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Department of Genomic Medicine

Cancer Immune Monitoring and Analysis Center

Division of Cancer Medicine

The University of Texas MD Anderson Cancer Center

THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
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SOP Agilent SureSelect$^{XT\ HS}$ Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library Protocol

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A. SCOPE

The scope of this SOP is to cover the automated sample processing using the Agilent NGS Workstation with the SureSelect^{XT} Low Input target enrichment workflow for samples to be sequenced using the Illumina paired-read sequencing platform. The procedure will cover both Whole Exome Sequencing and Targeted Sequencing library preparation.

B. SAFETY PRECAUTIONS

Wear appropriate personal protective equipment (PPE) when working in the laboratory. Follow signs labeled on the instrument to prevent physical injury.

C. SURESELECT TARGET ENRICHMENT OVERVIEW

Prior to sequencing, individual library preparations, hybridizations, and captures are performed. Depending on the target size of the SureSelect capture, multiple samples can be pooled and sequenced in a single lane using the 8-bp SureSelect^{XT} Low Input multiplex indexes. The SureSelect^{XT} Low Input Library preparation is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower quality DNA prepared from FFPE samples, using a DNA input range to 10 to 200 ng DNA. The figure below represents the sample preparation workflow for SureSelect^{XT} Low Input NGS Target Enrichment.

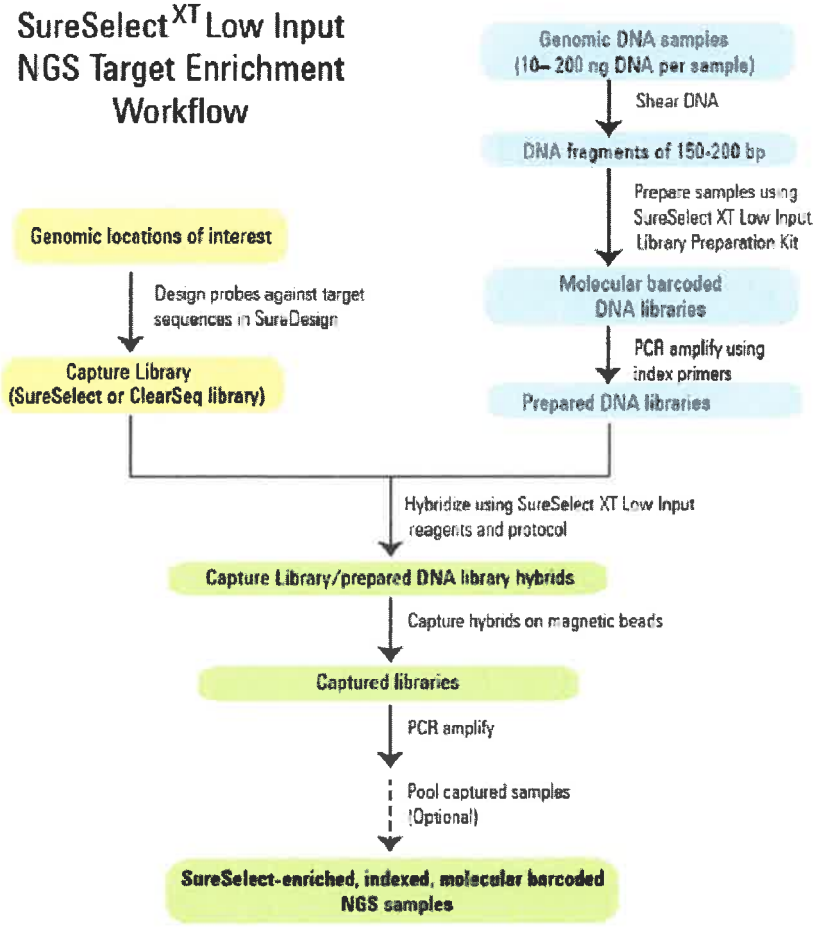


Figure 2 Overall sequencing sample preparation workflow.

