SOP Title:

IDT Targeted Capture Panel for Cell-Free DNA (cfDNA) Library Preparation Protocol, Single Plex Capture

1.0 Purpose and scope

This standard operating procedure (SOP) is for the preparation of cfDNA sequencing libraries using targeted custom gene panels from IDT. cfDNA isolated from plasma is used to generate pre-capture libraries using KAPA Hyper Prep library preparation reagents, IDT unique molecular indexes (UMIs), and IDT dual indexed sequencing adapters. Pre-capture libraries are sequenced to obtain copy number alterations and assess tumor purity and ploidy using ichorCNA [1]. Pre-capture libraries are hybridized to IDT custom pre-designed gene capture probe panels composed of 5'-biotinylated oligos, stringently washed with xGen hybridization wash solutions, enriched for target bait regions and amplified prior to sequencing on Illumina platforms. The procedure may be performed on a range of input amounts beginning at 10ng of cfDNA, but ideally greater than 50ng cfDNA and may be used with any IDT probe set [2]:

- IDT xGen Predesigned Gene Capture Pool
- IDT xGen Lockdown Probes

Related documents:

- xGen Predesigned Gene Capture Pools link to document.
- xGen Lockdown Probes link to document.
- Manufacturer-supplied protocol for library prep and post-capture amplification: KAPA Hyper Prep Kit (KR0961 –v6.17) link to document
- Manufacturer-supplied protocol for hybridization and capture: *xGen hybridization capture of DNA libraries* (Version 2), link to document
- xGen Duplex Seq Adapters- Tech Access, link to document

Related TGL documents:

- YYYY_MM_DD_SAMPLE SUBMISSION FORM_PI_Lastname_Firstname_TGL.xls
- 16_05_11_Kapa_Hyperprep_lot_tracking_sheet.xls
- 18_03_12_IDTCapture_Reagent_lot_tracking.xls
- SOP_Plasma_and_Buffy_Coat_Separation_From_Whole_Blood_Protocol_v1.0.2
- SOP_Purification_of_Circulating_Nucleic_Acids_from_Plasma_Protocol_v1.0.6
- SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc
- SOP_TapeStation_4200_V1.0
- SOP_KAPA_Library_Quantification_Illumina_Platforms_V1.0.2_production

Refer to section *1.6 Appendix: design notes* for information regarding the modifications and adjustments with respect to the manufacturer-supplied protocols that were incorporated in this SOP.

