Prewarm beads to room temperature.
- Vortex AMPure XP Beads until well dispersed, then add 50µl to the 50µl PCR reaction. Mix entire volume up ten times to mix thoroughly.
- Incubate at room temperature for 15 min.
- Place on magnetic stand for at least 5 min, until liquid appears clear.
- Remove and discard 95µl of the supernatant.
- Add 200µl freshly prepared 80% EtOH.
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
- Add 200µl freshly prepared 80% EtOH
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
- Air dry beads for 15 min, or until completely dry.
- Resuspend with 30µl Resuspension Buffer (from Illumina kit). Pipette entire volume up and down ten times to mix thoroughly.
- Incubate at room temperature for 2 min.
- Place on magnetic stand for 5 min, until liquid appears clear.
- Transfer 30µl of supernatant to new tube.

Bead Cleanup of PCR products – Repeat 2:

Repeat as above, but adding 24µl beads and elute in 10µl Resuspension Buffer at the end, transferring 10µl to a new tube.