PROTOCOL: Illumina Barcoded Paired-End Capture Library Preparation Using Non-index Adaptors and Phusion DNA Polymerase

This protocol provides instructions for preparing DNA paired-end capture libraries for targeted sequencing by Illumina platforms. It involves using the Covaris S2 system for shearing DNA samples, using the NEBNext End Repair, A-Tailing, and Ligation Modules with non-index adaptors for DNA modification, using the 2X Phusion High-Fidelity PCR Master Mix and index PCR primers for ligation-mediated PCR (LM-PCR),as well as using Nimblegen liquid probe sets for solution-based single capture target enrichment. Quality control standard or criteria for each major procedural step is also addressed here.

The whole process described in this protocol includes the following steps:
Pre-capture Library preparation
DNA fragmentation
DNA end repair
3'-end adenylation
Ligation with Illumina multiplexing PE adaptor
Pre-capture LM-PCR, purification and QC
NimbleGen solution capture
Post-capture LM-PCR, purification and QC

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Reagents

Item
NEBNext End-Repair Module, Cat# E6050L, NEB
NEBNext dA-Tailing Module, Cat# E6053L, NEB
NEBNext Ligation Module, Cat# E-6056L, NEB
Illumina Multiplexing PE Adaptor Oligos Note: All these primer oligos need to be HPLC purified. Illumina multiplexing PE adaptor oligo A 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT Illumina multiplexing PE adaptor oligo B 5'-phospho-GATCGGAAGAGCACACGTCT
Precapture LM-PCR primer IMUX-P1.0 (working concentration 50uM) 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T HPLC purified; *addition of phosphothioate bond.

Precapture LM-PCR barcoded primers (working concentration 50uM)

Note: All these primer oligos need to be HPLC purified. Addition of phosphothioate bond before addition of the last "T" as a preventative measure against degradation in storage.

IBC1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
IBC12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Hybridization Enhancing Oligos (working concentration 1mM)

Note: All of these primer oligos need to be HPLC purified. Addition of 3'dideoxycytisine bond in the end of oligos to prevent potential nucleotide extension during post-capture PCR.

HEO Multiplexing PE 1.0
5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT/3ddC

HEO Multiplexing IBC1-12
Same nucleotide sequences with the precapture LM-PCR barcoded primer IBCn.

Post-capture LM-PCR primers (working concentration 50uM)
Post-capture LM-PCR 1.1
5'-AATGATACGGCGACCACCGAGA

Post-capture LM-PCR 2.1
5'-CAAGCAGAAGACGGCATACGAG

HPLC purified.

Agencourt AMPure XP beads(60ml), Cat# A63882, Beckman Coulter
Phusion High-fidelity PCR Master Mix, Cat# M0531, NEB
Cot-1 DNA, Cat #15279-011, Invitrogen
NimbleGen liquid probes (e.g.Vcrome Rebal Exome Probes)
SeqCap EZ Hyb and Wash, 24 Rxn, Cat #05634261001, Roche.
Dynabeads M-270 Streptavidin, Cat# 65305, Invitrogen

Consumables

Item	Source	Catalog #
1.2% FLASH DNA gel cassette	Lonza/VWR	57023
Flash Gel Loading Dye (5X)	Lonza/VWR	50462
Flash Gel Marker 100 – 4kb	Lonza/VWR	50473
EtOH, 200 proof	Aaper	Stock room
Clear 12-strip 0.2ml PCR tubes w/ caps	Phenix	MPC-665
Caps Only 12-Strip Clear for MPC-655	Phenix	MPC-660
1.7 ml microtubes (MCT-175-B)	VWR	10011-720
10 ul Pipet tips	VWR	47449-868
200 ul pipet tips	VWR	22234-016
1000 ul pipet tips	VWR	16466-008
Shearing microtube	Covaris	520045
Nuclease-free Water	Qiagen	129114
Flashgel Starter Kit	VWR	95015-612

