DNA Library Preparation for Illumina Sequencing

DNA library preparation for Illumina sequencing – Genomic sequencing

Updated: 5/2013

1. gDNA preparation (55 ul of 20 ng/ul conc; ~ 1 ug of starting gDNA)

2. DNA fragmentation
   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 Corvaris (8th floor)
      i. Check no bubbles
      ii. Put the tube into the cylinder in Covaris
      iii. Settings:
          1. Temperature : 4 °C
          2. Duty cycle : 10%
          3. Intensity : 5.0
          4. Bursts per second : 200
          5. Duration time : 60 sec *
          6. Mode : Frequency sweep

   *Optimize fragmentation time

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
   2.5 ul 10mM dNTP mix
   2.5 ul NEB PNK (T4 PolyNucleotide Kinase; 5U/ul)
   3.5 ul NEB T4 DNApol (5U/ul)
   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

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 48.75 ul AMPure Beads to 65 ul reaction (0.75 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 112 ul, gently remove and discard clear sample without disturbing beads, some liquid may remain, this removes DNA below 300 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
   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 remove DNA above 400 bp.
   q. Add 88 ul AMPure Beads to 83 ul samples, gently pipette entire volume up and down 10 times
   r. Incubate at room temperature for 5 minutes
s. Place tubes in magnetic stand at room temperature for 5 minutes, sample should appear clear
i. 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 23.5 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
   vi. With tubes on stand, gently add 50 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 19.5 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 ul of clear sample to new tube, Can store at -20 *

* Run 2 ul of sample on a gel before ligating adaptors, use a high percent agarose gel and stain with syber gold, samples should appear as 300-400 bands

7. Ligate Adapters

a. Do not prepare reaction mixtures. Add adapters individually in each reaction tube.
   1X reaction (in 1.5ml tube)
   17 ul DNA (eluted in EB buffer)
   20 ul 2X Quick Ligation buffer
   1 ul Adapter (#1 ~ # 12 for each sample)
   2 ul NEB Quick ligase
   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. Remove tubes from stand and dry at room temperature for 5 minutes
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)
   - 2 ul Primer mix
   - 1 ul 10mM dNTPs
   - 0.5 ul DNA Polymerase (Phusion High-Fidelity)
   - 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 10 – 12 cycle (least cycle is preferred)
   - 60 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.

**Pictures:**

*Libraries before adaptor ligation*:

*“Picture taken before protocol was updated: with current protocol, bands should appear at 300-400, not 200-300”*
Final libraries with adaptor dimers present:

Perfect final libraries: