PROTOCOL: The complete TrTA-based multiplex capture sequencing protocol for SOLiD™ Platform

This protocol describes the process of utilizing AB 96 barcodes to multiplex target enrichment with NimbleGen microarrays or liquid-phase probes. Genomic or whole-genome-amplification (WGA) DNA is sheared by Covaris into small fragments with approximately average size of 125bp. The fragmented DNA then undergoes end repairing and 3'-end adenylation. Following A-tailing, DNA fragments are ligated to Mul-P1-adaptor and Mul-Int-adaptor. After ligation, DNA fragments with proper adaptor-insert structure are amplified during ligation-mediated PCR (LM-PCR) using Mul-pre-P1 primer and barcoded Mul-pre-BCn primer. For multiplexed sequence capture, individually barcoded DNA libraryies are pooled with equal molarity to make up a pre-capture library pool. The library pool can then be captured with either NimbleGen microarrays or solution probes. For initial experiment setup, we recommend researchers to use 1ug to 5ug input DNA. With more experience built up, researchers can use lower input DNA amount by carefully adjusting adaptor amount used in ligation step and LM-PCR cycle number.

The whole process described in this protocol includes the following steps:

1. DNA fragmentation
2. DNA end repair
3. 3'-end adenylation
4. Ligation with TrTA adaptors
5. Pre-capture LM-PCR, purification and QC
6. Make barcoded pre-capture library pool
7. Hybridization/Wash/Elution
8. Post-capture LM-PCR, purification and QC
9. Assessment of enrichment using qPCR

Reagents

Item
NEBNext End-Repair Module, Cat# E6050L, NEB
NEBNext dA-Tailing Module, Cat# E6053L, NEB
NEBNext Ligation Module, Cat# E-6056L, NEB
SPRI AMPure XP beads(60ml), Cat# A63882, Beckman Coulter
2X custom-made SOLiD Library High Fidelity Amplification Mix, Cat# A12125, Invitrogen
Cot-1 DNA, Cat #15279-011, Invitrogen
Vcrome Rebal Exome Probes
SeqCap EZ Hyb and Wash, 24 Rxn, Cat #05634261001, Roche.
Dynabeads M-270 Streptavidin, Cat# 65305, Invitrogen
QIAquick PCR Purification Kit, Cat# 28104, Qiagen

Oligonucleotide Sequences

Oligos		5'->3'
Adaptors	Mul-P1-Adapter Oligo A	CCTCTCTATGGGCAGTCGGTGAT
	Mul-P1-Adapter Oligo B	p-TCACCGACTGCCCATAGAGAGG
	Mul-Int-Adapter Oligo A	p-CGCCTTGGCCGTACAGCAG
	Mul-Int-Adapter Oligo B	CTGCTGTACGGCCAAGGCGT
Pre-capture LM-PCR primers	Mul-pre-P1	CCTCTCTATGGGCAGTCGGTGAT
	Mul-pre-BCn (n=1-96)	CTGCCCCGGGTTCCTCATTCTCT(10bp- Lifetech barcode)CTGCTGTACGGCCAAGGCG*T
3-oligo Hybridization Enhancing Oligo mixture	Mul-HEO1	CCTCTCTATGGGCAGTCGGTGAT/3ddC
	Mul-HEO2	CTGCCCCGGGTTCCTCATTCT/3ddC
	Mul-HEO3	CTGCTGTACGGCCAAGGCGT/3ddC
2-oligo Hybridization Enhancing Oligo mixture	Mul-HEO1	CCTCTCTATGGGCAGTCGGTGAT/3ddC
	Mul-pre-BCn – HEO (n=1-96)	CTGCCCCGGGTTCCTCATTCTCT(10bp-barcode)CTGCTGTACGGCCAAGGCGT/3ddC
Post-capture LM-PCR primers	Mul-post- P1	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGA*T
	Mul-post-P2	CTGCCCCGGGTTCCTCATTCT

*addition of phosphothioate bond.
/3'ddc: 3'-dideoxycytosine modification
Note: all oligos need to be HPLC purified.

