PROTOCOL: Illumina Paired-end Whole Exome Capture Library Preparation Using Full-length Index Adaptors and KAPA 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 DNA fragmentation, using the NEBNext End Repair, A-tailing, and Ligation Module kits for end modification, using the 2X KAPA HiFi HotStart ReadyMix for ligation-mediated PCR (LM-PCR),as well as using NimbleGen liquid probe sets for solution-based single or co-capturing target enrichment. Illumina full-length index adaptors are used in the ligation reaction. Quality control standard or criteria for each major procedural step is also addressed here

1. MATERIALS REQUIRED

1.1 Reagents

Item	Source	Catalog #	Storage Temperature
2X KAPA HiFi HotStart ReadyMix	KAPA	KK2601	-15°C to -25°C
A.C. E. Formamide 25ml, 99.5% High Purity (25 ml), Low Conductivity	ISC Bioexpress	K295	-15°C to -25°C
Cot-1 DNA (working concentration 1mg/mL)	Invitrogen	15279-011	-15°C to -25°C
Dynabeads M-270 Streptavidin	Invitrogen	65305	2°C-8°C
EB (Tris-Chloride)	Qiagen	19086	RT
EtOH 200 Proof	Aaper	111ACS200	RT
FlashGel Loading Dye (5X)	Lonza/VWR	50462	2°C-8°C
FlashGel Marker 100 – 4 kb	Lonza/VWR	50473	2°C-8°C
Hybridization Enhancing Oligos (working concentration 1 mM) HEO Multiplexing PE 1.0* and full-length HEO Multiplexing IBC1-12* * 3-ddC modification added.	Sigma	N/A	-15°C to -25°C
Illumina Index Adapters (working concentration 15 µM)	Sigma	N/A	-15°C to -25°C
NEBNext dA-Tailing Module Klenow Fragment (3'→5" exo-) 10X NEBNext dA-Tailing Reaction Buffer	NEB	E6053L	-15°C to -25°C
NEBNext End Repair Module 10X NEBNext End Repair Reaction Buffer NEBNext End Repair Enzyme Mix	NEB	E6050L	-15°C to -25°C
NEBNext Ligation Module Quick T4 DNA Ligase 5X NEBNext Quick Ligation Reaction Buffer	NEB	E-6056L	-15°C to -25°C
Nuclease-Free Water	Qiagen	129114	RT
Pre and Post-capture LM-PCR Primers (working concentration 50 µM) LM-PCR 1.1 LM-PCR 2.1	Sigma	N/A	-15°C to -25°C
SeqCap EZ Hyb and Wash, 96 Rxn 10X Wash Buffer I 10X Wash Buffer II	Roche	05634253001	-15°C to

10X Wash Buffer III 10X Stringent Wash Buffer 2X Hybridization Buffer Formamide (to replace component A, see above)) 2.5X Bead Wash Buffer			-25°C
SPRI AMPure XP Beads(60 ml)	Beckman Coulter	A63881	2°C-8°C
VCRome2.1 Rebal Exome Probes	HGSC design	N/A	-15°C to -25°C

1.2 Consumables

Item	Source	Catalog #
Agilent 7500 Chips	Agilent	5067-1506
Caps Only 12-Strip Clear for MPC-655	Phenix	MPC-660
Clear 12-Strip 0.2ml PCR Tubes with Caps	Phenix	MPC-665
FlashGel DNA Cassette (1.2%)	Lonza	57023
FlashGel Starter Kit	VWR	95015-612
Eppendorf tubes (1.7 ml) MCT-175-C	VWR/Axygen	10011-722
Pipette tips (1000 ul)	VWR	16466-008
Pipette tips (10 ul)	VWR	47449-868
Pipette tips (200 ul)	VWR	22234-016
Shearing microTube (6x16 mm) AFA Fiber with Snap-Cap	Covaris	520045