D. EQUIPMENT

- Agilent NGS Workstation Option B with VWorks software version 11.3.0.1195
- Thermal cycler and accessories
- P2, P10, P20, P200 & P1000 single channel pipettes
- P10, P100, & P200 multichannel pipette
- Vortex mixer
- Qubit fluorimeter
- Covaris model E220
- Microcentrifuge
- Plate or strip centrifuge
- 96-well plate mixer
- Agilent 4200 TapeStation
- Applied Biosystems Quant Studio 6
- Agilent PlateLoc Thermal Microplate Sealer
- Vacuum concentrator

- -80°C freezer, -20°C freezer, and 4°C cooler
- Magnetic separator

- Ice Bucket

E. MATERIALS

- Robotic Pipetting Tips (Sterile, Filtered, 250µL)
- Applied Biosystems MicroAmp Optical 96-well Reaction Plate
- Applied Biosystems MicroAmp EnduraPlate Optical 96-Well Fast Clear Reaction Plate with Barcode
- Tube cap strips, domed
- Eppendorf twin.tec full-skirted 96-well PCR plates
- Thermo Scientific Reservoirs
- Nunc DeepWell Plates, sterile, 1.3-mL well volume
- Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)
- Qubit Assay Tubes
- Eppendorf LoBind Tubes, 1.5-ml and 0.5-ml PCR clean
- P2, P10, P20, P200 & P1000 sterile, filtered tips
- Eppendorf P10 & P100 dual filtered, PCR clean and sterile tips
- Covaris 96 microTube Plate (P/N: 520078)
- 96-well plate foil seals
- 8-well tube strips and caps
- PPE: gloves, protective eyewear, and lab coat

F. REAGENTS

- SureSelect^{XT} Low Input Reagent + Capture Library Kit for Illumina (ILM) platforms, 96 reactions with Index Primers 1-96 or 96 reactions with Index Primers 97-192
- Agencourt AMPure XP Kit, 450 ml
- Dynabeads MyOne Streptavidin T1, 100 ml
- D1000 ScreenTape, Reagents, and Sample Buffer
- High Sensitivity D1000 ScreenTape, Reagents, and Sample Buffer
- KAPA Library Quantification Kit
- 1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- 100% Ethanol (Ethyl Alcohol, 200 proof)
- Nuclease-free water (not DEPC-treated)

G. PROCEDURAL NOTES

- DNA will be prepared using suitable purification system depending on sample source (fresh or FFPE).
- DNA integrity of FFPE DNA will be assessed using Agilent Genomic DNA ScreenTape assay DIN score. The DIN score will be used to determine input modifications.

Table 12 SureSelect XT Low Input DNA input modifications based on DNA Integrity Number (DIN) score

Protocol Parameter	non-FFPE Samples	FFPE Samples		
		DIN > 8*	DIN 3–8	DIN<3
DNA input for Library Preparation	10 ng to 200 ng DNA, quantified by Qubit Assay	10 ng to 200 ng DNA, quantified by Qubit Assay	Use at least 15 ng for more intact samples and at least 40 ng for less intact samples. Use the maximum amount of DNA available, up to 200 ng, for all samples. Quantify by Qubit Assay.	Use at least 50 ng for more intact samples and at least 100 ng for the least intact samples. Use the maximum amount of DNA available, up to 200 ng, for all samples. Quantify by Qubit Assay.

* FFPE samples with DIN>8 should be treated like non-FFPE samples for DNA input amount determinations.

H. SHEARING

Shearing steps depend on quality of DNA. High quality and FFPE DNA have different shearing requirements.

1. Determine input amount of DNA. Each sample should contain 10-200 ng gDNA.
2. Transfer DNA to wells in Covaris microTube 96 well plate.
3. Bring volume of DNA up to 50 ul with 1X Low TE Buffer.
4. Shear DNA using Covaris E-series instrument with SonoLab software v7 or later software using these settings:

Setting	High-quality DNA	FFPE DNA
Duty Factor	10%	10%
Peak Incident Power (PIP)	175	175
Cycles per Burst	200	200
Treatment Time	2 × 120 seconds	240 seconds
Bath Temperature	2° to 8° C	2° to 8° C

Use the steps below for two-round shearing of high-quality DNA samples only:

- Shear for 120 seconds
- Spin the microTUBE for 10 seconds
- Vortex the microTUBE at high speed for 5 seconds
- Spin the microTUBE for 10 seconds
- Shear for additional 120 seconds
- Spin the microTUBE for 10 seconds
- Vortex the microTUBE at high speed for 5 seconds
- Spin the microTUBE for 10 seconds

5. Once shearing complete, transfer 50 ul of sheared gDNA to an Eppendorf twin.tec 96-well plate.
6. Run sheared gDNA using High Sensitivity D1000 DNA Analysis kit. Target DNA fragment size is 150 to 200 bp.
7. Create input plate for library sample preparation. Input amount depends on quality of DNA. Transfer required amount to a new Eppendorf twin.tec plate and bring volume up to 50 ul using nuclease free water.

