Overview

This protocol is a combination of producing ds cDNA from your RNA samples and then utilizing Nextera Tagmentation to produce your libraries. Please remember to think about what information you are looking for from your RNA-Seq data, this protocol does not produce stranded libraries and the fragments are produced by random hexamers, so the RNA samples will need to be cleaned up by Ribozero to remove rRNA.

Notes – This has only been tested on around 500ng of Ribominus treated RNA, the lower limits of this protocol are currently unknown.

Validation of protocol

The nextera protocol for generating RNAseq libraries was tested. An analysis of the pilot experiments for this protocol is here:

First strand synthesis

Reagents required:

- DNase and Ribozero Treated RNA 500 ng
- Superscript III kit (Invitrogen, 18080-051)

Mix the following:

<table>
<thead>
<tr>
<th>Sample Tube</th>
<th>1x</th>
<th>24x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random Hexamers (50ng/µl)</td>
<td>1 µl</td>
<td>24 µl</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1 µl</td>
<td>24 µl</td>
</tr>
<tr>
<td>RNA sample</td>
<td>5 µl</td>
<td>-</td>
</tr>
</tbody>
</table>
Nuclease Free Water 3 µl 72 µl
TOTAL 10 µl 5 µl x 24 mastermix + 5 µl sample

Incubate in tube at 65 °C for 5 min then place on ice for at least 1 minute.

Prepare the following:

<table>
<thead>
<tr>
<th>cDNA Synthesis Mix</th>
<th>1x</th>
<th>24x</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT buffer</td>
<td>2 µl</td>
<td>48 µl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>4 µl</td>
<td>96 µl</td>
</tr>
<tr>
<td>DTT (0.1M)</td>
<td>2 µl</td>
<td>48 µl</td>
</tr>
<tr>
<td>RNAse OUT (40 U / µl )</td>
<td>0.5 µl</td>
<td>12 µl</td>
</tr>
<tr>
<td>Superscript III RT (200 U / µl )</td>
<td>0.5 µl</td>
<td>12 µl</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>1 µl</td>
<td>24 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>10 µl</td>
<td>10 µl x 24 mastermix</td>
</tr>
</tbody>
</table>

Add 10 µl of the master mix of the cDNA Synthesis Mix to each of the sample tubes.

Run the following incubation program

<table>
<thead>
<tr>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>10 min</td>
</tr>
<tr>
<td>50°C</td>
<td>50 min</td>
</tr>
<tr>
<td>85°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Chill immediately on ice for at least 5 minutes.

**Second strand synthesis**

Reagents required:

- 5x second-strand buffer ([Invitrogen, 10812-014](#))
- dNTPs (10mM) from Superscript III Kit
- Rnase H from Superscript III Kit
- Ecoli DNA ligase ([Invitrogen, 18052-019](#))
- Ecoli DNA polymerase I ([Invitrogen, 18010-017](#))
- MinElute PCR purification kit ([Qiagen, 28004](#))
- Centrifuge

Prepare the following:

<table>
<thead>
<tr>
<th>Second Strand Mix</th>
<th>1x</th>
<th>24x</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x SS buffer</td>
<td>8 µl</td>
<td>192 µl</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1 µl</td>
<td>24 µl</td>
</tr>
<tr>
<td>E. coli Ligase</td>
<td>0.5 µl</td>
<td>12 µl</td>
</tr>
<tr>
<td>DNA Polymerase I</td>
<td>2 µl</td>
<td>48 µl</td>
</tr>
<tr>
<td>RNase H</td>
<td>0.5 µl</td>
<td>12 µl</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>8 µl</td>
<td>192 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>20 µl</td>
<td>20 µl x 24 mastermix</td>
</tr>
</tbody>
</table>

Add 20 µl of the master mix of the Second Strand Mix to each of the sample tubes.

Incubate at 16 for 3 hours.
Mini-elute (see kit for instructions) at 10 µl with TE buffer. **Note:** This was done with a small set of samples (i.e., 8). Other purification methods should be considered for larger scale preps. Quantify with HS-DNA Qubit assay or the SYBR Green plate assay then proceed to Nextera protocol with 2.5 ng/µL of sample.

