Protocol Version 2.1

Preparation of SOLiD™ System Fragment Libraries for Targeted Resequencing using NimbleGen Microarrays or Solution Phase Sequence Capture

Intended Use

This protocol provides instructions for preparing DNA fragment libraries for targeted resequencing using NimbleGen Sequence Capture with the Applied Biosystems SOLiD ™ System sequencing platform. Briefly, 5 µg genomic DNA is sheared, end-repaired and ligated with BCM-HGSC designed truncated-TA (TrTA) adaptors. The library is amplified by pre-capture LM-PCR (Linker Mediated-PCR) and hybridized to NimbleGen microarrays (385K or 2.1M features) or Seqcap EZ solution probe libraries. After washing, amplification by post-capture LM-PCR and a qPCR-based quality check, the successfully captured DNA is ready for SOLiD ™ System sequencing.

References

1. Section 2.2, Prepare an express fragment library, *Applied Biosystems SOLiD™ 3 Plus System Library Preparation Guide (version Oct 2009).*
2. Chapters 7-9, 11 in *NimbleGen Arrays User's Guide - Sequence Capture Array Delivery (version 3.2).*
3. Chapters 5, 6, 8 in *NimbleGen SeqCap EZ Exome Library SR User's Guide (version 1.2).*

   Note: *Unless specifically indicated in this protocol, refer to the above manuals for lists of equipment, reagents and consumables necessary for this procedure.*

Oligonucleotide Sequences

Oligonucleotide ID	Sequence
SOLiD™ System Multiplex Library PCR Primer 1	5' –CCA CTA CGC CTC CGC TTT CCT CTC TAT GGG CAG TCG GTG AT- 3'
SOLiD™ System Library PCR Primer 2	5' –CTG CCC CGG GTT CCT CAT TCT- 3'
Hybridization enhancing oligo TrTA-A	5' - CCT CTC TAT GGG CAG TCG GTG AT/3ddC/ - 3'
Hybridization enhancing oligo SOLiD-B	5' - CTG CCC CGG GTT CCT CAT TCT /3ddC/ - 3'

Step 1: DNA Fragmentation

1. Fill a Covaris™ water bath to level 12 and degas for 30 minutes prior to shearing (request a water level sticker from Covaris™). When a tube is placed in the holder the water level should be at the base of the cap and the glass portion of the tube should be completely submerged.

2. Dilute the 5 µg DNA into 100 µL of 1× Low TE Buffer in a microcentrifuge tube.

   Note: *Sample amount is based on PicoGreen reading.*

3. Place a Covaris™ microTube 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.

4. Shear the DNA using the following Covaris™ S2 System shearing conditions.
   - Number of Cycles: **6**
   - Bath Temperature: **5 ºC**
   - Bath Temperature Limit: **30 ºC**
   - Mode: **Frequency sweeping**
   - Water Quality Testing Function: **Off**
   - Duty cycle: **20%**
   - Intensity: **5**
   - Cycles/burst: **200**
   - Time: **60 s**

5. Place the Covaris™ microTube into the loading station. While keeping the snap-cap on, insert a pipette tip through the pre-split septa and slowly remove the sheared DNA and transfer it into a new 1.5 mL microcentrifuge tube.

6. Check the fragment size using a 1.2 % Flash Gel DNA Cassette (Lonza, Cat. No. 57023). The average size should be approximately 125 bp for SOLiD v4 (see Fig. 1 for example gel picture).