I. SAMPLE PREPARATION

1. Prepare Agilent work station according to SureSelectXT_LI_ILM_vB1.0.1.VWForm. For Sample Preparation steps, use LibraryPrep_XT_LI_ILM_v.B1.0.1.rst runset.
2. Prepare appropriate volume of End Repair/dA-Tailing master mix using volumes listed on table below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
End Repair-A Tailing Buffer (bottle)	16 µl	204 µl	340 µl	476 µl	612 µl	884 µl	1768 µl
End Repair-A Tailing Enzyme Mix (orange cap)	4 µl	51 µl	85µl	119 µl	153 µl	221 µl	442 µl
Total Volume	**20 µl**	**255 µl**	**425 µl**	**595 µl**	**765 µl**	**1105 µl**	**2210 µl**

3. Prepare appropriate volume of Ligation master mix using volumes listed below:

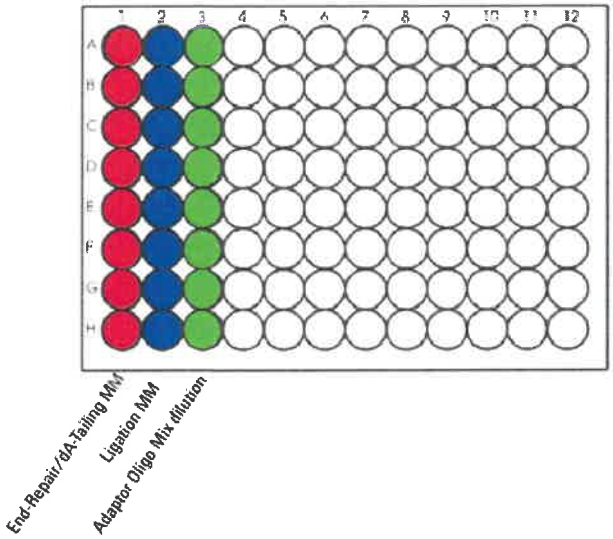
Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Ligation Buffer (bottle)	23 µl	293.3 µl	488.8 µl	684.3 µl	879.8 µl	1270.8 µl	2541.5 µl
T4 DNA Ligase (blue cap)	2 µl	25.5 µl	42.5 µl	59.5 µl	76.5 µl	110.5 µl	221.0 µl
Total Volume	**25 µl**	**318.8 µl**	**531.3 µl**	**743.8 µl**	**956.3 µl**	**1381.3 µl**	**2762.5 µl**

4. Prepare appropriate volume Adaptor Oligo Mix dilution according to volumes listed below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µl	42.5 µl	63.8 µl	85.0 µl	106.3 µl	143.5 µl	266.5 µl
Adaptor Oligo Mix (white cap)	5 µl	85.0 µl	127.5 µl	170.0 µl	212.5 µl	2287.5 µl	533.0 µl
Total Volume	**7.5 µl**	**127.5 µl**	**191.3 µl**	**255.0 µl**	**318.8 µl**	**430.5 µl**	**799.5 µl**

5. Prepare master mix source plate using Nunc DeepWell plate according to table below:

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
End Repair-dA Tailing master mix	Column 1 (A1-H1)	31.0 µl	52.0 µl	73.0 µl	94.0 µl	136.5 µl	273.0 µl
Ligation master mix	Column 2 (A2-H2)	36.0 µl	62.0 µl	88.0 µl	114.0 µl	169.0 µl	338.0 µl
Adaptor Oligo Mix dilution	Column 3 (A3-H3)	15.0 µl	22.5 µl	30.0 µl	37.5 µl	52.5 µl	97.5 µl



