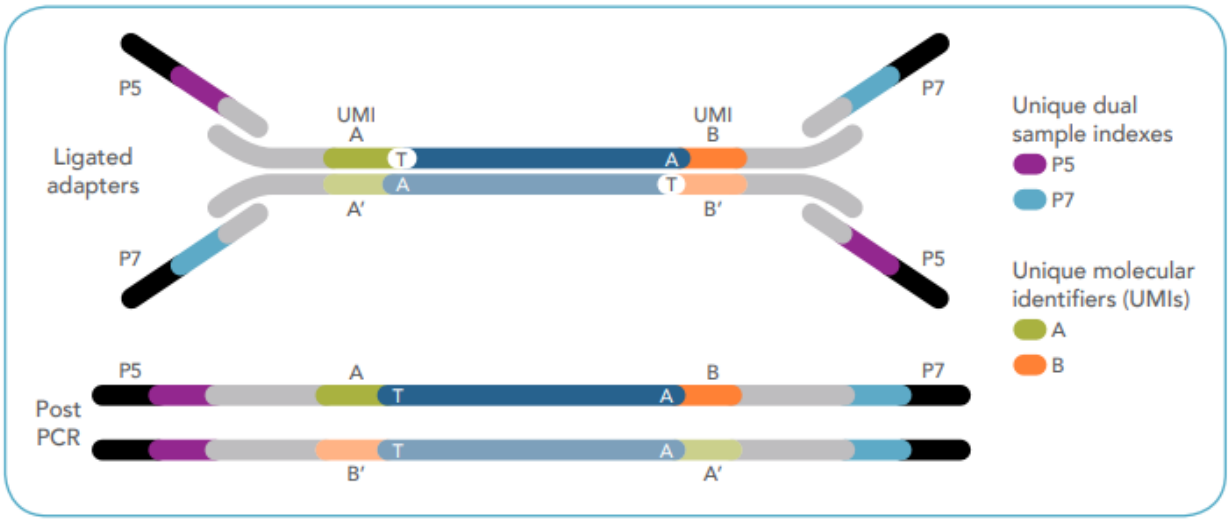
Plasma WGS Library Preparation - KAPA

Purpose

To define the procedure for plasma whole genome library preparation using the KAPA Hyper Prep Kit with IDT adapters and indexes.

Scope

Following Receipt and Extraction Quality Control (QC) checks, samples in the plasma whole genome workflow must undergo library preparation. This standard operating procedure (SOP) is for the preparation of cell free DNA (cfDNA) isolated from plasma to generate libraries using KAPA Hyper Prep library preparation reagents, IDT unique molecular indexes (UMIs), and IDT dual indexed sequencing adapters.



From IDT xGen® Duplex Seq Adapters—Tech Access product sheet.

Responsibilities

Management:
Review and update procedure, as required.

QA Manager:
Monitor quality output from this procedure.

Laboratory Staff:
Follow the SOP, document all required metrics, and report any non-conformances that may occur.