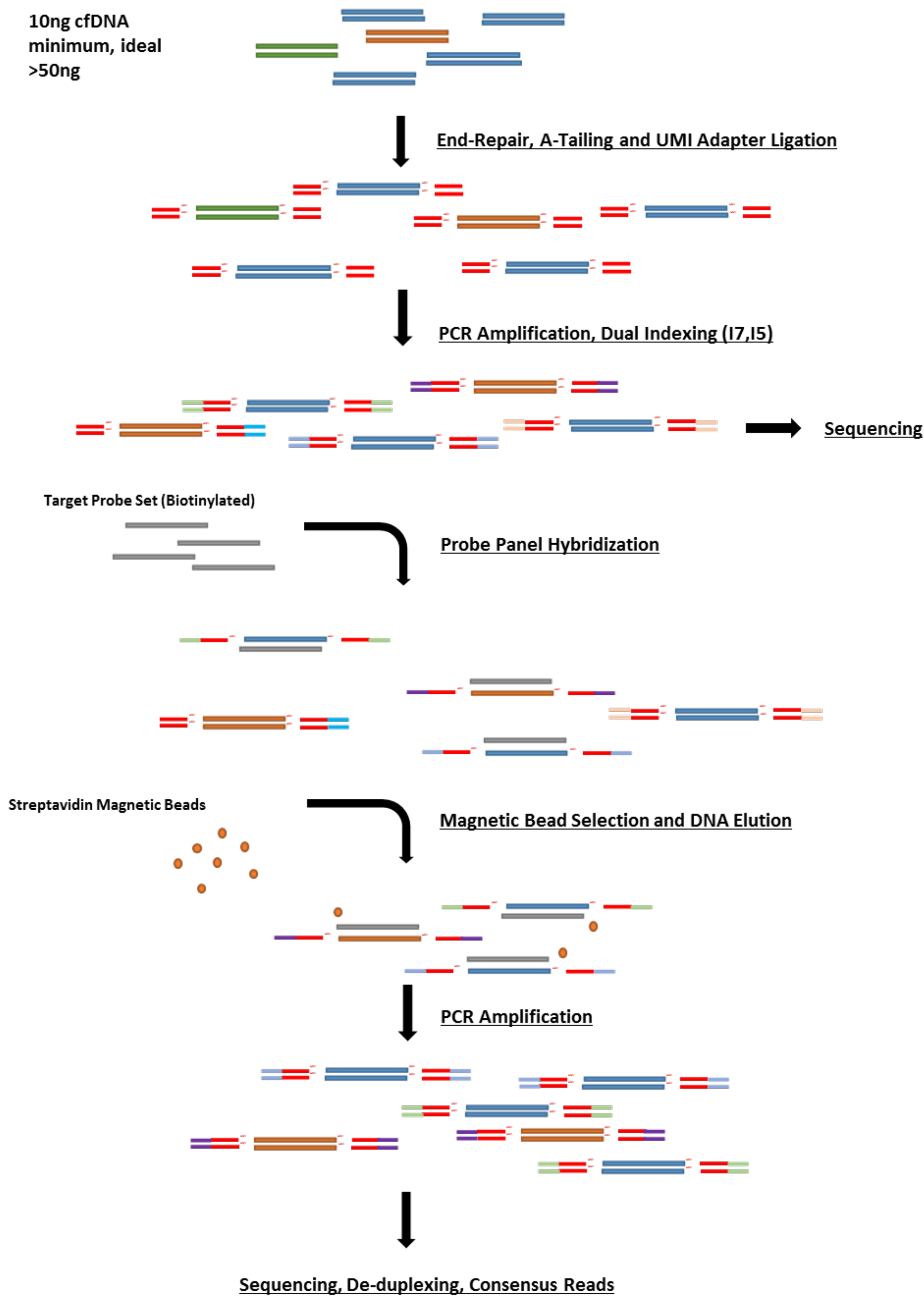


Figure 1: cfDNA Target Panel Capture with sWGS Workflow.

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); 4°C	5067-5365
	Genomic DNA reagents; 4°C	5067-5366
	High Sensitivity D1000 Screen Tape (7 tapes,112 samples); 4°C	5067-5584
	High Sensitivity D1000 Reagents; 4°C	5067-5585
	Plate Foil Seal	5067-5154
	96 Well plates, 150ul, conical, 25/pk	5042-8502
	Mx3000P Optical Strip Caps	401425
	Mx3000P Strip Tubes	401428
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); -20°C	7962363001 (KK8504)
	KAPA HiFi HS RM (6.25 mls, for post-capture PCR); -20°C	7958935001 (KK2602)
IDT	xGen Duplex Seq Adapter-Tech Access; 2nmol at 15uM, dual 3bp UMI; approximately 133.3ul (stock solution); -20°C	1080799
	xGen Duplex Seq Primers (duplex I5/I7 Indices for NextSeq); 4nmole Ultramer DNA Plate Oligo at 40uM or 100ul; Pre-mixed Amplification Primers; -20°C	IDT_cfMeDIP_xgen-duplex-seq-oligo-ordering-form.xlsx
	xGen® Hybridization and Wash Kit, 16 rxn or 96 rxn; Box 1, -20°C; Box 2, 4°C	1080577; 1080584
	Human Cot DNA; 150ul or 650ul;-20°C	1080768; 1080769
	xGen Universal Blockers-TS mix, 16 rxns or 96rxns; -20°C	1075474; 1075475
	xGen Predesigned Gene Capture Pools (16 rxn); -20°C	Custom
	IDTE, pH=8.0 10X2mL (dilution of probe pools if required); -20°C	11-01-02-05
	xGen Library Amplification Primer, 96 rxns; optional; -20°C	1077676
	Nuclease-Free Duplex Buffer; 10X2mL; -20°C	11-01-03-01

Beckman Coulter/Cedarlane	AMPure XP beads, 60mL; 4°C	A36881
ThermoFisher	Dynabeads M270 Streptavidin beads, 2ml or 10 mL; 4°C	65305; 65306
	1M Tris, pH=8.0, 100 mls, RT	AM9855G
	Qubit dsDNA HS Assay Kit; RT, 4°C	Q32854
	Qubit Assay Tubes	Q32856
EpigenDx	High Methylation Control (Human Genomic DNA); 5ug, 100ng/ul; -20°C	80-8061-HGHM5
College of American Pathologists	CAP Proficiency Test, Cell Free DNA; -80°C	CFDNA.2018
Commonly used reagents		
MedStore	Sigma Nuclease-free water	W4502-1L
	Axygen Snaplock Centrifuge Microtubes (clear), 1.5mL, 250/pk (10 pks/case)	MCT-150-L-C
MedStore/Greenfield Specialty Alcohols	Ethanol anhydrous 100% (brown bottle), case of 12X 500ml	P006EAAN
	Ethanol anhydrous 100%, 4X4L white jugs (cleaning only)	P016EAAN
Illumina	Resuspension Buffer (RSB); -20°C	Various Illumina kits

