SureSeq

A Sysmex Group Company

SureSeq NGS Library Preparation Handbook

SureSeq Handbook

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1 Introduction

The SureSeq™ NGS Hyb & Wash Kit has been optimised for use with SureSeq NGS Library Preparation Kit and SureSeq gene panel baits designed by Oxford Gene Technology (OGT™) in collaboration with recognised cancer experts to deliver accurate detection of somatic variants.

The SureSeq range is compatible with Illumina HiSeq™, NextSeq™ and MiSeq™ chemistries.

1.1 Storage

The kit should be used before the expiry date indicated on the kit label. The SureSeq NGS library preparation kit, SureSeq panel baits and SureSeq NGS Hyb & Wash Kit should be stored at

–20°C.

1.2 Safety

Handling of the SureSeq panel baits should be carried out by trained laboratory staff in accordance with good laboratory practice, using the correct protective equipment such as laboratory coats, safety glasses and gloves.
The OGT SureSeq NGS Hyb & Wash Kit (16) does contain chemicals which are potentially hazardous when mishandled, particular care should be given to both Formamide and the OGT Hybridisation Buffer.

Ensure that all Operators have familiarised themselves with the SDS and relevant Risk Assessments before proceeding with the Protocol.

1.3 Intended Use

Products in the SureSeq range are Research Use Only assays. Variants in non-coding regions of these genes will not be detected. This kit is designed to be used by suitably trained personnel using DNA extracted from a variety of tissues including blood and FFPE. Tables indicating the volume of reagents to be used for 16 reactions include excess for +1 reaction.

2 Equipment and reagents required

2.1 Equipment required (not supplied)

- Covaris® Focused-ultrasonicator or equivalent
- Agilent® 2200 TapeStation (cat. no. G2965A, or equivalent) and relevant reagents
- SpeedVac® (Thermo Fisher Scientific) or equivalent
- Thermal cycler (e.g. BioRad MJ Research DNA Engine PTC-200) or equivalent
- Laboratory vortex mixer + adapter for a standard microwell plate (e.g. IKA™ MS 3 Digital Vortex Mixer or BioShake iQ Thermomixer)
- Hot block for 1.5 ml tubes
- Microfuge for standard 1.5 ml tubes and 8-Strip PCR tubes
- Appropriate magnetic rack for 96–well microwell plates and 1.5 ml tubes
- Qubit® fluorometer (Life Technologies cat. no. Q32857)
- Thermo Scientific NanoDrop™
- 20-200 µl and 1-10 µl 8-Channel pipette
- Illumina® MiSeq, NextSeq or HiSeq

2.2 Reagents and consumables required (not supplied)

- OGT SureSeq NGS Library Preparation Kit (16) (OGT, cat. no. 500070) – with OGT SureSeq NGS Index Kit - Collection A (16) included OR OGT SureSeq NGS Library Preparation Kit (48) (OGT, cat. no. 500073) – with OGT SureSeq NGS Index Kit - Collection B (48) included
- OGT SureSeq NGS Hyb & Wash Kit (16) (OGT, cat. no. 500075)
- Covaris microTUBE AFA Fiber Snap-Cap (Covaris, cat. no. 520045)
- NEBNext® dsDNA Fragmentase® (New England Biolabs Inc, cat. no. MO348S)
- 0.5M EDTA pH 8.0 (Ambion, AM9260G)
- Dynabeads® M-270 Streptavidin (OGT, cat. no. 500080)
- Agencourt AMPure™ beads (OGT, cat. no. 500081)
- Molecular Biology Grade 100% Ethanol (Sigma Aldrich, cat. no. E7023 or equivalent)
- Molecular Biology Grade water (Sigma Aldrich, cat. no. W4502-1L or equivalent)
- Quant-iT™ dsDNA HS Assay Kit and Quant-iT dsDNA BR Assay Kit (Life Technologies, cat. no. Q32850, Q32854)
- Qubit Assay Tubes (Life Technologies, cat. no. Q32856)
- Agilent D1000 and High Sensitivity D1000 Reagents and ScreenTapes (cat. no. 5067-5582, 5067-5583, 5067-5584, 5067-5585)
- DNA LoBind Tubes (Eppendorf, cat. no. 022431021 or equivalent)
- 96-well microwell plates (Starlab, cat. no. E1403-0100) and 8-Strip PCR tubes (Starlab cat. no. A1402-3700) or equivalent
- Sequencing reagents required for the MiSeq / HiSeq (e.g. Illumina, cat. no. MS–102-2002, MS-102-2022, GD-401-3001, FC-401-3001)

- 5.0 M Sodium hydroxide solution, molecular biology-grade (e.g. Sigma-Aldrich, cat. no. S8263)
- 15 ml or 50 ml Falcon Tubes or similar

2.3 SureSeq Interpret Software

Fastq files generated from the Illumina sequencers can be analysed using the SureSeq Interpret Software and turned into interactive reports. The SureSeq Interpret Software is OGT's powerful, standalone data analysis package that is provided with the kit.

