High-Throughput Indexed Library Preparation And Pooled Agilent Exome Enrichment

For Illumina sequencing platform
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Reagents / Consumables

NEBNext Mastermix Set 1 (New England BioLabs, E6040)
DNA 1000 kit (Agilent, 5067-1504)
DNA High Sensitivity kit (Agilent, 5067-4626)
AMPure XP Kit (Beckman Coulter, A63880)
microTUBE with AFA fiber and snap cap (Covaris, 520045)
96-well 0.3mL skirtless PCR plates (E&K Scientific, 480096)
2.0mL RNase-free collection tube (Denville, C1734)
0.2mL RNase-free PCR tube (Ambion, AM12225)
MicroAmp 8-cap strips (Applied Biosystems, N8010535)
Elution Buffer (Qiagen, 19086)
PEG 8000 (Promega, V3011)
Sodium Chloride (Promega, H5271)
PCR Grade water
100% ethanol

Equipment

Agilent 2100 Bioanalyzer
Covaris Model S2 Adaptive Focused Acoustics
Manual Multichannel Pipette (20uL, 200uL)
96-well magnet separator
Microcentrifuge
Vortex mixer

Custom Oligonucleotides

Order oligos HPLC purified. * corresponds to phosphorothioate bond. /5Phos/ corresponds to phosphate group. This notation is consistent with the format for IDT.

P5_PCR 5'- AATGATACGGCGACCACCGAG 3'

P7_PCR 5'- CAAGCAGAAGACGGCATACGAG 3'

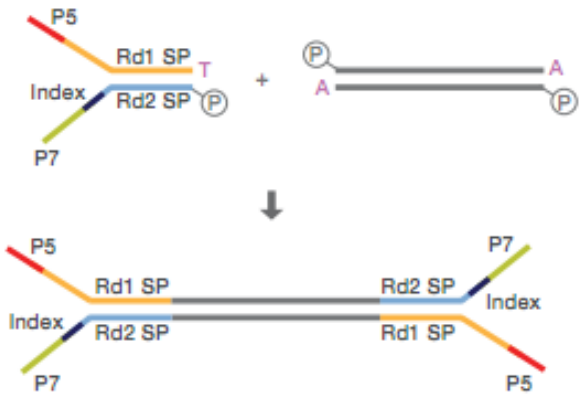
P5_adapter

5'- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC* T 3'

P7_adapter_#

5'- /5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCAC[Barcode]ATCTCGTATGCCGTCTT TGCTT*G 3'

#	Barcode
1	ATCACG
2	CGATGT
3	TTAGGC
4	TGACCA
5	ACAGTG
6	GCCAAT
7	CAGATC
8	ACTTGA
9	GATCAG
10	TAGCTT
11	GGCTAC
12	CTTGTA



Index-Adapter Preparation

Reconstitute oligonucleotides to 200 uM in 10 mM NaCl annealing buffer, then mix oligonucleotide pairs (P5_adapter, P7_adapter_#) at a 1:1 ratio (25 ul:25 ul). Heat solution to 95ºC for 5 minutes followed by cooling at a rate of 0.1ºC/s to 4ºC in a thermal cycler. Dilute with Elution Buffer to a working concentration of 15 uM.
Store at -20ºC.

Low-Level Pooling

Some sequencing experiments require the use of fewer than 12 index sequences in a lane with a high cluster density. In such cases, a careful selection of indexes is required to ensure optimum cluster discrimination by having different bases at each cycle of the index read. Illumina recommends the following sets of indexes for low-level pooling experiments.

Pool of 2 samples:

- Index #6 GCCAAT
- Index #12 CTTGTA

Pool of 3 samples:

- Index #4 TGACCA
- Index #6 GCCAAT
- Index #12 CTTGTA

Pool of 6 samples:

- Index #2 CGATGT
- Index #4 TGACCA
- Index #5 ACAGTG
- Index #6 GCCAAT
- Index #7 CAGATC
- Index #12 CTTGTA

20% PEG / 2.5M NaCl (for SPRI purification)

Add 10g PEG 8000 to a beaker containing 7.3g NaCl, bring the volume to 50mL with ddH20. Stir with a magnetic bar. Autoclave for 15 minutes, allow cooling at room temperature.

Genomic Library Preparation

Starting material: 260/280 ratio 1.7-1.9, 260/230 ratio >1.8

Note: Prepare all master mixes on ice. SPRI beads are retained within reaction mixture during enzyme treatments.

Fragment DNA

Fragmenting and size-selection are optimized for 2x100bp reads.

1ug DNA into Covaris microtube, total 50ul with Elution Buffer.