Consumables

Item	Source	Catalog #
2.2% 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	
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
TwinTech 384 well plate	VWR	12000-658
Flashgel Starter Kit	VWR	95015-612

Equipments and Devices

Item	Source	Catalog #
Epoch Multiplate Spectrophotometer	BioTek/Fisher	GR422
Epoch Multi-volume Plate	BioTek/Fisher	11-120-571
Covaris S2 Acoustic Shearer	Covaris	600028
P1000 pipette	Rainin	
P200 pipette	Rainin	
P10 pipette	Rainin	
Gel Imager PC 2000	BioRad	1708195
Thermomixer	Eppendorf	0022670000
Agilent Bioanalyzer 2100	Agilent	G2940CA
E-Bases	Invitrogen	EB-M03
Microcentrifuge 5424	Eppendorf	5424000410
AB GeneAmp Thermocycler	Applied Biophysics	N8050200
Thermocycler Sample Block Module	Applied Biophysics	4314443
Minifridge Chillers	Boekel/VWR	260009
Biotek Synergy HT Multi-Mode Microplate Reader	BioTek/Fisher	NC0127276
SpeedVac	Savant/Fisher	DNA120-115
Neodymium Magnetic Bar	KJ Magnetics	BZX0X08
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

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 2.2)*

Step 1: DNA Frag*mentation*

1. *Fill a Covaris™ water bath to level 12 and degas for* **3**0 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 1ug-5µg DNA into 50 µL of 1× Low TE Buffer in a microcentrifuge tube.

   Note: *Use either PicoGreen or Qubit to quantify DNA concentration instead of Nanodrop.*

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 50 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.

   CRITICAL STEP: Make sure all solution in the Covaris tube have been transferred into microcentrifuge tube. Perform a quick spin before transfer if needed.

6. Check the fragment size on a 1.2 % FlashGel DNA Cassette (Lonza, Cat. No. 57023) or an Agilent Bioanalyzer 2100 DNA Chip 7500. The peak mode should be at approximately 125 bp. (see Fig. 1 for example gel picture).

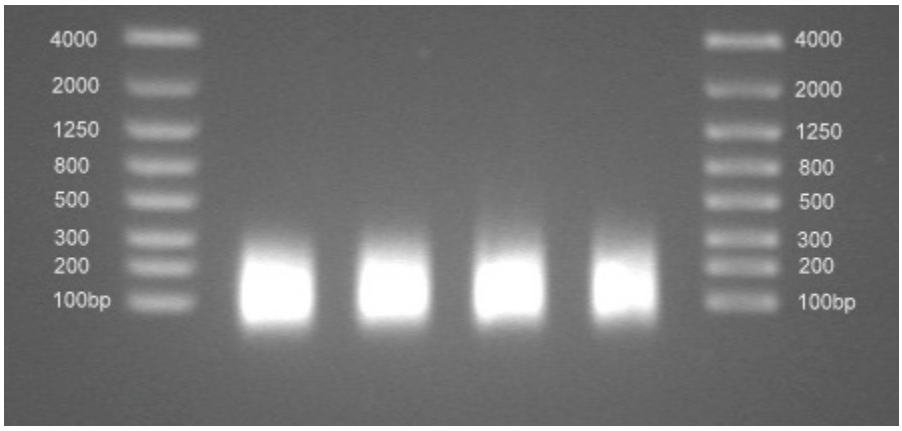

Fig. 1 Post-Covaris DNA size distribution on 1.2% FlashGel. Marker lane, FlashGel DNA marker (100bp-4kb, Cat. No. 50473, Lonza).

Step 2. DNA End Repair

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

Component	Volume (µL)
Sheared DNA	50
End-repair 10× buffer	9
End-repair enzyme mix	5
Nuclease-free water	26
Total	**90**

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

2. Incubate the mixture at 20°C 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 (162ul) and incubate for 10 minutes at room temperature on a rotator.
   b. Place the sample tube(s) in the Dynal MPC-S. After the solution clears, carefully remove the supernatant without disturbing the beads.
   c. After 3 times freshly prepared 70% ethanol wash, dry and elute with 40ul EB buffer.