Reagents and Consumables

Item Description	Vendor	Catalogue #
NA12878 DNA from LCL (gDNA positive control); GLCS_0002	Coriell	NA12878
Seraseq® Blood TMB Mix Score 7 (positive control) ; GLCS_0035	SeraCare	SER-0710-2087
KAPA Hyper Prep kit (96 rxns, no PCR module); -20°C	Roche	7962371001
KAPA Hyper Prep kit (96 rxns); -20°C (alternate kit if 7962371001 not available)	Roche	7962363001
KAPA HiFi HS RM (6.25 mls, for post-capture PCR); -20°C	Roche	7958935001
xGen Duplex Seq Adapter-Tech Access; 2 nmol at 15 µM, dual 3bp UMI; approximately 133.3 µl (stock solution); -20°C	IDT	1080799
xGen Duplex Seq Primers (duplex I5/I7 Indices for NextSeq); 4 nmole Ultramer DNA Plate Oligo at 20 µM, 200 µl; Pre-mixed Amplification Primers; -20°C	IDT	Listed here
Nuclease-Free Duplex Buffer; 10X2mL; -20°C	IDT	11-01-03-01
NucleoMag® NGS Clean-up and Size Select beads; 4°C or AMPure XP Beads; 4°C	Macherey-Nagel Beckman/Cedarlane	744970 A36881 (60 ml)
1M Tris, pH=8.0, 100 mls, RT	ThermoFisher	AM9855G
Qubit dsDNA HS Assay Kit; RT, 4°C	ThermoFisher	Q32854
Qubit Assay Tubes	ThermoFisher	Q32856
TapeStation High Sensitivity D1000 ScreenTape	Agilent	5067-5584
TapeStation High Sensitivity D1000 Reagents	Agilent	5067-5585
TapeStation High Sensitivity D1000 Ladder	Agilent	5067-5587
Sigma Nuclease-free water	MedStore/Sigma	W4502-1L
Snaplock Centrifuge Microtubes (clear), 1.5 mL, 250/pk (10 pks/case)	Various	MCT-150-L-C
Ethanol anhydrous 100% (brown bottle), case of 12X 500 ml	MedStore/Greenfield Specialty Alcohols	P006EAAN
Ethanol anhydrous 100%, 4X4L white jugs (cleaning only)	MedStore/Greenfield Specialty Alcohols	P016EAAN
Resuspension buffer (RSB); -20°C	Illumina	Various Illumina kits
FluidX 96-Format, 0.5ml External Thread, Next-Gen Dual-Coded Tube (Capped, bulk; 960 tubes)	Brooks Life Sciences	68-0701-10

Note: Plastic consumables may be substituted for an approved equivalent product

Equipment

Item Description	Vendor	Catalogue #
Vacufuge Plus or CentriVap Benchtop Vacuum Concentrator Eppendorf OR VWR	Eppendorf or VWR	022820001 or 89004-916
Centrifuge	Various	Various
c1000 thermal cycler	Bio-Rad	1841100
Mini-centrifuge	Various	Various
Pipettes (mechanical)	Various	Various

Vortex	Various	Various
Dynamag (magnetic rack)	Thermo Fisher	12321D
Dynamag-96 (96 well magnetic rack)	Thermo Fisher	123331D
Covaris M220	Covaris	COV-500295
Covaris M220: microTUBE-50 AFA Fiber Screw-Cap	Covaris	PN520166
Covaris M220: Holder XTU Insert microTUBE 50	Covaris	PN500488
Qubit	Life Technologies	Q33226
TapeStation 2200	Agilent	G2964AA
TapeStation 4200	Agilent	G2991AA
Applied Biosystems QuantStudio 3 Real-Time PCR System (96-well, 0.1mL blck)	Applied Biosystems	A28136

Variables and Observations to Record

Record
KAPA, IDT, index and other reagent lots
Quantity of input DNA (ng)
Qubit quantification (ng/µl) pre-capture and post capture library
% Adapter contaminant
Library size (bp) (Tapestation)

Batch Controls

A no template control (NTC) and positive DNA control must be included in each library synthesis batch. Buffy coat gDNA positive control recorded under the MISO project GLCS (MISO alias GLCS_0002) or alternatively SeraCare positive control under the MISO project GLCS (MISO alias GLCS_0035). Control libraries are not sequenced but are included in each library synthesis batch and recorded in a MISO batch within the respective MISO CAP project(s). Control libraries must be synthesized at the same time as a production library batch. The following library information is recorded and logged in MISO for all libraries, including the negative and positive controls:

1. IDT Dual Indices (8 bp I5-I7 Index Sequences)
2. Average fragment size measured by Tapestation (bp)
3. DNA concentration as measured by Qubit (ng/µl)

Important Considerations:

<u>Reagent Lots and Expiration</u>

- Prior to starting an assay, inspect reagents to ensure that they have not expired.