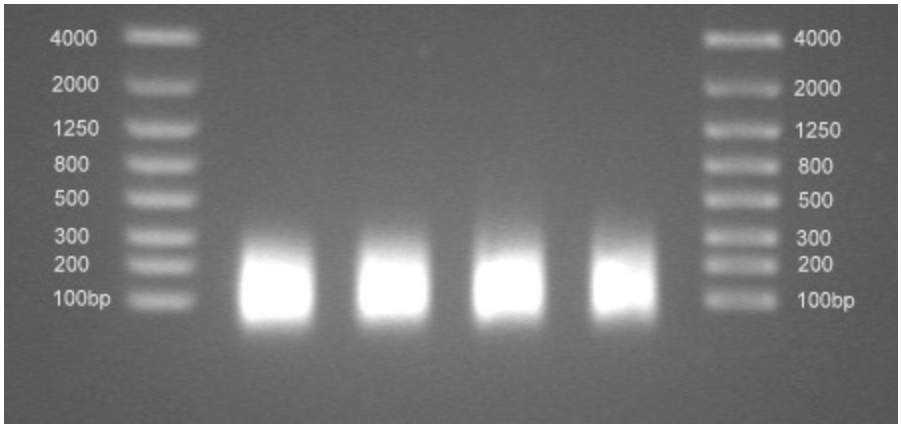

Fig. 1 Post-Covaris DNA size distribution on 1.2% Flash Gel. Marker lane, FlashGel DNA marker (100bp-4kb, Cat. No. 50473, Lonza).

7. Purify the DNA with QIAquick PCR Purification Kit (Cat. No. 28106). Elute with 30 ul of EB buffer.

Step 2. DNA End-Repair

1. Combine and mix the following components in an eppendorf tube:

Component	Volume (µL)
Sheared DNA	30
End-repair 10× buffer	10
End-repair enzyme mix	5
Nuclease-free water	55
Total	**100**

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

2. Incubate the mixture at 20°C for 30 minutes.

3. Purify the DNA with QIAquick PCR Purification Kit (Cat. No. 28106). Elute with 32 µl of EB buffer.

Step 3. 3' –Adenylation

1. Combine and mix the following components in an eppendorf tube:

Component	Volume (µL)
End-repaired DNA	32
dA-Tailing Reaction Buffer (10X)	5
Klenow Fragment (3'-5' exo$^-$)	3
Nuclease-free water	10
Total	**50**

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

2. Incubate the mixture at 37ºC for 30 min.

3. Purify the DNA with QIAquick PCR Purification Kit (Cat. No. 28106). Elute with 40 µl of EB buffer.

4. Quantitate using NanoDrop. Multiply the concentration by the total volume of sample---use this approximate value to calculate the amount of adaptor needed as below.

Step 4. Ligate P1 and P2 Adaptors to the DNA

1. Follow the instruction (page 25) in Section 2.2, Prepare an express fragment library, *Applied Biosystems SOLiD™ 4 System Library Preparation Guide (version March 2010)* to calculate the amount of adaptor needed for the reaction based on the quantity and average size of the recovered DNA in order to achieve 7.5 fold adaptor/insert ratio.

2. BCM-HGSC designed truncated-TA (TrTA) adaptor sequences are as follows:

Oligonucleotide ID	Sequence
TrTA-P1-Top TrTA-P1-Bottom	5' -CCT CTC TAT GGG CAG TCG GTG AT-3' 3'- GGA GAG ATA CCC GTC AGC CAC T^{p}-5'
TA-P2-Top TA-P2-Bottom	5'- ^{p}GA GAA TGA GGA ACC CGG GGC AG-3' 3'-TCT CTT ACT CCT TGG GCC CCG TC-5'

2. Combine and mix the components below.

Component	Volume (µL)
TrTA-P1 Adaptor (ds), 50 pmol/µL	variable
TA-P2 Adaptor (ds), 50 pmol/µL	variable
Quick Ligase 2× buffer	100
A Tailed DNA	38
Quick Ligase Enzyme	5
Nuclease-free water	variable
Total	**200**

From NEB Quick Ligation Kit (Cat. No. M2200L).

3. Incubate at room temperature for 30 minutes.

4. Purify the DNA with the Agencourt® XP® Beads (Cat. No. A63882).
   a. Add 1.8 volumes Agencourt® AMPure® beads to the sample and incubate for 10 minutes at room temperature on a rotator.
   b. Place the sample tube(s) in the magnetic rack. After the solution clears, remove the supernatant and discard.
   c. After 3 times freshly prepared 70% ethanol wash, dry and elute with 40ul EB buffer.
   d. Determine the yield by NanoDrop and run an Agilent Bioanalyzer 2100 DNA Chip 7500 to assure free adaptors have been removed (see Fig 2 for example).

