**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 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:

*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 µ</td>
<td>24 µ</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1 µ</td>
<td>24 µ</td>
</tr>
<tr>
<td>RNA sample</td>
<td>5 µ</td>
<td></td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>3 µ</td>
<td>72 µ</td>
</tr>
<tr>
<td>TOTAL</td>
<td>10 µ</td>
<td>5 µ x 24 mastermix + 5 µ 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 µ</td>
<td>48 µ</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>4 µ</td>
<td>96 µ</td>
</tr>
<tr>
<td>DTT (0.1M)</td>
<td>2 µ</td>
<td>48 µ</td>
</tr>
<tr>
<td>RNAse OUT (40 U / µl)</td>
<td>0.5 µ</td>
<td>12 µ</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](https://www.invitrogen.com))
- dNTPs (10mM) from Superscript III Kit
- Rnase H from Superscript III Kit
- Ecoli DNA ligase ([invitrogen, 18052-019](https://www.invitrogen.com))
- Ecoli DNA polymerase I ([invitrogen, 18010-017](https://www.invitrogen.com))
- MinElute PCR purification kit ([Qiagen, 28004](https://www.qiagen.com))
- 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](https://www.illumina.com)

**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>Product Name</td>
<td>Manufacturer</td>
<td>Stock Number</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Microseal ‘A’ film</td>
<td>Biorad</td>
<td>MSB-5001</td>
</tr>
<tr>
<td>Microseal ‘B’ film</td>
<td>Biorad</td>
<td>MSB-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
- 200ul and 10ul 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/l
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 $n \times 1.02 \times 1.25l$ of TD Buffer and $n \times 1.02 \times 0.25l$ 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), $c \times 1.02 \times 1.5l$ 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 $c \times 1.02 \times 1.88l$ (full plate = 23 l) of 2x KAPA master mix and $c \times 1.02 \times 1.25l$ of TD Buffer and $c \times 1.02 \times 0.25l$ of TDE1 in a PCR tube. Mix thoroughly by gently pipetting the mixture up and down 20 times
2. Distribute RMM into $r$ tubes (or a PCR strip), $c \times 1.02 \times 1.5l$ into each tube
3. With a multichannel pipette, distribute RMM into all wells of a fresh plate (“PCR plate”), 1.5l per well
4. With a multichannel pipette, transfer 1l of gDNA into the PCR 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
0.625 l (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.

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 2.5 l of CMMs into each well of the plate, so that each column receives the same N5xx index. Make sure that the column number corresponds to the N5xx index. Change tips after every transfer.
4. Place the tubes in the thermocycler and run the following program:
   - 98°C for 2:45 min
   - 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

5. Transfer 2.5 l of RMM into each well of the plate, so that each row receives the same N5xx index (final total volume per well = 7.5 l). 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.

6. Place the tubes on the magnetic stand and incubate for about 1 min to separate beads from liquid. DNA is now on the beads

7. Cover plate with Microseal 'A'. Mix well by gently pipetting up and down 20 times.

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 20 sec
   - 72°C for 2 min
   - 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 8.5 l of KAPA polymerase mix, n x 1.02 x 0.5 l of primer P1, and n x 1.02 x 0.5 l 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 9.5 l into each tube

12. Take the plate off the magnetic stand. Add 33 l 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

13. 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

14. 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

15. Place the plate on the magnetic stand and incubate for about 1 min to separate beads from liquid. DNA is now on the beads

Step 3. PCR Clean-up and size selection

Preparation

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 2.5 l of CMMs into each well of the plate, so that each column receives the same N7xx index. Mix well by gently pipetting up and down 20 times.
4. Place the tubes on the magnetic stand and incubate for about 1 min to separate beads from solution. Wait for the solution to become clear
5. 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
6. Place the plate 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
7. Let the plate air dry for approximately 5 min. Do not overdry the beads.
8. Take the plate off the magnetic stand. Add 33 l 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
9. 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
10. 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.