Equipments and Devices

Item	Source	Catalog #
Covaris S2 Acoustic Shearer	Covaris	600028
P1000 pipette	Rainin	L-1000
P200 pipette	Rainin	L-200
P10 pipette	Rainin	L-10
Gel Imager PC 2000	BioRad	1708195
Thermomixer	Eppendorf	0022670000
Agilent Bioanalyzer 2100	Agilent	G2940CA
Microcentrifuge 5424	Eppendorf	5424000410
AB GeneAmp Thermocycler	Applied Biophysics	N8050200
Thermocycler Sample Block Module	Applied Biophysics	4314443
Minifridge Chillers	Boekel/VWR	260009
SpeedVac	Savant/Fisher	DNA120-115
Nutator mixer	VWR	82007-202
DynaMag2 particle concentration rack	Invitrogen	123-21D

Before you start, make sure that you read carefully the following safety information:

- Wear lab coat, gloves, and protective goggles.

- Protect bench working area with absorbent bottom plasticized pad.

- Discard all materials in Biohazard bag. Discard all liquid in a Biohazard labeled plastic bottle that should be tightly closed and disposed of in the Biohazard bag when full.

References:

- Illumina Multiplexing_SamplePrep_Guide_1005361_D.
- Chapters 5, 6, 8 in *NimbleGen SeqCap EZ Exome Library SR User's Guide (Version 2.2).*

Precapture Library Preparation

1. DNA Fragmentation

a) Fill a Covaris™ water bath to the level of 12 in the Covaris S2 device and degas for 30-45 minutes prior to shearing your gDNA in the S2. When a microtube is placed in the tube holder, the water level should be at the base of the cap and the glass portion of the tube should be completely submerged. The water bath temperature should be between 6 ºC and 8 ºC.

b) Dilute 500ng-1µg of DNA (based on Picogreen) into 100 µl of 1xTE buffer in a microcentrifuge tube.

c) Place a Covaris™ microTube (6X16mm) into the loading station. Keep the cap on the tube and use a tapered pipette tip to slowly transfer the 100 µL of DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube. Bubbles will interfere with the acoustic field.

d) Shear the DNA using the following Covaris™ S2 System shearing conditions.
   1. Number of Cycles: **1 (80 seconds per cycle)**.
   2. Batch Temperatue: **6-8 ºC**
   3. Bath Temperature Limit: **<15 ºC**
   4. Mode: **Frequency sweeping**
   5. Water Quality Testing Function: **Off**
   6. Duty cycle: **10%**
   7. Intensity: **4**
   8. Cycles/burst: **200**
   9. Time: **80 s**

e) Place the Covaris™ microtube into the loading station. While keeping the snap-cap on, insert a pipette tip through the pre-split septa, slowly remove the sheared DNA and transfer it into a new 1.7 ml tube.

f) Load 1 µl of each sample and 2 µl of 100 bp – 4000 bp marker on a 1.2% FlashGel DNA Cassette (Lonza, Cat#: 57023). Gel running condition set as the following: Voltage 270, running time: 4 minutes. Gel imager (Bio-Rad) setting should be uniformed to the condition as described here. Condition of Bio-Rad gel imager: Filter 1 for Eth Bromide, auto expose of 0.6 s. Image transform: Higher: 3000, low: 200, Gamma: 1.00, Min: 79, Max: 4095.

Note: The average size should be approximately 300 bp and the majority of fragment sizes should be smaller than 500 bp (see Figure 1 gel picture for example). If majority size of sheared fragments is larger than 800 bp, re-shear the sample with 20 s increment each time for up to 2 additional cycles. The negative control should give no product.

