SOP Title:

FFPE Agilent SureSelect XT + KAPA Hyper Prep Human Exome Library Prep

1.0 Purpose and scope

This standard operating procedure (SOP) is for the preparation of Agilent SureSelect XT exome sequencing libraries from FFPE at PM-OICR TGL for use on Illumina sequencers.

The modified Agilent protocol uses KAPA Hyper Prep kits for the preparation of pre- and post- capture libraries, employing Agilent SureSelect^{XT} reagents for the hybridization and exome capture steps. The procedure requires 100ng of FFPE DNA as input material and can be used with the following capture libraries:

- SureSelect^{XT} Human All Exon v6 + COSMIC (64Mb)
- SureSelect^{XT} Human All Exon v5 + UTRs (74.6Mb)

Related documents:

- Manufacturer-supplied protocol for library prep and post-capture amplification: *KAPA Hyper Prep Kit for SureSelect^{XT} Target Enrichment* (KR1320 – v3.17), link to document
- Manufacturer-supplied protocol for hybridization and capture: *Agilent SureSelect^{XT} Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library* (Version C0, December 2016), link to document

Related TGL documents:

- YYYY_MM_DD_SAMPLE SUBMISSION FORM_PI_Lastname_Firstname_TGL.xls
- SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc
- 16_05_11_Kapa_Hyperprep_lot_tracking_sheet.xls
- 17_07_07_Agilent_Sureselect_XT_Library_kit_lot_control.doc
- SOP_TapeStation_4200_V1.0
- SOP_KAPA_Library_Quantification_Illumina_Platforms_V1.0.2_production

Refer to section *5.0 Appendix: design notes* for information regarding the modifications and adjustments with respect to the manufacturer-supplied protocols that were incorporated in this SOP.

2.0 Materials

Workspace		
	Library prep room for all the steps prior pre-capture PCR amplification	
	Post-PCR area for the pre-capture PCR amplification and all the subsequent steps	
Equipment and associated consumables		
Covaris (DMARK)	Covaris M220	
	Microtube-50 AFAF Fiber screw cap (25 or 250 tubes)	PN520166 or PN520167(case of 250)
Agilent	Agilent TapeStation 4200	
	Genomic DNA Screen Tape (7 tapes,112 samples)	5067-5365
	Genomic DNA reagents	5067-5366
	High Sensitivity D1000 Screen Tape (7 tapes,112 samples)	5067-5584
	High Sensitivity D1000 Reagents	5067-5585
Eppendorf	Vacufuge Plus (Speedvac)	022820001
Various	Thermal cycler (i.e. Applied Biosystems Veriti, BioRad T100, etc)	
Medstore	PCR STRIP(8), 0.2ml NEUTRAL, ATT FLAT CAP,120/pk	72.991.002
ThermoFisher	Dynamag (magnetic rack)	12321D
	Dynamag-96 (96 well magnetic rack)	123331D
Reagent kits		
Roche	KAPA Hyper Prep kit (96 rxns)	7962363001 (KK8504)
	KAPA HiFi HS RM (6.25 mls, for post-capture PCR)	7958935001 (KK2602)
Agilent	SureSelect Library Prep Kit	
	SureSelect Target Enrichment, Box 1	
	SureSelect Target Enrich. Kit, ILM Ind. Hyb Module Box 2	
	SureSelect XT Reagent Kit for 96 samples	G9611B
	SureSelect XTHuman All Exon V5 +UTR (96rxns) or SureSelect XT Human All Exon V6+ COSMIC (96 rxns)	5190-6213 5190-9308
Beckman Coulter/Cedarlane	AMPure XP beads	A36881 (60 ml)
ThermoFisher	Dynabeads MyOne Streptavidin T1	65602(10 ml)
	1M Tris, pH=8.0, 100 mls	AM9855G
	Qubit dsDNA HS Assay Kit	Q32854
	Qubit Assay Tubes	Q32856
Commonly used reagents		
MedStore/Greenfield Specialty Alcohols	Ethanol anhydrous 100% (brown bottle), case of 12X 500ml	P006EAAN
	Ethanol anhydrous 100%, 4X4L white jugs (cleaning only)	P016EAAN
Medstore	Nuclease-free water	W4502-1L

3.0 General Guidance:

- When opening a new kit, record all lot numbers in TGL master lot tracking sheet (R:\Lot_tracking_forms\17_05_23_Agilent_Sureselect_XT_lot_tracking_sheet.docx, associate all samples to the master lot tracking sheet using a letter key (“A”, “B”) in sample tracking sheet
- Do not mix and match reagents from multiple kits! Aliquot reagents where appropriate to minimize freeze thaw cycles, indicate freeze thaw with a dot on top of tube
- Before beginning work every day, wipe down all pipetors and bench surfaces with Peroxide wipes, then wipe with 70% ethanol (made from bulk ethanol, 4L)
- Buffers may be heated in Eppendorf thermal block, but ideally within PCR cycler in strip tube
- AMPureXP beads must be allowed to reach room temperature before use (30 mins at room temp)
- Be sure beads are thoroughly bound to magnetic rack when washing, loss of beads will reduce diversity. Before adding ethanol washes to beads, use a 10ul pipette to remove residual supernatant when using multichannel pipetors. This is critical for FFPE samples.
- Wet beads that contain residual ethanol prior to elution in water or RSB will interfere with reactions (beads should almost appear to be cracking from dryness). Always add RSB to dry beads before lifting from magnetic rack. Dried beads are very electrostatic and can “jump” out of the tube and be lost. Always visually confirm that you have re-suspended the dried beads entirely.
- Be sure not to carry over beads after elution, if beads do carryover, bind to magnetic rack and transfer again to fresh strip tube
- Record quants, dilutions, and other QC information in individual sample sheet
- Ethanol wash solutions should be made fresh every day and use molecular grade H20 and anhydrous ethanol (brown bottle only!), always use private ethanol and H20 aliquots to minimize risk of contamination between technicians
- When making master mixes a 10% overage should be sufficient
- 10 mM Tris can be made by diluting 100 ul of 1M stock in 9900 ul of molecular grade H20
- Enzyme solutions should be ‘flick’ mixed and briefly spun (minifuge) down prior to use, buffers should be vortexed and spun down
- Most reactions should be briefly spun to collect material at bottom of reactino well, especially when removed from thermocyclers
- Record all QC steps in original sample submission sheet, not running sample sheet, including MISO LIMS IDs, (LIB, LDI) master lot tracking references, protocol version and include your name so that user/tech can be traced in sample sheet
- Record and highlight all unusual observations, errors, or other issues in sample sheet

4.0 Procedure

Qubit material to verify concentration using Qubit HS dsDNA Assay kit and following SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx. If sample concentration is excessively high, dilute stock solution and re-qubit to a reasonable concentration.

Run Agilent Genomic Tapestation and record DIN if sample quantity is sufficient (5-10 ng/ul, 1ul). If sample quantity is low (<100ng), skip this step. Use appropriate concentration, do not go outside of tape concentration recommendation, or DIN will not be accurate.

PRE-PCR AREA

1. DNA Shearing (Covaris M220 instrument)
    a. Use Holder XTU Insert microTUBE 50 (PN500488)
    b. Use microTUBE-50 AFA Fiber Screw-Cap (PN520166)
    c. Fill reservoir to appropriate level with AFA water or milliQ water

1) Dilute 100 ng of FFPE DNA in 10mM Tris pH 8.0-8.5 to a total volume of 50µl.
2) Transfer sample to Covaris microTUBE 50 AFA tube through septum (Spin down briefly, no bubbles around fiber prior to shearing).
3) Load the tube into the sonication chamber, lower tube lever.
4) Run protocol: 50ul_shear_300bp (Peak Incident Power:75; Duty factor:10%; Cycles/burst: 200; Treatment Time: 90 seconds; Temp 20°C) (both FFPE and fresh frozen).
5) Briefly spin micro tube containing sheared DNA and transfer sheared DNA by "twisting off" cap and aliquoting into PCR tubes strip.

SAFE STOP POINT. Place in -20°C.

2. KAPA HyperPrep End Repair and A-Tailing (ER & AT)

Allow AMPure XP beads to warm to room temp for 30 minutes before use! Proceed immediately to ligation after incubation.

ER & AT Mix	**1x**
ER & AT Buffer	7
ER & AT Enzyme Mix	3
Total Mix-->	10
Fragmented FFPE DNA	50
Total Reaction--->	60

1) Prepare "ER & AT Mix" mastermix and keep on ice.
2) Add 10ul of the mix to the 50µl of sheared DNA.
3) Incubate in a thermal cycler as follows using program "End Repair":
   20°C for 30 min; 65°C for 30 min; 4°C HOLD.
   Heated lid: 85°C.

3. KAPA HyperPrep Adapter Ligation

(Minimize freeze thaw of adapters, ie 3-5X max)

1) Prepare “AL Mix” mastermix and keep on ice.
2) Add 50µl of the mix to the 60µl of ER & AT product.
3) Incubate in a thermal cycler as follows using program “Ligation”:
   20°C for 15 min Heated lid: off**; proceed immediately to next step.**

AL Mix	**1x**
Ligation buffer	30
DNA ligase	10
SureSelectXT Adapter Oligo (top stock)	10
Total Mix-->	50
ER & AT product	60
Total Reaction--->	110

4. Post-Ligation Cleanup

1) Add 88uL AMPureXP beads to each 110uL ligation reaction product.
2) Pipette to mix and incubate at room temperature for 15 minutes.
3) Place on magnet and allow solution to clear (5 minutes); Remove supernatant without disturbing beads. Use 10ul pipette to remove residual supernatant.
4) Add 200uL 80% EtOH while still on magnet and let sit for 30 seconds, then remove ethanol. Repeat for a total of 2 washes. Carefully remove and discard the supernatant.
5) Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
6) Re-suspend beads in 23µl of nuclease-free water and incubate for 2 min to elute the DNA. Visually confirm resuspension is complete.
7) Place the tubes on a magnet and incubate 5 minutes until the liquid is clear. Transfer 20ul of the clear supernatant to a new tube.

5. KAPA HyperPrep Pre-capture Library Amplification

1) Prepare "Pre-capture PCR mix" mastermix and keep on ice
2) Add 30µl of the mix to the 20µl of Adapter-ligated library.

Move to Post PCR Work Area

3) Incubate in a thermal cycler as follows and run program "PCR":
98°C for 45 sec; 12 cycles {98°C for 15 sec; 60C for 30 sec; 72°C for 30 sec}; 72°C for 1 min; 4°C HOLD**
Heated lid: 105°C ** note FFPE annealing and extension time is not the same as standard protocol!!!

Pre-capture PCR Mix	**1x**
2x KAPA HiFi mix (	25
SureSelect Primer (brown cap)	1.25
SureSelect Ilmn rev (clear cap)	1.25
PCR-grade water	2.5
Total Mix-->	30
Adapter-ligated library	20
Total Reaction--->	50

6. Post-amplification Cleanup

1) Add 50µL AMPureXP beads to each 50 µL PCR reaction product.
2) Pipette to mix and incubate at room temperature for 15 minutes.
3) Place on magnet and allow solution to clear (5 minutes); Remove supernatant without disturbing beads. Use 10ul pipette to remove residual supernatant.
4) Add 200uL 80% EtOH while still on magnet and let sit for 30 seconds, then remove ethanol. Repeat for a total of 2 washes. Carefully remove and discard the supernatant.
5) Dry the beads at room temperature for 15 min, or until all of the ethanol has evaporated.
6) Re-suspend beads in 34µl of nuclease-free water, or RSB and incubate for 2 min to elute the DNA. Visually confirm resuspension.
7) Place the tubes on a magnet and incubate until the liquid is clear (5 minutes). Transfer 32ul of the clear supernatant to a new tube.

SAFE STOP POINT. Place in -20°C.

7. Assess Quality and Quantity of the Pre-capture Library

1) Use Qubit HS DNA assay to quantify pre-capture library following SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx. A total of 500ng is required for further processing. If yield is <400ng, repeat library prep and pool material for hyb.
2) Optional: Use TapeStation High Sensitivity D1000 to verify that the library has an average region size of approx. 360bp with minimal adapter contamination. If adapter contamination, note in sample sheet.

8. Hybridize DNA Samples to Capture Library

1) SpeedVac 500ng of DNA library at <45°C for approx. 35 min to reduce volume to 3.4ul, or until no liquid is present at the bottom of the tube. If completely dry, re-suspend in 3.4 ul of nuclease free water, repeatedly aliquot suspension along lower half of tube to ensure all material is solubilized of wall of tubes after drying. DO NOT OVERDRY!

2) Prepare master mixes for Hyb. Buffer Mix (A), RNase Inhibitor Mix (B) and Block Mix reactions*:

Hyb. Buffer Mix (A)	1x
Hyb1 (orange cap)	6.63
Hyb2 (red cap)	0.27
Hyb3 (yellow cap)	2.65
Hyb4 (black cap)	3.45
Total Mix-->	13

RNase Inhibitor Mix (B)	1x
RNase block	0.5
Nuclease-free water	1.5
Total Mix-->	2

Capture Mix (A+B)	1x
Hyb Buffer Mix (A)	13
RNase Inhibitor Mix (B)	2
Capture Library (Probes)	5
Total Mix-->	20

*minimum of 4 rxn master mix to prevent pipetting error, upon new kit receipt, ALWAYS aliquot Capture library (probes) into 6 top stock tubes (approx. 82 ul each tube) for storage in -80°C. Make sure no precipitates are visible in solutions prior to making master mixes.

Block Mix	1x
Block1 (green cap)	2.5
Block2 (blue cap)	2.5
ILM Block3 (brown cap)	0.6
Total Mix-->	5.6

3) Combine Hyb Buffer Mix (A) and RNase Inhibitor Mix (B) to create Capture Mix (A+B).

4) To each 3.4µl of KAPA DNA library (step 1), add 5.6 ul of Block Mix, pipette to mix.
5) Incubate in a thermal cycler as follows using “HYB” program:
95°C for 5 min; 65°C HOLD (at least 5 min)
Heated lid: 105°C

6) While maintaining DNA + block mix at 65°C in the thermal cycler, add 20µl of Capture Mix (A+B), pipette to mix.

Hybridization tube	1x
DNA + block mix	9
Capture Mix (A+B)	20
Total volume-->	29

7) Continue the incubation in a thermal cycler as follows:
   65°C HOLD (at 16 hours)
   Heated lid: 105°C

9. Washing of Captured Exome

1) Prewarm SureSelect Wash Buffer 2 at 65°C (Eppendorf block or PCR strip tube)
2) Prepare streptavidin-coated magnetic beads as follows:
   i. Bring Dynabeads MyOne Streptavidin T1 magnetic beads to room temperature for 30 min.
   ii. Re-suspend beads by vortexing vigorously.
   iii. Dispense a volume of 50µl (x the number of captures) of beads in an Eppendorf tube.
   iv. Perform a total of 3 washes in 200µl (x the number of captures) of Binding Buffer.
   v. After removing the supernatant from the last wash, re-suspend in 200µl (x the number of captures) of Binding Buffer.
3) While maintaining the hybridization tube at 65°C, add hybridization reaction to 200µl of washed streptavidin beads using a multichannel pipette, mix well by slowly pipetting up and down and cap the wells. (NB add hyb to beads, not beads to hyb, order is important)
4) Incubate on a plate mixer (1400-1800rpm) at room temperature for 30 min.
5) Briefly spin, collect beads on a magnetic stand and discard the supernatant.
6) Re-suspend the beads in 200µl of Wash Buffer 1, mix by pipetting up and down and incubate at room temperature for 15 min.
7) Briefly spin, collect beads on a magnetic stand and discard the supernatant.
8) Wash the beads with Wash Buffer 2 (pre-warmed at 65°C, and maintain this temperature throughout the wash process to ensure stringency):
   i. Re-suspend beads in 200µl, mix well by pipetting up and down and cap the wells.
   ii. Incubate at 65°C for 10 minutes.
   iii. Capture the beads using a magnetic separator and discard the supernatant.
   iv. Repeat the process for a total of 3 washes in Wash Buffer 2 (pre-warmed at 65°C).
9) Re-suspend the beads in 19µl of nuclease-free water (Keep bead +H20 mix for on bead amplification!), and maintain on ice.

10. On-bead Post-capture Amplification

Post-capture PCR mix	1x
2x KAPA HiFi mix (KK2602)	25
Post-cap. Fwd primer (orange cap)	1
Total Mix-->	26
Indexing primer (unique)	5
Library-bead suspension	19
Total Reaction--->	50

1) Prepare "Post-capture PCR Mix" master mix.
2) Add 5µl of Indexing primer (unique to each library) to library bead suspension from step 9.
3) Add 26 µl of "Post-capture PCR mix" to library bead suspension and mix rxn by pipetting up and down.
4) Incubate bead suspension in a thermal cycler as follows using program "PCR 2":
   98°C for 45 sec; 10cycles {98°C for 15 sec; 60°C for 30 sec; 72°C for 30 sec}; 72°C for 1 min; 4°C HOLD**
   Heated lid: 105°C ** note FFPE annealing and extension time is not the same as standard protocol!!!

11. Final Cleanup

1) Add 90µL AMPureXP beads to each 50 µL PCR reaction product.
2) Pipette to mix and incubate at room temperature for 15 minutes.
3) Place on magnet and allow solution to clear (5 minutes); remove supernatant without disturbing beads. Use 10ul pipette to remove residual supernatant.
4) Add 200uL 80% EtOH while still on magnet and let sit for 30 seconds, then remove ethanol. Repeat for a total of 2 washes. Carefully remove and discard the supernatant.
5) Dry the beads at room temperature for 15 min, or until all of the ethanol has evaporated.
6) Re-suspend beads in 34µl of nuclease-free water, or RSB and incubate for 2 min to elute the DNA. Visually confirm resuspension.
7) Place the tubes on a magnet and incubate 5 minutes until the liquid is clear. Transfer 32ul of the clear supernatant to a new tube.

SAFE STOP POINT. Place in -20°C.

12. Assess Quality and Quantity of the Post-capture Library

1) Use Qubit HS dsDNA assay using TGL SOP to quantify post-capture library (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx.).
2) Use High Sensitivity D1000 screen tape and record average library size distribution (set region to 100bp- 1000bp). Average library size will be used to size correct the library after RT-qPCR quantification, and is used in LIMS sample IDs. Record Tapestation file ID in sample tracking sheet.

5.0 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of change
0.1	2017-03-20	First draft by Alberto Leon
1.2	2017-05-03	Edited by Dax
1.2.1	2017-05-03	Reviewed edited by Kayla
1.3	2017-06-30	Final Edits
1.3.1	2017-07-07	DT added lot control sheet
1.3.2	2017-07-17	Error corrected in oligo adapter in ligation 2.5ul changed to correct volume of 10ul