Step 3. 3' –end Adenylation

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

Component	Volume (µL)
End-repaired DNA	40
dA-Tailing Reaction Buffer (10X)	6
Klenow Fragment (3'-5' exo$^-$)	3
Nuclease-free water	11
Total	**60**

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

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

3. Purify the DNA with the Agencourt® XP® Beads (Cat. No. A63882).

   a. Add 1.8 volumes Agencourt® AMPure® beads to the sample (108ul) and incubate for 10 minutes at room temperature on a rotator.

   b. Place the sample tube(s) in the Dynal MPC-S. After the solution clears, carefully remove the supernatant without disturbing the beads.

   c. After 3 times freshly prepared 70% ethanol wash, dry and elute with 40ul EB buffer.

   d. Determine the yield by running Agilent Bioanalyzer 2100 DNA 7500 Chip (if use ≥ 500ng starting DNA) or High Sensitivity Chip (if use <500ng starting DNA).

Step 4. Ligation with TrTA adaptors

1. Based on the molarity and mass concentration information collected from 3.3d, calculate the amount of adapter needed in order to achieve 15 fold adapter/insert ratio.

   For example, for a precapture library with Molarity X nmol/l, mass concentration Y ng/ul, if Z ug of the DNA will be used for hybridization, the molar mass of each adaptor used in ligation = 15 x (Z x10^3 ng ÷ Y ng/ul x X nmol/10^6 ul) =15 x (ZX/Y) pmol

2. Oligonucleotide annealing.

   Mul-P1 adapter: Mul-P1-Adapter Oligo A + Mul-P1-Adapter Oligo B

   Mul-Int adapter: Mul-Int-Adaptor Oligo A + Mul-Int-Adaptor Oligo B

   Mix the paired oligonuclieotides at final concentration of 250uM in 1x Ligase buffer 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 5x to obtain 50uM working concentration.

3. Combine and mix the components below.

Component	Volume (µL)
Mul-P1 Adapter (ds), 50 pmol/µL	variable
Mul-Int Adapter (ds), 50 pmol/µL	variable
NEBNext Quick Ligase (5X)	18
A-Tailed DNA	40
Quick Ligase Enzyme	5
Nuclease-free water	variable
Total	**90**

4. Incubate at room temperature for 30 minutes.

5. Purify the DNA with the Agencourt® XP® Beads (Cat. No. A63882).
   a. Add 1.8 volumes Agencourt® AMPure® beads to the sample (162ul) and incubate for 10 minutes at room temperature on a rotator.
   b. Place the sample tube(s) in the Dynal MPC-S. After the solution clears, carefully remove the supernatant without disturbing the beads.
   c. After 3 times freshly prepared 70% ethanol wash, dry and elute with 42ul EB buffer.
   d. Assess yield and quality by running Agilent Bioanalyzer 2100 DNA 7500 Chip (if use ≥500ng starting DNA) or High Sensitivity DNA chip (if use <500ng starting DNA).
   e. CRITICAL STEP: Excess free adaptors will interfere with PCR amplification and should be removed.