**Library Prep via Nextera Tagmentation**

For more information on this Nextera protocol see Nextera Protocol for multiplexed genomic DNA libraries

**Consumables**

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nextera DNA Sample Preparation Kit (96 or 24 samples)</td>
<td>Illumina</td>
<td>FC-121-1031</td>
</tr>
<tr>
<td>Nextera Index Kit (96 indices, 384 samples)</td>
<td>Illumina</td>
<td>FC-121-1012</td>
</tr>
<tr>
<td>Microseal ‘A’ film</td>
<td>Biorad</td>
<td>MS-5001</td>
</tr>
<tr>
<td>Microseal ‘B’ film</td>
<td>Biorad</td>
<td>MS-1001</td>
</tr>
<tr>
<td>AMPure XP beads</td>
<td>Beckman Coulter</td>
<td>A63880</td>
</tr>
<tr>
<td>Primer P1 (AATGATACGGCGACCACCGA), purified with HPSF, at 10M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer P2 (CAAGCAGAAGACGGCATACGA), purified with HPSF, at 10M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Library Amplification Kit</td>
<td>KAPA Biosystems</td>
<td>KK2611/KK2612</td>
</tr>
</tbody>
</table>

**Labware**

- PCR plates. Use PCR plates that do not have a rim. On some thermocyclers (e.g., Biorad) a rim can prevent the lid from pressing tightly and uniformly on the plate which can cause evaporation and partial or complete loss of samples, especially in edge wells. VWR® 96-Well Thermal Cycling Plate from VWR (Cat. # 89049-178) seem to work fine for us
- 200µl and 10µl multichannel pipettes
- 96-well plate magnetic stand (e.g., Life Technologies, Cat. #123-31D)

**Step 1. Tagmentation of ds cDNA Preparation**

1. Prepare ds cDNA at concentration at 2.5ng/µl
2. Confirm the concentration by HS Qubit assay or with SYBR Green plate assay
3. Remove the TD, TDE1 and gDNA from the –20°C and thaw on ice
4. After thawing, mix all reagents and ds cDNA by gently vortexing

**Procedure (n samples = r rows, c columns )**

1. Make the Tagmentation Master Mix (TMM) by mixing n x 1.02 x 1.25µl of TD Buffer and n x 1.02 x 0.25µl of TDE1 in a PCR tube. Mix thoroughly by gently pipetting the mixture up and down 20 times
2. Distribute TMM into r tubes (or a PCR strip), c x 1.02 x 1.5µl into each tube
3. With a multichannel pipette, distribute TMM into all wells of a fresh plate (“tagmentation plate”), 1.5µl per well
4. With a multichannel pipette, transfer 1µl of gDNA into the tagmentation plate (total volume = 2.5µl per well). Mix by gently pipetting up and down 10 times. Change tips after every transfer.
5. Cover plate with Microseal ‘B’
6. Give the plate a quick spin to collect all liquid at the bottom (Sorvall or Allegra centrifuges, 1000 rpm for 1 min). Do not forget to balance the centrifuge.
7. Place the plate in the thermocycler and run the following program:
   - 55°C for 5 min
   - Hold at 10°C
Step 2. PCR (with reconditioning)

Preparation

1. Remove the KAPA polymerase mix (KAPA amplification kit KK2611/KK2612) and the indices from the −20°C and thaw at room temperature. I recommend storing the indices in aliquots in tube strips, so that they are accessible with a multichannel pipette.
2. After thawing, mix reagents and indices by vortexing.

Procedure (n samples = r rows, c columns )

1. Make r Row Master Mixes (RMMs), one for each row, by mixing c x 1.02 x 1.88l (full plate = 23 l) of 2x KAPA master mix and c x 1.02 x 0.625l (full plate = 7.65 l) of each of the N50x indices in each of r PCR tubes. Make sure to note which tube receives which index. Mix thoroughly by gently pipetting the mixture up and down 20 times.
2. Make c Column Master Mixes (CMMs), one for each column, by mixing r x 1.02 x 1.88l of 2x (full plate = 15.34 l) KAPA master mix and r x 1.02 x 0.625l (full plate = 5.1 l) of each of the N7xx indices into c PCR tubes. Make sure to note which tube receives which index.
3. Arrange the CMMs in a plate holder just above or below the PCR plate with tagmented genomic DNA.
4. With a multichannel pipette, transfer 2.5l of CMMs into each well of the plate, so that each column receives the same N7xx index. Make sure that the column number corresponds to the N7xx index. Change tips after every transfer.
5. Arrange the RMMs strip on a plate holder to the left or to the right of the plate containing tagmented DNA.
6. With a multichannel pipette, transfer 2.5 l of the RMMs into each well of the plate, so that each row receives the same N5xx index (final total volume per well = 7.5l). Make sure that the row number corresponds to the N5xx index. Mix by gently pipetting up and down 10 times. Change tips after every transfer.
7. Cover plate with Microseal ‘A’. Make sure to press well on each well, especially edge wells.
8. Give the plate a quick spin to collect all liquid at the bottom at 1000 rpm for 1 min.
9. Place the tubes in the thermocycler and run the following program:
   - 72°C for 3 min
   - 98°C for 2.45 min
   - 98°C for 15 sec
   - 62°C for 30 sec
   - 72°C for 1.30 min
   - Repeat steps (3–5) 8 times
   - Hold at 4°C