3.0 General Guidance:

- De-identified study codes must be used on all documentation.
- When opening a new kit, record all lot numbers in TGL master lot tracking sheet (R:\Lot_tracking_forms\16_05_11_Kapa_Hyperprep_lot_tracking_sheet.xls and 18_03_12_IDT_Capture_Reagent_lot_tracking.xls, 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.
- Buffers bottles should be mixed/swirled prior to each use.
- Record date of receipt, resuspension, 1st use, and ethanol/isopropanol addition directly on boxes, bottles, and tubes where appropriate.
- Use molecular grade H_20 and anhydrous ethanol (brown bottle only!). Always use personal stocks of ethanol and H_20 aliquots to minimize risk of contamination between technicians.
- 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.
- Residual ethanol on beads prior to elution in water or RSB may interfere with subsequent 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 again and transfer elution to fresh strip tube or microfuge tube.
- Record quants, dilutions, thermocycler, and other QC information in project running sheet.
- Ethanol wash solutions should be made fresh every day and use molecular grade H_20 and anhydrous ethanol (brown bottle), always use private ethanol and H_20 aliquots to minimize risk of contamination between technicians.
- When making master mixes, a 10% overage should be sufficient.
- 10 mM Tris may be made by diluting 100 ul of 1M stock in 9900 ul of molecular grade H_20.
- Enzyme solutions should be ‘flick’ mixed and briefly spun down (minifuge) prior to use, buffers should be vortexed and spun down.
- Most reactions should be briefly spun to collect material at bottom of reaction well, especially when removed from thermocyclers.
- Record all QC steps in project running sheet including MISO LIMS IDs LIB, LDI; master lot tracking references; protocol version; and technician performing the stage of the protocol.
- Record and highlight all unusual observations, errors, or other issues in sample sheet.
- cfDNA may be contaminated with genomic DNA from leukocyte lysis which may interfere with downstream informatics interpretation. Follow SOP_Purification_of_Circulating_Nucleic_Acids_from_Plasma_Protocol_v1.0.6 and genomic DNA Tape Station steps to confirm if genomic DNA contamination is present. Record levels of genomic contaminant (% fragments>1000bp) for all inputs. Verify sample contains a cfDNA peak centred around 150-160bp before beginning assay, if >20% consult with lab manager.
- A sheared genomic control may be utilized for assay development or training; see procedure below.
- TGL recommends IDT duplex seq UMI adapter mix combined with NextSeq compatible duplex seq indexing primers. The duplex seq UMI adapter is a double stranded forked short adapter that ligates to end repaired, A-tailed library; a 3bp UMI is added at both the 5’ end of an insert, and the 3’ end of the insert. Using ConsensusCruncher[3], unique molecular indexes (UMIs) from the sequencing library are utilized to suppress sequencer errors; duplicate reads are amalgamated into single-strand consensus sequences and combined into duplex consensus sequences. Singletons (reads lacking duplicate sequences) are corrected and combined with single-strand consensus sequences and collapsed to unique molecules.

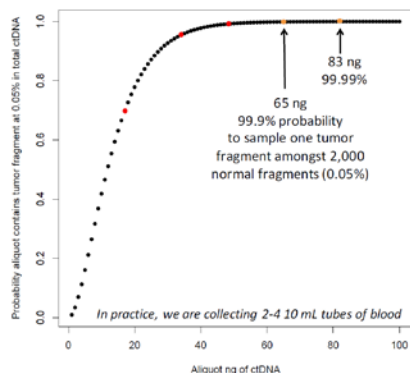
- Nuclease-Free Duplex Buffer (cat # 11-01-03-01) is recommended for dilution of UMI and indexing adapters if necessary.
- IDT recommends extended hybridization times (up to 16 hours) for small panels (<1000 probes) or for GC rich designs.
- In step 7. “Hybridize DNA Samples to Capture Library”, 2X Hybridization Buffer may contain salt crystals, if crystals are observed, heat the tube at 65°C, shaking intermittently to solubilize; it may take several hours to solubilize!

3.1 Safety and Precautions

- Ensure proper PPE is worn during this protocol including lab coat and latex/nitrile gloves. Safety glasses are recommended, but optional. Report exposure or injury to OICR Senior Health and Safety Officer and/or Manager. Change gloves if soiled.
- Before beginning work every day, wipe down all pipettes and bench surfaces with peroxide wipes, then wipe with 70% ethanol (made from bulk ethanol, 4L).
- Full MSDS information is available here: https://msdsmanagement.msdsonline.com/9f815ba5-279c-40ea-854a-8f022ffe62b3/ebinder/?nas=True

3.2 Panel Validation Considerations and Limits of Assay

- A minimum of 1 reference tumor genomic (gDNA) and a maximum of 3 tumor gDNAs with matched buffy coat gDNA are required for validation of all ctDNA panels. These tumors should have known/documented VAFs in the range of 50% and be represented/targeted in the ctDNA panel; if the VAFs are not documented, these tumors may need to be sequenced to determine inherent frequencies (*see tables below).
- For each patient plasma series, a sheared buffy coat normal control is recommended for germline variant filtering (50X). If clonal hematopoiesis is of interest, 1000X depth of buffy coat gDNA is required.
- cfDNA may be limiting within a study cohort and define assay limit of detection (LOD). Ideally plasma is pooled from 3-4 Streck or K3 EDTA tubes per time point or approximately 10 mls of plasma. Be aware of the following constraints that scale with lower input cfDNA [2]:



- Sensitivity for a mutation is limited to 0.2% (fraction of reads) due to sequencer error rates inherent to Illumina technology. Sequencing errors occur at approximately 0.1% of reads. Informatics tools like Consensus Cruncher [3] may suppress/correct these errors post sequencing.
- Different tumors may have varying amounts of detectable ctDNA and specific fragments within the total pool of cfDNA, see article [2].
- For 5% limit of detection (LOD), 10,000X coverage of probe space results in 5,000X de-duplicated depth of coverage; ideal if you have approximately 10 ng of cfDNA available per sample.
- For 1% LOD, 20,000X coverage of probe space results in 10,000X de-duplicated depth of coverage; ideal if you have approximately 40-100ng of cfDNA available per sample.
- To define a panel's LOD for a set of variants, a dilution series must be conducted. Sheared genomic tumor (gDNA) with a 50% variant allele frequency (VAF) is serial diluted in a background of sheared, matched buffy coat gDNA. More thorough validations may incorporate up to 3 tumor sets and/or increasing technical replicates up to 3. These considerations must be balanced against the cost/budget of your study. The sequencing of larger panels may make more comprehensive validation cost prohibitive.
- If you have a minimum of ≥10 ng of cfDNA available for your cohort, a 5% tumor ctDNA VAF LOD may be inferred. Sequencing probe set depth of coverage must be a minimum of 10,000X to achieve 5,000X de-duplicated reads. The following serial dilution set is suggested:

				sheared genomic	
		dilution (%)	ctDNA target panel assay input (ng)	sheared tumor gDNA (ng)	sheared buffy coat DNA (ng)
Control*	Stock	100	10	0	10
Control*	Stock	100	10	10	0
1	Dilution	10	10	3	27
2	Dilution	1	10	prior dilution (3 ng); use volume equivalent	27
3	Dilution	0.1	10	prior dilution (3 ng); use volume equivalent	27
4	Dilution	0.01	10	prior dilution (3 ng); use volume equivalent	27
			minimum total DNA (ng)	13	118
			ideal total DNA required for triplicate (ng)	39	354

- If you have a minimum of ≥40 ng of cfDNA available for most of your cohort, a 1% tumor ctDNA VAF LOD may be inferred. Sequencing probe set depth of coverage must be a minimum of 20,000X to achieve 10,000X de-duplicated reads. The following guidance for serial dilutions is suggested. The following serial dilution set is suggested:

				sheared genomic	
		dilution (%)	cfDNA target panel assay input (ng)	sheared tumor gDNA (ng)	buffy coat sheared DNA (ng)
Control*	Stock	100	50	0	50
Control*	Stock	100	50	50	0
1	Dilution	10	50	7	63
2	Dilution	1	50	prior dilution (7 ng); use volume equivalent	63
3	Dilution	0.1	50	prior dilution (7 ng); use volume equivalent	63
4	Dilution	0.01	50	prior dilution (7 ng); use volume equivalent	63
5	Dilution	0.001	50	prior dilution (7 ng); use volume equivalent	63
6	Dilution	0.0001	50	prior dilution (7 ng); use volume equivalent	63
			Minimum Total DNA (ng)	77	378
			Ideal Total DNA required for triplicate (ng)	231	1134

4.0 Procedure

If cfDNA is received from external labs, always qubit cfDNA to verify concentration using the Qubit HS dsDNA Assay kit and following SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx. For all cfDNA, run a genomic tape following SOP_TapeStation_4200_V1.0, record genomic contaminant level for all cfDNA. Genomic tapes require a concentration of 10-100ng/ul, 1ul. If sample concentration is out of range (>100ng/ul), dilute stock and re-qubit. Ideally, cfDNA should be in the range of 0.2-10ng/ul, ie pipetting more than 1 ul of cfDNA into a library synthesis reaction. If cfDNA per mL of plasma is >25ng/mL, or plasma appeared red in colour, confirm there is no genomic DNA contamination by running an Agilent Genomic ScreenTape.

PRE-PCR AREA

1.0 Component Preparation

1. Dilute UMI adapter.
    a. Add 100 µl of xGen Duplex Seq Adapter-Tech Access (IDT PN#1080799, stock 15uM) to 400 µl Nuclease-Free Duplex Buffer (IDT PN#11-01-03-01). Aliquot 55 µl (≈10 samples), 3µM working stocks. Discard after 3 freeze thaws. Track freeze thaws with a black marker, dotting top of aliquot tube to indicate thaws.

2. Dilute xGen Universal Blockers-TS mix.
    a. Add 288 µl of IDTE, pH=8.0 (PN11-01-02-05) to 32 ul of xGen Universal Blockers-TS mix (PN1075474, 16rxns), or a 1 in 10 dilution of stock blocker mix. Aliquot to 21 µl stocks (≈10 samples) and freeze at -20°C. Discard after 3 freeze thaws. Track freeze thaws with a black marker, dotting top of aliquot tube to indicate thaws.