6. Prepare purification reagents by adding 140ul of homogenous AMPure XP beads per well into a separate Nunc DeepWell plate.
7. Prepare a reservoir containing 15 ml of nuclease free water and a separate reservoir containing 45 ml of freshly prepared 70% ethanol.
8. Set-up prepared plates according to set up on LibraryPrep_XT_LI_ILM_v.B1.0.1.rst runset.
9. Press start when set-up complete and allow LibraryPrep runset to complete.
10. Once LibraryPrep runset complete, prepare workstation for amplification of adaptor-ligated libraries according to Pre-CapPCR_XT_LI_ILM_v.B1.0.1.pro.
11. Pre-program thermal cycler with PCR steps below. Refer to the next table for Pre-Capture PCR cycle number recommendations:

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see Table 24)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 50 µL.

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	100 to 200 ng	8 cycles
	50 ng	9 cycles
	10 ng	11 cycles
FFPE sample DNA	100 to 200 ng*	11 cycles
	50 ng	12 cycles
	10 ng	14 cycles

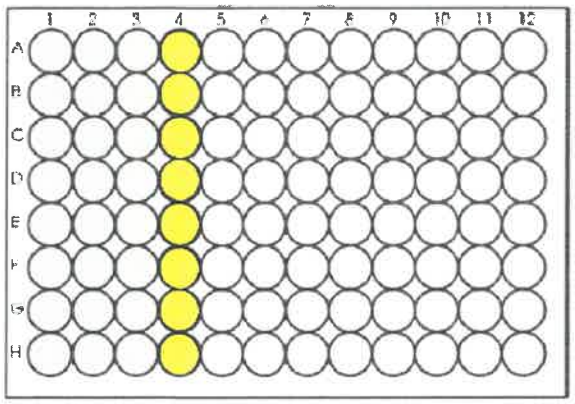
* qPCR-determined quantity of amplifiable DNA or DIN value-adjusted amount of input DNA

12. Prepare appropriate volume of pre-capture PCR mix according to table below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	6.5 µl	82.9 µl	138.1 µl	193.4 µl	248.6 µl	359.1 µl	718.3µl
5× Herculase II Reaction Buffer (clear cap)	10 µl	127.5 µl	212.5 µl	297.5 µl	382.5 µl	552.5 µl	1105 µl
100 mM dNTP Mix (green cap)	0.5 µl	6.4 µl	10.6 µl	14.9 µl	19.1 µl	27.6 µl	55.3 µl
Forward Primer (brown cap)	2 µl	25.5 µl	42.5 µl	59.5 µl	76.5 µl	110.5 µl	221.0 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	12.8 µl	21.3 µl	29.8 µl	38.3 µl	55.3 µl	110.5 µl
Total Volume	**20 µl**	**255.1 µl**	**425.0 µl**	**595.1 µl**	**765 µl**	**1105 µl**	**2210 µl**

13. Dispense pre-capture PCR mix to same Nunc DeepWell master mix source plate that was used for the LibraryPrep runset. Use volumes below:

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Pre-Capture PCR Master Mix	Column 4 (A4-H4)	31.0 µl	52.0 µl	73.0 µl	94.0 µl	136.5 µl	273.0 µl



14. Prepare the pre-capture PCR indexing primer plate. Use the appropriate SureSelect XT Low Input Index primers. In each well of a ½ PCR plate, combine 2 ul of the specific indexing primer (1-96 or 97-192) to the assigned sample well with 3 ul of nuclease free water.
15. Set-up prepared plates according to set up on Pre-CapPCR_XT_LI_ILM_v.B1.0.1.pro.
16. Press start when set-up complete and allow Pre-CapPCR runset to finish.
17. Once Pre-CapPCR runset is complete, transfer PCR plate to thermal cycler with appropriate program.
18. Set up workstation according to AMPureXP_XT_LI_ILM_v.B1.0.1.pro:Pre-Capture PCR. Use AMPure beads left over from LibraryPrep runset.
19. Once Pre-PCR thermal cycling program complete, transfer PCR plate to assigned position on workstation.
20. Press start to begin AMPureXP_XT_LI_ILM_v.B1.0.1.pro:Pre-Capture PCR runset.
21. Once runset complete, assess the quantity and quality of DNA. Use Agilent 4200 TapeStation instrument and the Agilent D1000 DNA Analysis kit.
22. Store pre-captured libraries in 4°C overnight or -20°C for long term storage. Otherwise proceed to hybridization steps.