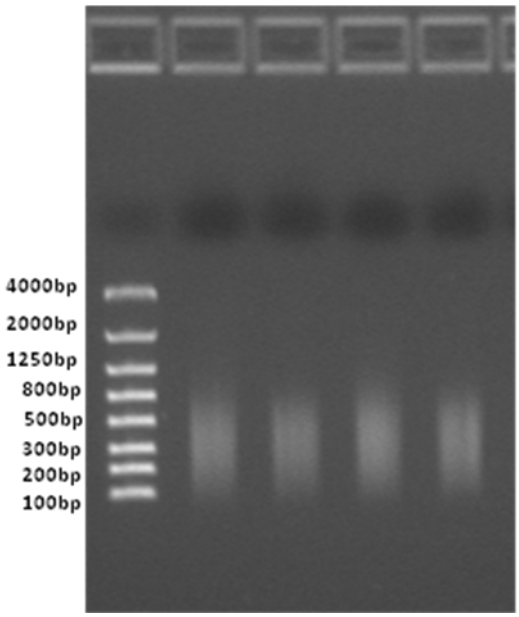

Figure 1 Post-Covaris DNA size distribution on 1.2% Flash Gel. Marker lane: FlashGel DNA marker (100 bp – 4000 bp, Cat. No. 50473, Lonza).

g) Purify by adding 1.8X Agencourt AMPure XP beads and binding by rotating on a Nutator mixer for about 10 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant (and save). Wash 3 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash. Add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 5 minutes to let the beads dry. Elute DNA in 52 µl of Buffer EB.

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2. DNA End Repair

a) Combine and mix the following components in a 1.7 ml tube:

Component	Volume (µL)
Sheared DNA	50.0
End-repair 10× buffer*	9.0

End-repair enzyme mix*	5.0
Nuclease-free water	26.0
Total	90.0

*From NEBNext End-Repair Module (Cat. No. E6050L).

b) Incubate the mixture at 20°C for 30 minutes at bench top cooler.

c) Purify by adding 1.8X Agencourt AMPure XP beads and binding by rotating on a Nutator mixer for about 10 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant (and save). Wash 3 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash. Add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 5 minutes to let the beads dry. Elute DNA in 42 µl of Buffer EB.

3. 3'-end Adenylation

a) Combine and mix the following components in a 1.7 ml tube:

Component	Volume (µL)
End-repaired DNA	40.0
NEBNext™ dA-Tailing Reaction Buffer (10X)*	6.0
Klenow Fragment (3'-5' exo⁻)*	3.0
Nuclease-free water	11.0
Total	60.0

*From NEBNext dA-Tailing Module (Cat. No. E6053L).

b) Incubate the mixture at 37ºC for 30 min in a calibrated thermomixer.

4) Purify by adding 1.8X Agencourt AMPure XP beads and binding by rotating on a Nutator mixer for about 10 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant (and save). Wash 3 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash. Add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 5 minutes to let the beads dry. Elute DNA in 42 µl of Buffer EB.

4. Ligate Adaptors to the DNA

Oligonucleotide annealing:

Illumina multiplexing PE adapter oligo A and Illumina multiplexing PE adapter oligo B should be ordered as HPLC purified.

Mix the paired oligonuclieotides at final concentration of 300uM in 1x Ligase buffer (from NEBNext Ligation Module, Cat# E6056-L) and run with the following annealing program on thermocycler: 95°C for 5 min, 80°C for 3 min, 70°C for 3 min, 60°C for 3 min, 50°C for 3 min, 40°C for 3 min, 30°C for 3 min, 20°C for 3 min and 4°C hold. Dilute 20x to obtain 15uM working concentration. Aliquot annealed adaptor into eppendorf tubes and store them in -20°C freezer.

a) Combine and mix the components below

Component	Volume (µL)
Nuclease-free water	22.0
Illumina Multiplexing PE Adaptors (15 µM concentration)	5.0
Quick Ligase 5X buffer*	18.0
A-Tailed DNA	40.0
Quick Ligase Enzyme*	5.0
Total	90.0

- From NEB (Cat. No. E-6056L).

b) Incubate at room temperature for 30 minutes.

c) Purify by adding 1.1X Agencourt AMPure XP beads and binding by rotating on a Nutator mixer for about 10 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant (and save). Wash 3 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash. Add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 5 minutes to let the beads dry. Elute DNA in 52 µl of Buffer EB.

