NEXTFLEX Human Whole Exome

Library Preparation Target Enrichment

Enzymatic Preparation / Enhanced Hybridization

KIT CONTAINS:12, 48, or 96 RXNS

USER MANUAL FOR:
#NOVA-6010-EVAL
#NOVA-6010-48
#NOVA-6010-96

Human Whole Exome -
Library Preparation Target Enrichment
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GENERAL INFORMATION

Materials Provided

Box 1. Library Preparation #1 Storage: -20°C

Product Name	1 sample (µL)
EP-ER/A Buffer	3
EP-ER/A Enzyme	6
Ligase Buffer	12
Ligation Enzyme	4

Box 2. Library Preparation #2 Storage: -20°C

Product Name	1 sample (µL)
UDI-Primer 1-192	5
Short Adapter	10

Box 3. Target Capture Solution #1 Storage: -20°C

Product Name	1 sample (µL)
Block #1	2.5
Block #2	2.5
Block #3 (D)	0.6
Target Capture Probe #2	1.0
Post Capture PCR Forward Primer	2.5
Post Capture PCR Reverse Primer	2.5

Box 4. Target Capture Solution #2 Storage: RT

Product Name	1 sample (µL)
Wash Buffer #1	800.0
Wash Buffer #2	500.0
Wash Buffer #3	1500.0
Hyb Buffer	24.0
Hyb Buffer Enhancer	3.0

Box 5. Target Capture Solution #3 Storage: -80°C

Product Name	1 sample (µL)
Target Capture Probe #1 (HWE)	6.0

Pouch Products Storage: See below

Product Name	Storage Condition
Clean-up Bead	4°C
NM Bead #1	4°C
NM Bead #2 (NM Bead Binding Buffer)	RT
Polymerase	-20°C
Streptavidin Bead	4°C

Materials Required (Not Provided)

Reagents & Consumables

Product Name	Suggested Supplier	p/n
Nuclease-free water (not DEPC-treated)	General lab supplier	-
Ethanol (200 proof)	General lab supplier	-
Isopropanol	General lab supplier	-
Conical centrifuge tubes (15 mL or 50 mL)	General lab supplier	-
P10, P20, P200, and P1000 pipettes	General lab supplier	-
Multichannel pipette	General lab supplier	-
Sterile, nuclease-free aerosol barrier pipette tips	General lab supplier	-
0.2 mL PCR Tube Strips, Low Profile and Cap Strips	General lab supplier	-
Powder-free gloves	General lab supplier	-
DNA LoBind Tubes, 1.5 mL PCR clean	Eppendorf	022431021 or equivalent
Microtubes 1.5ml Clear, Sterile	AXYGEN	MCT-150-C-S or equivalent
Eppendorf™ twin.tec™ 96-well LoBind PCR Plates, Skirted	Eppendorf	0030129512 or equivalent
Microseal 'B' adhesive seals	Bio-Rad	MSB-1001 or equivalent

Equipment

Product Name	Suggested Supplier	p/n
LabChip® GX Touch™ Instrument	Revvity	CLS138162 or equivalent
Magnetic separator	Revvity	CMG-301 or equivalent
Thermal Cycler	General lab supplier	-
Micro-centrifuge	General lab supplier	-
Vortex mixer	General lab supplier	-

Before You Begin

It is recommended to read the manual carefully to understand the experimental procedure. In particular, read and follow the bold text.

Notice

The results of this experiment can be affected by the quality of the DNA. We recommend using high-quality DNA samples. However, low-quality samples (e.g., FFPE tissue extracted gDNA) show variable efficiency due to chemical damage and fragmentation, and its small amounts. Therefore, we recommend additional experimental optimization when you prepare the NGS library with these samples.

Directions for the use of this product

1. This product should be used for research use only.
2. Handle specimens cautiously to avoid potential infections.
    A. Handle reagents cautiously and avoid contact with any area of the user's skin, eyes, and mucous membrane. In the event of reagent contact, rinse with water immediately to remove. If the reagent spills from the cartridge, dilute with water and follow the duly accredited treatment.
    B. All blood serum materials from human sources should be handled carefully and treated as potential infectious sources.
3. Genomic DNA extracted from bio-specimens such as blood, saliva, FFPE, and fresh tissue should be used as sample specimens.
4. Reagents should be used by the suggested Usage and Volume within the expiry date.
5. Reagent storage should be carried out at their suggested temperature and condition. Temperature conditions are listed below:
6. Room temperature (RT, 15-25°C), Freezer(-20°C), Deep freezer (-80°C)
7. For disposal of used or expired reagents, please contact an authorized waste disposal company in accordance with relevant laws and regulations.
8. Be cautious with specific allergenic reagents.
9. Please maintain a nuclease-free environment during the experiment. Wear a lab coat, disposable gloves, and protective goggles. If you have any problems, questions, or related concerns, please email us at ngs@revvity.com

PROTOCOL OVERVIEW

	Workflow	Time
NGS Library Preparation	Enzymatic preparation	1 hr
	Adapter ligation	20 mins
	Purification of sample using Clean-up Bead	40 mins
	Amplification of the Adapter-Ligated Library	20 mins
	Purification using NM Bead	40 mins
	Pre-capture pooling of samples	40 mins
Hybridization & Amplification	Prepare libraries and reagents for hybridization	1 hr
	Hybridize target capture probes with DNA library	16 hr
	Selection of target captured library by using streptavidin bead	1.5 hr
	Post-capture PCR amplification, Purification, and QC	1.5 hr
Sequencing on an Illumina® platform		

SAMPLE PREPARATION

NGS LIBRARY PREPARATION

STEP 1: Enzymatic Preparation

! NOTE: Follow the protocol in this section for human genomic DNA inputs ≥ 50 ng to construct libraries with an average insert size of ~300 bp, for targeted enrichment and direct sequencing, respectively. Fragmentation times provided are for high-quality samples. Optimization of fragmentation time may be required for samples of compromised quality (e.g., FFPE).

PREPARATION

Library Preparation Box #1	10x EP-ER/A buffer 5x EP-ER/A Enzyme	Keep cool on ice (or cooling block)

PROCEDURE

1. Set the program into a thermal cycler (see the details in Table 4 and 5).
2. Adjust the DNA sample volume to 21 µL as below in Table 1, if necessary. The input amount should be ≥ 50 ng of DNA.

Table 1. Adjusting the Volume per Sample

21 - x	Nuclease-free water
x	DNA Sample
21 µL	TOTAL

3. Transfer the entire DNA sample into a new 8-strip PCR tube (or 96-well plate).
4. Prepare Enzymatic Prep Master Mix in the 8-strip PCR tube as described in Table 2 (for multiple samples, prepare the Enzymatic Prep Master Mix of EP-ER/A Buffer and EP- ER/A Enzyme in a new 1.5 mL LoBind tube including at least 5% of additional volume).