1.3 Equipments and Devices

Item	Source	Catalog/Model #
ABI 96-Well GeneAmp® PCR System 9700	Applied Biosystems.	9700
Agilent Bioanalyzer 2100	Agilent	G2940CA
Covaris S2 Acoustic Shearer	Covaris	600028
Covaris S2 MicroTube Holder	Covairs	500114
DynaMag™-2 Magnet	Invitrogen	123-21D
DynaMag™-96 side Magnet	Invitrogen	123-31D
Gel Imager PC 2000	BioRad	1708195
Microcentrifuge 5424	Eppendorf	5424000410
Minifridge Chillers	Boekel/VWR	260009
Neodymium Magnetic Bar	KJ Magnetics	BZX0X08
Pipette (P10) Single Channel	Rainin	L-10
Pipette (P1000) Single Channel	Rainin	L-1000
Pipette (P200) Single Channel	Rainin	L-200
Speed Vacuum (DNA Concentrator)	Savant/Fisher	DNA120-115
Thermocycler Sample Block Module	Applied Biophysics	4314443
Thermomixer	Eppendorf	0022670000

1.4. Oligonucleotide Sequences

All oligonucleotides listed here should be purified by HPLC.

Illumina index adaptor oligos (*addition of phosphothioate bond before addition of the last "T")

Illu-A-ID 1	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ATC ACG ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 2	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CGA TGT ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 3	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC TTA GGC ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 4	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC TGA CCA ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 5	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ACA GTG ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 6	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GCC AAT ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 7	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CAG ATC ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 8	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ACT TGA ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 9	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GAT CAG ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 10	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC TAG CTT ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 11	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GGC TAC ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-ID 12	/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CTT GTA ATC TCG TAT GCC GTC TTC TGC TTG
Illu-A-universal	5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T

Note: Illu-A-IDn (n=1-12) and Illu-A-universal oligos should be pre-annealed using the following protocol:
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. Record the date.

Full-length Blocking oligos (all oligos carry 3'-ddC modification to prevent primer extension in the downstream PCR)

IMUX-HEO1 (5'->3')
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

IMUX-HEO-IBCn (n=1-12) (5'->3')

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

LM-PCR primer 1.0
5'-AATGATACGGCGACCACCGAGA

LM-PCR primer 2.0
5'-CAAGCAGAAGACGGCATACGAG

2. POLICIES

2.1 Before you start, make sure to read the laboratory safety manuals and follow the instructions in the safety manuals.

2.2 Before starting the library preparation, make sure that all primers, adaptors, enzyme kits, probes, Agilent Bioanalyzer reagents are ready to use.

2.3 A negative control (ddH2O) should be included during preparation. A reagent blank control should also be used for PCR setups. The negative and reagent blank controls should give no product at every stage.

2.4 Genomic DNA should be stored at 4ºC refrigerator for short time storage. Pre-capture final library and capture final library should be stored at -20ºC freezer at a designated box.

3. PROCEDURES

3.1 Pre-capture Library Preparation

3.1.1 DNA Fragmentation

1. Fill a Covaris™ water bath to the level of 12 in the Covaris S2 device and pre-cool the water bath and degas for 30 minutes. The water bath temperature should be between 6 ºC and 10 ºC.

During the pre-cooling and degas, do the following things:
A. Dilute 500ng of sample DNA with nuclease-free water and bring the final volume to 80ul.
B. Transfer the diluted DNA sample into a Covaris microTube (Cat # 520045).

2. After degas, place the sample Covaris™ microTube into the loading station.

3. Shear the DNA using the following Covaris™ S2 System shearing conditions.

- Number of Cycles: **2 (60 seconds per cycle)** (if sample quality is poor, start with 1 cycle and check on a gel).
- Batch Temperatue: **6-10 ºC**
- Bath Temperature Limit: **<15 ºC**
- Mode: **Frequency sweeping**
- Water Quality Testing Function: **Off**
- Duty cycle: **10%**
- Intensity: **5**
- Cycles/burst: **200**
- Time: **180s**

4. After shearing, remove the microTube from the machine. Load 1 µl of sheared sample and 3 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.