J. HYBRIDIZATION

1. Determine input amount of DNA for hybridization reaction. Each reaction requires 500-1000 ng of prepared DNA in a volume of 12 ul. To calculate input divide required input (500-1000ng) by the concentration of DNA determined by D1000 DNA analysis.
2. Aliquot input DNA to Eppendorf twin.tec plate.
3. Use vacuum concentrator to dry samples at ≤ 45°C. Reconstitute each dried sample with 12 ul of nuclease free water.
4. Set up workstation according to Hyb_XT_LI_ILM_v.B1.0.1.pro runset.

5. Program thermal cycler with hybridization cycling steps:

Segment Number	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	10 minutes
3	Hold for NGS workstation steps†	1	65°C	Hold
4	Hybridization	60	65°C‡	1 minute
			37°C	3 seconds
5	Hold until start of Capture**	1	65°C†	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 30 µl (final volume of hybridization reactions during cycling in Segment 4).

† Samples are transferred to the NGS Workstation during this Hold step when prompted by the VWorks software.

‡ Reducing the hybridization temperature to 60°C (Segments 4 and 5) may improve coverage for AT-rich regions of some libraries.

** Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on page 95.

6. Prepare the appropriate volume of Block master mix on ice according to table below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 8 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µl	31.9 µl	53.1 µl	74.4 µl	95.6 µl	138.1 µl	276.3 µl
SureSelect XT HS and XT Low Input Blocker Mix (blue cap)	5.0 µl	63.8 µl	106.3 µl	148.8 µl	191.3 µl	276.3 µl	552.5 µl
Total Volume	**7.5 µl**	**95.6 µl**	**159.4 µl**	**223.1 µl**	**286.9 µl**	**414.4 µl**	**828.8 µl**

7. Prepare the appropriate volume of Hybridization Buffer master mix at room temperature according to the table below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 8 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µl	53.1 µl	74.4 µl	95.6 µl	116.9 µl	159.4 µl	297.5 µl
SureSelect Fast Hybridization Buffer (bottle)	6.0 µl	127.5 µl	178.5 µl	229.5 µl	280.5 µl	382.5 µl	714.0 µl
Total Volume	**8.5 µl**	**180.6 µl**	**252.9 µl**	**325.1 µl**	**397.4 µl**	**541.9 µl**	**1011.5 µl**

8. Prepare the appropriate volume of Capture Library Master Mix. Volumes will depend on the target size of the capture library bait:

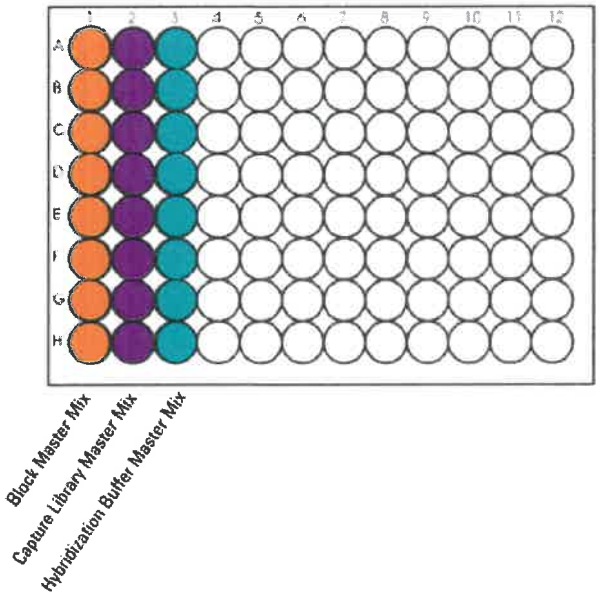
Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µl	76.5 µl	114.8 µl	153.0 µl	191.3 µl	306.0 µl	592.9 µl
RNase Block (purple cap)	0.5 µl	8.5 µl	12.8 µl	17.0 µl	21.3 µl	34.0 µl	65.9 µl
Capture Library	2.0 µl	34.0 µl	51.0 µl	68.0 µl	85.0 µl	136.0 µl	263.6 µl
Total Volume	**7.0 µl**	**119.0 µl**	**178.6 µl**	**238.0 µl**	**297.6 µl**	**476.0 µl**	**922.3 µl**