! IMPORTANT – Because enzymes are active at room temperature and may fragment DNA to undesired sizes. Ensure that the Enzymatic Prep Master Mix is mixed thoroughly and is kept on ice until placed in the thermal cycler.

Table 2. Composition of Enzymatic Prep Master Mix

3 µL	EP-ER/A Buffer	Enzymatic Prep Master Mix
6 µL	EP-ER/A Enzyme	
9 µL	TOTAL	

5. Add 9 µl of Enzymatic Prep Master Mix to each 8-strip PCR tube (containing the DNA sample), mix well using a pipette, and spin down the tube.

! IMPORTANT– Combine it on ice and mix well.

Table 3. Composition of Enzymatic Prep Mix

9 µL	Enzymatic Prep Master mix
21 µL	Sample
30 µL	TOTAL

6. Put the tubes on thermal cycler and run the following program in Table 4.

Table 4. Thermal Cycler Program (lid temperature: 70°C)

1 min	4°C	! *IMPORTANT – Desired time for fragmentation depends on the condition of the initial input DNA amount. See Table 5.*
Desired time (Table 5)	32°C	
30 min	65°C	
hold	4°C	

Table 5. Desired Time for Fragmentation

Average insert size	Desired fragmentation time (min.) for DNA
~300 bp	18 ± 5

! IMPORTANT – The optimal fragmentation times differ between enzyme production lots. Please find the fragmentation time-size table in your kit located inside the lid of Library Preparation box #1. The fragmentation times are based on the fragmentation of 200 ng of DNA with DIN≥9.

! IMPORTANT – This kit can be applied to damaged samples such as FFPE. However, it should be optimized to an appropriate fragmentation time. We recommend that FFPE samples have a shorter fragmentation time depending on the level of degradation and starting size distribution.

7. After running the thermal cycler program, prepare the Ligation Master Mix immediately for the Adapter Ligation step.

! IMPORTANT – Fragmented samples can be kept at 4°C for no more than one hour.

! IMPORTANT – Proceed with the next step right away.

STEP 2: Adapter Ligation

PREPARATION

Library Preparation Box #1	Ligation buffer Ligation Enzyme	Keep cool on ice (or cooling block)
Library Preparation Box #2	Short Adapter	Thawing on ice

PROCEDURE

1. Add 10 µL of Short Adapter directly into the PCR tube (DNA sample) where Enzymatic Preparation was performed. Mix the tube well on the vortex mixer and pulse-spin down.

   Table 6. Addition of Adapter to make DNA sample & Adapter

30 µL	ER/A-tailed Sample	Mix Sample and Adapter first
10 µL	Adapter	
40 µL	TOTAL	

2. Prepare Ligation Master Mix (ligation, Buffer, ligation Enzyme and nuclease-free water) in a new 1.5 mL LoBind tube as described in Table 7.

   Table 7. Composition of Ligation Mix

4 µL	Nuclease-free water	The viscosity of reagents is high, mix well using vortex mixer
12 µL	Ligation Buffer	
4 µL	Ligation Enzyme	
20 µL	TOTAL	

3. Add 20 µL of Ligation Master Mix into the PCR tube (DNA sample & Short Adapter, 40 µL). Mix the sample tube well using a vortex mixer and pulse-spin down.

! IMPORTANT – Combine it on ice and mix well.

! IMPORTANT – The final reaction volume is 60 µl.

4. Incubate the Ligation mixture at 20°C for 20 minutes in thermal cycler (lid temperature: 40°C).

! IMPORTANT – Proceed with the next step right away.

STEP 3: Purification of sample Using Clean-up Bead

PREPARATION

Clean-up Bead	Keep the bead at room temperature for at least 30 minutes before use. Store the bead at 4°C after use
80% ethanol solution	Using freshly made 80% ethanol solution is preferable

PROCEDURE

1. Vortex Clean-up Bead to achieve a homogeneous state.
2. Add 48 µL Clean-up Bead to a new 1.5 ml LoBind Tube (0.2mL PCR tube or 96-well plate are also available) and transfer the A-tailed DNA (60 µL) to the tube. Mix well with a pipette and pulse-spin down the tube. Incubate at room temperature for 5 minutes.

! IMPORTANT – Avoid spinning down the bead too strongly to prevent precipitation.

Table 8. Volumes of Clean-up Bead (0.8x)

Reagent	Volume Ratio (Bead : Sample)
Clean-up Bead	0.8 : 1 (e.g., 48 µL : 60 µL)

3. Put the tube in the magnetic separator for 3-5 minutes until the solution is clear.
4. Keep the tube in the magnetic separator and discard the supernatant

! IMPORTANT – During Steps 4 to 6, be careful not to touch the bead while you remove the supernatant.

5. Keeping the tube in the magnetic separator, add 500 µL (180 µL for PCR tube or 96-well plate) of fresh 80% ethanol to each tube.
6. Incubate for 30 seconds at room temperature, then discard the ethanol.
7. Repeat wash steps 5-6 once.
8. Spin down, put the tube in the magnetic separator and discard residual ethanol.
9. Dry the sample tube at room temperature for 2 minutes or until residual ethanol has completely evaporated.

! IMPORTANT – Excessive drying of the bead pellets until cracks occur causes a decrease in elution efficiency. Proceed with step 10 before the crack appears.

10. Add 22 µL of nuclease-free water to the sample and thoroughly mix with a pipette. Pulse-spin down and incubate the sample at room temperature for 2 minutes.
11. Put the tube in the magnetic separator for 2 minutes until the solution is clear.
12. Transfer the supernatant (20 µL) to a new 1.5 mL LoBind Tube and keep the supernatant for the next step.

! STOP POINT - If not immediately continuing to the next step, samples can be stored at -20°C.

STEP 4: Amplification of the Adapter-Ligated Library

! NOTE: Determine the appropriate index primers for each sample (See Table 12. Index sequences).

! IMPORTANT - Use different index primer pairs if the samples are sequenced in the same sequencing run.

PREPARATION

Polymerase	Polymerase	Keep cool on ice (or cooling block)
Library Preparation Box #2	UDI-Primer (Unique Dual Index)	Thawing on ice Keep cool on ice (or cooling block)

**The name of each primer differs between kits. Please find the index primers in your kit located in Library Preparation box #2.*

PROCEDURE

1. Set the program into a thermal cycler (see the details in Table 10).
2. Prepare new 0.2 mL PCR tubes (or 96-well PCR plates) and PCR mix as described in Table 9 (add 5% of the additional volume of reagents for multiple libraries).