3. Dilute xGen Predesigned Gene Capture Pool.
    a. Add 576 µl of IDTE, pH=8.0 (PN11-01-02-05) to 64 ul of xGen Predesigned Gene Capture Pool, 16rxns, or a 1 in 10 dilution of probe pool. Aliquot to 41 µl (≈10 samples) stocks and freeze at -20°C. Discard after 3 freeze thaws. Track freeze thaws with a black marker, dotting top of aliquot tube to indicate thaws.

4. Shear “cfDNA” genomic control using Covaris M220 instrument.
    a. Use Holder XTU Insert microTUBE 50 (PN500488) and microTUBE-50 AFA Fiber Screw-Cap (PN520166). Fill Covaris M220 reservoir with milliQ water to level that submerges the microtube to the area indicated by the arrow.

    b. Dilute genomic control (EpigenDx, 80-8061-HGHM5) in 10 mM Tris pH 8.0-8.5 to a total volume of 50 µl.
        i. 10 µl Control Human High Methylated DNA (1000ng) + 40 µl 10 mM Tris pH 8.0-8.5
    c. Transfer sample to Covaris microTUBE 50 AFA tube through septum (Spin down briefly, no bubbles around fiber prior to shearing). Load the tube into the sonication chamber.
    d. Run protocol: 50ul_shear_150bp (Peak Incident Power: 75; Duty factor: 10%; Cycles/burst: 200; Treatment Time: 360 seconds; Temp 20°C).
    e. Briefly spin micro tube containing sheared DNA, twist off cap and transfer sheared DNA to new 1.5 mL tube.
    f. Verify size distribution (expected 150bp peak) on Agilent TapeStation using High Sensitivity D1000 tape and SOP_TapeStation_4200_V1.0, record.
    g. Qubit using SOP_Qubit_Assay_for_Nucleic_Acid_Quantification_V1.0.

h. Dilute sheared DNA to a concentration of 2.5ng/ul or 45 ul in 360 ul of 10mM Tris pH 8.0-8.5.
i. Aliquot and store at -20°C.

1.1 cfDNA Prepartion of Libraries – Day 1

3) Add minimum of 10ng (ideal >50ng) of each cfDNA sample into separate tubes. 50 ng of sheared "cfDNA" or CAP cfDNA may be used in pilot assays. Either add H_2O or speed vac for a final volume of 50µL.

1.1.1 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.

4) Prepare "ER & AT Mix" mastermix and keep on ice.
5) Add 10ul of the mix to the 50µl of cfDNA.
6) 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.

ER & AT Mix	1x
ER & AT Buffer	7
ER & AT Enzyme Mix	3
Total Mix-->	10
cfDNA or sheared "cfDNA" or CAP cfDNA	50
Total Reaction--->	60

1.1.2 KAPA HyperPrep UMI Adapter Ligation

Minimize freeze thaw of UMI adapter, i.e. 3X max.

7) Prepare "AL Mix" master mix and keep on ice.
8) Add 50µl of the mix to the 60µl of ER & AT product.
9) Incubate in a thermal cycler as follows using program "CTLigation":
   20°C for 2 hours Heated lid: off**; proceed immediately to next step.**

AL Mix	1x
Ligation buffer	30
DNA ligase	10
xGen Duplex Seq Adapter (3uM UMI pool)	5.0
H_2O	5.0
Total Mix-->	50
ER & AT product	60
Total Reaction--->	110

1.2 Post-Ligation Cleanup

10) Remove samples from thermal cycler. Quick Spin.
11) Vortex AMPure XP beads to thoroughly re-suspend, then add 88uL AMPureXP beads to each 110uL ligation reaction product (Final Volume 198uL).
12) Pipette to mix and incubate at room temperature for 15 minutes.

13) Place tube on magnet and allow solution to clear (5 minutes); remove supernatant without disturbing beads. Use 10ul pipette to remove residual supernatant.
14) 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 using a 10ul pipette.
15) Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
16) Re-suspend beads in 22µl of RSB and incubate for 2 min to elute the DNA. Visually confirm resuspension is complete.
17) 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.

1.3. KAPA HyperPrep Pre-capture Library Amplification and Indexing

Libraries are amplified to include Illumina sequencing adapters with dual indexes. **Record the I5 and I7 indexes used on the sample tracking sheet.** Use optimized I7-I5 index combinations. *For >50ng of cfDNA input, 5 ul of xGEN Duplex Seq primer pair will result in a library with minimal residual adapter. If using <20ng, use 1.5 ul of xGEN Duplex Seq primer pair and add 3.5 ul of H_20.

Pre-capture PCR Mix	**1x**
2x KAPA HiFi mix	25
xGEN Duplex Seq primer pair (20uM)	5*
H_20	0
Total Mix-->	30
Adapter-ligated library	20
Total Reaction--->	50

18) Prepare "Pre-capture PCR mix" master mix and keep on ice.
19) Add 30µl of the mix to the 20µl of Adapter-ligated library.