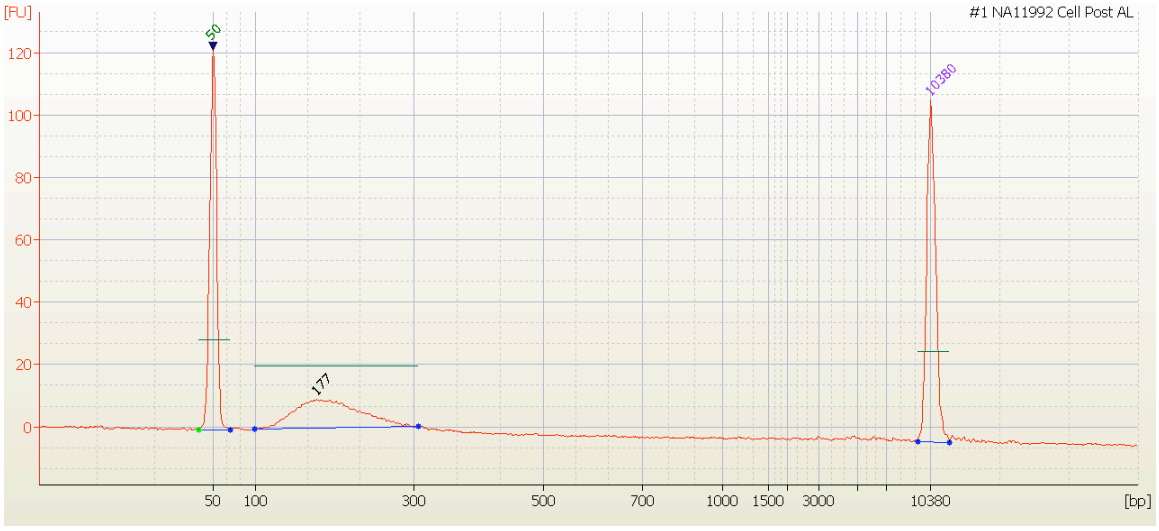

Fig. 2 Example Post-ligation DNA sample on Agilent Bioanalyzer 2100 DNA 7500 Chip. 500ng starting DNA was used.

Step 5. Pre-capture LM-PCR, Purification and QC

1. Set up PCR reaction.

Component	Volume (µL)
Adaptor ligated DNA	40
2X custom-made SOLiD Library High Fidelity Amplification Mix [a]	85
H2O	38
Mul-pre-P1 primer (50 µM)	3.5
Mul-pre-BCnbarcoded primer (50 µM)	3.5
Total	**170**

a. If the 2X custom-made SOLiD Library High Fidelity Amplification Mix is not available, researchers can use other enzyme mixes designed for NextGen library preparations such as 2xPhusion master mix (Cat# F-531L, Finnzymes Phusion High Fidelity Master Mix kit).

2. Mix well and immediately pipet 85 µL of the PCR mix into each of two 0.2 µl PCR amplification strip tubes.

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

Step	Stage	Temp	Time
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)*	N/A	N/A
7	Final Extension	70 ºC	5 min
8	Hold	4 ºC	hold

Recommended PCR cycle numbers for different amounts of starting DNA

Starting DNA amount	PCR Cycle
5ug	6
2ug	7
1ug	8
500 ng	9
250ng	10
100ng	12

** The recommended PCR cycles shown in the table are based on our in-house tests. Due to variation in sample quality and procedure handling, more or less cycle numbers may be used. We recommend checking the amplified product with gel electrophoresis to assure adequate amplification.* See Fig.3 for example of adequate amplification.

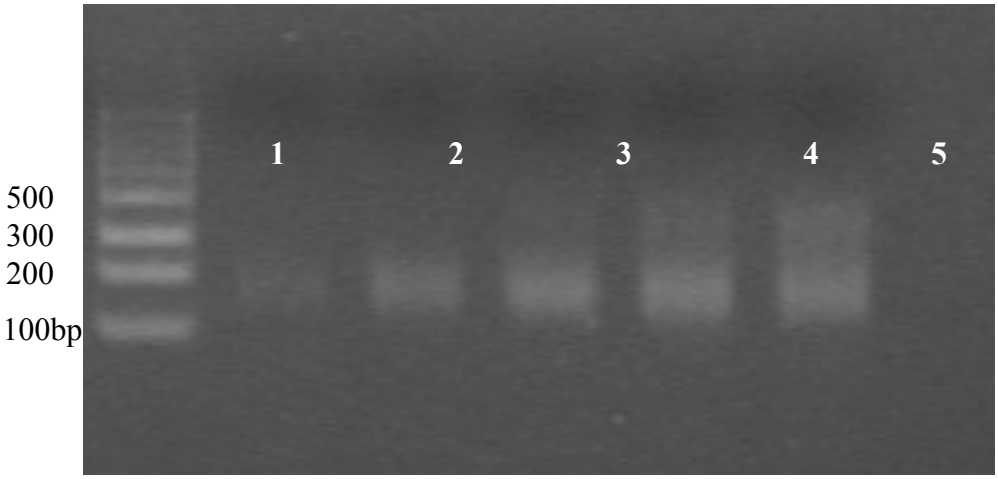

Fig. 3 Adequate *vs* over-amplified LM-PCR on 1.2% Flash Gel. Marker lane, FlashGel DNA marker (100bp-4kb); Lane 1, Ligation product is amplified with less than optimal cycle number. Lane 2-3 ,Ligation product is amplified with appropriate cycle number. The cycle numbers used in lane 4 and 5 lead to over amplification demonstrated by the presence of large amplicons.