Table 9. Composition of PCR mix

25 µL	Polymerase
5 µL	UDI-Primer 1-192 (select one)
20 µL	Adapter-ligated DNA Library
50 µL	TOTAL

3. Add 25 µL of the Polymerase to each tube.

! IMPORTANT – Combine all reagents on ice.

4. Add 5 µL of each UDI-Primer to the individual tube.

! IMPORTANT – Check and record the appropriate Unique dual index per sample.

5. Add 20 µL of Adapter-ligated DNA library to each PCR tube, mix well and spin down.
6. Put the tubes on a thermal cycler and run the PCR program shown in Table 10 (see the number of PCR cycles in Table 11). Lid temperature: 105°C.

Table 10. PCR Program

45 sec	98°C
15 sec	98°C
30 sec	65°C
1 min	72°C

Repeat step 2 to step 4
(Total PCR cycles, See in Table 11)

10 min	72°C
hold	4°C

Table 11. The Number of PCR Cycles

Genomic DNA amount (ng), from page 9 - Step 1	Total PCR Cycles
500 ng ≤ gDNA amount	3-4
100 ng ≤ gDNA amount < 500 ng	5-6
50 ng ≤ gDNA amount < 100 ng	7-8
gDNA amount < 50 ng	9-10

** The quality of gDNA can affect PCR efficiency.*

Table 12. UDI Index Sequences (1 of 3)

Index	i5 Sequence	i7 Sequence	Index	i5 Sequence	i7 Sequence
UDI Primer 1	ATATGCGC	CTGATCGT	UDI Primer 37	GATAGGCT	TGTGACTG
UDI Primer 2	TGGTACAG	ACTCTCGA	UDI Primer 38	AGTGGATC	GTCATCGA
UDI Primer 3	AACCGTTC	TGAGCTAG	UDI Primer 39	TTGGACGT	AGCACTTC
UDI Primer 4	TAACCGGT	GAGACGAT	UDI Primer 40	ATGACGTC	GAAGGAAG
UDI Primer 5	GAACATCG	CTTGTCGA	UDI Primer 41	GAAGTTGG	GTTGTTCG
UDI Primer 6	CCTTGTAG	TTCCAAGG	UDI Primer 42	CATACCAC	CGGTTGTT
UDI Primer 7	TCAGGCTT	CGCATGAT	UDI Primer 43	CTGTTGAC	ACTGAGGT
UDI Primer 8	GTTCTCGT	ACGGAACA	UDI Primer 44	TGGCATGT	TGAAGACG
UDI Primer 9	AGAACGAG	CGGCTAAT	UDI Primer 45	ATCGCCAT	GTTACGCA
UDI Primer 10	TGCTTCCA	ATCGATCG	UDI Primer 46	TTGCGAAG	AGCGTGTT
UDI Primer 11	CTTCGACT	GCAAGATC	UDI Primer 47	AGTTCGTC	GATCGAGT
UDI Primer 12	CACCTGTT	GCTATCCT	UDI Primer 48	GAGCAGTA	ACAGCTCA
UDI Primer 13	ATCACACG	TACGCTAC	UDI Primer 49	ACAGCTCA	GAGCAGTA
UDI Primer 14	CCGTAAGA	TGGACTCT	UDI Primer 50	GATCGAGT	AGTTCGTC
UDI Primer 15	TACGCCTT	AGAGTAGC	UDI Primer 51	AGCGTGTT	TTGCGAAG
UDI Primer 16	CGACGTTA	ATCCAGAG	UDI Primer 52	GTTACGCA	ATCGCCAT
UDI Primer 17	ATGCACGA	GACGATCT	UDI Primer 53	TGAAGACG	TGGCATGT
UDI Primer 18	CCTGATTG	AACTGAGC	UDI Primer 54	ACTGAGGT	CTGTTGAC
UDI Primer 19	GTAGGAGT	CTTAGGAC	UDI Primer 55	CGGTTGTT	CATACCAC
UDI Primer 20	ACTAGGAG	GTGCCATA	UDI Primer 56	GTTGTTCG	GAAGTTGG
UDI Primer 21	CACTAGCT	GAATCCGA	UDI Primer 57	GAAGGAAG	ATGACGTC
UDI Primer 22	ACGACTTG	TCGCTGTT	UDI Primer 58	AGCACTTC	TTGGACGT
UDI Primer 23	CGTGTGTA	TTCGTTGG	UDI Primer 59	GTCATCGA	AGTGGATC
UDI Primer 24	GTTGACCT	AAGCACTG	UDI Primer 60	TGTGACTG	GATAGGCT
UDI Primer 25	ACTCCATC	CCTTGATC	UDI Primer 61	CAACACCT	TGGTAGCT
UDI Primer 26	CAATGTGG	GTCGAAGA	UDI Primer 62	ATGCCTGT	CGCAATCT
UDI Primer 27	TTGCAGAC	ACCACGAT	UDI Primer 63	CATGGCTA	GATGTGTG
UDI Primer 28	CAGTCCAA	GATTACCG	UDI Primer 64	GTGAAGTG	GATTGCTC
UDI Primer 29	ACGTTCAG	GCACAACT	UDI Primer 65	CGTTGCAA	CGCTCTAT
UDI Primer 30	AACGTCTG	GCGTCATT	UDI Primer 66	ATCCGGTA	TATCGGTC
UDI Primer 31	TATCGGTC	ATCCGGTA	UDI Primer 67	GCGTCATT	AACGTCTG
UDI Primer 32	CGCTCTAT	CGTTGCAA	UDI Primer 68	GCACAACT	ACGTTCAG
UDI Primer 33	GATTGCTC	GTGAAGTG	UDI Primer 69	GATTACCG	CAGTCCAA
UDI Primer 34	GATGTGTG	CATGGCTA	UDI Primer 70	ACCACGAT	TTGCAGAC
UDI Primer 35	CGCAATCT	ATGCCTGT	UDI Primer 71	GTCGAAGA	CAATGTGG
UDI Primer 36	TGGTAGCT	CAACACCT	UDI Primer 72	CCTTGATC	ACTCCATC

Table 12. UDI Index Sequences (2 of 3)