√ **QC #1** The average size should be approximately 200-300 bp and the majority of fragment sizes should be smaller than 500 bp (see example in **Figure 1**). If majority size of sheared fragments is larger than 500 bp, re-shear the sample with 20 s increment each time for up to 2 additional cycles. The negative control should give no product.

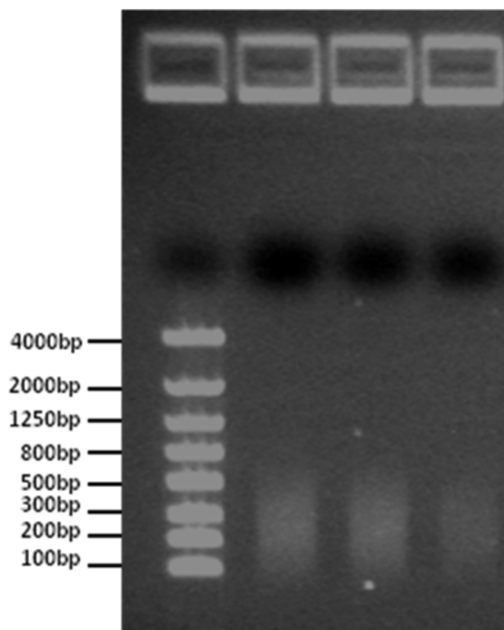


Figure 1. 1.2% Flash Gel electrophoresis of post-shearing DNA along with 100bp-4kb DNA ladder.

5. After verifying that the sheared fragment size is correct, transfer the sample DNA (~76 ul in volume) from the Covaris microTube to a 1.7ml Eppendorf tube.

3.1.2 DNA End-Repair

1. Combine and mix the following components in the sample tube:

Component	Volume (µL)
Sheared DNA	76.0
End-repair 10× buffer*	9.0
End-repair enzyme mix*	5.0
Total	90.0

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

2. Incubate the mixture at 25°C for 20 minutes at a bench top thermomixer.

During the incubation, do the following things:
A. Thaw Adenylation reagents on ice.
B. Prepare another thermomixer at 37°C for adenylation incubation.

3. Purify with 1.8x SPRI AMPure XP beads as the following:

(3a). Add 162ul SPRI AMPure XP beads in the tube.

(3b). Mix the DNA/Beads on thermomixer in the highest speed (1400rpm) for 5 minutes at RT.

(3c). Placing the tube into the DynaMag™-2 magnet device for 2 minutes until the solution clears. Remove and discard the supernatant.

(3d). Wash two times with freshly prepared 70% ethanol. Keep the sample tube in the DynaMag™-2 magnet device during the washing and avoid disturbing the bead pellet. After washing, leave the tube in the DynaMag™-2 magnet device for 2 minutes at RT, then elute DNA in 52 µl nuclease-free H2O.

3.1.3 3' –Adenylation

1. Combine and mix the following components in the sample tube:

Component	Volume (µL)
End-repaired DNA	51.0
NEBNext™ dA-Tailing Reaction Buffer (10X)*	6.0
Klenow Fragment (3'-5' exo⁻)*	3.0
Total	60.0

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

2. Incubate the mixture at 37ºC thermomixer for 20 min.

During the incubation, thaw Ligation reagents on ice.

3. Purify with 1.8X SPRI AMPure XP beads by performing step 4.1.2 (3a) through (3d). And elute in 64ul nuclease-free H2O.