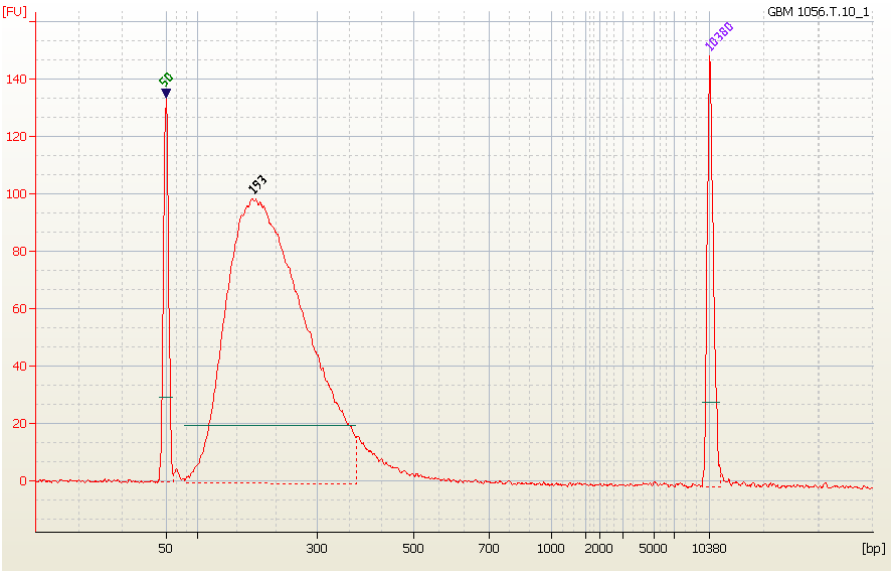

Fig. 2 Example Post-ligation DNA sample on Agilent Bioanalyzer 2100 DNA 7500 Chip. A second round of bead purification has been performed.

Step 5. Nick-translation and Pre-capture PCR amplification

1. Prepare the PCR master mix.

Component	Volume (µL)
Adaptor ligated DNA	38
1.1 X Platinum PCR Supermix HiFi	400
TrTA-P1 primer (50 µM)	10
Library PCR Primer 2 (50 µM)	10
Nuclease-free water	42
Total	**500**

Platinum PCR Supermix HiFi (Invitrogen Cat. No. 12532-016)

2. Pipet 125 µL the PCR master mix into each of four 0.2 µL PCR amplification strip tubes.

3. Run the PCR cycling program listed in the table below:

Step	Stage	Temp	Time
1	Nick Translate	72 ºC	20 min
2	Initial Denature	95 ºC	5 min
3	Denature	95 ºC	15 sec
4	Anneal	60 ºC	15 sec
5	Extend	70 ºC	1 min
6	Go to Step 3 (variable 6~8 cycle)*	N/A	N/A
7	Final Extension	70 ºC	5 min
8	Hold	4 ºC	indefinite

** The number of cycles should be determined empirically. Due to variation in sample handling and amplification efficiency, it is recommended to check the amplified product using gel electrophoresis starting at 6 cycles to assure adequate amplification but without over-amplification.* See Fig.3 for example adequate amplification.

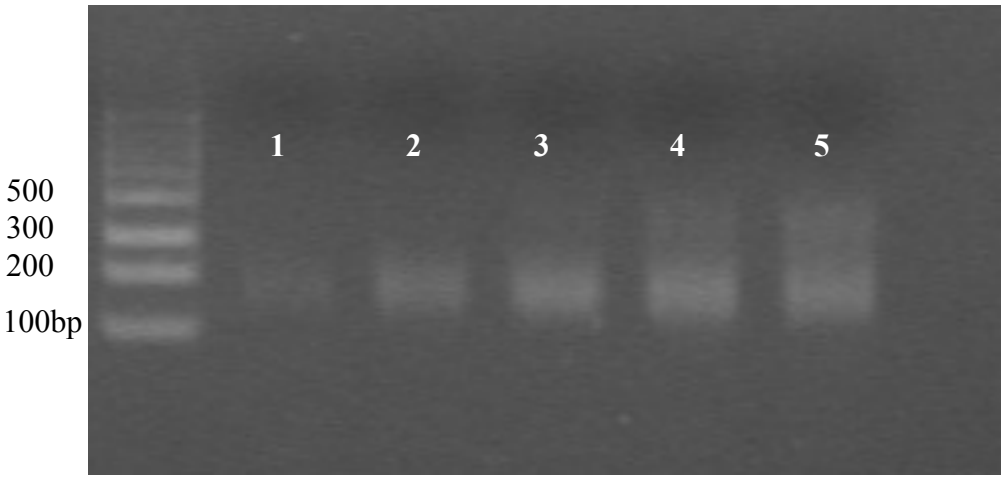

Fig. 3 Adequate *vs* over-amplified LM-PCR on 1.2% Flash Gel. Marker lane, FlashGel DNA marker (100bp-4kb); Lane 1 to 3 are considered appropriate cycle numbers for adequate amplification. The cycle numbers represented in lane 4 and 5 lead to over amplification demonstrated by the presence of large amplicons.