Index	i5 Sequence	i7 Sequence	Index	i5 Sequence	i7 Sequence
UDI Primer 73	AAGCACTG	GTTGACCT	UDI Primer 109	CGAGGAAT	GCTACTTC
UDI Primer 74	TTCGTTGG	CGTGTGTA	UDI Primer 110	GTATGGCA	TGACTTGC
UDI Primer 75	TCGCTGTT	ACGACTTG	UDI Primer 111	AGGTCTGA	TCGCACTA
UDI Primer 76	GAATCCGA	CACTAGCT	UDI Primer 112	CTCATTGC	CCTATAGG
UDI Primer 77	GTGCCATA	ACTAGGAG	UDI Primer 113	ACATGAGG	CCGTGAAT
UDI Primer 78	CTTAGGAC	GTAGGAGT	UDI Primer 114	TGTCGTAG	GTCACCTT
UDI Primer 79	AACTGAGC	CCTGATTG	UDI Primer 115	CGTCTTCA	GGATGCTA
UDI Primer 80	GACGATCT	ATGCACGA	UDI Primer 116	GTCGCTAA	TTACCGTG
UDI Primer 81	ATCCAGAG	CGACGTTA	UDI Primer 117	TCCTGGTA	TGGTCAGT
UDI Primer 82	AGAGTAGC	TACGCCTT	UDI Primer 118	CGTCACTA	CTTGAAGG
UDI Primer 83	TGGACTCT	CCGTAAGA	UDI Primer 119	TGACGTCT	CGATTAGG
UDI Primer 84	TACGCTAC	ATCACACG	UDI Primer 120	CATGCGAA	AACGCAGT
UDI Primer 85	GCTATCCT	CACCTGTT	UDI Primer 121	AAGCTCCT	GACAACTC
UDI Primer 86	GCAAGATC	CTTCGACT	UDI Primer 122	AGGTAACG	CTTGAGCA
UDI Primer 87	ATCGATCG	TGCTTCCA	UDI Primer 123	CAAGCTTG	CCATCAAG
UDI Primer 88	CGGCTAAT	AGAACGAG	UDI Primer 124	TCCTCATG	AGTCATCG
UDI Primer 89	ACGGAACA	GTTCTCGT	UDI Primer 125	CACCGATA	TGAACCTG
UDI Primer 90	CGCATGAT	TCAGGCTT	UDI Primer 126	TGTAAGCG	TATCCTGC
UDI Primer 91	TTCCAAGG	CCTTGTAG	UDI Primer 127	GAACGGAT	ACCTAGTC
UDI Primer 92	CTTGTCGA	GAACATCG	UDI Primer 128	GACCTAGA	TAGGCTGA
UDI Primer 93	GAGACGAT	TAACCGGT	UDI Primer 129	ATCGGTGT	CATTAGGC
UDI Primer 94	TGAGCTAG	AACCGTTC	UDI Primer 130	TGAGACCA	GCTGTTGA
UDI Primer 95	ACTCTCGA	TGGTACAG	UDI Primer 131	ACGACACT	TTCACAGC
UDI Primer 96	CTGATCGT	ATATGCGC	UDI Primer 132	CGGACTAA	ATGGTCAC
UDI Primer 97	ATCTGCCT	AGACTGTC	UDI Primer 133	TAGCATCC	TCATTCGG
UDI Primer 98	ATTCTGCG	CAGGTTCA	UDI Primer 134	AAGTTGGC	AGGCGTAA
UDI Primer 99	ATCAAGCC	AACTCGTG	UDI Primer 135	GATCCTCA	AGATACGG
UDI Primer 100	GACTGACT	GCAGATTC	UDI Primer 136	GTGACCAA	AATGGCCT
UDI Primer 101	CAGAATCG	CAAGGATC	UDI Primer 135	GATCCTCA	AGATACGG
UDI Primer 102	TGCTATCC	CCAACAGT	UDI Primer 136	GTGACCAA	AATGGCCT
UDI Primer 103	CTCCGTAT	TCACCTGA	UDI Primer 137	CAGATGAG	CGACAAGT
UDI Primer 104	GAGTAGAC	CGGAACTT	UDI Primer 138	TAACGACC	ACACGTAC
UDI Primer 105	AGCCAATC	TACTGCTG	UDI Primer 139	CCAGTGAT	AACAGTCG
UDI Primer 106	GCCAAGTA	TCAGACAG	UDI Primer 140	GCTCAAGA	TACATGCG
UDI Primer 107	GTGCACAT	GTAATGCC	UDI Primer 141	AGAGAGCT	GCATCACA
UDI Primer 108	AGCAAGTG	CCAAGCAT	UDI Primer 142	AGATCCAC	CTTATCCG

Table 12. UDI Index Sequences (3 of 3)

Index	i5 Sequence	i7 Sequence	Index	i5 Sequence	i7 Sequence
UDI Primer 143	CCTGACAA	CGTAAGGT	UDI Primer 169	AACGCAGT	CATGCGAA
UDI Primer 144	CCGTATCT	GACGTGAT	UDI Primer 170	CGATTAGG	TGACGTCT
UDI Primer 145	GACGTGAT	CCGTATCT	UDI Primer 171	CTTGAAGG	CGTCACTA
UDI Primer 146	CGTAAGGT	CCTGACAA	UDI Primer 172	TGGTCAGT	TCCTGGTA
UDI Primer 147	CTTATCCG	AGATCCAC	UDI Primer 173	TTACCGTG	GTCGCTAA
UDI Primer 148	GCATCACA	AGAGAGCT	UDI Primer 174	GGATGCTA	CGTCTTCA
UDI Primer 149	TACATGCG	GCTCAAGA	UDI Primer 175	GTCACCTT	TGTCGTAG
UDI Primer 150	AACAGTCG	CCAGTGAT	UDI Primer 176	CCGTGAAT	ACATGAGG
UDI Primer 151	ACACGTAC	TAACGACC	UDI Primer 177	CCTATAGG	CTCATTGC
UDI Primer 152	CGACAAGT	CAGATGAG	UDI Primer 178	TCGCACTA	AGGTCTGA
UDI Primer 153	AATGGCCT	GTGACCAA	UDI Primer 179	TGACTTGC	GTATGGCA
UDI Primer 154	AGATACGG	GATCCTCA	UDI Primer 180	GCTACTTC	CGAGGAAT
UDI Primer 155	AGGCGTAA	AAGTTGGC	UDI Primer 181	CCAAGCAT	AGCAAGTG
UDI Primer 156	TCATTCGG	TAGCATCC	UDI Primer 182	GTAATGCC	GTGCACAT
UDI Primer 157	ATGGTCAC	CGGACTAA	UDI Primer 183	TCAGACAG	GCCAAGTA
UDI Primer 158	TTCACAGC	ACGACACT	UDI Primer 184	TACTGCTG	AGCCAATC
UDI Primer 159	GCTGTTGA	TGAGACCA	UDI Primer 185	CGGAACTT	GAGTAGAC
UDI Primer 160	CATTAGGC	ATCGGTGT	UDI Primer 186	TCACCTGA	CTCCGTAT
UDI Primer 161	TAGGCTGA	GACCTAGA	UDI Primer 187	CCAACAGT	TGCTATCC
UDI Primer 162	ACCTAGTC	GAACGGAT	UDI Primer 188	CAAGGATC	CAGAATCG
UDI Primer 163	TATCCTGC	TGTAAGCG	UDI Primer 189	GCAGATTC	GACTGACT
UDI Primer 164	TGAACCTG	CACCGATA	UDI Primer 190	AACTCGTG	ATCAAGCC
UDI Primer 165	AGTCATCG	TCCTCATG	UDI Primer 191	CAGGTTCA	ATTCTGCG
UDI Primer 166	CCATCAAG	CAAGCTTG	UDI Primer 192	AGACTGTC	ATCTGCCT
UDI Primer 167	CTTGAGCA	AGGTAACG			
UDI Primer 168	GACAACTC	AAGCTCCT			

! IMPORTANT – Proceed with the next step right away.