3.1.4 Ligation of Illumina Index Adaptors

1. Combine and mix the components in the sample tube:

Component	Volume (µL)
Illumina Index Adaptors (15 µM concentration)	5.0
Quick Ligase 5X buffer*	18.0
A-Tailed DNA	62.0
Quick Ligase Enzyme*	5.0
Total	90.0

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

2. Incubate at room temperature for 20 minutes.

During the incubation, do the following things:
A. Thaw Kapa PCR enzyme mix on ice.
B. Set up PCR cycling condition (see below)

3. Purify with 1.0x SPRI AMPure XP beads by performing step 4.1.2 (3a) through (3d). Elute DNA in 58µl nuclease-free H2O and transfer eluted DNA (~56ul in volume) into 0.2ml PCR strip tubes. Register the well ID in spreadsheet.

3.1.5 Pre-capture Ligation-Mediated PCR (LM-PCR)

1. Add and mix the following PCR reagents to the PCR strip well:

Component	Volume (µL)
2X KAPA HiFi HotStart ReadyMix	60.0
LM-PCR primer 1.0 (50 µM)	2.0
LM-PCR primer 2.0 (50 µM)	2.0
Total reaction volume	120.0

2. Seal the wells with caps. Place them in the ABI GeneAmp PCR System 9700/Veriti for amplification enrichment. PCR condition is described below:

(1) 45" @ 98ºC
(2) 15" @ 98ºC
(3) 30'' @ 60ºC
(4) 30'' @ 72ºC

Repeat step (2) to (4) for total <u>6 cycles</u>

(5) 1' @ 72ºC
(6) HOLD @ 4ºC
(7) END

3. Load 1 µl of sample into one lane of a 1.2% FlashGel with 3 µl Marker. See example in **Figure 2**.

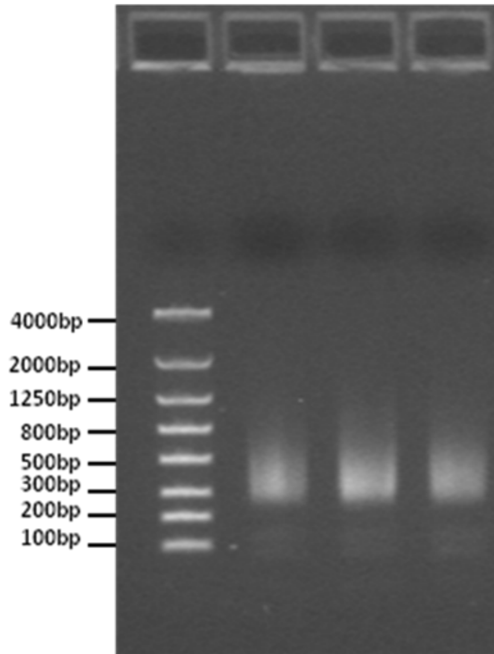
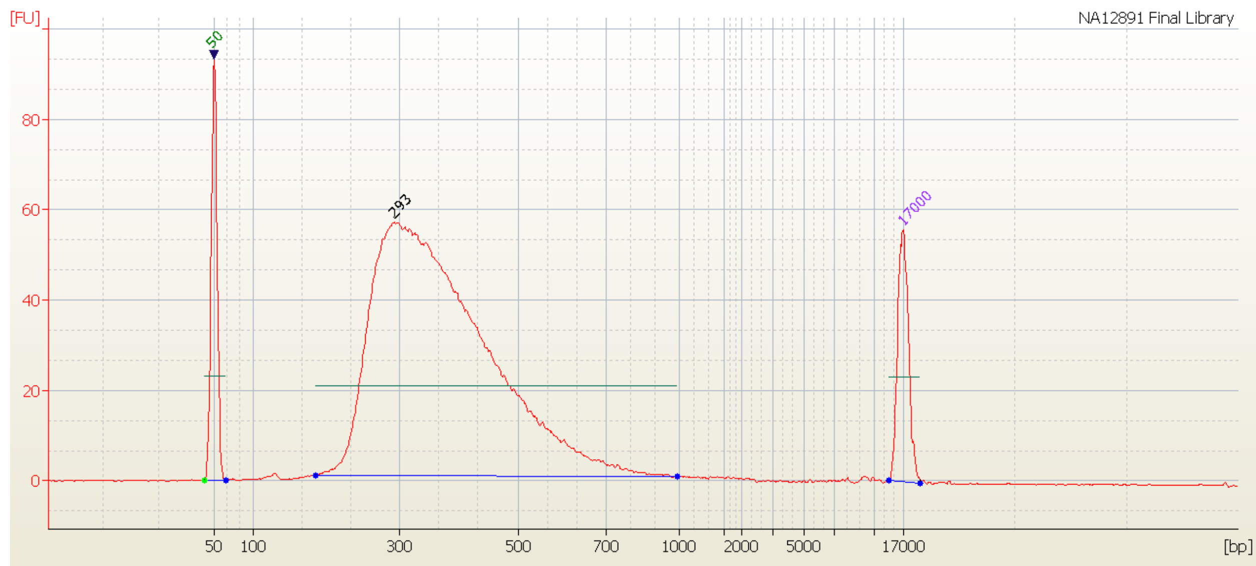