3 Workflow overview

For ordering information about OGT products visit www.ogt.com.

The following section contains instructions for sample library production specific to the Illumina sequencing platform. For each sample, individual library preparations, hybridisations, and captures are performed. The samples are then tagged by PCR with an index (barcode) sequence and prepared for sequencing.

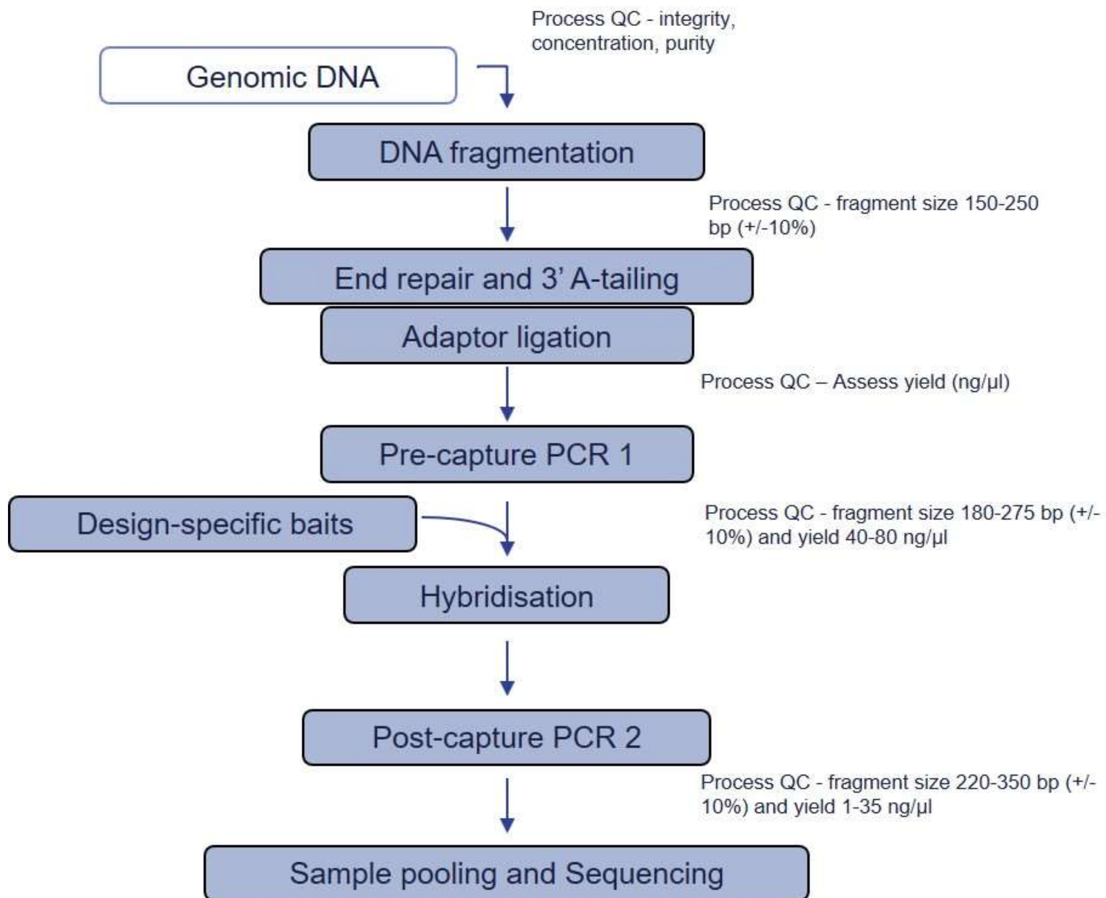


Figure 1: Workflow of sample library preparation indicating the expected DNA fragment size at each step of the procedure.

4 Sample requirements

The recommended amount of input DNA for all OGT panels is 1 µg but have been shown to work with as little as 50 ng high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE sample. Data quality can be adversely affected by the quality of DNA samples, see Sample Quality Control for further details.