STEP 5: Purification Using NM Bead

NOTE: The NM bead have a distinct application use that is different from the Clean-up Bead. Please be sure to use each separately and not to mix up the two.

PREPARATION

NM Bead	Keep the bead at room temperature for at least 30 minutes before use. Store the bead at 4°C after use
Binding buffer	Store the buffer at Room temperature
100% isopropanol	Store the buffer at Room temperature
80% ethanol solution	Using freshly made 80% ethanol solution is preferable

PROCEDURE

1. Thoroughly vortex NM Bead to achieve a homogeneous state.
2. Add 75 µL of binding buffer, 45 µL of 100% isopropanol, and 22.5 µL of NM Bead to a new 1.5 mL LoBind tube and add 50 µL of amplified sample to the tube. Mix well with a pipette and pulse-spin down the tube. Incubate at room temperature for 5 minutes.

! IMPORTANT – Avoid spinning down the NM bead too strongly to prevent precipitation.

Table 13. Volumes of NM Bead

75 µL	Binding Buffer
45 µL	100% isopropanol
22.5 µL	NM Bead
50 µL	Amplified sample
192.5 µL	TOTAL

3. Keep the tube in the magnetic separator for 5 minutes, then discard the supernatant.

! IMPORTANT – During Steps 3 to 6, be careful not to touch the bead while you remove the supernatant.

4. Keeping the tube in the magnetic separator, add 500 µL of fresh 80% ethanol to each sample tube.
5. Incubate for 30 seconds at room temperature and then discard the 80% ethanol
6. Repeat 80% ethanol wash (steps 5-6).
7. Spin down, put the tube in a magnetic separator, and discard residual ethanol.
8. Dry the sample tube at room temperature for more than 2 minutes until residual ethanol has completely evaporated.

! IMPORTANT – Excessive drying of the bead pellets until cracks occur causes a decrease in elution efficiency. Proceed with step 10 before the crack appears.

9. Add 12 µL of nuclease-free water to the sample and mix well with a pipette. Pulse-

spin down and incubate the sample at room temperature for 5 minutes.

10. Put the tube in the magnetic separator for 2 minutes until the solution is clear.
11. Transfer the supernatant (10 µL) to a new 1.5 mL LoBind Tube.

! STOP POINT - If not immediately continuing to the next step, samples can be stored at -20°C.

STEP 6: Pre-capture Pooling of samples

PREPARATION

Clean-up Bead	Keep the bead at room temperature for at least 30 minutes before use. Store the bead at 4°C after use
80% ethanol solution	Using freshly made 80% ethanol solution is preferable

PRE-CAPTURE POOLING GUIDE

After the purification with NM bead, the volume of each sample should be 10 µL. For the next step, we recommend pooling 4 samples into a single pooling library (see Table 14 below).

Table 14. Pooling Guide according to the Number of Samples

Number of samples	Total Volume (µL)
4	40 µL

PROCEDURE

1. Thoroughly vortex Clean-up Bead to achieve a homogeneous state.
2. Add each DNA library sample (10 µL each) to a new 1.5 mL LoBind tube according to Table 14. Mix it with the same volume of Clean-up Bead (See Table 15). Mix well with a pipette and pulse-spin down the tube. Incubate at room temperature for 5 minutes.

! IMPORTANT – Avoid spinning down the bead too strongly to prevent precipitation.

Table 15. Volumes of Clean-up Bead (1x)

Reagent	Volume Ratio (Bead : Sample)
Clean-up Bead	1 : 1 (e.g., 40 µL : 40 µL)

3. Put the tube in the magnetic separator for 3-5 minutes until the solution is clear.
4. Keep the tube in the magnetic separator and discard the supernatant

! IMPORTANT – During Steps 4 to 6, be careful not to touch the bead while you remove the supernatant.

5. Keeping the tube in the magnetic separator, add 500 µL of fresh 80% ethanol to each tube.
6. Incubate for 30 seconds at room temperature, then discard the ethanol.
7. Repeat wash steps 5-6 once.
8. Spin down, put the tube in the magnetic separator and discard residual ethanol.
9. Dry the sample tube at room temperature for 2 minutes or until residual ethanol has completely evaporated.

! IMPORTANT – Excessive drying of the bead pellets until cracks occur causes a decrease in elution efficiency. Proceed with step 10 before the crack appears.

10. Add 12 µL of nuclease-free water to the sample and thoroughly mix with a pipette. Pulse-spin down and incubate the sample at room temperature for 2 minutes.
11. Put the tube in the magnetic separator for 2 minutes until the solution is clear.
12. Transfer the supernatant (10 µL) to a new 1.5 mL LoBind Tube and keep it for the

next step.

! STOP POINT - If not immediately continuing to the next step, samples can be stored at -20°C.

HYBRIDIZATION & WASHING

Recommendation

We recommend that you perform a pilot test to determine if experimental conditions are suitable for hybridization.

* Prepare PCR plates or tubes suitable for your thermal cycler. After sealing the PCR plates or tubes with sealing tape or PCR caps, incubate 27 µL of nuclease-free water at 65°C for 2 hours (lid temperature is 105°C).
* Ensure that there is no extensive evaporation. If the evaporation volume does not exceed 3.4 µL, the hybridization can be performed with the materials you used in the pilot test.