Figure 2. 1.2% Flash Gel electrophoresis of pre-capture LM-PCR amplification product. Running with 100bp-4kb DNA ladder.

√ **QC #2** If the intensity indicates that the product is considerably weak after a total of 8 cycles of PCR amplification, the whole process should be repeated.

4. Transfer the PCR product into a 1.7 ml tube. Purify the sample with 1.5x SPRI AMPure XP beads by repeating sub-steps from step 4.1.2 (3a) through (3d). Elute DNA with 60 µl **molecular grade water.**

√ **QC #3** Run 1 µl sample on an Agilent Bioanalyzer 2100 DNA 7500 Chip to check size distribution and quantitate PCR product (see example below). Normally 3-5ug PCR products can be generated.



3.2. Liquid Exome Sequence Capture

3.2.1 Thaw Exome Probes

Remove the appropriate number of VCRome 2.1 Rebalanced probe (manufactured by NimbleGen, 4.5 µl /100 ng exome probes per capture) from the -20°C freezer and allow them to thaw on ice.

3.2.2 Prepare the Hybridization Samples

1. Add 10 µl of 1 mg/ml Cot-1 DNA and 3 µg of pre-capture library to the 1.7ml hybridization tube.

2. Add 0.65 µl of each 1,000 µM Hybridization Enhancing Oligos (HEOs) [HEO1 and HEO-IBCn (n=1-12), see **2.4 Oligonucleotide Sequences**].
***Using full-length blocking oligos is critical for capture efficiency**.
***for co-capture experiments, pre-mix HEO-IBCn at equal amount and add 650 pmoles of the mixed blockers to the hybridization tube (along with 650 pmoles of HEO1) .**

3. Close the tube lid and make a hole in the cap of the tube with an 18-20 gauge or smaller needle.

4. Dry the pre-capture library/Cot-1 DNA/ HE Oligos mix at a DNA vacuum concentrator on high heat setting (65 °C ± 2 ºC) for about 30-60 minutes depending on volume.
*** Do not overdry the hybridization sample.**

5. To each dried-down sample tube, add:
   7.5 µl of 2X Hybridization Buffer
   3.0 µl Formamide*

* **Aliquot Formamide for single usage to avoid freeze and thaw cycle. Do not use component A in the hybridization kit.**

The sample tube should now contains the following components:

Components	Solution Capture
Cot-1 DNA	10.0 µg (dried)
Pre-capture library DNA	3.0 µg (dried)
1,000 µM HE Oligos	650 pmoles of HEO1 and 650 pmoles of HEO-IBCn or HEO-IBC mix (dried)
2X Hybridization Buffer	7.5 µl
Formamide	3.0 µl
Total volume	10.5 µl

6. Cover the hole in the tube cap with a “Tough Spots” sticker.

7. Vortex the pre-capture library/Cot-1 DNA/HE Oligos plus Hybridization Cocktail (2X Hybridization Buffer + Formamide) for 10 seconds and quick spin down.