4.1 Sample thoughput

- Low-throughput (LT) protocol: all incubations are performed in 0.2 ml tubes. Post incubation, each reaction volume is transferred to a fresh 1.5 ml tube and sample clean-up performed using the volumes highlighted in blue (marked with ♦). All wash steps are performed using a magnetic rack capable of holding 1.5 ml tubes.
- High-throughput (HT) protocol: all incubations are performed in 0.2 ml tubes or a non-skirted PCR plate. Post incubation, the sample purification is performed in the same 0.2 ml tube or non-skirted PCR plates using volumes highlighted in red (marked with •). All wash steps are performed using a magnetic rack, capable of holding 0.2 ml tubes or a 96-well non-skirted PCR plate (0.2 ml volume).

5 Sample Quality Control

Testing sample integrity, concentration and purity:

- Concentration — Use Invitrogen Qubit (or similar)
- DNA integrity — Use Agilent Genomic DNA ScreenTape (cat. no. 5067-5365) and Genomic DNA Reagents (cat. no. 5067-5366) (or similar)
- Purity — Use Thermo Scientific NanoDrop (or similar)

Determination of the concentration of gDNA sample is mandatory for all samples prior to starting the SureSeq NGS Library Preparation protocol. In addition, we highly recommend that further quality control (QC) assessments are carried out for lower-quality DNA from FFPE samples.

5.1 Concentration — Qubit

5.1.1 Assessing DNA concentration

1. Prepare the Qubit working solution by diluting the Qubit reagent 1:200 in Qubit buffer.
2. Load 190 µl of Qubit working solution into each of the tubes used for standards and 199 µl of Qubit working solution into each of the tubes used for samples.
3. Add 10 µl of each Qubit standard and 1 µl of sample to the appropriate tubes.
4. Mix by vortexing for 2–3 sec being careful not to generate bubbles.
5. Incubate the tubes at room temperature for 2 min.
6. Measure DNA concentrations following the onscreen prompts.

5.2 DNA integrity — Genomic DNA TapeStation

5.2.1 Assessing DNA integrity

1. Add 3 µl of Genomic DNA Ladder into the first tube/well of the strip tube or plate.
2. Add 10 µl of Genomic DNA Sample Buffer to as many additional tubes/wells as required.
3. For each sample under assessment, add 1 µl of DNA sample to 10 µl of Genomic DNA Sample Buffer. Seal all the tubes/wells.
4. Vortex the tubes or plate for 5 sec.
5. Briefly spin down to consolidate the sample to the bottom of the tubes/wells.
6. Load strip of tubes or plate into the Agilent 2200 TapeStation.
7. Highlight the required samples on the controller software and fill in the sample names in the sample sheet.
8. Select “Start” and provide a filename to save your results.
9. Check that the electropherogram shows that the integrity of the Genomic DNA is intact with an even distribution and peak height >1000 bp.

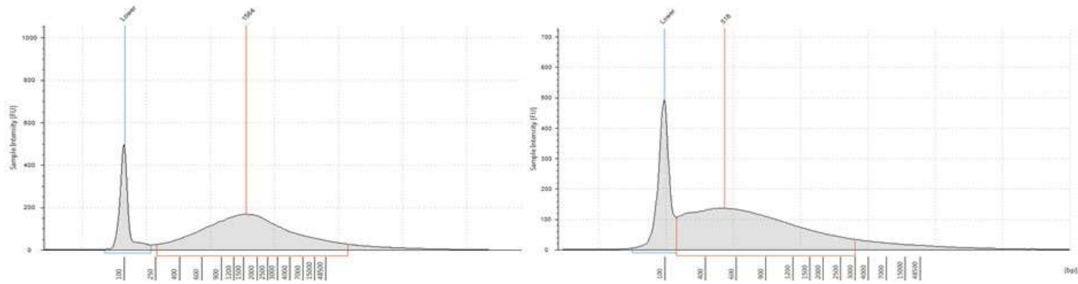


Figure 2: Assessment of DNA integrity using Genomic DNA ScreenTape. Electropherogram on the left shows an intact FFPE sample with an even distribution and a peak height >1000 bp, the electropherogram on the right shows a degraded FFPE sample with a peak height <1000 bp.

5.3 Purity — NanoDrop

5.3.1 Assessing DNA purity

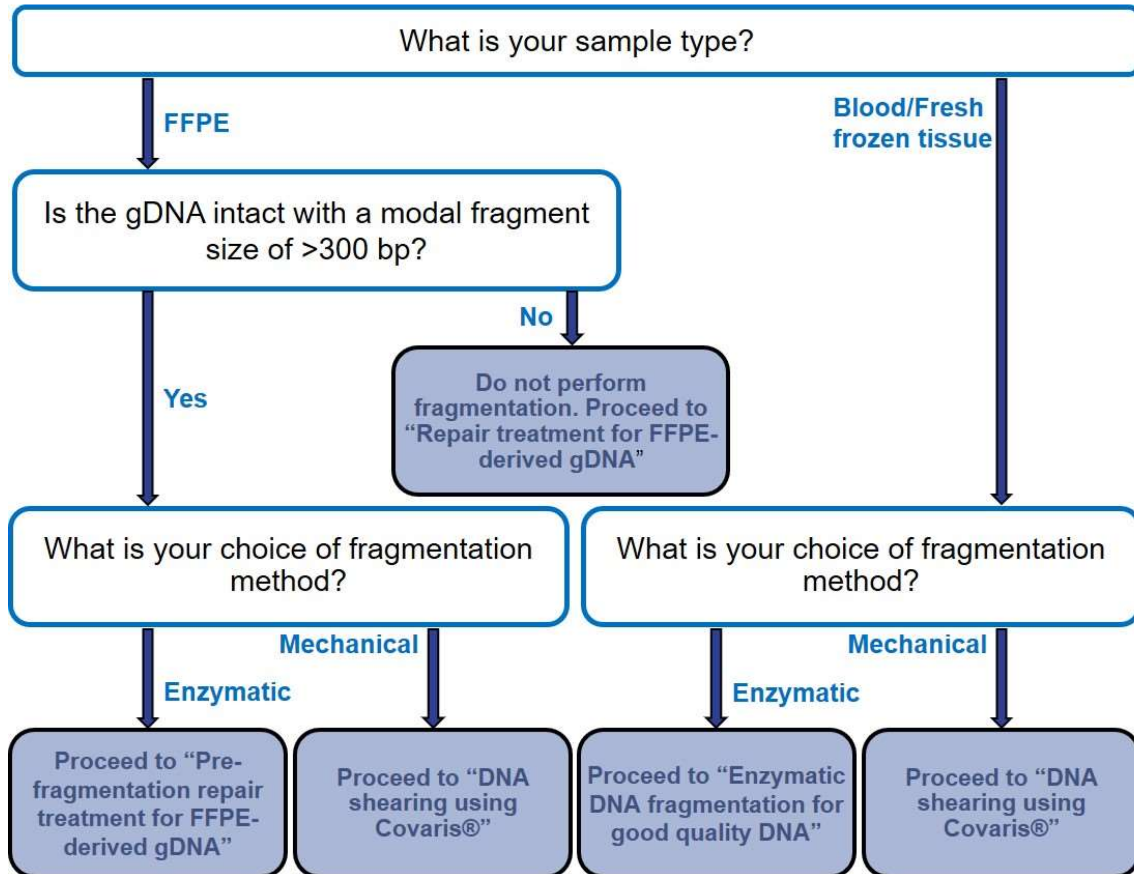
1. Load 1 µl of each sample onto the pedestal.
2. Click “Measure”.
3. Record the readings for 260/230, 260/280 and the concentration (ng/µl).

An OD 260/280 ratio of 1.8 to 2.0 and OD 260/230 ratio of 1.5 to 1.8 is recommended. Use of DNA samples with lower ratios may result in poor performance.

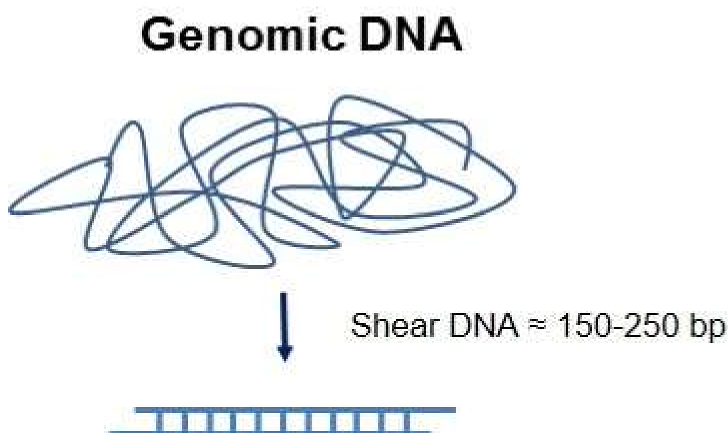
6 DNA fragmentation

This handbook includes options for DNA fragmentation by mechanical shearing and enzymatic DNA fragmentation prior to library preparation.

Prepare gDNA according the instructions below.



7 Enzymatic DNA fragmentation for good quality gDNA



Genomic DNA is enzymatically fragmented using double-stranded Fragmentase to prepare fragments of suitable size. We recommend that NEBNext dsDNA Fragmentase is used with the OGT SureSeq panel protocol. Post-enzymatic treatment, the DNA fragments should have a size distribution with a peak at between 150-250 bp.

Estimated time: 20 min for 8 - 24 samples. Hands-on time: 10 min.

7.1 Preparation

- Take the AMPure XP beads out of the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Remove the Fragmentase Reaction Buffer from storage (–15°C to –25°C). Allow to thaw to room temperature. Do not place on ice.
- Remove the dsDNA Fragmentase from storage (–15°C to –25°C). Mix for 3 sec on a vortex mixer prior to use, spin down and place on ice.
- Have the 0.5 M EDTA on the bench ready for step 7.

7.2 DNA Fragmentation

1. Prepare DNA with nuclease-free water in 0.2 ml tubes to a total volume of 16 µl. Keep the DNA sample on ice.
2. Prepare the Fragmentation Master Mix as shown in Table 1. Keep all reagents on ice.

Reagent	1x library (µl)	___ x library (µl)	16x library (µl)
DNA sample	16	-	-
10x Fragmentase Reaction Buffer v2	2		34

Reagent	1x library (µl)	___ x library (µl)	16x library (µl)
dsDNA Fragmentase	2		34
TOTAL	**20**		**68**

Table 1 Fragmentation Master Mix

Note: Ensure the enzyme is mixed well on a vortex mixer for 3 sec prior to use.

3. Mix well on a vortex mixer.
   Note: When a master mix is prepared for multiple samples, it is recommended that the mix is added to the samples using a multichannel pipette. If no master mix is prepared, then ensure that the fragmentation buffer is always added first to the reaction tube.
4. Add 4 µl of Fragmentation Master Mix to each well or tube, vortex the mixture for 3 sec and spin down. Keep the samples on ice.
5. Preheat the thermocycler to 37°C.
6. Place the samples on the thermocycler and incubate at 37°C for 30 min.
7. Add 5 µl 0.5 M EDTA and pipette mix to stop the reaction.
8. Proceed immediately to post-fragmentation purification.

7.3 Post-fragmentation purification

Estimated time: 40 min for 8 - 24 samples.

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 45 µl of homogenous AMPure XP beads to each fragmented DNA sample (in either 1.5 ml LoBind tubes or 0.2 ml tubes/96-well plate). Mix well using a vortex mixer (1.5 ml tube) or pipetting up and down at least 10 times (0.2 ml tubes/plate). Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand/rack whilst adding ♦ 500 µl (or • 200 µl) of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for approximately 1 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 29 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove 27 µl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.

13. The quality and quantity of DNA obtained after fragmentation should be assessed using the High Sensitivity Agilent 2200 TapeStation, Agilent Bioanalyser or similar. An example of a good DNA trace is shown in Figure 3. Ensure a distribution with a peak height of 150-250 bp is achieved. Set up the machine and prepare the tape, samples and ladder following the manufacturer's instructions.

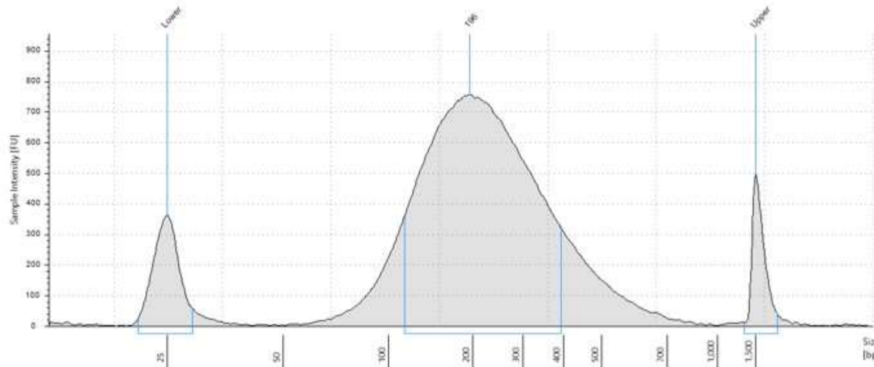


Figure 3: Analysis of fragmented DNA using an Agilent High Sensitivity D1000 ScreenTape assay. Size distribution with a peak between 150 to 250 bp (+/– 10%).

OPTIONAL STOPPING POINT: If the samples are not to be used immediately for the end repair step, store them at 4°C overnight or at –20°C for long-term storage. If continuing proceed to End repair and 3' end 'A' Tailing

8 Pre-fragmentation repair treatment for FFPE-derived gDNA

Estimated time: 60 min for 8 - 16 samples. Hands-on time: 35 min.

8.1 Preparation

- Remove the SureSeq FFPE Repair Mix reagents (white cap) from storage (–15°C to –25°C). Place the repair mix enzyme (RM500079) on ice and allow the repair buffer (RB500079) to thaw to room temperature.

8.2 Prepare SureSeq FFPE Repair Master Mix

- To process multiple samples, prepare a master mix on ice. The volume of each reagent to add to the master mix for processing 16 samples (including excess) is shown below as an example.
- For multiple samples, prepare the reaction mix as shown in Table 2. Mix well on a vortex mixer.

8.3 Repair damaged DNA

1. Prepare DNA with nuclease-free water in 0.2 ml tubes to a total volume of 16 µl.
2. Prepare the FFPE repair master mix as shown in Table 2.

Reagent	1x library (µl)	___ x library (µl)	16 x library (µl)
FFPE gDNA sample	16	-	-
Repair buffer (RB500079) (white cap)	2		34
Repair mix enzyme (RM500079) (white cap)	2		34
TOTAL	**20**		**68**

Table 2 SureSeq FFPE Repair Master Mix

3. Add 4 µl of the reaction master mix to each well or tube.
4. Add 16 µl of each DNA sample to the relevant well or tube.
5. Mix by pipetting 10 times, remembering to change pipette tips between samples.
6. Incubate at 20°C for 15 min (no heated lid).

8.4 Post-repair purification

Estimated time: 40 min for 8–16 samples.

1. Use only room temperature AMPure XP beads.

2. Mix the AMPure XP bead solution well so that the reagent appears homogeneous and consistent in colour.
3. Add 60 µl of homogeneous AMPure beads to each repaired DNA sample. Mix well by pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic rack and wait for the solution to clear (which should take approximately 3-5 min).
5. Keep the tube in the magnetic rack. Do not touch the beads whilst carefully removing 75 µl of the cleared solution.
6. Continue to keep the tube in the magnetic rack whilst adding 200 µl of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 9 and step 10).
9. Return the tube or plate to the magnetic rack and wait 1 min. Remove any remaining ethanol using a P20 pipette and tip, being careful to not touch the bead pellet.
10. Dry the samples on a 37°C heat block for 3–5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
    IMPORTANT: Do not over-dry as this will decrease yield.
    Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
11. Add 18 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer and incubate for 3 min at room temperature.
12. Spin the tube briefly, place on the magnetic stand and leave for 2–3 min until the solution is clear.
13. Remove 16 µl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
    OPTIONAL STOPPING POINT: If the samples are not to be used immediately for the fragmentation step, store them at 4°C overnight or at –20°C for long-term storage. If continuing proceed to Fragmentation of FFPE samples using Fragmentase.

9 Fragmentation of FFPE samples using Fragmentase

Genomic DNA is enzymatically fragmented using double-stranded Fragmentase to prepare fragments of suitable size for use with the Illumina NGS system. Post-enzymatic treatment, the DNA fragments should have a size distribution with a peak between 230-300 bp.

Estimated time: 5 - 25 min.

9.1 Preparation

- Take the AMPure XP beads out of the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Remove the Fragmentase Reaction Buffer from storage (-15C to -25C). Allow to thaw at room temperature.
- Remove the dsDNA Fragmentase from storage (-15 to -25C). Mix for 3 sec on a vortex prior to use, spin down and place on ice.
- Have the 0.5 M EDTA at the bench ready for step 7.
- Fill an ice box with ice.

9.2 Choosing fragmentation conditions

- Fragmentation length will vary depending on the quality of the input DNA.
- Use Table 3 to determine the appropriate incubation lengths:

DNA Integrity Score	Modal fragment length (bp)	Severity of damage	Incubation length (min)
> 5.5	> 3000	Mild	25
2.5 – 5.5	1000 – 3000	Moderate	20
1.5 - 2.5	300 - 1000	Poor	5
< 1.5	> 300	Severe	No fragmentation

Table 3 Incubation times for FFPE depends on the severity of DNA damage as measured by DNA score and/or modal fragment length

1. Prepare the Fragmentation Master Mix as shown in Table 4. Keep all reagents on ice.

Reagent	1x library (µl)	___ x library (µl)	16 x library (µl)
DNA sample	16	-	-
10x Fragmentase Reaction Buffer v2	2		34
dsDNA Fragmentase	2		34

Reagent	1x library (µl)	___ x library (µl)	16 x library (µl)
TOTAL	20		**68**

Table 4 Fragmentation Master Mix

2. Mix well on a vortex mixer.
   Note: When a master mix is prepared for multiple samples, it is recommended that the mix is added to the samples using a multichannel pipette. If no master mix is prepared, then ensure that the fragmentation buffer is always added first to the reaction tube.
3. Add 4 µl of Fragmentation Master Mix to each well or tube, vortex the mixture for 3 sec and spin down. Keep the samples on ice.
4. Preheat the thermocycler to 37°C.
5. Place the samples on the thermocycler and incubate at 37°C for the length indicated in Table 3.
6. Add 5 µl 0.5 M EDTA and pipette mix to stop the reaction.
7. Proceed immediately to post-fragmentation purification.

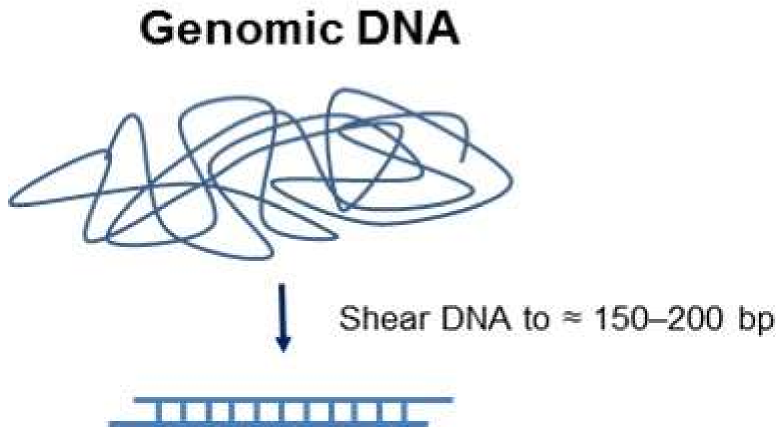
9.3 Post-fragmentation purification

Estimated time: 40 min for 8 - 24 samples.

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 45 µl of homogenous AMPure XP beads to each fragmented DNA sample (in either 1.5 ml LoBind tubes or 0.2 ml tubes/96-well plate). Mix well using a vortex mixer (1.5 ml tube) or pipetting up and down at least 10 times (0.2 ml tubes/plate). Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand/rack whilst adding ♦ 500 µl (or • 200 µl) of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for approximately 1 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 29 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove 27 µl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
13. The quality and quantity of DNA obtained after fragmentation should be assessed using the High Sensitivity Agilent 2200 TapeStation, Agilent Bioanalyser or similar. Ensure a distribution with a peak height of 230-300 bp is achieved. Set up the machine and prepare the tape, samples and ladder following the manufacturer's instructions.

OPTIONAL STOPPING POINT: If the samples are not to be used immediately for the end repair step, store them at 4°C overnight or at –20°C for long-term storage. If continuing proceed to End repair and 3' end 'A' Tailing

10 DNA shearing using Covaris



Genomic DNA is mechanically sheared using a sonicator to prepare fragments of suitable size for use with the Illumina NGS system. The sheared DNA fragments should have a size distribution with a peak at between 150-200 bp.

Estimated time: 6 min shearing per sample.

10.1 Preparation

- Take the AMPure XP beads out of the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Refer to the Covaris instrument user guide for set up. For example, (for a Covaris S2/E210 System);
    - Fill the Covaris tank with fresh deionised water to level 12 on the fill line label.
    - When a Covaris microTUBE is inserted ensure the water covers the visible glass part of the tube.
    - Set the chiller temperature to 4°C.
    - Open the Covaris control software. Degassing should start automatically but if not, select the Degas button. Degas the instrument for at least 30 min before use.

10.2 Shear the DNA

1. Prepare DNA with 1x TE Buffer in a 1.5 ml LoBind tube to a total volume of 130 µl or 50 µl depending on starting amount and type of gDNA. See Table 5 for details.
2. Put a Covaris microTUBE into the loading and unloading station. Keep the cap on the tube.
3. Use a tapered pipette tip to slowly transfer the DNA sample through the pre-split septum. Be careful not to introduce a bubble into the bottom of the tube.
4. Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 5. The target peak for base pair size is 150 to 200 bp.