Move to Post PCR Work Area

20) 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

1.3.1 Post-amplification Cleanup

21) Add 50µL AMPureXP beads to each 50 µL PCR reaction product.
22) Pipette to mix and incubate at room temperature for 15 minutes.
23) Place on magnet and allow solution to clear (5 minutes); remove supernatant without disturbing beads. Use 10ul pipette to remove residual supernatant.

24) 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. Use 10ul pipette to remove residual supernatant.
25) Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
26) Re-suspend beads in 34µl of nuclease-free water, or RSB and incubate for 2 min to elute the DNA. Visually confirm re-suspension.
27) 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.

1.3.2. Assess Quality and Quantity of the Pre-capture Library (Quality Control)

28) Use Qubit HS DNA assay to quantify pre-capture library following SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx. A total of 500ng (400ng minimum) is required for further processing. If yield is <400ng, repeat library prep and pool material for hybridization.
29) Use High Sensitivity D1000 screen tape and record average library size distribution of ≈270bp by setting the TapeStation region to 160bp- 1000bp. Record % adapter contaminant or the region <160bp. If % adapter contaminant exceeds 10%, consult with manager before proceeding. 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.
30) Quantify a small portion of the pre-capture library for sWGS sequencing, preserving >500 ng (400 ng minimum) of stock library for targeted capture following SOP_KAPA_Library_Quantification_Illumina_Platforms_V1.0.2_production.
31) Pre-capture libraries are sequenced for library quality and copy number analysis. Multiple libraries may be pooled together, a minimum of 20,000 clusters is required for analysis (0.001X) on MiSeq Nano, V2 chemistry and reagents 150bpX8bpX8bpX150bp. Pause library prep until library insert size is verified. Pass libraries with mean insert size >140bp, fail if <140bp. Notify manager of QC alert for libraries with reads per start point (RSP) >2.0.

1.4. Hybridize Genepool/Panel Probes to Pre-Capture Library

If continuing capture after drying (step 35), take out hybridization reagents to thaw. xGen 2X Hybridization Buffer may contain salt crystals, if crystals are observed, heat the tube at 65°C, shaking intermittently to solubilize; it may take <u>several hours to solubilize</u>!*

32) Prepare Blocker Master mix with Human Cot DNA and xGen Blocking Oligos
33) Add 7 uL of the Blocker Master Mix to each 1.5mL microtube.
34) Add 500 ng of each library* in a tube containing the Blocker Master Mix.
35) Dry the mixture down in a speedvac.

Blocker Master Mix	1x
Human Cot DNA	5
xGen Blocking Oligos (diluted)	2
Total Mix-->	7
Pre-Capture library (500ng)	Variable

SAFE STOP POINT. Tubes can be stored at room temperature overnight, or -20°C for longer.

36) Prepare a hybridization master mix with: xGen 2X Hybridization Buffer, xGen Hybridization Buffer Enhancer, diluted xGen Lockdown Panel or diluted custom probes, and nuclease-free water. Pipette to mix.
37) Add 17 uL of Hyb Master Mix to each tube containing dried DNA. Ensure Hyb Master Mix is in contact with dried DNA pellet, and pipette to mix.
38) Cap tubes, and incubate at room temperature for at least 5 mins.
39) Pipette to mix and briefly spin down tubes.
40) Transfer entire volume to labelled low-bind PCR tubes, and spin down.
    Place samples in thermocycler and run the HYB program:
    95°C for 30 sec; 65°C 4 hr; 65°C HOLD 100°C heated lid**

Hybridization MM	1x
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
xGen Lockdown Panel or custom probes (diluted)	4
H_20	1.8
Total Mix-->	17

1.4.1. Prepare Streptavidin Beads and Hybridization Wash buffers

Take out Streptavidin (Dynabeads M-270) beads to warm to room temperature for 30 minutes prior to use. xGEN 10X Wash Buffer 1 may be cloudy, heat bottle at 65°C prior to preparing diluted wash mix. Maintain 1X wash buffer 1 and 1X stringent wash buffer in second thermal cycler at **65°C HOLD** 70°C heated lid.**

41) Prepare the following xGen buffers to create 1x working solutions:

Component	H_2O (uL)	Buffer (uL)	Total per Reaction	Storage
xGen 2X Bead Wash Buffer	150	150	300	Room temp
xGen 10X Wash Buffer 1	225	25	250	Aliquot 110 ul per well (or hyb pool), and maintain at 65°C in thermal cycler.* Keep excess for later washes.
xGen 10X Wash Buffer 2	135	15	150	Room temp
xGen 10X Wash Buffer 3	135	15	150	Room temp
xGen 10X Stringent Wash Buffer	270	30	300	Aliquot 160ul per well, 2 wells per hyb pool and maintain at 65°C in thermal cycler.

Always maintain diluted wash buffer 1 and stringent wash buffer at 65°C throughout washes. The working solutions may be stored at room temperature for up to 4 weeks.