8. Denature the pre-capture library/Cot-1 DNA/HE Oligos/Hybridization Cocktail at 95°C for 10 minutes in a thermomixer.

9. Quickly spin the pre-capture library/Cot-1 DNA/HE Oligos/Hybridization Cocktail at Eppendorf centrifuge 5430 (2000 rpm) at room temperature.

10. Mix the denatured hybridization sample with 4.5 µl exome probe in a 0.2 ml strip tube well. Mix by pipetting.

The hybridization reaction should now include the following components:

Components	Solution Capture
Cot-1 DNA	10.0 µg (dried)
Pre-capture Library	1.0 µg (dried)
1,000 µM HE Oligos	650 pmoles of HEO1 and 650 pmoles of HEO-IBCn or HEO-IBC mix (dried)
2X Hybridization Buffer	7.5 µl
Formamide	3.0 µl
Exome Library (VCRome 2.1)	4.5 µl
Total volume	15.0 µl

11. Incubate the mixture in an ABI 96-Well 9700 GeneAmp PCR machine at 47°C for 64-72 hours with the heated lid turned on.

3.2.3 Washing and Elution of Captured DNA

Prepare Sequence Capture and Bead Wash Buffers

1. Dilute 10X Wash Buffers (I, II, III and Stringent) and 2.5X Bead Wash Buffer to create 1X working solutions as following.

Amount of Concentrated Buffer	Amount of PCR Grade Water	Total Volume of 1X Buffer*
2 ml - 10X Stringent Wash Buffer	18 ml	20 ml
2 ml - 10X Wash Buffer I	18 ml	20 ml
1 ml - 10X Wash Buffer II	9 ml	10 ml
1 ml - 10X Wash Buffer III	9 ml	10 ml
200 µl 2.5X Bead Wash Buffer	300 µl	500 µl
* Store working solutions at room temperature for up to 2 weeks.		

2. Preheat the following wash buffers in an ABI 96-Well 9700 GeneAmp PCR machine at 47°C. (Option: the buffers can be pre-heated during hybridization incubation)
For each capture:
2 X 200 µl of Stringent Wash Buffer
175 µl of Wash Buffer I

Prepare the Streptavidin Dynabeads

1. Allow the Streptavidin Dynabeads warm to room temperature for 15 minutes prior to use.

2. Mix the beads thoroughly by vortexing.

3. Aliquot 100 µl of beads for each capture into a single 1.5 ml tube (i.e. for 1 capture use 100 µl beads and for 4 captures use 400 µl beads, etc.). Enough beads for 5 captures can be prepared in a single tube.

4. Place the tube in a DynaMag™-2 magnet device. When the liquid becomes clear (should take less than 5 minutes), remove and discard the liquid and be careful to leave all of the beads in the tube. Any remaining traces of liquid will be removed with subsequent wash steps.

5. While the tube is in the DynaMag™-2 magnet device, add twice the initial volume of beads of 1X Bead Wash Buffer (i.e. for 1 capture use 200 µl of buffer and for 4 captures use 800 µl buffer, etc.).

6. Remove the tube from the DynaMag™-2 magnet device and vortex for 10 seconds.

7. Place the tube back in the DynaMag™-2 magnet device to bind the beads. Once liquid turns clear, remove and discard the liquid.

8. Repeat above steps from 5 - 7 for a total of 2 washes.

9. After removing the buffer following the second wash, re-suspend the beads with 1X hybridization buffer containing Formamide (see below recipe).

7.5ul 2x hybridization buffer
3.0ul Formamide
4.5ul ddH2O
* **Aliquot Formamide for single usage to avoid freeze and thaw cycle.**

10. Aliquot 15-20 µl of re-suspended beads into 0.2 ml strip tube well and preheat it in an ABI 96-Well 9700 GeneAmp PCR machine at 47°C for 5 minutes.