Note: (Optional) Assess yield and quality by running Agilent Bioanalyzer 2100 DNA 7500 Chip (refer to Agilent 2100 Bioanalyzer User's Guide for Molecular Assays). Make sure there is no excess free adaptor's peak in the eluted DNA sample.

5. Enrichment by LM-PCR Amplification

a) Prepare the following PCR mix: using only A or B column instructed as follow for Invitrogen enzyme mix or NEB phusion enzyme mix accordingly

Component	Volume (µL)
DNA library	50.0
Nuclease-free water	6.0

2X Phusion HiFi PCR Master Mix	60.0
Precapture LM-PCR primer IMUX-P1.0 (50 µM)	2.0
Precapture LM-PCR barcoded primer IBCn (n=1-12) (50 µM)	2.0
Total	120.0

b) Vortex gently and transfer mix into a 0.2 ml PCR strip tubes. Seal the wells with caps.

c) Place them in the ABI GeneAmp PCR System 9700/Veriti for amplification enrichment. PCR condition:

(1) 30'' @ 98ºC
(2) 10" @ 98ºC
(3) 30" @ 60ºC
(4) 30'' @ 70ºC
(5) Repeat step (2) to (4) for a total of 6 cycles
(6) 5' @ 70ºC
(7) HOLD @ 4ºC

d) Load 1 µl of sample into one lane of a 1.2% FlashGel to check amplification.

e) Purify by adding 1.1X Agencourt AMPure XP beads and binding by rotating on a Nutator mixer for about 10 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant (and save). Wash 3 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash. Add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 5 minutes to let the beads dry. Elute DNA in 42 µl of Buffer EB.

f) Run an Agilent Bioanalyzer 2100 DNA Chip 7500 to check size distribution and quantity of PCR product. Make sure there is no excess primer left in the eluted sample (See Figure 2 for example). You should have enough DNA (>1ug) for capture hybridization.