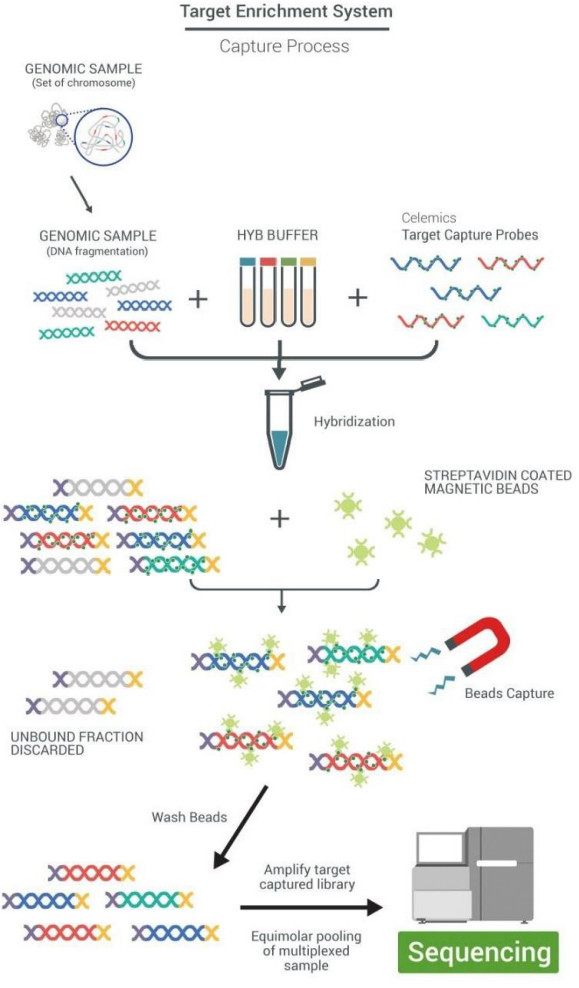

Figure 1. The Process of target captured enrichment system

STEP 1: Enhanced Hybridization of the Library

NOTE:

- Thaw the Target Capture Probe #1 on ice slowly.
- Do not vortex Target Capture Probe #1 (tapping or pipetting is allowed).
- Hyb Buffer should be pre-warmed at 37°C before use.
- Maintain the sample at 65°C during the whole hybridization process.

PREPARATION

Target Capture Solution Box #1	Block #1	Thawing on Ice Keep cool on ice (or cooling block)
	Block #2	
	Block #3 (D)	
	Target Capture Probe #2	
Target Capture Solution Box #2	Hyb Buffer	Pre-warmed at 37°C until the solution is clear
	Hyb Buffer Enhancer	Keep on room temperature
Target Capture Solution Box #3	Target Capture Probe #1	Thawing on Ice Keep cool on ice (or cooling block)

TARGET CAPTURE PROBE PREPARATION

For Whole Exome Sequencing kit, the target capture probe is supplied in powder form. Please check your reaction number and add the appropriate volume of nuclease-free water as described below

▶ Target Capture Probe; resuspension of the powdered probe

The powdered target capture probe must be dissolved with nuclease-free water before use. Add Nuclease-free water (7.2 µL per hybridization reaction) to Target Capture probe #1 (HWE) as shown below and mix well by pipetting (Do not use Vortex Mixer) to dissolve the reagent.

* Keep the eluted Target Capture probe #1 (HWE) on ice.
* Once you dissolve the powder target capture probe, store the remaining probe solution at -80°C after use.
* Each pre-capture pooled library mix will be treated as 1 Hyb. reaction.
    - For 12 Hyb. reaction kit
    - Add 86.4 µL of nuclease-free water into the tube.
    - For 24 Hyb. reaction kit (composed of 12 reactions x 2 tubes) Add 86.4 µL of nuclease-free water into each tube.

PROCEDURE

Hybridization needs three reagents: 1) Block Mix with DNA library, 2) Hybridization Buffer Mix, and 3) Capture Library Mix. The three reagents are sequentially incubated in the thermal cycler and mixed for a hybridization reaction.

1. Set the program into a thermal cycler (see the details in Table 19).
2. Preparation of Block Mix with DNA library.
    a. Take 10 µL of pre-capture pooled sample to a new 8-strip tube (or 1st column of 96- well PCR plate, see figure 2).
    b. Prepare Block Mix as shown in Table 16.

Table 16. Block Mix

2.5 µL	Block #1
2.5 µL	Block #2
0.6 µL	Block #3 (D)
5.6 µL	TOTAL

    c. Add 5.6 µL of Block Mix to each sample tube. (The total volume is 15.6 µL)
    d. Mix well, Seal, and Spin down the 8-strip PCR tube (96-well PCR plate).
    e. Keep the PCR tube (or plate) on ice before hybridization.
3. Preparation of Hybridization Buffer Mix.
    a. Confirm the Hyb Buffer had pre-warmed at 37°C before use. If any precipitates are remains, keep incubating at 37°C until the solution is clear.
    b. Prepare Hyb Buffer Mix as shown in Table 17 and load it into a new 8-strip PCR tube. Mix-well gently pipetting and seal the tube completely and spin down.
        * Do not place the Hyb Buffer tube on ice. Keep the tube at RT.
        * When using 96-well plate, do not put the Hybridization Buffer Mix on 96-well plate at this step.
        * When Hyb buffer and Hyb Buffer Enhancer are mixed, it turns into a cloudy white solution, however, this does not affect your final capture product.

Table 17. Hyb Buffer Mix

24 µL	Hyb Buffer
3 µL	Hyb Buffer Enhancer
27 µL	TOTAL

4. Preparation of Capture Library Mix.

    * Caution - Do not vortex Target Capture Probe #1 and Capture Library Mix

    a. Prepare Capture Library Mix as shown in Table 18. Mix well with gentle pipetting.

Table 18. Capture Library Mix

6 µL	Target Capture Probe #1 (HWE)
1 µL	Target Capture Probe #2
7 µL	TOTAL

    b. Put 7 µL of Capture Library Mix into a new 8-strip PCR tube and seal it completely. (Spin down if needed)
    c. Keep the Capture Library Mix tube on ice.

* When using 96-well plate, do not put the Capture Library Mix on 96-well plate at this step.

5. Perform hybridization reaction on a thermal cycler described in Table 19.
   a. Input the following program into a thermal cycler (lid temperature: 105°C).

Table 19. Hybridization PCR Program

5 min	95°C
hold	65°C

   b. Put the Block Mix with DNA library (from step 1) tube (or plate) in the thermal cycler and start the Hybridization PCR program in Table 19.

   ! IMPORTANT – Keep the tube on thermal cycler until 'step i'

   c. Once the temperature reaches 65°C, put the Hyb Buffer Mix (from step 2) tube into the thermal cycler and incubate at 65°C for 3 minutes.

*When using a 96-well PCR plate, transfer Hyb Buffer to an empty column (wells without Block Mix, see figure 2) and quickly seal the PCR plate.

   ! IMPORTANT – Keep the tube on thermal cycler until 'step i'

   d. After 3 minutes (incubation of Hyb Buffer at 65°C), put the Capture Library Mix (from step 3) PCR tube into the thermal cycler and incubate at 65°C for 2 minutes.

      * When using a 96-well PCR plate, transfer 7 µL Capture Library Mix to an empty column (wells without Block Mix and Hyb Buffer Mix, see figure 2) and completely seal the PCR plate quickly.

   e. After step 4-d, there should be three PCR tubes (or 3 lanes of reagents in a plate) in the thermal cycler.

