Modified Protocol for Illumina Paired-End Library Construction

(Lazinski & Camilli, manuscript in preparation)

Introduction

The ends of the resulting library are identical to those produced by the Illumina Paired-End Kit. The library generated by this method can be sequenced with the standard Illumina sequencing primers used for paired-end or genomic sequencing.

Steps and Materials Needed

1. Shearing
   - Nebulizer (Invitrogen, Part # 45-0072)
   - Shearing Buffer (40% glycerol + 10mm Tris pH 8 + 1mm EDTA)
   - Dextran (30 mg/ml, Sigma # 9577)
   - KAc solution (3M potassium acetate + 6M acetic acid)
   - Isopropanol
2. Blunting
   - Neb Quick Blunting Kit (Part # E1201S)
3. 3' A Addition
   - NEB Klenow (exo-) (Part # M0212S)
4. Adapter Ligation and Sample Purification
   - NEB Quick Ligation Kit (Part # M2200S)
   - 2.5 uL OLJ131/OLJ137 annealed adapter mix @ 50 uM (see text)
   - Qiagen QIAquick Gel Extraction Kit (cat # 28706 or equivalent)
5. PCR Amplification
   - 10mM dNTP mix
   - 10x Easy A PCR buffer (included with Easy A PCR enzyme below)
   - 1 uL OLJ139 @ 30 uM (see text)
   - 1 uL OLJ140 @ 30 uM (see text)
   - 1 uL Easy A PCR enzyme (Strategene cat# 600404)
   - PCR purification kit (Strategene cat# 400773 or Qiagen equiv.)

1. Shearing

Place 5-30 ug of genomic DNA (vol. = 5-200 uL) in a nebulizer together with 795-600 mL shearing buffer so that the final volume is 800 mL. Nebulize at room temperature for 4 minutes @ 35 psi.

Spin nebulizer @ 450g for 2 minutes.

Collect and measure sample volume and transfer to an eppendorf tube. Typically, it will be > 450 mL and < 600 mL; some volume loss is inevitable due to evaporation and possible leaks in the Nebulizer.

Add 3 uL of 30mg/ml Dextran as carrier (NOT dextran sulfate), 1/10th vol. of KAc solution, vortex, then add 1 volume Isopropanol, vortex, put on metal surface at -80 for 5 min, thaw and spin @ 20,000 G for 5 min.

Decant sup. Spin @ 20K G for 5 sec. and carefully use a pipette to remove remaining liquid without disturbing the pellet. Wash pellet with 800 µL of 70% ethanol, vortex, spin @ 20K G for 1 min. Decant sup. Spin @ 20K G for 5 sec. and carefully use a pipette to remove remaining liquid without disturbing the pellet. Dry pellet in rotovac for 2 min (or air dry for 10 min). Resuspend pellet in 10mM tris pH 8.0 or Qiagen elution buffer at a final concentration of 333ng/uL (assuming 100% recovery); i.e. if you started with 5ug, resuspend in 15uL, if you started with 30 ug, resuspend in 90 uL.

2. Blunting

Uses NEB Quick Blunting Kit.

In a PCR Tube, Add 15uL (5ug) sheared genomic DNA + 2uL 10X Blunting Buffer + 2uL 1mM dNTP + 1mL Blunting Enzyme Mix. In a thermolcycler, incubate @ 20°C for 60 min., then heat inactivate @ 75°C for 30 min., then chill @ 4°C.

3. 3' A addition

To the above tube, add 1uL NEB Klenow (exo-) and 0.5 uL 100mM dATP. Vortex, spin, and incubate in thermalcycler for 45 min @ 37°C, followed by 30 min @ 75°C, followed by 4°C chill.

4. Adapter Ligation and Sample Purification

Uses NEB Quick Ligation Kit.

