SAMPLE PREPARATION FOR RNA-SEQ

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BACKGROUND

- For preparation of RNA samples for RNA-seq

NOTES

- Wear goggles, gloves and coat

EQUIPMENT

- Equipment:
  - Vortex
  - Centrifuge
  - Pipettes and Tips, Tubes
  - Aligent 2100 Bioanalyzer
- Materials:
  - Aligent RNA 6000 Kit
  - RiboZero Magnetic Kit – Human/Mouse/Rat (Epicentre MRZH11124)
  - RNA Clean XP Kit
  - Turbo DNase
  - Superscript III First-Strand Synthesis System
  - RNase H
  - NEB Buffer 2
  - Klenow fragment DNA polymerase
  - Ampure XP
  - 100% Ethanol
  - RNAse-free water

PROTOCOL

1. Check RNA integrity using the Aligent 2100 Bioanalyzer (get training from Jane-Ann)
   a. Preparatory work
      i. Aliquot 1µL of each RNA sample into a new 1.5mL microcentrifuge tube
      ii. Sign-in on the sign-in sheet located beside the Bioanalyzer
      iii. Remove reagents from fridge/freezer and thaw at room temperature for 30 minutes
      iv. Turn the heat block on to 70°C
      v. Clean Bioanalyzer electrodes
         1. Fill “RNase Zap” electrode cleaner with 350µL RNase Zap; shake chip

2. Open the machine lid and place electrode cleaner into the Bioanalyzer in the correct position; gently close lid using two hands and leave for 1 minute
3. Fill "dH_2O" electrode cleaner with 350µL of dH_2O; shake chip
4. Open the machine lid, remove the "RNase Zap" chip and insert the "dH_2O" chip; gently close lid using two hands and leave for 1 minute
5. Open the machine lid, remove the "dH_2O" chip
6. Keep lid open for 30 seconds for the water on the electrodes to evaporate before closing the lid

b. Prepare RNA 6000 Nano Gel
   i. Pipette 500µL of RNA 6000 nano gel matrix into spin filter
   ii. Centrifuge gel matrix at 1500g for 10 minutes at room temperature
   iii. Aliquot 65µL into a new 0.5mL tube
   iv. The remainder can be stored at 4°C and can be used within 1 month of preparation
c. Prepare gel-dye mix
   i. Vortex the RNA 6000 nano dye for 10 seconds
   ii. Add 1µL of RNA 6000 nano dye to the 65µL filtered gel
   iii. Vortex solution well and spin at 13000g for 10 minutes at room temperature
d. Prepare samples and ladder
   i. Heat denature the samples (1µL) and ladder (1µL) at 70°C for 2 minutes
   ii. Quick spin all samples to remove condensation on the lid
   iii. Keep samples at room temperature until ready to use
e. Load the gel-dye mix
   i. Place a new RNA 6000 nano chip on the chip priming station
   ii. Pipette 9µL of the gel-dye mix into the well marked (G)
   iii. Press down plunger until it is held by the clip after closing the priming station
   iv. Wait 30 seconds and then release clip (plunger should move at least 0.3mL upwards within 1 second; if not, the chip may not be properly pressurized and may need to be reprimed)
   v. Pipette 9µL of the gel-dye mix into the other 2 "G" wells
f. Load the RNA 6000 nano marker
   i. Pipette 5µL of RNA 6000 nano marker into each sample well and the ladder well
g. Load the ladder and the samples
   i. Add 1µL of ladder into the ladder well
   ii. Add 1µL of sample into each sample well and 1µL of the marker into each unused well
   iii. Vortex the loaded chip on IKA vortexer for 1 minute at 2400rpm
   iv. Run the chip in the Aligent 2100 bioanalyzer within 5 minutes
   v. Save results onto computer
h. Cleanup
   i. Remove chip immediately after run is complete
   ii. Fill "dH_2O" electrode cleaner with 350µL of dH_2O; shake chip
   iii. Open the machine lid and insert the "dH_2O" chip; gently close lid using two hands and leave for 1 minute
   iv. Open the machine lid, remove the "dH_2O" chip
   v. Keep lid open for 30 seconds for the water on the electrodes to evaporate before closing the lid

vi. Empty contents of the "RNase Zap" and "dH_2O" chips into the garbage but save the chips to reuse next time
vii. Turn off the Bioanalyzer, computer and heating block
viii. Complete the sign-in sheet with remaining information

i. Analysis
i. Ensure RNA has a high RNA Integrity Number (preferably >8)