Bead Resuspension Mix	1x
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
H_2O	5.8
Total Mix-->	17

42) Prepare Bead Resuspension Mix in a low-bind tube as follows:
43) Vortex room temperature streptavidin beads thoroughly and aliquot 50 uL X n number of captures into a 1.5 mL low bind tube.
44) Add 100 uL X n of bead wash buffer per capture and pipette up and down 10 times to mix.
45) Place tubes on a magnetic rack to fully separate (~1 min) and discard the supernatant.
46) Remove the tubes from the magnet and repeat steps 4 and 5 two more times for a total of three washes.
47) Re-suspend the beads in 17 uL Xn of Bead Resuspension Mix. Mix thoroughly to prevent any beads from drying in the tube. Quick spin if needed.
48) Aliquot 17 uL of re-suspended beads into labeled PCR strip tube (1 for each capture).

1.4.2. Biotin Probe Capture

49) Heat 1x Wash buffer 1 (one well per hyb) and 1x Stringent Wash Buffer (two wells per hyb) in thermocycler at 65°C (2nd thermocycler, minimum incubation, 15 minutes).
50) After the 4 hour hyb incubation (step 40), take the strip tube out of the thermal cycler.

51) Stop the HYB program, and start the WASH program.
    65°C HOLD 70°C heated lid.**
52) Add 17 uL of prepared streptavidin bead resuspension (step 48) into PCR tube containing hyb.
53) Vortex to re-suspend and quick spin.
54) Return PCR tubes back to thermocycler for 45 min. It's ok to place tubes in thermocycler before lid has cooled to 70°C.
55) Gently vortex the tubes every 10-12 minutes.
56) Remove tubes after 45 minutes and proceed to heated washes.

1.4.3. Washing of Captured Gene Pool/ Probe set

Heated washes:

57) Transfer 100 uL of heated Wash Buffer 1 to the hyb (34ul), pipette to mix 10 times (pipette set to 120ul). * Try to minimize bubble formation.
58) Place tube on magnetic rack for 1 minute and discard the supernatant.
59) Remove tube from magnet, and add 150 uL of heated Stringent Wash Buffer and pipette to mix being careful to avoid bubble formation.
60) Incubate for 5 minutes at 65°C in thermocycler.
61) Place sample on magnet for 1 minute and discard supernatant.
62) Repeat steps 3 to 5 for a total of two stringent washes.

Room temperature washes:

63) Add 150 uL of room temperature Wash Buffer 1 and vortex thoroughly.
64) For 2 minutes, alternate between vortexing for 30 secs, and resting for 30 secs.
65) Spin down briefly, place on magnet for 1 minute and discard supernatant.
66) Add 150 uL of Wash Buffer 2 and vortex thoroughly.
67) For 2 minutes, alternate between vortexing for 30 secs, and resting for 30 secs.
68) Spin down briefly, place on magnet for 1 minute and discard supernatant.
69) Add 150 uL of Wash Buffer 3 and vortex thoroughly.
70) For 2 minutes, alternate between vortexing for 30 secs, and resting for 30 secs.
71) Spin down briefly, place on magnet for 1 minute and discard supernatant.
72) Remove residual Wash Buffer 3 using 10 ul pipette.
73) Re-suspend beads in 20 uL of nuclease-free water, pipetting 10X to mix; bead slurry will be used for on-bead amplification.

1.5. Post-Capture On-Bead PCR

of PCR cycles may vary depending on panel size and sample multiplexing, see appendix #2.

Amplification MM	1x
2X KAPA HiFi HotStart ReadyMix	25
xGen Library Amplification Primer	1.25
H_20	3.75
Total Mix-->	30
Post-capture bead slurry	20
Total Reaction--->	50

74) Prepare the Amplification Master Mix:
75) Add 30 uL of amplification mix to each hyb pool tube and run the "Post-Cap PCR" program: **98°C for 45 sec; 12 cycles {98°C for 15 sec; 60°C for 30 sec; 72°C for 30 sec}; 72°C for 1 min; 4°C HOLD****

SAFE STOP POINT. Amplified libraries may be stored at -20°C overnight, preferable to proceed to Post-Capture PCR Cleanup.

1.5.1. Post-Capture PCR Cleanup

76) Add 75µL AMPureXP beads to each 50 µL PCR reaction product.
77) Pipette to mix and incubate at room temperature for 10 minutes.
78) Place on magnet and allow solution to clear (5 minutes); remove supernatant without disturbing beads. Use 10ul pipette to remove residual supernatant.
79) Add 125uL 80% EtOH while still on magnet and let sit for 60 seconds, then remove ethanol. Repeat for a total of 2 washes. Carefully remove residual ethanol with a 10ul pipette.
80) Dry the beads at room temperature for 1-3 min, or until all of the ethanol has evaporated.
81) Re-suspend beads in 34µl of nuclease-free water, or RSB and incubate for 2 min to elute the DNA. Visually confirm resuspension.
82) 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.