4. Purify the DNA with the Agencourt® XP® Beads (Cat. No. A63882).
   a. For each sample, pool two PCR reaction tubes into one 1.5 ml LoBind tube.
   b. Add 1.8 volumes Agencourt® AMPure® beads to the sample tube and incubate for 10 minutes at room temperature on a rotator.
   c. Place the sample tube(s) in the Dynal MPC-S. After the solution clears, carefully remove the supernatant without disturbing the beads.
   d. After 3 times wash with reshly prepared 70% ethanol, dry and elute with 40ul Molecular Grade H2O.
   e. Assess yield and quality by running Agilent Bioanalyzer 2100 DNA 7500 Chip. Fig. 4 for example. Record Molarity and Mass concentration for each sample.

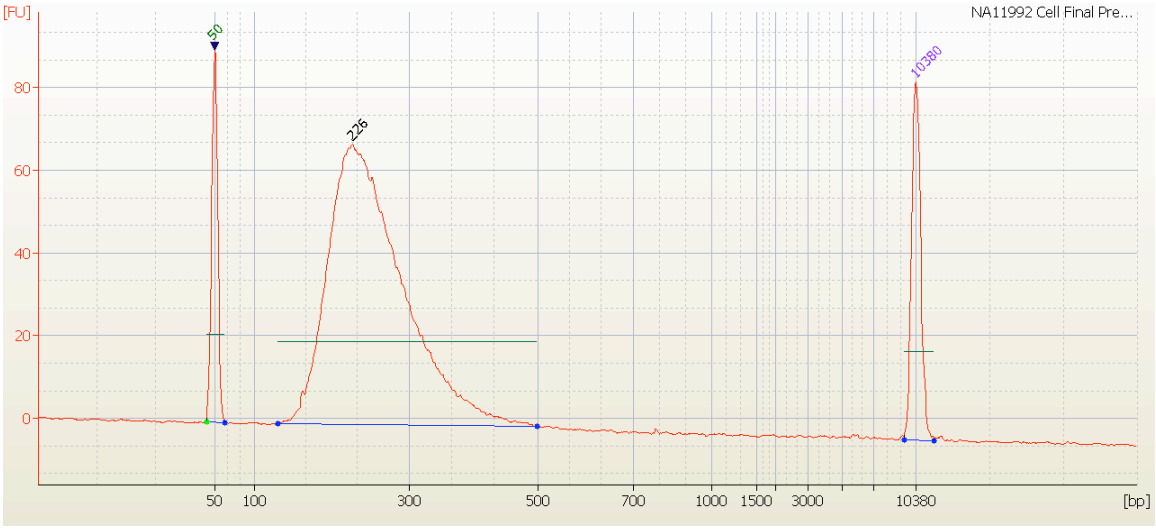

Fig. 4 Example Pre-capture LM-PCR DNA library on Agilent Bioanalyzer 2100 DNA 7500 Chip. 500ng starting DNA was used and post-ligation product was amplified by 9 cycles PCR.

Step 6. Make Barcoded Pre-capture Library Pool

To achieve optimal color balance during sequencing run, barcodes should be used 4 in a set (for example, 4-plex using barcodes 1-4, 5-8, 9-12…; 8-plex using barcode 1-8, 9-16…; 12-plex using 1-12 etc).The pool should be made with equal molar presentation for each barcoded pre-capture library. This should be based on the Molarity reading of each sample generated by Agilent Bioanalyzer. If average size difference among the samples is small (e.g. <20 bp), mass concentration may also be used.

Note: depending on the target size, researchers should use their own discretion to decide the extent of multiplexing and quantity of the DNA used in hybridization. In general, increasing DNA amount in hybridization reaction helps improve capture metrics; and this is especially true if the probe has a large size of target and a large number of barcodes are multiplexed in one capture. For 4-plex and 6-plex whole exome capture, we recommend at least 500ng/per barcode DNA should be used. But for small regional probes, smaller amounts of DNA could be used in the hybridization.