4. Clean up the samples using QIAquick PCR Purification Kit (Cat. No.28106) and elute in 100 µl total using molecular grade water.
5. Quantitate the amplification products by NanoDrop and PicoGreen. Run an Agilent Bioanalyzer 2100 DNA Chip 7500 to check size distribution and assure the primers have been removed (See Fig. 4 for example).

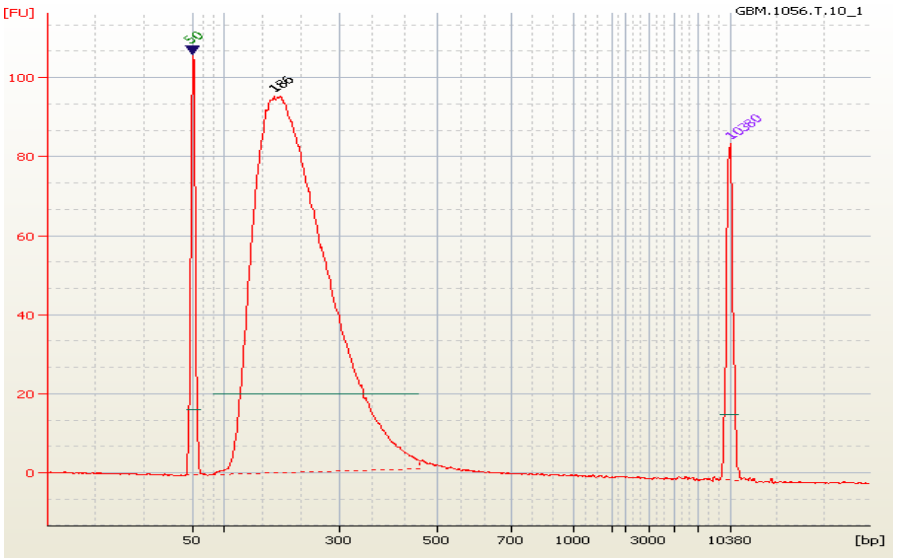

Fig. 4 Example Pre-capture LM-PCR DNA library on Agilent Bioanalyzer 2100 DNA 7500 Chip.

Step 6. Sequence Capture

Using NimbleGen Microarrays

Follow Chapters 7-9 in the *NimbleGen Arrays User's Guide - Sequence Capture Array Delivery (version 3.2)* with the following exceptions:

a. Use 4 µg pre-capture library for microarray hybridization.
b. Chapter 7, step 2.3. Add 0.65 µl Hybridization enhancing oligo TrTA-A (1000uM) and 0.65ul Hybridization enhancing oligo SOLiD-B (1000uM) into each library DNA/COT1 sample. Dry the samples in a SpeedVac on high heat (60 ºC).
c. Proceed directly to Step 6 of this protocol (Post-capture LM-PCR, Purification and QC).

Using NimbleGen SeqCap EZ Solution Probes

Follow Chapters 5 and 6 in the *NimbleGen SeqCap EZ Solution Probes SR User's Guide (version 1.2)* with the following exceptions:

a. Use 2 µg pre-capture library for solution phase hybridization.
b. Chapter 5, step 2. Replace PE-HE1 and PE-HE2 with Hybridization enhancing oligo TrTA-A and Hybridization enhancing oligo SOLiD-B.
c. Chapter 6, step 5.16. Add 80ul PCR grade water instead of 50ul.
d. Proceed directly to Step 6 of this protocol (Post-capture LM-PCR, Purification and QC).