- Expired reagents may not be used for validated clinical assays but may be used for RUO assays at the discretion of the Production Manager.
- Lot numbers for critical reagents must be recorded on MISO.

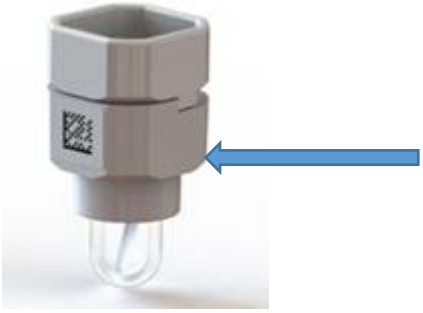
Other Considerations:

- 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.
- Buffer 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_2O and anhydrous ethanol (brown bottle only!). Always use personal stocks of ethanol and H_2O aliquots to minimize risk of contamination between technicians.
- AMPureXP (or NucleoMag) beads must be allowed to reach room temperature before use (30 mins at room temp).
- Be sure beads are thoroughly bound to the magnetic rack when washing; loss of beads will reduce diversity. Before adding ethanol washes to beads, use a 10 µl 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 the 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 the magnetic rack again and transfer elution to fresh strip tube or microfuge tube.
- Ethanol wash solutions should be made fresh every day and use molecular grade H_2O and anhydrous ethanol (brown bottle), always use private ethanol and H_2O 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 µl of 1M stock in 9900 µl of molecular grade H_2O.
- 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 the bottom of the reaction well, especially when removed from thermal cyclers.
- A sheared genomic control is used for a positive assay control

Procedure

Component Preparation

1. Dilute UMI adapter.
    a. Add 100 µl of xGen Duplex Seq Adapter-Tech Access (IDT PN#1080799, stock 15 µM) to 400 µl Nuclease-Free Duplex Buffer (IDT PN#11-01-03-01). Vortex to mix, centrifuge briefly and aliquot to 55 µl (≈10 samples), 3 µM working stocks. Discard after 3 freeze thaws. Track freeze thaws with a black marker, dotting top of aliquot tube to indicate thaws.
2. Shear genomic positive control (GLCS_0002 or GLCS_0035) using the Covaris M220 instrument.
    a. Shearing is described in detail in the TM. Covaris M220 E220 Use and Maintenance SOP, located on the QMS.
    b. 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 in the figure to the right.



Positive Control

    a. Dilute 20 ng of Positive control (GLCS_0002 or GLCS_0035) in 10 mM Tris pH 8.0-8.5 to a final volume of 50 µl.
    b. Transfer samples to Covaris microTUBE 50 AFA tube through septum (Spin down briefly, ensure there are no bubbles around fiber prior to shearing). Load the tube into the sonication chamber.
    c. Run protocol: 50ul_shear_150bp (Peak Incident Power: 75; Duty factor: 10%; Cycles/burst: 200; Treatment Time: 360 seconds; Temp 20°C).
    d. Briefly spin micro tube containing sheared DNA, twist off cap and transfer sheared

cfDNA/gDNA Preparation of Libraries

1) Prepare samples, NTC and positive control. Aliquot input amount of 10 ng of cfDNA (**not sheared**), or 20 ng of **sheared** gDNA (gDNA positive control) sheared as described in Component Preparation) into separate tubes.
Aliquot 50 µl of H_2O as a no template control (NTC). Add H_2O or speed vac samples to a final volume of 50 µl.
Note: the validated input range is 10-50 ng. The default input is listed above, however, Management may specify a different input within this range as required.

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

ER & AT Mix	1x (µl)
ER & AT Buffer	7
ER & AT Enzyme Mix	3
Total Mix	10
cfDNA or sheared "gDNA"	50
Total Reaction	60

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

Proceed immediately to ligation after incubation.

