Nextera Based RNASeq using ds cDNA from polyDT primers

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 polyDT, so the ds cDNA will not include RNA's without poly-A tails.

Notes – This has only been tested on around 1000ng of Dnasse Treated Total 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:

![Nextera R...tocol.pdf](Nextera_R...tocol.pdf)

First strand synthesis
Reagents required:
- DNase Treated RNA
- 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>Oligo(dT)20 (50 uM)</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>
<tr>
<td>Nuclease Free Water</td>
<td>3 µl</td>
<td>72 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>10 µl</td>
<td>5 µl x 24 mastermix + 5 µl sample</td>
</tr>
</tbody>
</table>

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>
</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>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 (ie 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>
</tbody>
</table>
Microseal ‘A’ film  
Biorad  
MSB-5001

Microseal ‘B’ film  
Biorad  
MSB-1001

AMPure XP beads  
Beckman Coulter  
A63880

Primer P1 (AATGATACGGCGACCACGAG), purified with HPSF, at 10M  
Primer P2 (CAAGCAGAAGACGGCATACGA), purified with HPSF, at 10M

Library Amplification Kit  
KAPA Biosystems  
KK2611/KK2612

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 1.25l of TD Buffer and n x 1.02 x 0.25l 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 1.5l into each tube
3. With a multichannel pipette, distribute TMM into all wells of a fresh plate (‘tagmentation plate’), 1.5l per well
4. With a multichannel pipette, transfer 1l of gDNA into the tagmentation plate (total volume = 2.5l 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

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

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
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 H20 or 37ml of 95% EtOH to 13ml H20.

Procedure (n samples = r rows, c columns, 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 c x 1.05 x 1 x V l (full plate, 171 rxn = 214 l) of beads into r PCR tubes or a PCR strip
4. Using a multichannel pipette, transfer V l (full plate, 171 rxn = 17 l) of beads into each well containing the PCR product. Mix well by gently pipetting up and down 20 times. Repeat steps (2–4) 4 times
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. Repeat for a total of 2 washes
8. Remove the remaining ethanol with P10 pipette
9. Let the plate air dry for approximately 5 min. Do not overdry the beads
10. 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
11. 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
12. 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.
beads are accidentally pipetted, resuspend them back, wait for the solution to clear up, and repeat.