Step 7. Hybridization/Wash/Elution

A. When 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 pool for microarray hybridization.

   Note: depending on the target size and the extent of multiplexing, more pooled library DNA (up to 8 ug) may be used in hybridization reaction in order to achieve optimal capture performance. However, as a general recommendation, we suggest to start the test run with 4 ug DNA in hybridization.

b. Chapter 7, step 2.3. If use 3-pieces universal HEOs, add 0.65 µl Mul-HEO 1, 2, 3 (1000uM) into each library DNA/COT1 sample. If use full-length barcoded HEOs, add 0.65 µl Mul-HEO1 (1000uM) and 0.65/N µl individual Mul-pre-BCn-HEO (1000uM; N: number of barcodes multiplexed in one capture). Dry the samples in a SpeedVac on high heat (60 ºC).

   Note: 3-pieces HEO mix can be universally used for any index combination. However, using full-length barcoded blocking oligos give rise to improved capture efficiency.

c. Chapter 9, step 3.14. Keep the volume of each eluate to 50 ul. The eluted DNA can be stored in -20 ºC until use or proceed to Step 8 of this protocol (Post-capture LM-PCR, Purification and QC).

B. When using NimbleGen SeqCap EZ Solution Probes

Follow Chapters 5 and 6 in the *NimbleGen SeqCap EZ Exome Library SR User's Guide (Version 2.2),* with the following exceptions:

a. Use 1-2 µg pre-capture library for solution phase hybridization.

   Note: depending on the probe target size and the extent of multiplexing, quantity of DNA in hybridization can be increased for optimal capture performance. As a general recommendation, we suggest to start the test run with 1 ug DNA in hybridization for regional capture probe and 2 ug for whole exome capture probes.

b. Use 50 ug COT-1for each hybridization.

   Note: 5ug COT1 specified in the NimbleGen protocol may work equally well but since it has not been thoroughly tested in HGSC, we are still listing 50ug COT1 usage here.

c. Chapter 5, step 2. If use 3-pieces universal HEOs, add 0.65 µl Mul-HEO 1, 2, 3 (1000uM) into each library DNA/COT1 sample. If use full-length barcoded HEOs, add 0.65 µl Mul-HEO1 (1000uM) and 0.65/N µl individual Mul-pre-BCn-HEO (1000uM; N: number of barcodes multiplexed in one capture).
   Note: 3-pieces HEO mix can be universally used for any index combination. However, using full-length barcoded blocking oligos give rise to improved capture efficiency.

d. Chapter 6, step 4. Add 50ul PCR grade water to the beads. The beads/captured DNA combination can be stored at -20 ºC or proceed directly to Step 8 of this protocol (Post-capture LM-PCR, Purification and QC).

Step 8. Post-capture LM-PCR, Purification and QC

1. Set up PCR reaction.

Component	Volume (µL)
Captured and eluted DNA eluted (Microarray) ***– or –*** Beads plus captured DNA (SeqCap EZ)	50
2X custom-made SOLiD Library High Fidelity Amplification Mix[a]	60
Mul-post-P1 (50 µM)	1.5
Mul-post-P2 (50 µM)	1.5
H2O	7
Total	**120**

a. If the 2X custom-made SOLiD Library High Fidelity Amplification Mix is not available, researchers can use other enzyme mixes designed for NextGen library preparations such as 2xPhusion master mix (Cat# F-531L, Finnzymes Phusion High Fidelity Master Mix kit).

2. Mix well and immediately pipet 120 µL of the PCR mix into each of two 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 (variable12-14cycle)*	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).
   a. For each sample, pool two PCR reaction tubes into one 1.5 ml LoBind tube.
   b. Add 1.8 volumes Agencourt® AMPure® beads to the sample tube and incubate for 10 minutes at room temperature on a rotator.
   c. Place the sample tube(s) in the Dynal MPC-S. After the solution clears, carefully remove the supernatant without disturbing the beads.
   d. After 3 times wash with freshly prepared 70% ethanol, dry the beads and elute with 40ul Molecular Grade H2O.

5. Run an Agilent Bioanalyzer 2100 DNA Chip 7500 to check product size and concentration.

Step 9. 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 2.2)* 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 different probes. The captured DNA sample is now ready for sequencing using the SOLiD™ 4 sequencing system.

(end)