KAPA HyperPrep UMI Adapter Ligation

AL Mix	1x (µl)
Ligation buffer	30
DNA ligase	10
xGen Duplex Seq Adapter (3uM UMI pool)	5
Molecular Grade H_2O	5
Total Mix	50
ER & AT product	60
Total Reaction	110

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

5) Prepare "AL Mix" master mix and keep on ice.
6) Add 50 µl of the mix to the 60 µl of ER & AT product. Pipette to mix, centrifuge briefly.
7) Incubate in a thermal cycler as follows using program "LIG":
   20°C for 2 hours Heated lid: **OFF**
8) **At least 30 mins** before the end of the 2 hour incubation take Ampure XP beads out to warm to room temperature.

Once the 2 hour incubation is complete, **proceed immediately to post-ligation cleanup.**

Post-Ligation Cleanup

9) Remove samples from the thermal cycler. Quick Spin.

10) Vortex beads to thoroughly re-suspend, then add 88 µl of beads to each 110 µl ligation reaction product (Final Volume 198 µl).
11) Pipette to mix and incubate at room temperature for 15 minutes.
12) Place tube on magnet and allow solution to clear (5 minutes); remove supernatant without disturbing beads. Use 10 µl pipette to remove residual supernatant.
13) Add 200 µl 80% EtOH while still on the 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 10 µl pipette.
14) Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
15) Re-suspend beads in 22 µl of nuclease-free water or RSB and incubate for 2 min to elute the DNA. Visually confirm resuspension is complete.
16) Place the tubes on a magnet and incubate 5 minutes until the liquid is clear. Transfer 20 µl of the clear supernatant to a new tube.

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, and enter in MISO when creating the library.** Use optimized I7-I5 index combinations.

Pre-capture PCR	1x (µl)
2x KAPA HiFi mix	25
xGEN Duplex Seq primer pair (20uM)	5*
Molecular Grade H_2O	0
Total Mix	30
Adapter-ligated library	20
Total Reaction	50

*For >20 ng of input, 5 µl of xGEN Duplex Seq primer pair will result in a library with minimal residual adapter. If using <20 ng input, use 1.5 µl of xGEN Duplex Seq primer pair and add 3.5 µl of H_2O.

17) Add 25 µl of KAPA HiFi mix to the adapter-ligated library "Pre-capture PCR" and keep on ice.
18) Add **5 µl of xGEN Duplex Seq primer pair** **see note above*. Pipette to mix, centrifuge briefly.
19) Incubate in a thermal cycler as follows and run program "PRECAP_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

Post-amplification Cleanup

20) Add 50 µl AMPureXP (or NucleoMag) beads to each 50 µl PCR reaction product.
21) Pipette to mix and incubate at room temperature for 15 minutes.
22) Place on magnet and allow solution to clear (5 minutes); remove supernatant without disturbing beads. Use 10 µl pipette to remove residual supernatant.
23) Add 200 µl 80% EtOH while still on the magnet and let sit for 30 seconds, then remove ethanol. Repeat for a total of 2 washes. Carefully remove and discard the supernatant. Use 10 µl pipette to remove residual supernatant.
24) Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
25) Re-suspend beads in 34 µl of nuclease-free water, or RSB and incubate for 2 min to elute the DNA. Visually confirm re-suspension.
26) Place the tubes on a magnet and incubate until the liquid is clear (5 minutes). Transfer 32 µl of the clear supernatant to a new tube.

SAFE STOP POINT. Place labeled tubes containing precapture libraries in -20°C.

Quality Control: Assess Quality and Quantity Use Qubit HS DNA assay to quantify pre-capture library following TM. Genomics Qubit Fluorometric Quantitation. Use High Sensitivity D1000 screen tape and TM. High Sensitivity TapeStation Assay SOP, 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 the manager before proceeding. Record TapeStation file ID in sample tracking sheet.

Important note: Please record QC observations in the Qubit/TapeStation verification logs located in each instrument's lab binder

LIMS Entries

1. For general MISO instruction and training, see TM. LIMS Usage - MISO.

Plasma WGS Libraries (PG)

2. Propagate a MISO "PG" library (LIB) from the respective gDNA/cfDNA aliquot. If using the MISO bulk edit function, ensure that the order of samples has not been changed in MISO when propagating samples. Enter the following information for each precapture library:

- Matrix Barcode: Use barcode scanner while column is selected
- Box Alias and Position: Use Box Search column to find correct Box Alias
- Creation Date: Defaults to current date, but set to date of library creation
- SOP: Set to Plasma WGS Library Preparation- KAPA (see version at the footer of SOP)
- Thermal Cycler
- GroupID: Do not alter the groupID unless you are certain that it is required (e.g. for re-validation). Group ID is inherited and has been set during sample accessioning. GroupIDs are used to determine which entries are top-ups: See "What is Group ID?"
- Design: Set to **PG**

- Platform: Set to Illumina
- Type: Set to Paired End
- Index Kit: Set to **GRP TGL Dual Index UD Set 1 or 2**
- Has **UMIs: Set to True**
- Index 1: Set to the index used for the library. LIMS will autofill Index 2.
- Kit: Set to KAPA Hyper Prep
- Kit Lot: The lot number of the KAPA Hyper Prep Kit
- QC Status: Set to Ready if passed, see QM. Quality Control and Calibration Procedures. Include QC note if required.
- Size (bp): Determined during quality control (1.3.2)
- Volume: Set to 30 µl, unless elution volume was less than expected (pre capture library, 32 µl for elution, 2 µl QC)
- Conc.: Qubit concentration of precapture library

3. Propagate Library Aliquot. Prepare a 20 µL aliquot at 5 ng/µL of the stock library and propagate a matching MISO Library Aliquot from the MISO Library. Check "Create New Box" and provide all details, including the barcode using a scanner.

a. Box Position: Match parent positions here

b. QC Passed: Set to True

c. Conc. 5 ng/µL

d. Volume: 20 µL

e. Parent ng Used: Record the amount of stock library used to create the dilution

f. Parent Vol. Used: Record the volume (µL) of stock library used to create the dilution

g. GroupID: Do not enter a groupID unless you are certain it is required (e.g. for revalidation). Group ID is inherited and has been set during sample accessioning.

i. GroupID is used by the analysis pipeline to determine which samples/libraries belong together, e.g. to determine which entries are top-ups: https://wiki.oicr.on.ca/x/6ARdBw

h. Creation Date: Defaults to current date, but set to date of dilution/aliquot creation

i. Prior to saving Libraries, check that all information is correct and that the MISO entry matches the LIMs tracking sheet

All Stock Libraries

4. Add Library QC to all stock libraries that were prepared during the same library synthesis batch:

a. Select all stock libraries prepared together in the same batch.
b. Select Add QCs: In the pop-up window,
   i. Enter 2 QCs per Library: Average Library Size (TapeStation or Fragment Analyzer) and Concentration (Qubit)
   ii. Select the name of the instrument used for each QC

iii. Add 2 Controls per QC (Positive control, No Template Control).

c. Fill in the 2 QC areas per library, ensuring each individual library has both QC values entered.
d. Control 1: Assign as positive control with the lot number of the control used and indicate if the control passes.
e. Control 2: Assign as negative control, enter the lot number of nuclease-free water used and indicate if control passes.
f. When all QC metrics have been entered, review to ensure all QC values are paired to the correct Library, and then Save.

5. Attach batch QC files to all stock libraries that were prepared during the same library synthesis batch:

a. Select all stock libraries prepared together in the same batch.
b. Select Attach Files: Upload New Files.
c. Category:
    i. Scanned PDF of LIMS tracking sheet (worksheet batch)
    ii. Qubit csv file
    iii. TapeStation run report
d. Choose files: Attach the appropriate file corresponding to the category previously selected. Only one file can be uploaded at a time.
e. Upload.
f. Repeat previous steps until all four Batch QC files have been uploaded.

6. After all QC has been entered and the appropriate files have been uploaded, library aliquots may be added to the following MISO workset for sequencing:

a. Move the Library aliquot tubes into the "Miseq box 2" and update the locations in MISO.
b. Add the LDI for every sample passed on for sequencing into the Workset- GRP MiSeq QC Queue (For CAP-stream NovaSeq 2x151) in MISO.
c. Send an email to GenomicsLibrarySubmissions@oicr.on.ca with the LDI's that were added to the Workset to alert the sequencing team that there are CAP libraries pending sequencing.

Appendix 1: 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.

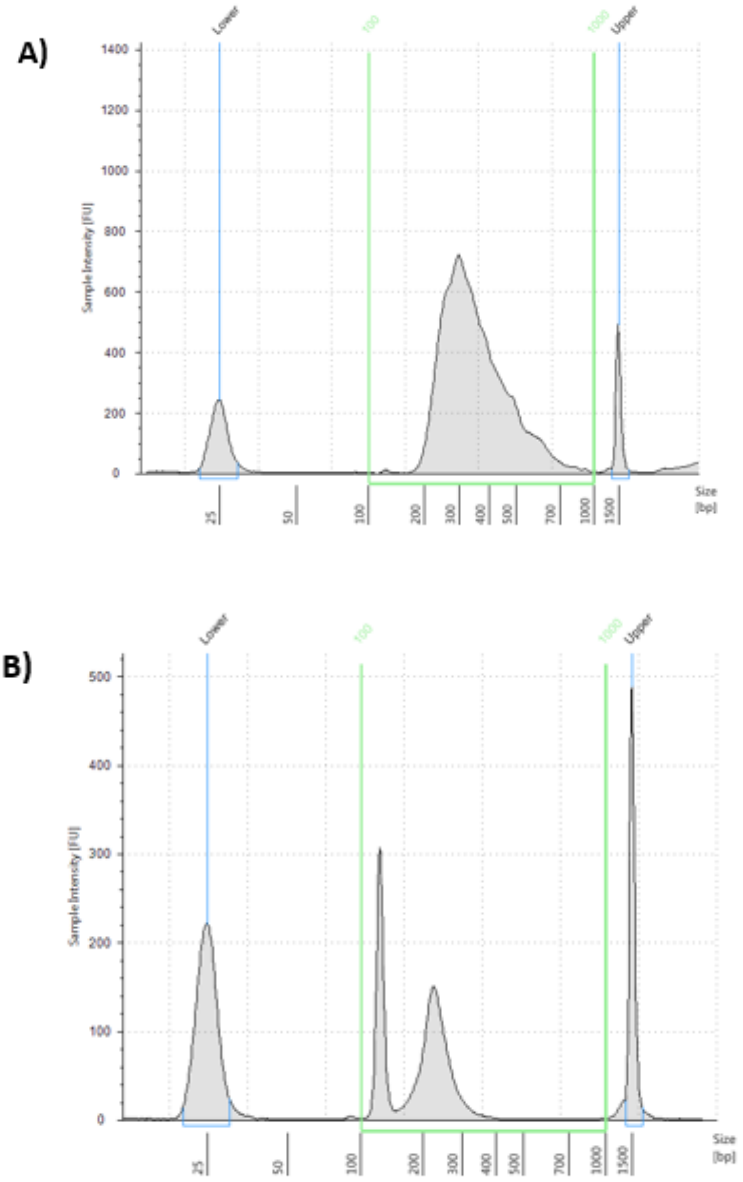


Figure 1: High Sensitivity D1000 TapeStation trace of a samples with A) no adapter contamination and B) heavy adapter contamination at ~130-160 bp.

Version History

Version	Description of Changes
1.1	• Change log introduced to document 2025-03-05 • Updated section headed "LIMS Entries" subpoint 4 (adding QC) to include entering 2 QCs per library (Average Library Size (TapeStation or FA) and Concentration(Qubit)) as well as selecting the name of the instrument used

	• Added a note under section headed “Quality Control: Assess Quality and Quantity” reminding users to record observations in the appropriate lab binder