Hybrid Capture Protocol 1

Preparation of Illumina library prior to array OR solution capture

Shearing samples to 100-300bp

For array capture, dilute 20 µg genomic DNA to a total volume of 100 µl with water.
For solution capture, dilute 3 µg genomic DNA to a total volume of 100 µl with water.

1. Mix and transfer to a 6mmx16mm AFA fibre vial (Covaris cat. no. 520031).

2. Seal the tube using an 8mm metal crimp seal cap (Covaris, cat no. 520028) and crimping tool.

3. Shear with a Covaris, using the settings:

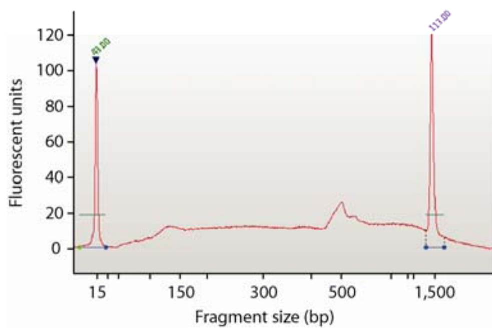
Duty cycle	20 %
Intensity	5
Cycle/burst	200
Time	120 sec

4. Remove the sample from the machine. Open the vial and transfer the sample into a fresh 1.5 ml Eppendorf lo-bind tube. Keep samples on ice.

5. Run 1 µl on an Agilent Bioanalyzer 2100 chip to check the quantity of fragmented DNA and to confirm the success of the fragmentation.

Impure DNA may shear badly. If there is any doubt about the purity of the sample, perform an ethanol precipitation before shearing (Figure 1).

a) without ethanol precipitation



b) with ethanol precipitation

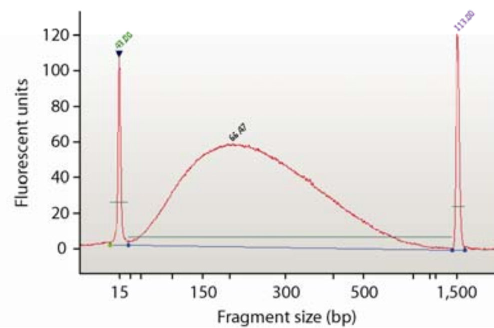


Figure 1: Impure DNA sample sheared using identical Covaris settings a) before and b) after ethanol precipitation.

Purification after Fragmentation

Use one column for each 10 µg of DNA. For 10-20 µg DNA, divide equally between two columns. Elute with 45 µl EB per 5 µg bound DNA.

1. Add 5 x volume of Buffer PB to the fragmented DNA (for 100 µl of fragmented DNA add 500 µl buffer). Vortex.

2. Pipette 750 µl of mix per column. Pipette slowly and make sure that all the liquid comes out of the pipette tip. Centrifuge the samples for 1 minute at 13,000 rpm in a benchtop centrifuge.

3. Discard the flow-through from the collection tube. Repeat step 2 if you have any buffer mix left.

4. To wash, add 750 µl of Buffer PE to each of the columns. Centrifuge for 1 minute as above. Discard the flow-through and centrifuge again for a further minute.

5. Leave the tubes in a rack with the lids open to dry for 2 minutes.

6. Transfer the column to a clean labelled 2 ml lo-bind Eppendorf tube. For each 5 µg of bound DNA, add 47 µl of EB buffer to the centre of the column and leave for 1 minute. Centrifuge for 1 minute as above.

7. Combine eluates, if applicable. You should have a volume of 45 µl for every 5 µg in the original sample (approximately 2 µl of buffer is retained by the column).

End Repair

This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and E. coli DNA polymerase I Klenow fragment. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

1. Prepare a master mix containing the following reaction mix per 5 µg sample, plus a 10 % excess (round the mass of sample UP to the nearest 5 µg):

Water	30 µl
10x T4 DNA ligase buffer with 10mM ATP	10 µl
10mM dNTP mix	4 µl
3U/ µl T4 DNA polymerase	5 µl

5U/ µl Klenow DNA polymerase	1 µl
10U/ µl T4 PNK	5 µl

2. Mix, and aliquot 55 µl of master mix into each sample tube containing the 45 µl of eluate from the previous step. Mix well and spin down.

3. Incubate for 30 minutes at room temperature (20-25 °C).

4. Clean up using one QIAquick PCR column for up to 10 µg of DNA, as described above. Elute in 34 µl EB per 5 µg DNA. This gives ~32 µl of eluate, because approximately 2 µl of buffer is retained by the column.

Addition of 'A' Bases to the 3' End of the DNA Fragments

This protocol adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single 'T' base overhang at their 3' end.