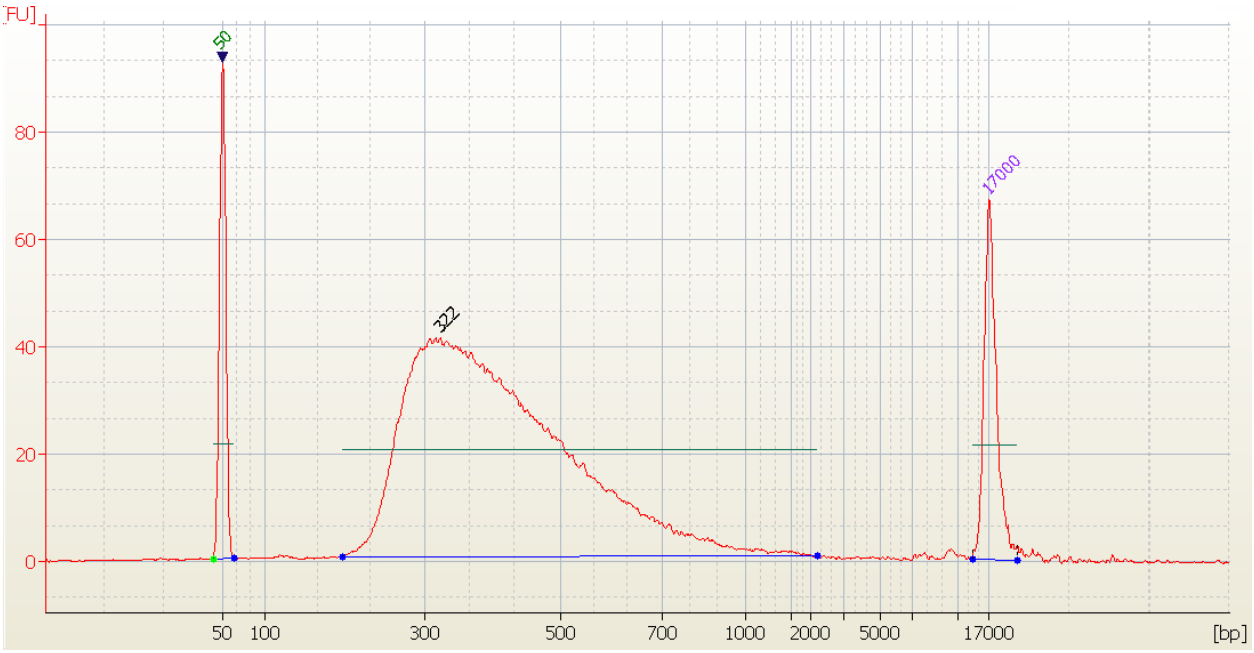

Figure 2 Example of Illumina paired end pre-capture DNA library on Agilent Bioanalyzer 2100 DNA 7500 Chip.

Nimblegen Solution Capture

1. Prepare for Hybridization

   Remove the appropriate number of probe (4.5 µl probe/100 ng probe per precapture library) from the -20°C freezer and allow them to thaw on ice.

2. Hybridization/SA beads capture/washing/elution

   a) Add 50 µl of 1 mg/ml Cot-1 DNA and 1 µg of precapture library to a new 1.5 ml tube.

   b) Add 0.65 µl of each 1,000 µM Hybridization Enhancing Oligos (HEOs) [HEO Multiplexing PE 1.0 and HEO Multiplexing IBCn (n=1-12), see reagent table] to the precapture library plus Cot-1 DNA.
   Note: using full-length hybridization enhancing oligos is critical for optimal capture efficiency.

   c) Close the tube's lid and make a hole in the top of the tube's cap with an 18-20 gauge or smaller needle.

d) Dry the precapture library/Cot-1 DNA/ HE Oligos at a DNA vacuum concentrator on high heat setting (65 °C) for about 45-60 minutes*.
   - Do not overdry the DNA/blocker.

e) To each dried-down precapture library/Cot-1 DNA/HE Oligos, add:
   7.5 µl of 2X Hybridization Buffer
   1.0 µl Formamide*

   * Aliquot formamide for single usage to avoid freeze and thaw multiple times.

The tube with the precapture library/Cot-1 DNA/ HE Oligos should now contains the following components:

Components	Solution Capture
Cot-1 DNA	50.0 µg (dried)
Precapture library	1.0 µg (dried)
1,000 µM HE Oligos	0.65 µl (650 pM each, dried)
2X Hybridization Buffer	7.5 µl
Formamide	3.0 µl
Total	10.5 µl

Cover the hole in the tube's cap with a "Tough Spots" sticker.

Follow Chapters 5 and 6 in the *NimbleGen SeqCap EZ Exome Library SR User's Guide (Version 2.2)* to perform hybridization/capture/washing/elution steps; At step 4 in the Chapter 6, add 50ul PCR grade water to the beads.

Post-Capture PCR Amplification

1. Prepare the following PCR mix in 0.2ml tubes:

Component	Volume (µL)
DNA library	50.0
2X Phusion HiFi PCR Master Mix	60.0
Post-capture LM-PCR 1.1 (50 µM)	1.5
Post-capture LM-PCR 2.1 (50 µM)	1.5
PCR-grade H2O	7
Total	120.0

2. Quick spin the strip tube at Eppendorf centrifuge 5430 (2000 rpm) for 30 seconds at room temperature. Place the tube in the ABI GeneAmp PCR System 9700/Veriti machine for amplification under the following conditions:
   (1) 30'' @ 95ºC
   (2) 10" @ 95ºC
   (3) 30" @ 60ºC,
   (4) 30'' @ 70ºC
   (5) Repeat steps (2)-(4) for a total of 12 cycles
   (6) 5' @ 70ºC
   (7) HOLD @ 4ºC

3. After amplification is done, load 1 µl of reaction into one lane of a 1.2% FlashGel to check amplification.

4. Purify by adding 1.1X Agencourt AMPure XP beads and binding by rotating on a Nutator mixer for about 10 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant (and save). Wash 3 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash. Add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 5 minutes to let the beads dry. Elute DNA in 42 µl of Buffer EB.