   NOTE: Ensure that the lid is tight and that it is heated during incubation
10. Make Reconditioning PCR Master Mix (RMM), by mixing n x 1.02 x 8.5l of KAPA polymerase mix, n x 1.02 x 0.5l of primer P1, and n x 1.02 x 0.5l of primer P2. Mix thoroughly by gently pipetting the mixture up and down 20 times.
11. Distribute RMM into r tubes (or a PCR strip), c x 1.02 x 9.5l into each tube
12. With a multichannel pipette, transfer 9.5 l of RMM into each well of the plate (final PCR volume 17l). Mix by gently pipetting up and down 10 times. Change tips after every transfer.
13. Cover plate with Microseal ‘A’
14. Give the plate a quick spin to collect all liquid at the bottom at 1000 rpm for 1 min.
15. Place the tubes in the thermocycler and run the following program:
   - 95°C for 5 min
   - 98°C for 20 sec
   - 62°C for 20 sec
   - 72°C for 30 sec
   - Repeat steps (2–4) 4 times
   - 72°C for 2 min
   - Hold at 4°C

   NOTE: Ensure that the lid is tight and that it is heated during incubation

Step 3. PCR Clean-up and size selection

Preparation

1. Bring AMPure XP beads to room temperature
2. Prepare fresh 70% ethanol from absolute ethanol in a sterile reservoir. You will need n x 400l of 70% ethanol for washing (full plate = 50ml). Prepare that by adding either 35ml 100% EtOH to 15 ml of H2O or 37ml of 95% EtOH to 13ml H2O.
Procedure \((n \text{ samples} = r \text{ rows}, c \text{ columns}, \text{PCR volume } V)\)

1. Centrifuge the plate to collect all liquid (1000 rpm for 1 min)
2. Vortex beads for 30 sec to ensure that they are evenly dispersed
3. Transfer \(cx1.05 \times 1 \times V \text{ I (full plate, } 17l \text{ rxn} = 214 l)\) of beads into \(r\) PCR tubes or a PCR strip
4. Using a multichannel pipette, transfer \(V \text{ I (full plate, } 17l \text{ rxn} = 17 l)\) of beads into each well containing the PCR product. Mix well by gently pipetting up and down 20 times. The color of the mixture should appear homogeneous after mixing. Change tips between columns
5. Incubate at room temperature for 5 min. DNA is now on the beads
6. Place the plate on the magnetic stand and incubate for about 1 min to separate beads from solution. Wait for the solution to become clear
7. While the plate is on the magnetic stand, aspirate clear solution from the plate and discard. Do not disturb the beads. If beads are accidentally pipetted, resuspend them back, wait for the solution to clear up, and repeat
8. While the plate is on the magnetic stand, dispense 200\(l\) of 70\% ethanol into each well and incubate for 30 seconds at room temperature. Aspirate out ethanol without disturbing the beads and discard. Repeat for a total of 2 washes
9. Remove the remaining ethanol with P10 pipette.
10. Let the plate air dry for approximately 5 min. Do not overdry the beads.
11. Take the plate off the magnetic stand. Add 33l of 10mM Tris-HCl (pH 8) to each well of the plate. Carefully resuspend the beads by mixing 10-15 times. Incubate for 2 min at room temperature. DNA is now in the solution
12. Place the plate back onto the magnetic stand and incubate for about 1 min to separate beads from solution. Wait for the solution to become clear
13. While the plate is on the magnetic stand, aspirate clear solution from the plate and transfer to a fresh plate. Do not disturb the beads. If beads are accidentally pipetted, resuspend them back, wait for the solution to clear up, and repeat.