2. rRNA depletion using RiboZero kit
a. Bead-washing procedure
i. Pipette 225µL of magnetic beads (after vortexing) into a 1.5mL tube, per sample
ii. Place tube on magnetic stand for 1 minute
iii. Remove supernatant
iv. Add 225µL of RNase-free H_2O; vortex or pipette up and down
v. Place tube on magnetic stand for 1 minute
vi. Remove supernatant
vii. Remove tube from magnetic stand
viii. Add 65µL of magnetic bead resuspension solution and vortex
ix. Store at room temperature until ready

b. Treatment of RNA sample
i. Determine volume of RNA to use
1. Based upon the highest RNA concentration in your samples, calculate a volume that contains either 1-2.5µg or 2.5-5µg of RNA

ii. In a RNase-free tube, mix:

x µL	RNase-free H2O
4µL	RiboZero reaction buffer
y µL	RNA sample (max 28µL if input is 1-2.5µg, max 26µL if input is 2.5-5µg)
z µL	RiboZero RNA removal sol'n (8µL for 1-2.5µg RNA, 10µL for 2.5-5µg RNA)
40 µL	Total

iii. Mix by pipetting gently
iv. Incubate at 68°C on a heat block for 10 minutes
v. Store at room temperature for 15 minutes

c. rRNA removal
i. Add 40µL (all) of treated RNA sample to magnetic beads and, without changing pipette tips, pipette up and down 10x, then vortex sample
ii. Place sample at room temperature for 10 minutes, mixing every couple of minutes
iii. Vortex at max speed for 5s
iv. Place at 50°C (on heating block) for 7 minutes (avoid significant condensation)
v. Place on magnetic stand for 1 minute
vi. Carefully remove supernatant (containing RNA, around 85-90µL) and transfer to new microcentrifuge tube

3. RNA Clean XP Kit purification
a. Add 180µL of RNA clean beads to depleted RNA
b. Mix by pipetting up and down 10x gently
c. Incubate at room temperature for 15 minutes
i. During incubation, prepare fresh 80% EtOH

d. Place tube on magnetic stand for 5 minutes
e. Remove and discard supernatant, do not disturb the bead

f. While still on stand, add 200µL of freshly prepared 80% EtOH
g. Incubate for 30s on stand, then remove supernatant without disturbing beads
h. Repeat 80% EtOH wash
i. Remove supernatant, allow beads to dry on stand for 10 minutes
j. Take tube off stand, add 32µL H_2O to elute RNA
k. Thoroughly resuspend beads by gently pipetting 10 times
l. Incubate at room temperature for 2 minutes
m. Place on magnetic stand for 5 minutes (or until solution is clear)
n. While still on stand, collect supernatant into a new tube and place on ice
o. Aliquot 2µL from each 32µL sample into a new tube for use with the Bioanalyzer (give samples to Christine in the Farncombe institute to analyze using the Pico kit)
p. Continue or store at -80°C

4. Turbo DNase digestion
    a. Add the following to a RNase-free tube:

30 µL	rRNA-depleted RNA (collected from previous step)
1.5 µL	Turbo DNase
3.5 µL	10x buffer

    b. Mix contents with pipette
    c. Incubate for 30 minutes at 37°C
5. RNA Clean XP Kit purification (same as Step 3)
    a. Same as step 2 but with 70µL beads (note: 2x volume of reaction)
    b. Elute with 12µL H_2O
    c. Continue or freeze at -80°C
6. 1st strand cDNA synthesis
    a. Add the following to a RNase-free PCR tube:

10 µL	RNA from previous step
1 µL	dNTPs
1 µL	Random hexamers
12 µL	Total

    b. Place at 65°C on thermal cycler for 5 minutes
    c. In order, prepare the following mix (per sample):

2 µL	10x RT Buffer
4 µL	$MgCl_2$
2 µL	DTT
1 µL	RNase Out
1 µL	SSC III
10 µL	Total

    d. Add the 10µL mix to the first mixture
    e. Incubate for 10 minutes at room temperature
    f. Incubate for 50 minutes at 50°C and then 5 minutes at 85°C in a thermal cycler
    g. Continue or freeze at -80°C
7. Ampure XP DNA purification
    a. To the 22µL of the SSCIII mix from the previous step, add 44µL Ampure XP beads
    b. Mix by pipetting 10 times
    c. Incubate at room temperature for 5 minutes

i. Prepare fresh 80% EtOH in the meantime

d. Place tubes on magnetic stand and let stand for 1 minute
e. Remove supernatant from each sample and discard
f. Add 200µL of 80% EtOH to each sample and incubate for 30s
g. Remove supernatant and repeat 200µL 80% EtOH wash
h. Air dry beads for 10-15 minutes (preferably until you can see cracks in the bead)
i. Remove tubes from the magnetic stand and elute DNA by adding 22µL H_2O to beads
j. Mix well by pipetting
k. Place tubes back on stand until solution is clear
l. Transfer 22µL supernatant to a new tube
m. Continue or freeze at -80°C