IMPORTANT

Following processes must proceed very quickly and efficiently.

The high reaction temperature can cause the evaporation of reagents.

It will decrease the hybridization efficiency.

For reducing the reagents evaporation, please prepare

1. A new cap (or a new sealing film) and set the volume of a pipette (or a multi pipette) to 16 µL.
2. See Figure 2 before proceeding. When using the 96-well PCR plate, transfer reagents into the well (or lane) containing Capture Library Mix.

   f. Open the thermal cycler and remove the three PCR tube caps (or sealing film).

   g. Slowly pipette Hyb Buffer Mix up and down 2-3 times and transfer 18 µL into Capture Library Mix PCR tube (or PCR plate) and mix well by pipetting up and down 2-3 times (Figure 2. (1)).

   h. Transfer 15.6 µL (entire amount) of Block Mix with DNA library into Capture Library Mix PCR tube (or well) that contains reagents (Hyb Buffer and Capture Library Mix) and mix well by pipetting up and down 2-3 times (Figure (2)).

i. Quickly seal the PCR tube (or PCR plate) with a new cap (or a new sealing film) completely and incubate at 65°C as described in Table 20.

* In this step, remove empty PCR tubes from this step.

Table 20. The Reaction Temperature and Time by the Hybridization Method

Hybridization method	Temperature	Time
Standard	65°C	16 hours

j. Make sure that the PCR tube (or PCR plate) is completely sealed and the lid temperature is 105°C.

* The volume of the hybridization mixture will be 30 to 32 µL, depending on the degree of evaporation during incubation.

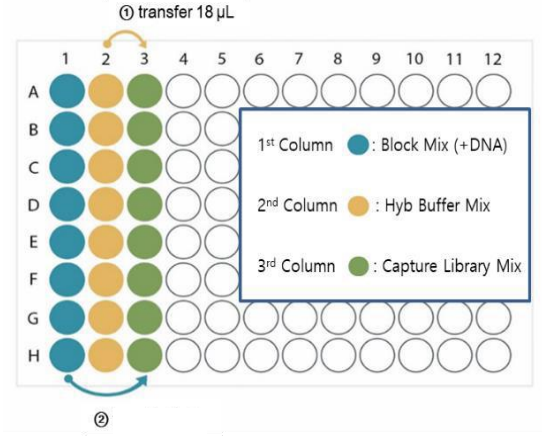

Figure 2. Mix all reagents in the 96-well PCR plate

STEP 2: Preparation of Streptavidin Bead

PREPARATION

Target Capture Solution Box #2	Wash Buffer #1	Keep on Room temperature
	Wash Buffer #3	Pre-heat at 70°C
	Streptavidin bead	Store at 4°C after use.

PROCEDURE

1. Pre-heat Wash Buffer #3 at 70°C in a heat block for the next step, Step 3. Selection of the Target Captured Library.
2. Vortex Streptavidin bead on a vortex mixer to achieve a homogeneous state.
3. Prepare a new 1.5 mL LoBind tube and add 50 µL of the Streptavidin bead to the tube.
4. Wash the Streptavidin bead as follow:
    a. Add 200 µL of Wash Buffer #1 to the tube containing 50 µL of the streptavidin bead.
    b. Mix well on the vortex mixer and spin down briefly.
    c. Put the tube in the magnetic separator until the solution is clear.
    d. Keep the tube in the magnetic separator and discard the supernatant (be careful not to touch the bead while you remove the supernatant).
    e. Repeat steps a - d two more times (total 3 times).
    f. Add 200 µL of Wash Buffer #1 to the bead.

! IMPORTANT - Proceed with the next step right away.

STEP 3: Selection of the Target Captured Library

PREPARATION

Target Capture Solution Box #2	Wash Buffer #2	Keep on Room temperature
	Wash Buffer #3	Pre-heat at 70°C

PROCEDURE

1. Keep the sample-hybridization mixture from Step 1. Enhanced Hybridization of the Library in a thermal cycler at 65°C.
2. Gently unseal the PCR tube (or PCR plate) in the thermal cycler and immediately transfer the sample-hybridization mixture to the bead solution (from Step 2. Preparation of Streptavidin Bead).
3. Invert the tube 3-5 times to mix them thoroughly and rotate the sample with a rotator (25 rpm) for 30 minutes at room temperature.

IMPORTANT - Do not vortex the tube vigorously, as it may damage the Target Capture Probe.

4. Spin down briefly.
5. Put the tube in a magnetic separator until the solution is clear. Carefully remove the supernatant.
6. Take the tube out of the magnetic separator and add 500 µL (180 µL for PCR tube or 96- well plate) of Wash Buffer #2 to each tube. Mix well on a vortex mixer and pulse-spin down.
7. Incubate the sample for 15 minutes at room temperature.
8. Briefly mix the sample on the vortex mixer and pulse-spin down. Put the tube in the magnetic separator until the solution is clear and remove the supernatant.
9. Rinse the bead with Wash Buffer #3 (pre-heated from 2.2 Preparation of Magnetic Bead).
    a. Add 500 µL (180 µL for PCR tube or 96-well plate) of pre-heated Wash Buffer #3 to each tube and mix well on a vortex mixer. Spin down briefly.
    b. Incubate the solution for 10 minutes at 70°C in a heat block.
    c. Briefly mix the sample on the vortex mixer and pulse-spin down.
    d. Put the tube in the magnetic separator until the solution is clear. Carefully remove the supernatant.
    e. Repeat steps a-d twice with Wash Buffer #3.

! IMPORTANT – Rinse the bead with Wash Buffer #3 3 times.

    f. Spin down the tube for 3-5 seconds and put the tube in the magnetic separator to remove residue.

! IMPORTANT - Wash Buffer #3 must be completely removed.

    g. Add 30 µL of nuclease-free water to each tube and mix well on a vortex mixer. Spin down briefly.
    h. On-Bead Capture Pool is prepared.

! STOP POINT - If not immediately continuing to the next step, samples can be stored at -20°C.

AMPLIFICATION OF TARGET CAPTURED LIBRARY

STEP 1: Amplification of the Captured Library

PREPARATION

Polymerase	Polymerase	Keep cool on ice (or cooling block)
Target Capture Solution Box #1	Post Capture PCR Forward Primer Post Capture PCR Reverse Primer	Thawing on ice Keep cool on ice (or cooling block)

PROCEDURE

1. Set the program into a thermal cycler (see the details in Table 22).
2. Prepare new 1.5 mL tube and make PCR Master Mix (Polymerase, Nuclease-free water, Post Capture PCR Forward Primer, Post Capture PCR Reverse Primer) as described in Table 21 (add 5% of the additional amount of reagents for multiple libraries).