1. Prepare a master mix containing the following reaction mix per 5 µg sample, plus a 10 % excess (round the mass of sample UP to the nearest 5 µg):

10x Klenow buffer	5 µl
1 mM dATP	10 µl

2. Mix, and aliquot 15 µl of master mix into each sample tube containing the 32 µl of end-repaired sample. Mix well and spin down.

3. Add 3 µl 5 U/ µl Klenow exo (3' to 5' exo minus). Mix and spin down.

4. Incubate for 30 minutes at 37 °C in a hot block.

5. Clean up using one QIAquick MinElute columns per 5 µg of DNA, eluting in 12 µl of EB buffer per column, in a 1.5 ml lo-bind Eppendorf tube. This gives ~10 µl of eluate, because approximately 2 µl of buffer is retained by the column.

Ligation of Adapters to DNA Fragments

This protocol ligates adapters to the ends of the DNA fragments. The procedure uses a 10:1 molar ratio of adapter to DNA insert, based on a starting quantity of 5 µg of DNA

before fragmentation. The quantities below are given per 5 µg of DNA. Adjust as appropriate.

1. Prepare a master mix containing the following reaction mix per 5 µg sample, plus a 10 % excess (round the mass of sample UP to the nearest 5 µg):

2 x DNA ligase buffer	25 µl
Illumina PE Adapter oligo mix	10 µl

2. Mix and aliquot 30 µl master mix into each sample tube containing the 10 µl of A-tailed sample. Mix and spin down.
3. Add 10 µl 2,000 U/ µl T4 DNA ligase. Mix and spin down.
4. Incubate for 15 minutes at room temperature (20-25 °C).
5. Clean ligated samples and C_0t1 DNA with SPRI beads, eluting in 50 µl water. Use a 5 x excess of C0t1 for array capture and a 20 x excess for solution capture.

Alternatively, if doing a pre-hyb PCR (see below), clean up using a QIAquick PCR column as described above, eluting in 50 µl EB.

SPRI bead cleanup

Allow SPRI beads to come to room temperature for at least 30 minutes. Reagents need to be mixed well prior to use and should appear homogeneous and consistent in colour.

1. Add 90 µl of SPRI beads per 50 µl of adapter ligated sample in a 1.5 ml Lo-bind Eppendorf tube.
2. Vortex and leave at room temperature for 5 minutes.
3. Place tubes in a magnetic rack.
4. Leave for 5 minutes or until sample is clear.
5. Carefully remove the clear solution from the tubes and discard.
6. Dispense 700 µl of 70 % ethanol into each tube while in the magnetic rack taking care not to disturb the magnetic beads. Aspirate and discard ethanol.
7. Repeat the ethanol wash once again (total of two washes).

8. Dry the samples on a heat block (keep the lid of the tube open) at 37 °C for 5 to 10 minutes or until the residual ethanol has evaporated.

9. Add 50 µl of molecular biology grade water, vortex and incubate at room temperature for 2 minutes.

10. Place tubes into the magnetic rack and leave for 2-3 minutes or until sample is clear.

11. Carefully remove the water and retain in a new 1.5 ml lo-bind Eppendorf tube.

12. Repeat step 9-12 once more, retaining the water in the same 1.5 ml lo-bind tube. Total volume of elute should be 100 µl.

13. Centrifuge the eluate at 13,000 rpm in a bench top centrifuge for 10 minutes

14. Transfer the sample to a new 1.5 ml lo-bind Eppendorf tube leaving behind any precipitated beads.

15. Quantify 1 µl of the library using an Agilent DNA 1000 chip on a Bioanalyzer 2100 and proceed to hyb, following the manufacturer's recommended protocols.

Optional step: pre-hyb PCR

Performing a small number of PCR cycles before hybridisation can improve robustness, particularly for clinical samples, and will simplify sample indexing. Amplify each 50 µl adapter-ligated library by dividing between 4 PCR reactions.

1. Prepare a master mix containing the following reaction mix per sample, plus a 10 % excess:

10 µM PE2.1	10 µl
10 µM PEV2.2	10 µl
2 x Phusion HF master mix	100 µl
water	30 µl

2. Mix and aliquot 150 µl of master mix into each 50 µl adapter-ligated library. Mix and spin down.

3. Aliquot into four 200 µl PCR tubes (50 µl each), and perform the following temperature cycling:

98 °C	2 minutes	
98 °C	20 seconds	
65 °C	30 seconds	x 6 cycles
72 °C	30 seconds	
72 °C	5 minutes	
4 °C	indefinitely	

4. Combine all 4 reactions and clean up with SPRI beads (see above), adding 360 µl beads to the 200 µl PCR reactions, and eluting in 50 µl water.

Quantify 1 µl of library using an Agilent DNA 1000 chip on a Bioanalyzer 2100 and proceed to hybridization (Hybrid Capture Protocol 2a or 2b).