DNA Library Preparation for Amplicon Miseq Sequencing

DNA library preparation for Amplicon Miseq sequencing – v2 2x250 option

Updated: 5/2014

1. Amplicon DNA preparation (55 ul of 20 ng/ul conc; ~ 1 ug of starting amp DNA)
2. DNA fragmentation: target length is ~ 250 bp (200-300 bp range)
   a. Prepare “MicroTube (6 x 16mm), AFA Fiber with Snap-Cap” for each DNA sample (Covaris™)
   b. Fragment ~ 1 ug DNA (55 ul of 20 ng/ul) using Covaris (8th floor)
      i. Check no bubbles
      ii. Put the tube into the cylinder in Covaris
      iii. Settings:
           Temperature: 5 °C
           Duty cycle: 10%
           Intensity: 5.0
           Bursts per second: 200
           Duration time: 240 sec *
           Mode: Frequency sweeping

*Optimize fragmentation time

3. Blunting DNA (End Repair)
   a. Prepare the following reaction mix on ice.
      1X Reaction (in 1.5 ml tube)
      50 ul DNA (~ 1ug) *
      6.5 ul 10X NEB ligation buffer with 10mM dATP Biology B0202S
      2.5 ul 10mM dNTP mix Invitrogen y02256
      2.5 ul NEB PNK (T4 Polynucleotide Kinase; 5U/ul) Biolab M0201S
      3.5 ul NEB T4 DNApol (5U/ul) Biolab M0203S
      65 ul Total volume
   b. Mix gently but thoroughly, and centrifuge briefly
   c. Incubate 30 min at 20 °C.

*You can run remainder of fragmented DNA on a gel, to confirm DNA was fragmented correctly

Test selection range with different ratio Beads:DNA
* Sonicate 50 ul of FY4 gDNA (200 ng/ul) at 40 sec only: smeared from 100 bp to 1 kb; then diluted to 400ul

<table>
<thead>
<tr>
<th>ID</th>
<th>Ratio</th>
<th>Beads (ul) A</th>
<th>DNA (ul)</th>
<th>Expected</th>
<th>My Own Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.65</td>
<td>32.5</td>
<td>50</td>
<td>Beads bind to &gt; 500 bp</td>
<td></td>
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<tr>
<td>2</td>
<td>0.70</td>
<td>35</td>
<td>50</td>
<td>Beads bind to &gt; 400 bp</td>
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<tr>
<td>3</td>
<td>0.75</td>
<td>37.5</td>
<td>50</td>
<td>Beads bind to &gt; 300 bp</td>
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<tr>
<td>4</td>
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<td>40</td>
<td>50</td>
<td>Beads bind to &gt; 250 bp</td>
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<tr>
<td>5</td>
<td>0.85</td>
<td>42.5</td>
<td>50</td>
<td>Beads bind to &gt; 200 bp</td>
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</tr>
<tr>
<td>6</td>
<td>0.90</td>
<td>45</td>
<td>50</td>
<td>Beads bind to &gt; 200 bp</td>
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</tr>
<tr>
<td>7</td>
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<td>47.5</td>
<td>50</td>
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</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>50</td>
<td>50</td>
<td>Beads bind to &gt; 150 bp</td>
<td></td>
</tr>
</tbody>
</table>

a. Resuspend magnetic beads by thoroughly shaking the bottle, aliquot the amount that you will need for all your samples and wait until they reach room temperature, always mix beads well before pipetting.
b. Add A ul AMPure Beads to 50 ul of sonicated DNA; gently pipette entire volume up and down 10 times
c. Incubate at room temperature for 5 minutes
d. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear
e. Gently remove and discard clear sample without disturbing beads, some liquid may remain, this removes target DNA below XXX bp
f. With tubes on stand, gently add 500 ul freshly prepared 80% ethanol to each tube and incubate at room temperature for 30
seconds, gently remove ethanol by pipette
g. Repeat step (f) for a total of 2 ethanol washes, ensure all ethanol is removed (use large pipette while tubes are on stand and small pipette immediately when tubes are removed individually from stand)
h. Remove tubes from stand and dry at room temperature for 5 minutes
i. Re-suspend dried beads with 51 ul water, gently pipette entire volume up and down 10 times, ensure beads are no longer attached to the side of the well
j. Incubate at room temperature for 5 minutes
k. Place tubes in magnetic stand at room temperature until sample appears clear – 5 minutes
l. Gently transfer 50 ul of clear sample to new tube; gel running

> RESULT (2% agarose gel + EtBr staining)