To the above tube, add 26.5 uL NEB 2x Quick Ligase buffer + 2.5 uL NEB quick ligase + 2.5 uL 50uM OLJ131/OLJ137 adapter mix (referenced below). Incubate @ 20℃ for 45 min. Split into two equal aliquots; one will serve as a backup should something go wrong when the second is gel purified. Add 3 uL 10x loading buffer to the second aliquot and run the sample on a 2% Agarose gel with ½ X TAE buffer and 1 ug/mL EtBr. Compare the resulting smear to 100 bp ladder and cut out region corresponding to between 400-500 bp. Recover DNA from gel slice using Qiagen QIAquick Gel Extraction Kit and resuspend DNA in 50 uL 10mM tris pH 8.0 or Qiagen elution buffer.

OLJ131:
5′AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT**T**C**C**GATCT3′
where the 2 underlined and bold positions are locked nucleic acids (LNA)

OLJ137:
5′**P**–GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCGTCTTCTGCTTG
where the 5' end is phosphorylated.

Above are synthesized by IDT and used without purification.

Equal aliquots of 100uM OLJ131 and 100uM OLJ137 are mixed, heated to 95℃ and then slow cooled to <40℃ to make the 50µM OLJ131/OLJ137 adapter mix.

(A note about the locked nucleic acid modifications: Notice that 13 of the final 14 nt of OLJ131 are complementary to the first 13 nt of OLJ137. Upon annealing, the oligos form a 13 nt duplex that has an unpaired 3' T overhang. This T overhang is complementary to the 3' A overhang added to the DNA sample in step (3) and facilitates the intended ligation event while precluding undesired adapter-adapter ligation. For the adapter to function properly, the 13 nt duplex must form. 2 LNA modifications have been introduced into OLJ131 to increase stability. Although these modifications function as intended, this increase in stability may be unnecessary. However, an adapter that completely lacks LNA modifications has not been tested.)

5. PCR amplification

10 uL DNA from step 4
2 uL 10mM dNTP mix
5 uL 10x Easy A PCR buffer
1 uL OLJ139 @ 30 uM (referenced on next page)
1 uL OLJ140 @ 30 uM (referenced on next page)
30 uL H2O
1 uL Easy A PCR enzyme

50 uL final vol.

PCR conditions,
2 min @ 95℃ preheat,
Then do 15 cycles of:
95℃ for 30 sec, 65℃ for 30 sec, 72℃ for 60 sec
After final cycle, incubate for an additional 4 min @ 72℃ then chill @ 4℃.

Use Qiagen or Stratagene PCR cleanup kit.

Verify 10% of sample relative to 100bp marker on a 2% agarose gel and SAVE THE PICTURE to send to TUCF.

If no product (ranging between 400-500 bp) is observed, you may repeat PCR with 20 cycles. If no product is still observed, then something went terribly wrong and you should start from scratch!

Note: 20 cycles is about as high as one would like to go. Assuming that after PCR, 2.5 ug of 400 nt product were obtained, this would be equal to 5.7×10^{12} molecules. Assuming that the PCR was working perfectly and that there was 2.0 fold amplification at each step, 20 cycles = 1,048,576 fold amplification. In this case, there would be 5,436,666 initial molecules amplified by the PCR. Since a single lane on the Illumina Genome Analyzer II can sequence in excess of 5 million molecules, the library complexity should be at least as high as that value.

If after 15 cycles, the product is very bright and there are additional products smaller than 400 bp but bigger than the primers, repeat PCR with only 10 cycles.

Once a good library is obtained, use a Nanodrop to quantitate library concentration and dilute a small aliquot such that you have at least 10 uL of a 20 nM solution (20 pmol/mL). In your calculation, assume that all inserts are 400 bp and that a bp is 660 daltons; hence 264 ng = 1pmol and 52.8ng in a total vol. of 10uL should be provided.

OLJ139:
5’AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA3’

OLJ140:
5’CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC3’

Both PCR oligonucleotides are unmodified.