Duty cycle: 10%; Intensity: 5.0; Bursts/s: 200; Duration: 120s

Transfer 50ul volume into 0.3ml skirtless PCR plate; add End Repair Master Mix

End Repair Master Mix		
Sheared DNA	50ul	
Elution Buffer	35ul	
End Repair buffer	10ul	
End Repair enzyme mix	5ul	
Total	100ul	Incubate 30 min @ 20ºC

Stopping point: Store at -20ºC

Aliquot 150ul of AMPure XP solid-phase reversible immobilization (SPRI) beads into 0.3ml skirtless PCR plate well.
Use a 200ul multichannel pipette to mix full volume 15 times; incubate at room temperature for 2 minutes.
Place on 96-well plate magnet for 4 minutes. Remove supernatant and discard supernatant.
With new tips and with plate on the magnet, add 100ul of 80% ethanol.
Incubate 30 sec on plate magnet. Remove, discard ethanol (without disturbing beads).
Place on thermal cycler for 2 minutes at 37C.
Elute directly into 3' Adenylation master mix; pipette mix 15 times to re-suspend.
Seal plate with 8-well cap strips.

3' Adenylation Master Mix		
Elution Buffer	42ul	
dA-tailing rxn buffer	5ul	
Klenow fragment	3ul	
Total	50ul	Incubate 30 min @ 37ºC

Note: Continue directly into clean-up and ligation of indexed-adapters.

Dispense 90ul of 20% PEG, 2.5M NaCl into the sample plate.
Multichannel pipette mix full volumes 15 times; incubate at room temperature for 2 minutes.
Place on 96-well plate magnet for 4 minutes. Remove supernatant and discard supernatant.
With new tips and with plate on the magnet, add 100ul of 80% ethanol.
Incubate 30 sec on plate magnet. Remove and discard ethanol (without disturbing the beads).
Place on thermal cycler for 2 minutes at 37ºC.
Elute directly into Indexed-Adapter master mix; pipette mix 15 times to re-suspend.
Seal plate with 8-well cap strips.

Indexed-Adapter Master Mix

Elution Buffer	32ul	
Quick ligation buffer	10ul	
Quick T4 DNA ligase	5ul	
Indexed-Adapter mix	3ul (add to each sample independently)	
Total	50ul	Incubate 15 min @ 20ºC

Stopping point: Store at -20ºC

Dispense 90ul of 20% PEG, 2.5M NaCl into the sample plate.
Multichannel pipette mix full volumes 15 times; incubate at room temperature for 2 minutes.
Place on 96-well plate magnet for 4 minutes. Remove supernatant and discard supernatant.
With new tips and with plate on the magnet, add 100ul of 80% ethanol.
Incubate 30 sec on plate magnet. Remove and discard ethanol (without disturbing the beads).
Place on thermal cycler for 2 minutes at 37ºC.
Add 20ul of EB; pipette mix 15 times to re-suspend.
Seal wells with 8-cap strip.

Invitrogen E-Gel Size-Selection

Load 20uL of adapter-ligated sample into each well. Do not use the outside wells in order to avoid edge effects. Load **25ul** of EB to the collection wells. Run the 2% Size-selection program for a total of 21:40 minutes. Collect full volume for each sample (~10ul) and transfer to a 0.3ml skirtless PCR plate.

Genomic Library Amplification

Size-selected sample	10ul
EB	26.5ul
5X Herculase II Buffer	10ul
dNTP mix	0.5ul
Herculase II Polymerase	1ul
P5_PCR, 50uM (1uM Final)	1ul
P7_PCR, 50uM (1uM Final)	1ul
Total	50ul

Profile

Step 1	98ºC	2 min
Step 2	98ºC	30 sec
Step 3	60ºC	30 sec
Step 4	72ºC	1 min
Step 5	Repeat Step 2 through Step 4 for a total of 10 cycles	
Step 6	72ºC	5 min
Step 7	4ºC	forever

Dispense 90ul of SPRI beads to each well of sample plate.
Multichannel pipette mix full volumes 15 times; incubate at room temperature for 2 minutes.
Place on 96-well plate magnet for 4 minutes. Remove supernatant and discard supernatant.
With new tips and with plate on the magnet, add 100ul of 80% ethanol.
Incubate 30 sec on plate magnet. Remove and discard ethanol (without disturbing the beads).
Place on thermal cycler for 2 minutes at 37ºC.
Add 30ul of Elution Buffer; pipette mix 15 times to re-suspend.
Transfer full volume to a clean PCR plate or collection tube.
Store at -20C

Run samples on Bioanalyzer DNA 1000 chip to determine fragment size and concentration.

Pooled Exome Enrichment

In this protocol, genomic libraries representing multiple individuals are pooled together and enriched in a single exome capture. Libraries are combined evenly based on molarity from the Bioanalyzer assay. A total of **3 pMol** prepared libraries are placed into a single enrichment.

To enrich 3 individuals in a single exome capture, 1 pMol of each library are pooled and lyophilized in a vacuum concentrator. A novel indexed blocking scheme (see below) is used to prevent non-specific pull down and maintain index integrity. The Indexed Blocking Reagent (IBR) replaces SureSelect Block #3 during hybridization.

Indexed Blocking Oligos

P5_b1_f

5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCT 3'

P5_b1_r

5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3'

P7_b1_f 5' CAAGCAGAAGACGGCATACGAG 3'

P7_b1_r 5' CTCGTATGCCGTCTTCTGCTTG 3'

P7_b2_f 5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT/3ddC/ 3'

P7_b2_r 5' AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Order oligos HPLC purified. /3ddC/ corresponds to 3' dideoxyCytosine. This notation is consistent with the format for IDT.