4. Gel-Free size selection using AMPure Beads
   a. Resuspend magnetic beads by thoroughly shaking the bottle, aliquot the amount that you will need for all your samples and wait until they reach room temperature, always mix beads well before pipetting.
   b. Add 52 ul AMPure Beads to 65 ul reaction (0.8 ratio), gently pipette entire volume up and down 10 times
   c. Incubate at room temperature for 5 minutes
   d. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear
   e. Set pipette to 115 ul, gently remove and discard clear sample without disturbing beads, some liquid may remain; this removes DNA below 200 bp
   f. With tubes on stand, gently add 500 ul freshly prepared 80% ethanol to each tube and incubate at room temperature for 30 seconds, gently remove ethanol by pipette
   g. Repeat step (f) for a total of 2 ethanol washes, ensure all ethanol is removed (use large pipette while tubes are on stand and small pipette immediately when tubes are removed individually from stand)
   h. Remove tubes from stand and dry at room temperature for 5 minutes
   i. Re-suspend dried beads with 52 ul water, gently pipette entire volume up and down 10 times, ensure beads are no longer attached to the side of the well
   j. Incubate at room temperature for 5 minutes
   k. Place tubes in magnetic stand at room temperature until sample appears clear – 5 minutes
   l. Gently transfer 50 ul of clear sample to new tube
   m. Add 35 ul AMPure Beads to 50 ul samples (0.7 ratio), gently pipette entire volume up and down 10 times
   n. Incubate at room temperature for 5 minutes
   o. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear
   p. Do not discard sample. Transfer 83 ul to new tube. Do not disrupt bead pellet or transfer beads. The beads bind and removes DNA above 300 bp
   q. Add 88 ul AMPure Beads to 83 ul samples, gently pipette entire volume up and down 10 times (this is to concentrate above DNA samples)
   r. Incubate at room temperature for 5 minutes
   s. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear
   t. Once solution clears, gently remove and discard 164 ul clear sample without disturbing beads, some liquid may remain
   u. With tubes on stand, gently add 500 ul freshly prepared 80% ethanol to each tube and incubate at room temperature for 30 seconds, gently remove ethanol by pipette
   v. Repeat step u for a total of 2 ethanol washes, ensure all ethanol is removed (use large pipette while tubes are on stand and small pipette immediately when tubes are removed individually from stand)
   w. Remove tubes from stand and dry at room temperature for 5 minutes
   x. Re-suspend dried beads with 33.5 ul water, gently pipette entire volume up and down 10 times, ensure beads are no longer attached to the side of the well
   y. Incubate at room temperature for 5 minutes
   a. Place tubes in magnetic stand at room temperature until sample appears clear – 5 minutes
   aa. Transfer 23 ul of clear sample to new tube. Can store at -20

5. Adenylate 3' Ends (Add “A” overhang to DNA ends)
   a. Prepare the following reaction mix on ice.
      1X reaction (in 1.5ml tube)
      23 ul DNA after size selection
      3 ul 10X NEB buffer 2
      1 ul 10mM dATP (1:10 of 100mM stock)
      3 ul Exo(-) Klenow (5U/ul)
      30 ul Total volume
   b. Incubate 30 min at 37 °C (dry heating machine)
   c. Purify samples using AMPure Beads.
      i. Resuspend magnetic beads by thoroughly shaking the bottle, aliquot the amount that you will need for all your samples and wait until they reach room temperature; always mix beads well before pipetting.
      ii. Add 30 ul AMPure Beads to 30 ul samples (1 ratio), gently pipette entire volume up and down 10 times
      iii. Incubate at room temperature for 5 minutes
      iv. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear
      v. Once solution clears, gently remove and discard 58 ul clear sample without disturbing beads, some liquid may remain; remove DNA below ~ 100 bp
      vi. With tubes on stand, gently add 500 ul freshly prepared 80% ethanol to each tube and incubate at room temperature for 30 seconds, gently remove ethanol by pipette
      vii. Repeat step (vi) for a total of 2 ethanol washes, ensure all ethanol is removed (use large pipette while tubes are on stand and small pipette immediately when tubes are removed individually from stand)
      viii. Remove tubes from stand and dry at room temperature for 5 minutes
      ix. Re-suspend dried beads with 19.5 ul water, gently pipette entire volume up and down 10 times, ensure beads are no
**Ligate Adapters**

6. Ligate Adapters

a. Do not prepare reaction mixtures. Add adapters individually in each reaction tube.