Table 44 Preparation of Capture Library Master Mix for Capture Libraries ≥3 Mb, 8 rows of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µl	25.5 µl	38.3 µl	51.0 µl	63.8 µl	102.0 µl	197.6 µl
RNase Block (purple cap)	0.5 µl	8.5 µl	12.8 µl	17.0 µl	21.3 µl	34.0 µl	65.9 µl
Capture Library	5.0 µl	85.0 µl	127.5 µl	170.0 µl	212.5 µl	340.0 µl	658.8 µl
Total Volume	**7.0 µl**	**119.0 µl**	**178.6 µl**	**238.0 µl**	**297.6 µl**	**476.0 µl**	**922.3 µl**

9. Prepare the master mix source plate using an Eppendorf twin.tec plate at room temperature. Refer to tables below for appropriate volumes and plate configuration.

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Eppendorf twin.tec Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Block master mix	Column 1 (A1-H1)	11.0 µl	19.0 µl	27.0 µl	34.9 µl	50.9 µl	102.7 µl
Capture Library master mix	Column 2 (A2-H2)	14.0 µl	21.4 µl	28.9 µl	36.3 µl	58.6 µl	114.4 µl
Hybridization Buffer master mix	Column 3 (A3-H3)	19.9 µl	29.0 µl	38.0 µl	47.0 µl	65.1 µl	123.8 µl



10. Load the Bravo deck with prepared plates according to the Hyb_XT_LI_ILM_v.B1.0.1.pro.

11. Begin the runset. Once liquid handling steps complete, place PCR plate in thermal cycler once prompted by the software.
12. The Bravo will complete aliquoting the Capture Library and Hybridization Buffer master mixes. When liquid handling complete, another prompt will appear. Remove PCR plate from thermal cycler and place back on Bravo deck.

13. Allow runset to complete liquid handling steps. Once complete, another prompt will appear. Place PCR plate back on thermal cycler as instructed by prompt and allow PCR hybridization cycling to complete.
14. While DNA is in hybridization, prepare workstation for capture and wash steps. Use SSELCapture&Wash_XT_LI_ILM_v.B1.0.1.rst runset to set up workstation.
15. Prepare the Dynabeads streptavidin beads by combining the components listed in the table below. The beads must be washed by vortexing the mixture and allowing the liquid to separate from the beads with a magnetic separator and discarding the supernatant. These steps must be repeated for a total of 3 washes.

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Dynabeads MyOne Streptavidin T1 bead suspension	50 µl	425 µl	825 µl	1225 µl	1.65 ml	2.5 ml	5.0 ml
SureSelect Binding Buffer	0.2 ml	1.7 ml	3.3 ml	4.9 ml	6.6 ml	10 ml	20 ml
Total Volume	**0.25 ml**	**2.125 ml**	**4.125 ml**	**6.125 ml**	**8.25 ml**	**12.5 ml**	**25 ml**

16. Once washes complete, re-suspend the washed Dynabeads with the appropriate volume of Binding Buffer. Refer to table below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Binding Buffer	0.2 ml	1.7 ml	3.3 ml	4.9 ml	6.6 ml	10 ml	20 ml

17. Aliquot 200 ul of washed Dynabeads for each well processed to Nunc DeepWell source plate.
18. Prepare an Eppendorf twin.tec plate labeled Wash #1. Aliquot 160 ul of SureSelect Wash Buffer 1 for each well to be processed.
19. Prepare a Nunc DeepWell source plate labeled Wash #2. Aliquot 1150 ul of SureSelect Wash Buffer 2 for each well to be processed.
20. Load prepared plates according to SSELCapture&Wash_XT_LI_ILM_v.B1.0.1.rst runset setup.
21. Determine if PCR hybridization cycling steps complete before starting Capture and Wash runset. When runset started, a prompt will appear to retrieve plate from thermal cycler and place on assigned position on deck.
22. Allow SSELCapture&Wash_XT_LI_ILM_v.B1.0.1.rst runset to complete. Store completed plate on ice and proceed to Post-Capture PCR steps.