Indexed Blocking Reagent (IBR)

Reconstitute oligos to 300uM with PCR Grade water. Combine equal volumes of each oligo to make 50uM indexed blocking reagent. Store at -20ºC

Hybridize the Pooled Library

Three components of hybridization reaction:

Hybridization Buffer
SureSelect Block Mix
Exome Capture (plus RNAse dilution)

Hybridization Buffer

Reagent	Volume (13ul/capture)
SureSelect Hyb #1	25ul
SureSelect Hyb #2	1ul
SureSelect Hyb #3	10ul
SureSelect Hyb #4	13ul
Total	49ul

Prepare in a 1.5mL RNAse-free microfuge tube. Warm to 65ºC with a heat block, placed near the hybridization thermal cycler.

SureSelect Block Mix

Reagent	Volume per capture
SureSelect Block #1	2.5ul
SureSelect Block #2	2.5ul
IBR	0.6ul
Total	5.6ul

Prepare in 0.2ml RNase-free PCR tube. Keep on ice. This will become the combined genomic library and block mix. Be mindful when sealing the cap; a crunched lid will allow volume to evaporate during hybridization.

Capture Library Mix

Reagent	Volume per capture
Exome library	5ul
PCR Grade water	1ul
RNase Block	1ul
Total	7ul

Prepare in 0.2ml RNase-free PCR tube. Keep on ice.

Hybridization Reaction

Reconstitute the lyophilized genomic libraries with 3.4ul of PCR Grade water. Transfer full volume to the SureSelect block mix. Mix by pipetting.

Load the combined genomic library and block mix onto the thermal cycler and run the following program:

Step 1	95ºC	5 min
Step 2	65ºC	∞

Use a heated lid; once the combined genomic library and block mix has been at 65ºC for 10 min, place the capture library mix on the thermal cycler. After 2 additional minutes, aliquot 13ul of the hybridization buffer (at 65ºC on heat block) to the capture library mix and immediately transfer the total volume to the combined genomic library and block mix. Add 1 drop of PCR grade mineral oil to the hybridization. Incubate at 65ºC for 24-72 hours.

Wash and Amplify the Exome Enriched library

Note: SureSelect Wash Buffer #2 must be warmed to 65ºC for at least 30 mins.

Prepare Strept-avidin Beads

For each capture; add 100ul of Dynal magnetic beads to a 2.0mL RNase-free collection tube. Add 200ul of SureSelect Binding Buffer, mix and vortex. Place on magnetic separator, remove and discard the supernatant. Repeat for a total of 3 washes.

Resuspend in 200ul SureSelect Binding Buffer

Wash the exome enrichment

Transfer 29ul of hybridization mixture (bottom layer) and add to strept-avidin beads. Incubate on a Nutator for 30 minutes at room temperature.

Place on a magnetic separator and discard the supernatant. Add 500ul of SureSelect Wash Buffer #1, vortex and incubate for 15 minutes at room temperature.

Place on a magnetic separator and discard the supernatant. Resuspend in 500ul of prewarmed Wash Buffer #2. Vortex and incubate at 65ºC for 10 minutes with a heat block (add water to the bottom of each well). Place on a magnetic separator and discard the supernatant. Repeat for a total of (3) 500ul washes.

Resuspend in PCR master mix and transfer to 0.3ml skirtless PCR plate.

Exome Library Amplification

EB	36.5ul
5X Herculase II Buffer	10ul
dNTP mix	0.5ul
Herculase II Polymerase	1ul
P5_PCR, 50uM (1uM Final)	1ul
P7_PCR, 50uM (1uM Final)	1ul
Total	50ul

Profile

Step 1	98ºC	2 min
Step 2	98ºC	30 sec
Step 3	60ºC	30 sec
Step 4	72ºC	1 min
Step 5	Repeat Step 2 through Step 4 for a total of 8 cycles	
Step 6	72ºC	5 min
Step 7	4ºC	forever

Dispense 90ul of SPRI beads to each well of sample plate.
Add full volume of PCR reaction (including strept-avidin beads) and pipette mix 15 times; incubate at room temperature for 2 minutes.
Place on 96-well plate magnet for 4 minutes. Remove supernatant and discard supernatant.
With new tips and with plate on the magnet, add 100ul of 80% ethanol.
Incubate 30 sec on plate magnet. Remove, discard ethanol (without disturbing beads).
Place on thermal cycler for 2 minutes at 37ºC.
Add 30ul of Elution Buffer; pipette mix 15 times to re-suspend.
Transfer full volume to a 0.3ml skirtless PCR plate.
Store at -20ºC

Run samples on Bioanalyzer High-Sensitivity chip to determine fragment size and concentration.

Submission to sequencing facility

Samples processed through a pooled-enrichment are combined with other pooled-enrichments to create a single mixture representing many individuals, each with a unique barcode. It is best to pool libraries evenly based on molarity from the Bioanalyzer High-Sensitivity assay. This mixture is diluted to 10nM and submitted for sequencing across multiple channels.