1X reaction (in 1.5 ml tube)
- 17 ul DNA (eluted in EB buffer)
- 20 ul 2X Quick Ligation buffer
- 1 ul Adapter ([20 uM; #1 ~ #12 for each sample, final 0.5 uM])
- 2 ul NEB Quick ligation

40 ul Total volume

b. Incubate 20 min at 23 °C (RT)

c. Purify samples using Agencourt® AMPure® XP beads (to remove adapter dimers : 120 bp)
   i. Resuspend magnetic beads by thoroughly shaking the bottle, aliquot the amount that you will need for all your samples and wait until they reach room temperature; always mix beads well before pipetting
   
   ii. Add 120 ul of H₂O to 40 ul of ligated sample (final : 0.25X Ligation buffer)
   
   iii. Add .85 volume of beads as the sample (136 ul first round, 17 ul second round), gently pipette entire volume up and down 10 times.
   
   iv. Incubate at room temperature for 5 minutes

   v. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear

   vi. Once solution clears, gently remove and discard clear sample (294 ul first round, 35 second round) without disturbing beads, some liquid may remain

   vii. With tubes on stand, gently add 500 ul freshly prepared 80% ethanol to each tube and incubate at room temperature for 30 seconds, gently remove ethanol by pipette

   viii. Repeat step 7 for a total of 2 ethanol washes, ensure all ethanol is removed (use large pipette while tubes are on stand and small pipette immediately when tubes are removed individually from stand)

   ix. Re-suspend dried beads with water (18 ul : first round, 15.5 ul : second round), gently pipette entire volume up and down 10 times, ensure beads are no longer attached to the side of the well

   x. Incubate at room temperature for 5 minutes

   xi. Place tubes in magnetic stand at room temperature until sample appears clear – 5 minutes

    xii. Transfer purified samples to a new tube (17.5 ul : first round, 15 ul : second round)

    xiii. Add 2.5 ul 2X Ligation buffer (20 ul in total), and repeat from step 3 to step 13 (final volume is 15 ul in water). Can store at -20 °C

**Amplify DNA fragments (PCR)**

7. Amplify DNA fragments (PCR)

a. Prepare the following reaction mix on ice.

1X reaction (in PCR tube)
- 10 ul 5X Buffer (Phusion High-Fidelity) NEB B0518S
- 2 ul Primer mix DG#366/DG#367 (10uM)
- 1 ul 10mM dNTPs Invitrogen y02256
- 0.5 ul DNA Polymerase (Phusion High-Fidelity) NEB M0530S
- 21.5 ul dH₂O
- 15 ul DNA sample

50 ul Total volume

b. PCR setting
- 2 min 98 °C
- 30 sec 98 °C
- 30 sec 65 °C Repeat **8 cycles** only
- 15 sec 72 °C
- 4 min 72 °C
- Hold 4 °C

c. Purify samples using AMPure Beads.
   i. Resuspend magnetic beads by thoroughly shaking the bottle, aliquot the amount that you will need for all your samples and wait until they reach room temperature; always mix beads well before pipetting.

   ii. Add 50 ul AMPure Beads to 50 ul samples (1 ratio), gently pipette entire volume up and down 10 times

   iii. Incubate at room temperature for 5 minutes

   iv. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear

   v. Once solution clears, gently remove and discard 98 ul clear sample without disturbing beads, some liquid may remain

   vi. With tubes on stand, gently add 500 ul freshly prepared 80% ethanol to each tube and incubate at room temperature for 30 seconds, gently remove ethanol by pipette

   vii. Repeat step 6 for a total of 2 ethanol washes, ensure all ethanol is removed (use large pipette while tubes are on stand and small pipette immediately when tubes are removed individually from stand)

   viii. Remove tubes from stand and dry at room temperature for 5 minutes

   ix. Re-suspend dried beads with 20 ul water, gently pipette entire volume up and down 10 times, ensure beads are no longer attached to the side of the well

   x. Incubate at room temperature for 5 minutes
xi. Place tubes in magnetic stand at room temperature until sample appears clear – 5 minutes
xii. Transfer 19.5 ul of clear sample to new tube, Can store at -20 °C

d. Gel validation of the DNA PCR product; run 2 ul of sample on a gel use a high percent agarose gel and stain with EtBr after gel has run. Samples should appear as 400-500 bands. Run gel long enough to get good separation of low molecular weights, increase contrast and look for the appearance of bands at ~120bp. If adaptor dimmers are present additional cleanup is necessary.

8. Characterize library: Run Agilent Bioanalyzer with 1 ul of sample to accurately estimate average fragment size, measure DNA concentration using Qubit

9. Measure library concentration for optimal cluster density using qPCR, pool libraries.