5.0 Quality Control

5.1. Assess Quality and Quantity of the Post-capture Library Pool

83) Use Qubit HS dsDNA assay using TGL SOP to quantify post-capture library (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx.).
84) Use High Sensitivity D1000 screen tape and record average library size distribution of ≈270bp by setting the TapeStation region to 160bp- 1000bp. Record % adapter contaminant or the region <160bp. If adapter contaminant exceeds 10% notify manager before proceeding. 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. See appendix 3 for representative traces with adapter contaminant.

85) Quantify sequencing library pools with RT-qPCR following protocol SOP_KAPA_Library_Quantification_Illumina_Platforms_V1.0.2_production.

5.2 Pre-Analytic Quality Control (QC)

All pre-capture and captured ctDNA libraries must undergo pre-analytic QC prior to deep sequencing on the NextSeq550, HiSeq2500, or NOVA platforms. Ideally a minimum of 24 and a maximum of 96 sequencing libraries from any type of library preparation may be multiplexed together, assuming unique indexes. Multiplexed libraries will be run on a MiSeq Nano Reagent Kit V2, 150bpX8bpX8bpX150bp.

Check the following QC results and pass/fail each sample prior to deep sequencing:

- On target reads (%) >70%.
- Reads per start point (RSP) (convert this information to duplication rate with the following equation: dup rate= 1 – (1/RSP): Fail if dup rate is >60%.
- Adapter contaminant <10%

Appendix 1: IDT xGEN Duplex Seq Adapter Index List (Library amplification primers)

- UMI indexing and I5-I7 indexing-amplification primers are from IDT. See IDT_8nt_UDI_Index_List_V1.xlsx for well location specific I5-I7 barcode indexes, see IDT-8nt-UDI-Primer-MixPlate.xlsx for amplification primer sequences.

Appendix 2: Adapter Contamination

The following figures depict the difference between samples with and without adapter contamination using High Sensitivity D1000 Tapes on the TapeStation 4200. The expected library size distribution for cfDNA libraries is ≈270bp, adapters may appear around 130-160bp.

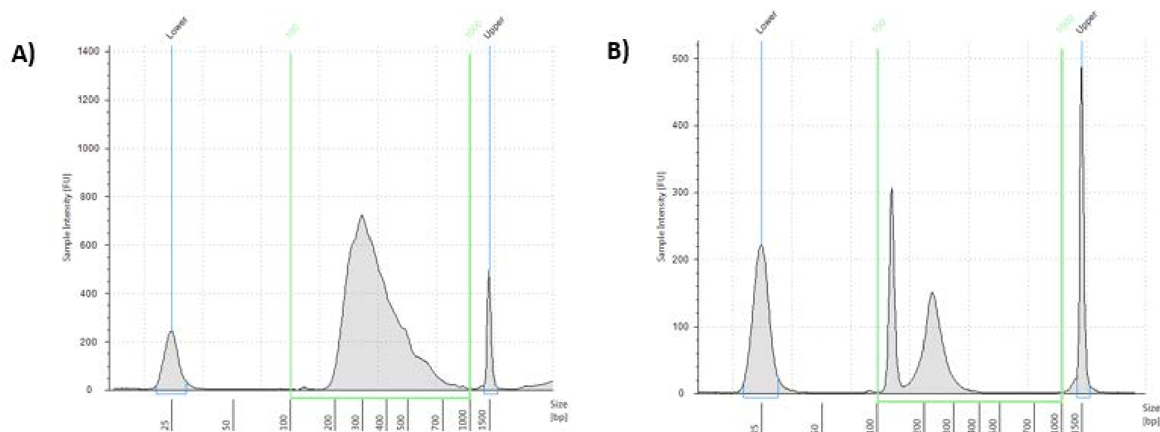


Figure 2: High Sensitivity D1000 Tapestation trace of a samples with no adapter contamination (a) and B) heavy adapter contamination at ~130-160 bp.

Appendix 4: MA38 Panel Details

- MA38 Panel; 3939 probes, 10 cycles.

1.6 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of change
1.0	2019-02-27	First draft by Madhuran Thiagarajah
1.01	2019-03-11	Edited by Dax Torti
1.02	2019-04-10	DT, increased input amounts, increased amount of amplification primer in PCR rxn.
1.03	2019-06-24	Updated to include pre-capture sWGS, revised input amounts, recommendations, updated article references. Updated workflow image. Revised protocol for individual captures with IDT reagent dilutions.

1.04	2019-08-07	DT-updated guidance on determining LOD, changed protocol title. Adjusted post capture PCR cycles to 12 from 10 to increase yield for single capture reactions.

1. Adalsteinsson, V.A., et al., *Scalable whole-exome sequencing of cell-free DNA reveals high concordance with metastatic tumors.* Nat Commun, 2017. **8**(1): p. 1324.
2. Kis, O., et al., *Circulating tumour DNA sequence analysis as an alternative to multiple myeloma bone marrow aspirates.* Nat Commun, 2017. **8**: p. 15086.
3. Wang, T.T., et al., *High efficiency error suppression for accurate detection of low-frequency variants.* Nucleic Acids Res, 2019.