K. POST-CAPTURE SAMPLE PROCESSING

1. Prepare workstation according to Post-CapPCR_XT_LI_ILM_v.B1.0.1.pro runset.
2. Pre-program thermal cycler with Post-Capture PCR thermal cycler program. Number of cycles depends on capture library target size.

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	9 to 14 See Table 58 for recommendations based on Capture Library size	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Capture Library Size/Description	Cycles
Libraries <0.2 Mb	14 cycles
Libraries 0.2–3 Mb (includes SSel XT HS and XT Low Input ClearSeq Comp Cancer)	12 cycles
Libraries 3–5 Mb	10 cycles
Libraries >5 Mb (includes SSel XT HS and XT Low Input Human All Exon V6 and Clinical Research Exome V2 libraries)	9 cycles

3. Prepare the appropriate volume of post-capture PCR Master Mix according to volumes on table below:

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	12.5 µl	159.4 µl	265.6 µl	371.9 µl	478.1 µl	690.6 µl	1328.1 µl
5× Herculase II Reaction Buffer (clear cap)	10 µl	127.5 µl	212.5 µl	297.5 µl	382.5 µl	552.5 µl	1062.5 µl
100 mM dNTP Mix (green cap)	0.5 µl	6.4 µl	10.6 µl	14.9 µl	19.1 µl	27.6 µl	53.1 µl
SureSelect Post-Capture Primer Mix (clear cap)	1 µl	12.8 µl	21.3 µl	29.8 µl	38.3 µl	55.3 µl	106.3 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	12.8 µl	21.3 µl	29.8 µl	38.3 µl	55.3 µl	106.3 µl
Total Volume	**25 µl**	**318.8 µl**	**531.3 µl**	**743.8 µl**	**956.3 µl**	**1381.3 µl**	**2656.3 µl**

4. Prepare the master mix source plate by adding the appropriate volume to all wells of column one of a Nunc DeepWell plate.

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
PCR Master Mix	Column 1 (A1-H1)	36.0 µl	62.0 µl	88.0 µl	114.0 µl	166.0 µl	322.0 µl

5. Load the Bravo deck with prepared plates according to the Post-CapPCR_XT_LI_ILM_v.B1.0.1.pro runset.
6. Allow runset to start. Place PCR plate in thermal cycler once prompted by software.
7. During Post-Capture PCR thermal cycling, prepare the workstation according to AMPureXP_XT_LI_ILM_v.B1.0.1.pro:Post-Capture PCR.
8. Prepare a Nunc DeepWell source plate with 55 ul of homogenous AMPure XP beads per well.
9. Prepare a reservoir containing 15 mL of nuclease-free water and a separate reservoir with 45 mL of freshly prepared 70% ethanol.
10. Load the prepared plates according to the Post-CapPCR_XT_LI_ILM_v.B1.0.1.pro runset.
11. Once Post-PCR thermal cycling program complete, transfer PCR plate to assigned position on workstation.
12. Press start to begin AMPureXP_XT_LI_ILM_v.B1.0.1.pro:Post-Capture PCR runset.

13. Once runset complete, assess the quantity and quality of DNA. Use Agilent 4200 TapeStation instrument and the Agilent High Sensitivity 1000 DNA Analysis kit.

14. Use concentration and region molarity from High Sensitivity D1000 analysis to normalize samples for qPCR quantification.
15. Quantify libraries by preparing samples with KAPA Biosystems library quantification kit and running qPCR on Applied Biosystems Quant Studio 6.
16. Combine the libraries such that each index-tagged sample is present in equimolar amounts using this equation:

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where $V(f)$ is the final desired volume of the pool,

$C(f)$ is the desired final concentration of all the DNA in the pool (typically 4 nM–15 nM or the concentration of the most dilute sample)

is the number of indexes, and

$C(i)$ is the initial concentration of each indexed sample

17. Once libraries are combined into a final pool, assess the quality and quantity of the pool using the Agilent 4200 TapeStation and the Agilent High Sensitivity D1000 DNA Analysis kit.
18. Quantify the final pool using the KAPA Biosystems library quantification kit and running qPCR on Applied Biosystems Quant Studio 6 to determine size adjusted concentration.

Genomics Laboratory

Curtis Gumbs, Scientific Manager, Genomic Medicine

[Signatures] and [dates]

4/18/18