Bind DNA to the Streptavidin Dynabeads

1. Without moving the hybridization samples off the ABI 96-Well 9700 GeneAmp PCR machine, transfer the preheated Streptavidin Dynabeads into hybridization reactions.

2. Mix gently by pipetting up and down 10 times.

3. Incubate the beads/DNA mixture in the ABI 96-Well 9700 GeneAmp PCR machine at 47°C for 45 minutes. Mix each sample by pipetting up and down 10 times at 15 minutes intervals.

Wash the Streptavidin Dynabeads and Elute Bound DNA

(**DynaMag™-96 side magnet device** can be utilized to replace the Neodymium Magnetic Bar in the washing steps. In such cases, quickly handing when transferring 0.2 ml strip tubes from PCR machine to the device is strongly recommended)

1. After the 45-minute incubation, while samples are still in the ABI 96-Well 9700 GeneAmp PCR machine, add 175 µl of preheated 1X Wash Buffer I to the Streptavidin Dynabeads plus Bound DNA.

2. Gently mix each sample mixture with multi-channel pipettes by pipetting up and down 10 times.

3. Place the Neodymium Magnetic Bar behind the strip of tubes to bind the beads while it is still in the ABI 96-Well 9700 GeneAmp PCR machine. Remove and discard the liquid once it turns clear.

4. Add 200 µl of preheated 1X Stringent Wash Buffer. Gently mix each sample mixture with multi-channel pipettes by pipetting up and down 10 times.

5. Incubate sample mixture at 47°C for 5 minutes in the ABI 96-Well 9700 GeneAmp PCR machine.

6. Place the Neodymium Magnetic Bar behind the strip of tubes to bind the beads while the tube is still in the ABI 96-Well 9700 GeneAmp PCR machine. Remove and discard the liquid once it turns clear.

7. Repeat Steps 5 - 7 one more time for a total of two washes using preheated 1X Stringent Wash Buffer .

8. Add 200 µl of room temperature 1X Wash Buffer I and gently mix by pipetting up and down 10 times while leaving it at RT for total 2 minutes.

9. Place the strip tubes inside the DynaMag™-96 side magnet device to bind the beads. Remove and discard the liquid once it turns clear.

10. Add 200 µl of room temperature 1X Wash Buffer II and gently mix it by pipetting up and down 10 times while leaving it at RT for total 1 minute.

11. Place the strip tubes inside the DynaMag™-96 side magnet device to bind the beads. Remove and discard the liquid once it turns clear.

12. Add 200 µl of room temperature 1X Wash Buffer III and gently mix it by pipetting up and down for 30 seconds.

13. Place the strip tubes inside the DynaMag™-96 side magnet device to bind the beads. Remove and discard the liquid once it turns clear.

14. Elute the captured DNA by adding 56 µl PCR grade water to each sample tube and mix.

15. Store the beads plus captured samples at -20°C freezer or proceed to post capture PCR amplification.

3.3 Post-Capture LM-PCR Amplification

1. Thaw 2X Custom HiFi Platinum Taq PCR mix on the ice.

2. Prepare the following PCR mix:

Component	Volume (µL)
DNA library	56
2X KAPA HiFi HotStart ReadyMix	60
LM-PCR 1.0 (50 µM)	2
LM-PCR 2.0 (50 µM)	2
Total reaction volume	120

3. 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 using the following condition:

(1) 45" @ 98ºC
(2) 15" @ 98ºC
(3) 30'' @ 60ºC
(4) 30'' @ 72ºC

Repeat step (2) to (4) for total 10-12 cycles

(5) 1' @ 72ºC
(6) HOLD @ 4ºC
(7) END

4. After amplification is done, load 1 µl of reaction into one lane of a 1.2% FlashGel with 3 µl markers. See example in **Figure 3.**

