

## **Protocol: Paired-End RNA Sequencing of rRNA-depleted MTB RNA**

Adapted from Illumina Paired End Sequencing protocol

rRNA-depleted RNA is obtained by using the Epicentre Ribo-zero RNA Removal Gram-Positive kits.

### **1. First-strand cDNA synthesis: start with rRNA-depleted mRNA**

- 1.1. Use standard protocol for cDNA synthesis using random hexamers and Fermentas Maxima reverse transcriptase (same protocol for qRT-PCR)

Prepare the mRNA and random hexamers mix. Total volume is 10  $\mu$ l.  
Heat denature using Program: 70c-50c. Place samples on ice

Prepare the RT Mix.

<u>RT Mix</u>	<u>1x</u>
5x buffer	3 $\mu$ l
10mM dNTPs	1 $\mu$ l
Ribolock Inhibitor	0.5 $\mu$ l
Maxima RT	0.5 $\mu$ l
Water	1.5 $\mu$ l

Aliquot 10  $\mu$ l of RT mix into each sample tube from above.  
Total rxn. volume = 20 $\mu$ l

PCR Parameters: use standard cDNA synthesis program  
(Thermacycler program: cDNA-ss3-mtb)

- 1.2. Immediately proceed to Second-strand cDNA synthesis

### **2. Second-strand cDNA synthesis and Covaris DNA-Shearing**

- 2.1. Use Invitrogen DNA polymerase I and Fermentas RNaseH enzymes plus Fermentas 10x RNaseH buffer in the reaction

1 <sup>st</sup> -strand cDNA	20 $\mu$ l
Nuclease-free water	56 $\mu$ l
10x RNaseH buffer	10 $\mu$ l
<u>25mM dNTPs</u>	<u>1.2 <math>\mu</math>l</u>

Prepare enzyme mix:

For 1x reaction, 0.8  $\mu$ l RNaseH and 12  $\mu$ l DNA Pol I; aliquot 12.8  $\mu$ l into each sample above.

- 2.2. Incubate at 16 $^{\circ}$ c for 2.5 hrs, use closed-lid thermacycler.
- 2.3. Purify ds cDNA using Agencourt AMPure XP beads. Follow Agencourt protocol. Elute in 50  $\mu$ l water volume.

- 2.4. Fragment the ds cDNA using the Covaris S2 instrument. Follow Covaris setup instructions.

Covaris settings (reference Agilent SureSelect manual):

<u>Setting</u>	<u>Value</u>
Duty Cycle	10%
Intensity	5
Cycles per burst	200
Time	3 min.
Temp.	4°C
Set Mode	Frequency sweeping

- 2.5. Purify fragmented cDNA with the QIAquick PCR purification kit, elute sample in 30µl EB
- 2.5.1. add 300 µl PB buffer to the sample
  - 2.5.2. transfer to a QIAquick column, spin dry
  - 2.5.3. add 750 µl PE buffer to the sample, spin dry
  - 2.5.4. transfer column to a new waste collection tube, spin 2 min.
  - 2.5.5. elute in 30 µl EB

**3. Perform End Repair of fragmented ds cDNA:** physical shearing methods produces heterogeneous ends comprising 3'-overhangs, 5'-overhangs, and blunt ends. This step uses T4 DNA polymerase and Klenow to blunt-end the overhangs. T4 PNK is used to concurrently phosphorylate the 5'-ends. 5x NEB Quick Ligation buffer contains ATP and is recommended by NEB to be used in end-repair reactions

- 3.1. Use in heating block or thermocycler, total reaction volume is 100 µl.
- 3.2. Prepare the following reaction mix in a 1.5 ml eppendorf tube:

<u>Reagent</u>	<u>Volume</u>
cDNA sample	30 µl
Nuclease-free water	44 µl
10x NEB T4 Ligation buffer	10 µl
10mM dNTPs	4 µl
T4 DNA polymerase	5 µl
Klenow enzyme	1 µl
T4 PNK	5 µl
10mg/ml BSA	1 µl

Note: enzymes purchased from NEB, concentrations may differ from supplied kits and need to work out final concentrations in reaction

- 3.3. Incubate reaction mix at 20°C for 1 hr.
- 3.4. Purify sample using QIAquick PCR kit, elute sample in 37 µl EB

**4. Add 'A' Bases to the 3' end of the cDNA Fragments:** this step adds a single 'A' base to the 3' end of the blunt phosphorylated cDNA fragments using Klenow enzyme (3' to 5' Exo - ) polymerase activity. This step prepares the fragment for ligation to the adapters which have a single 'T' base overhang at their 3' end.

- 4.1. Use NEBNext dA-tailing Module, total reaction volume is 50  $\mu$ l
- 4.2. Prepare the following reaction mix in a 1.5 ml eppendorf tube:

<u>Reagent</u>	<u>Volume</u>
cDNA sample (1-5 $\mu$ g)	37 $\mu$ l
10x NEBNext dA-Tailing buffer	5 $\mu$ l
Klenow exo -	3 $\mu$ l
Nuclease-free water	5 $\mu$ l

- 4.3. Incubate reaction mix at 37°C for 30 min.
- 4.4. Purify sample in using Qiagen MinElute PCR purification kit, elute sample in 25  $\mu$ l EB
  - 4.4.1. add 250  $\mu$ l PB buffer to the sample
  - 4.4.2. transfer to minelute column, spin dry
  - 4.4.3. add 750  $\mu$ l PE buffer to the column, spin dry
  - 4.4.4. transfer column to a clean waste collection tube, spin for 2 min.
  - 4.4.5. elute in 25  $\mu$ l EB buffer

**5. Ligate Adapters to cDNA Fragments:** uses a 10:1 molar ratio of adapter to cDNA, based on a starting quantity of 3  $\mu$ g original cDNA amount. This step adds distinct 5' and 3' sequences to the cDNA, preparing it for pcr library amplification.