Step 7. Post-capture LM-PCR, Purification and QC

1. Prepare the PCR master mix.

Component	Volume (µL)
Captured and eluted DNA eluted (Microarray) ***– or –*** Beads plus captured DNA (SeqCap EZ)	80
1.1 X Platinum PCR Supermix HiFi	400
Multiplex Library PCR Primer 1 (50 µM)	10
Library PCR Primer 2 (50 µM)	10
Total	**500**

2. Mix well and immediately pipet 125 µL of the PCR master mix into each of 4 0.2 µl PCR amplification strip tubes.

   Note: *When following the SeqCap EZ protocol (solution hybridization), the captured DNA remains attached to streptavidin beads after washing and is not eluted. The beads/captured DNA combination is used directly as template in the LM-PCR reactions.*

3. Run the PCR cycling program listed in the table below:

Step	Stage	Temp	Time
1	Initial Denature	95 ºC	5 min
2	Denature	95 ºC	15 sec
3	Anneal	60 ºC	15 sec
4	Extend	70 ºC	1 min
5	Go to Step 2 (variable12-18 cycle)*	N/A	N/A
6	Final Extension	70 ºC	5 min
7	Hold	4 ºC	indefinite

** The number of cycles should be determined empirically. Due to variation in sample handling and amplification efficiency, it is recommended to check the amplified product using gel electrophoresis starting at 12 cycles to assure adequate amplification but without over-amplification (see Fig.3 for example adequate amplification).*

4. Purify the DNA with the Agencourt® XP® Beads (Cat. No. A63882). Elute final library with 100ul EB buffer.

5. Quantitate the purified DNA with NanoDrop and PicoGreen. Run an Agilent Bioanalyzer 2100 DNA Chip 7500 to check product size distribution and verify that the primers have been removed (see Fig.5 for example).

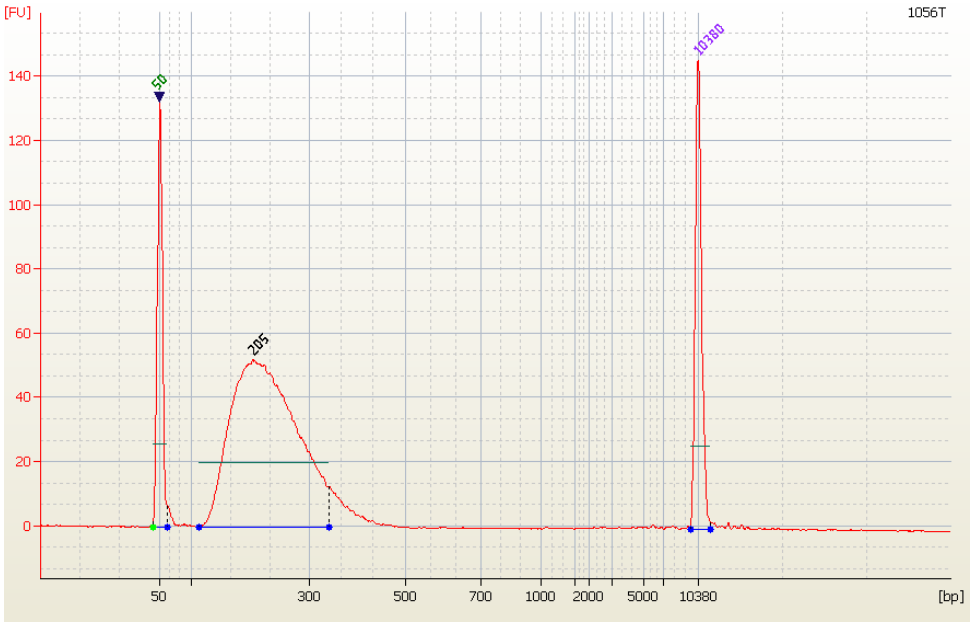

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Fig. 5 Example Post-capture LM-PCR DNA library on Agilent Bioanalyzer 2100 DNA 7500 Chip

Step 8. Assessment of Enrichment using qPCR

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 1.2)* for qPCR instruction.

An average delta Ct of ≥ 7.0 typically indicates successful enrichment of the NimbleGen control loci, although this value can vary significantly between Microarray and EZ Exome (solution) capture experiments. The captured DNA sample is now ready for sequencing using the SOLiD™ 3 Plus sequencing system.

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