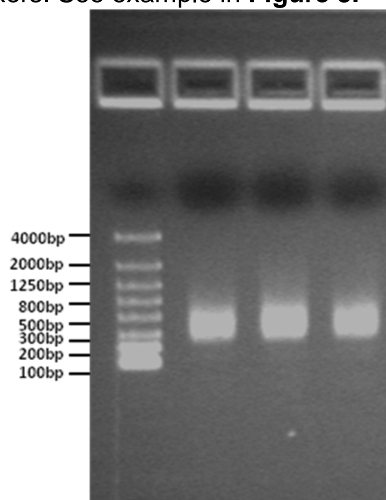
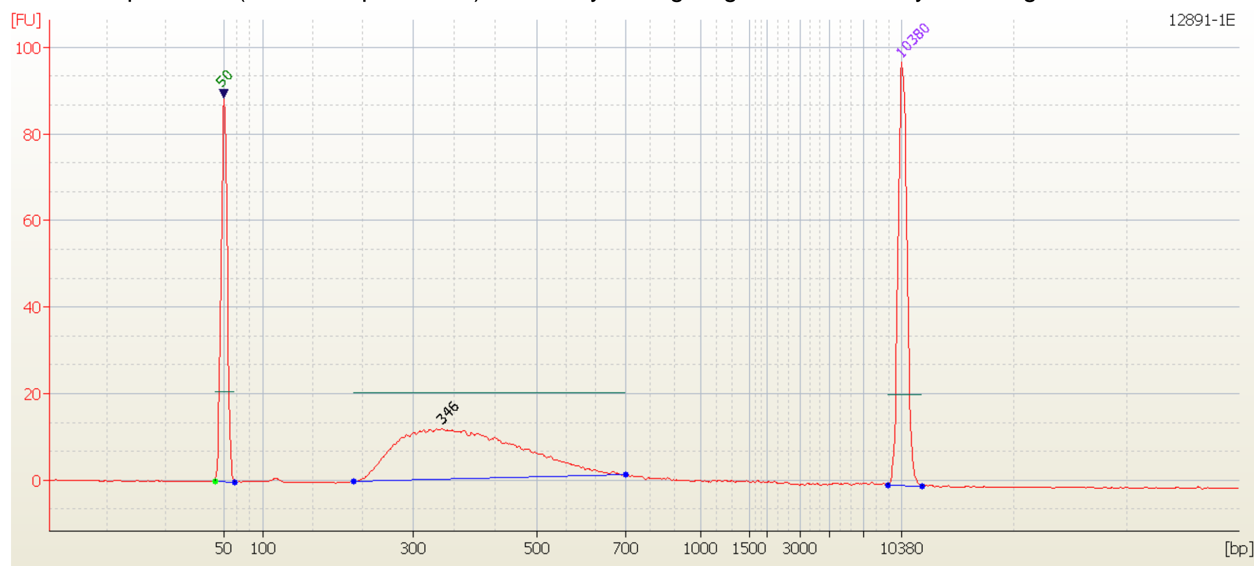


Figure 3. 1.2% Flash Gel electrophoresis of purified post-capture LM-PCR amplification product. Running with 100bp-4kb DNA ladder.

√ **QC #4** If the band intensity is too low after 12 cycles of PCR amplification, the capture process may need to be repeated.

5. Transfer the samples into 1.7 ml tube. Purify the samples by adding 1.5X SPRI AMPure XP beads. Elute DNA in 40 µl of nuclease-free water..

√ **QC #5** Run an Agilent Bioanalyzer 2100 DNA 7500 Chip to check size of distribution and quantify PCR products (see example below). Normally 500ng-1ug enriched library can be generated.



Optional: SYBR green-based qPCR could be performed to evaluate capture efficiency. If the average delta Ct of the four loci is greater than 6, the sample can be passed for sequencing. Otherwise, repeat steps starting from **4.2 Liquid Exome Sequence Capture**.

(End)