<u>ds DNA (nt)</u>	<u>g/mol</u>	<u>1 <math>\mu</math>g (pmol)</u>	<u>1<math>\mu</math>g (molecules)</u>
100	60,900	16.42	$9.89 \times 10^{12}$
200	121,000	8.21	$4.95 \times 10^{12}$
300	182,000	5.48	$3.30 \times 10^{12}$
500	303,000	3.29	$1.98 \times 10^{12}$

- 5.1. Use NEBNext Quick Ligation Module will decrease total incubation time to 15 minutes. However, if using the regular NEB ligation kit, **incubate samples at 25°C for 1 hr.**
- 5.2. Prepare the following reaction mix in a 1.5 ml eppendorf tube:  
Dilute adapters to 50  $\mu$ M (from 100  $\mu$ M original stock).

<u>Reagent</u>	<u>Volume</u>
cDNA sample	25 $\mu$ l
10x NEB T4 Ligation buffer	5 $\mu$ l
Ligation adapters (50 $\mu$ M)	3.3 $\mu$ l
T4 DNA ligase	5 $\mu$ l
Nuclease-free water	up to 50 $\mu$ l

Note: assume 3  $\mu\text{g}$  of fragmented cDNA and fragment size avg. 300nt equals  $\sim 16.44\text{pmol}$ , so should use  $\sim 164\text{pmol}$  of ligation adapters mix (50 $\mu\text{M}$ ).

- 5.3. There are 2 sets of ligation adapters, based on 2 reference papers
  - 5.3.1. Gnirke, et al. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. Nature Biotechnology, Feb.09.  
- ligation adapters: Oligo D and GD 7 (March 2010 order)
  - 5.3.2. Bentley, et al. Accurate Whole Human Genome Sequencing using Reversible Terminator Chemistry. Nature, Nov. 06.  
- ligation adapters: GD3 and PS-Ad#1
- 5.4. Purify ligated cDNA using QIAquick MinElute kit, elute sample in 20  $\mu\text{l}$  EB

6. **Gel Size-selection:** size-select fragments of interest on a low-range agarose gel. Suggest purifying  $\sim 200\text{-}300\text{bp}$  region, though other regions can be co-purified for library generation. Leave adequate space between samples when purifying multiple samples on the same gel.
  - 6.1. Use a low-range agarose gel such as NuSieve, prepare 150ml 2% gel with final 1x TBE concentration.
  - 6.2. Use ethidium bromide at 400ng/ml concentration (i.e., 60  $\mu\text{g}$  EtBr to 150 ml of 1x TBE-agarose)
  - 6.3. Load 5  $\mu\text{l}$  of NEB Quick Load 100 bp DNA ladder on both sides of sample, leave sufficient space to prevent sample contamination
  - 6.4. Run gel at 120 V for 60 min.
  - 6.5. Use QIAquick gel extraction kit to purify desired size-fragment range, elute in 30  $\mu\text{l}$  EB.
7. **Enrich cDNA Fragments:** enrich ligated cDNA fragments using PCR to amplify as well as to add specific sequences to the 3' and 5' ends.
  - 7.1. Use Phusion DNA polymerase from Finnzymes (NEB)
  - 7.2. Amount of ligated cDNA to take into PCR depends on the initial input DNA quantity; we typically start with  $\sim 1\ \mu\text{g}$  cDNA before shearing
  - 7.2. Prepare the following reaction, total volume is 50  $\mu\text{l}$

<u>Reagent</u>	<u>Volume</u>
ds cDNA (size-selected)	5 $\mu\text{l}$
5x Phusion HF buffer	20 $\mu\text{l}$
10mM dNTPs	2.5 $\mu\text{l}$
Amplification primers 1.0 (25 $\mu\text{M}$ )	1 $\mu\text{l}$
Amplification primers 2.0 (25 $\mu\text{M}$ )	1 $\mu\text{l}$
Phusion DNA polymerase (2U/ $\mu\text{l}$ )	1 $\mu\text{l}$
nuclease-free water	up to 100 $\mu\text{l}$

Note: Finnzymes recommended final primer concentration is 0.5 $\mu\text{M}$ , but it can

be varied 0.2-1.0  $\mu\text{M}$ . Keep primer stocks at 100  $\mu\text{M}$ , dilute to 25  $\mu\text{M}$  for use.

7.3. PCR parameters: use program '**cdna-amp**'

a. 98°C – 3 min.

b. 15 cycles:

98°C – 40 sec

65°C – 30 sec

72°C – 30 sec

c. 72°C – 5 min.

4°C – HOLD

7.4. Use Agencourt AMPure XP beads to purify PCR product. Follow Agencourt protocol. To exclude products < 200bp, use Agencourt beads to PCR volume at a 1:1 ratio. Elute in 30  $\mu\text{l}$  water volume.

7.5 Run samples on an Agilent bioanalyzer to QC before sequencing.