8. 2^{nd} strand cDNA synthesis
    a. Add the following to a nuclease-free PCR tube:

22 µL	1^{st} strand cDNA from previous step
1 µL	RNase H
3 µL	dNTPs (10mM)
3 µL	#2NEB Buffer
1 µL	Klenow fragment (DNA polymerase)
30 µL	Total

    b. Incubate at 16°C for 2 hours in a thermal cycler
    c. Continue or freeze at -80°C
9. Ampure XP DNA purification
    a. Same as step 7 but with 60µL beads
    b. Elute with 50µL H_2O
    c. Continue or freeze at -80°C
10. Covaris sonication (Covaris S220)
    a. Use the following protocol for 50µL samples, 150 base pairs
        i. Peak power: 175W
        ii. Duty factor: 20%
        iii. Cycles/burst: 200
        iv. Time: 155s
    b. Continue or freeze at -80°C
11. End repair
    a. Make master mix (per sample)

10 µL	10x Buffer
5 µL	NEB End Repair Enzyme Mix
35 µL	Sterile (nuclease-free) H_2O
50 µL	Total

    b. Add the 50µL master mix to each 50µL fragmented cDNA sample
    c. Place in thermal cycler for 30 minutes at 20°C
12. Ampure XP DNA Purification
    a. Same as step 7 but with 160µL beads (1.6x volume)
    b. Elute with 42µL H_2O
    c. Continue or freeze at -80°C

13. dA-tailing
    a. Add the following to a nuclease-free PCR tube:

42 µL	End-repaired cDNA
5 µL	dA-tailing reaction buffer
3 µL	Klenow fragment
50 µL	Total

    b. Place in thermal cycler for 30 minutes at 37°C
14. Ampure XP DNA purification
    a. Same as step 7 but with 90µL beads (1.8x volume)
    b. Elute with 25µL H_2O
    c. Continue or freeze at -80°C
15. Adapter ligation
    a. Add the following to a nuclease-free PCR tube:

25 µL	dA-tailed cDNA
10 µL	Adapters for Illumina
10 µL	Ligase buffer (5x)
5 µL	Ligase
50 µL	Total

    b. Place in thermal cycler for 15 minutes at 20°C
    c. Add 3µL of USER enzyme to each sample
    d. Place in thermal cycler for 15 minutes at 37°C
16. Ampure XP DNA purification
    a. Same as step 7 but with 50µL beads (1.0x volume)
    b. Elute with 34µL H_2O
    c. Continue or freeze at -80°C
17. PCR amplification with indexes
    a. Create the following master-mix (per sample)

0.25 µL	Taq polymerase
5 µL	10x Taq buffer
1.5 µL	$MgCl_2$ (50mM)
1 µL	dNTPs (10mM)
2.5 µL	Universal Primer (25µM)
3.25 µL	H_2O
13.5 µL	Total

    b. Add the master mix to the 34µL cDNA sample
    c. Add 2.5µL of a unique index primer (25µM) to each sample
        i. For the index primers, use NEBNext Index Sets 1 and 2, or design custom primers
    d. Amplify samples with the following PCR reaction:

i.	Initial denaturation	94°C	3 minutes	
ii.	Denaturation	94°C	45 seconds	4-8 cycles
iii.	Annealing	65°C	30 seconds	
iv.	Extension	72°C	30 seconds	
v.	Final extension	72°C	10 minutes	
vi.	Hold	4°C		

18. Ampure XP DNA purification

a. Same as step 7 but with 50µL beads (1.0x volume)
b. Elute with 27µL H_2O
c. Continue or freeze at -80°C

19. Quality Check and RNA sequencing run
    a. Provide samples to Christine King in the Farncombe Metagenomics Facility, who will:
        i. Run a Bioanalyzer run (DNA chip) to validate the size of your cDNA library (fragments are expected to be approximately ~260 base pairs) and to rule out the possibility of adapter-dimers in your library
        ii. Run a qPCR reaction to quantify the concentration of your samples
        iii. Sequence your samples using an Illumina HiSeq machine