5. Run an Agilent Bioanalyzer 2100 DNA Chip 7500 to check size distribution and quantity of PCR product. Make sure there is no excess primer left in the eluted sample (see Figure 3 for example).

6. Perform SYBR green-based qPCR with known four loci assays to evaluate capture efficiency. Follow Chapter 11, qPCR on LM-PCR Amplified Samples, in *NimbleGen Arrays User's Guide - Sequence Capture Array Delivery (version 3.2)* and Chapter 8, Measurement of Enrichment Using qPCR, in *NimbleGen SeqCap EZ Exome Library SR User's Guide (Version 2.2)* for qPCR instruction.

7. Dilution of Final Library for HiSeq Sequencing Run.

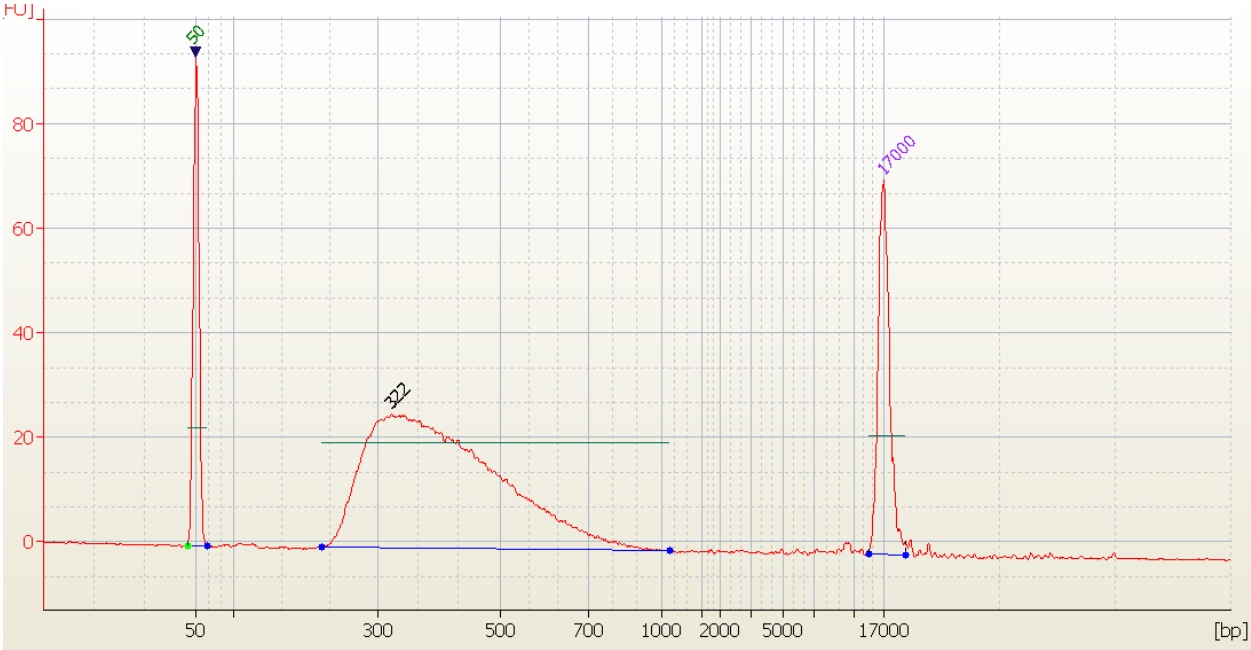

Figure 3: Agilent bioanalyzer results of final library

(end)