Table 21. Composition of PCR Master Mix

25 µL	Polymerase
5 µL	Nuclease-free water
2.5 µL	Post Capture PCR Forward Primer
2.5 µL	Post Capture PCR Reverse Primer
35 µL	TOTAL

3. Add 35 µL of the PCR master mix to each PCR tube.

! IMPORTANT – Combine all reagents on ice.

4. Thoroughly mix the On-Bead Capture Pool to a homogeneous state and add 15 µL of the On-Bead Capture Pool to the PCR tube.

Table 22. Composition of PCR Mix

35 µL	PCR Master Mix
15 µL	On-Bead Capture Pool
50 µL	TOTAL

5. Mix well and spin down briefly.
6. Run the thermal cycler with the PCR program shown in Table 23. (see the number of PCR cycles in Table 24). Lid temperature: 105°C.

Table 23. PCR program

45 sec	98°C
15 sec	98°C

30 sec	60°C
1 min	72°C
Repeat step 2 to step 4 (Total PCR cycles, See in Table 24)	
10 min	72°C
hold	4°C

Table 24. The Number of PCR Cycles

Target Size	Total Cycles
Target Size ≥ 1 Mb	14

! IMPORTANT - Proceed with the next step right away.

STEP 2: Purification of Sample Using Clean-up Bead

PREPARATION

Clean-up Bead	Keep the bead at room temperature for at least 30 minutes before use. Store the bead at 4°C after use
80% ethanol solution	Using freshly made 80% ethanol solution is preferable

PROCEDURE

1. Vortex Clean-up Bead to achieve a homogeneous state.
2. Add 90 µL Clean-up Bead to a new 1.5 ml LoBind Tube and add 50 µL of amplified captured DNA library to the tube. Mix well with a pipette and pulse-spin down the tube. Incubate at room temperature for 5 minutes

! IMPORTANT – Avoid spinning down the bead too strongly to prevent precipitation.

Table 25. Volumes of Clean-up Bead (1.8x)

Reagent	Volume Ratio (Bead : Sample)
Clean-up Bead	1.8 : 1 (e.g., 90 µL : 50 µL)

3. Put the tube in the magnetic separator for 3-5 minutes until the solution is clear.
4. Keep the tube in the magnetic separator and discard the supernatant

! IMPORTANT – During Steps 4 to 6, be careful not to touch the bead while you remove the supernatant.

5. Keeping the tube in the magnetic separator, add 500 µL of fresh 80% ethanol to each tube.
6. Incubate for 30 seconds at room temperature, then discard the ethanol.
7. Repeat wash steps 5-6 once.
8. Spin down, put the tube in the magnetic separator and discard residual ethanol.
9. Dry the sample tube at room temperature for 2 minutes or until residual ethanol has completely evaporated.

! IMPORTANT – Excessive drying of the bead pellets until cracks occur causes a decrease in elution efficiency. Proceed with step 10 before the crack appears.

10. Add 32 µL of nuclease-free water to the sample and thoroughly mix with a pipette. Pulse-spin down and incubate the sample at room temperature for 2 minutes.
11. Put the tube in the magnetic separator for 2 minutes until the solution is clear.
12. Transfer the supernatant (30 µL) to a new 1.5 mL LoBind Tube and keep the supernatant for the next step.

! STOP POINT - If not immediately continuing to the next step, samples can be stored at -20°C.

STEP 3: Assess Quality and Quantity of Each Library

Check the quality and quantity of the captured library with available instruments.

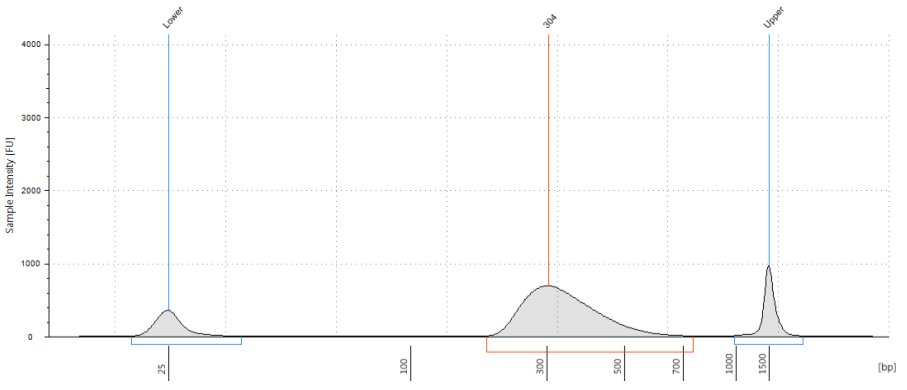

Figure 3. The example electrophoresis diagram of the captured library.

Make sure that the size of the amplified captured DNA library is 120-150 bp larger than the initial fragmented DNA.

SEQUENCING LIBRARY POOLING (OPTIONAL)

In this step, all target captured pre-pooled samples are pooled in one tube. The index of each sample must not overlap each other.

Consider target size, the quantity of data needed, and the concentration of every sample.

1. Create the spreadsheet as shown below in Table 26.
2. Enter the values of each sample in columns 1, 2, 3, 4, 5, and 6 of the spreadsheet.
3. Calculate Column 7 by the below calculation. (Column 8 (Gb)*(30 nmol))/(Column 5)

   *30 nmol is an example value for this manual. You can change this value depending on your sample's concentration.
4. Mix each sample with calculated volumes
5. After pooling, perform sequencing according to the running protocol of the sequencing equipment.

Table 26. Example of a Spreadsheet for Sample Pooling

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8
Sample name	Containing samples	i5 Index	i7 Index	Conc (nmol)	Volume of Sample (µL)	Vol. for Pooling (µL)	Needed Data (Gb)
Pre-capture pool 1	Pool1-A	501	701	91	30	6.59	20
	Pool1-B	502	702				
	Pool1-C	503	703				
	Pool1-D	504	704				
Pre-capture pool 2	Pool2-E	501	705	45.3	30	13.25	20
	Pool2-F	502	706				
	Pool2-G	503	707				
	Pool2-H	504	708				
Pre-capture pool 3	Pool3-I	505	701	77.2	30	7.77	5
	Pool3-J	506	702				
	Pool3-K	507	703				
	Pool3-L	508	704				
Pre-capture pool 4	Pool4-M	505	705	61.8	30	9.71	5
	Pool4-N	506	706				
	Pool4-O	507	707				
	Pool4-P	508	708				

* The particular example in Table 26 indicates that the volume for pooling (Column 7) is calculated by dividing 30 nmol by each Pre-capture pool concentration (Column 5). However, 30 nmol is an example and the amount of nmol can be changed according to the user's convenience.

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