Settings	Value for S2/E210 or S220/E220		Value for S2/E210 or S220/E220	Value for M220
Sample type	DNA from fresh/frozen tissue		DNA from FFPE tissue	DNA from all tissue types
Sample input (ng)	200-1000 ng	<200 ng	Up to 1000ng	Up to 1000ng
Sample volume (µl)	130	50	50	130
Duty Cycle/Factor	10%	10%	10%	20%
Intensity/Peak Incident Power (W)	5/175	5/175	5/175	50 (W)
Cycles per Burst	200	200	200	200
Time	6 cycles of 1 min each	180 sec	120 sec	150 sec
Temperature	4–7°C	4–7°C	4–7°C	20°C

Table 5 Covaris shear settings for the S2/E210, S220/E220, or M220 Focused ultrasonicator

Note: For all other models or sonicators, see manufacturer's instructions for settings to achieve a target peak between 150-200 bp.

5. Place the microTUBE into an appropriately sized tube adapter and spin in a picofuge for 5 sec to collect all liquid in the bottom of the microTUBE.
6. Put the Covaris microTUBE back into the loading and unloading station.
7. While keeping the snap-cap on, insert a tapered pipette tip through the pre-split septa, and then slowly remove the sheared DNA.
   IMPORTANT: If starting with less than 500 ng it is important to collect all liquid in the microTUBE.
8. Transfer the sheared DNA into a new 1.5 ml LoBind tube.
   Note: Purification post-shearing is the same for both LT and HT protocols.
9. If the sample type is DNA from FFPE tissues, proceed to FFPE repair, if not proceed to Post-shear purification (Covaris®).

11 Repair treatment for FFPE-derived gDNA

Estimated time: 60 min for 8-16 samples. Hands-on time: 35 min.

11.1 Preparation

- Remove the SureSeq FFPE Repair Mix reagents (white cap) from storage (–15°C to –25°C). Place the repair mix enzyme (RM500079) on ice and allow the repair buffer (RB500079) to thaw to room temperature.

11.2 Prepare SureSeq FFPE Repair Master Mix

- To process multiple samples, prepare a master mix on ice. The volume of each reagent to add to the master mix for processing 16 samples (including excess) is shown below as an example.
- For multiple samples, prepare the reaction mix as shown in Table 6. Mix well on a vortex mixer.

11.3 Repair damaged DNA

1. Prepare the FFPE repair master mix as shown in Table 6.

Reagent	1x library (µl)	___ x library (µl)	16x library (µl)
Sheared/degraded DNA sample	50	-	-
TE	3.5		59.5
Repair buffer (RB500079) (white cap)	6.5		110.5
Repair mix enzyme (RM500079) (white cap)	2		34
TOTAL	**62**		**204**

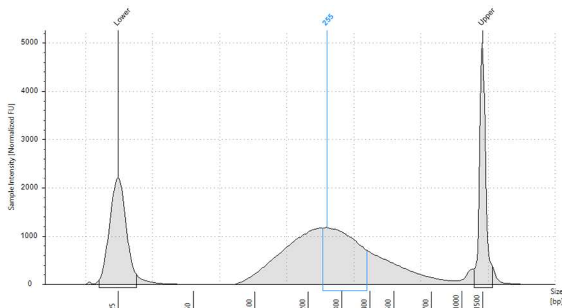
Table 6 SureSeq FFPE Repair Master Mix

2. Add 12 µl of the reaction master mix to each well or tube.
3. Add 50 µl of each DNA sample to the relevant well or tube.
4. Mix by pipetting 10 times, remembering to change pipette tips between samples. Incubate at 20°C for 15 min (no heated lid).

11.4 Post-repair purification for FFPE gDNA

Estimated time: 40 min for 8 - 16 samples

1. Use only room temperature AMPure XP beads.
2. Mix the AMPure XP bead solution well so that the reagent appears homogeneous and consistent in colour.

3. Add 186 µl of homogeneous AMPure beads to each repaired DNA sample. Mix well by pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic rack and wait for the solution to clear (which should take approximately 3-5 min).
5. Keep the tube in the magnetic rack. Do not touch the beads whilst carefully removing 240 µl of the cleared solution.
6. Continue to keep the tube in the magnetic rack whilst adding 200 µl of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 9 and step 10).
9. Return the tube or plate to the magnetic rack and wait 1 min. Remove any remaining ethanol using a P20 pipette and tip, being careful to not touch the bead pellet.
10. Dry the samples on a 37°C heat block for 3–5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
    IMPORTANT: Do not over-dry as this will decrease yield.
    Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
11. Add 29 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer and incubate for 3 min at room temperature.
12. Spin the tube briefly, place on the magnetic stand and leave for 2–3 min until the solution is clear.
13. Remove 27 µl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
14. Optional: *The quality and quantity of DNA obtained after shearing should be assessed using the Agilent 2200 TapeStation, Agilent Bioanalyzer or similar. An example of a good DNA trace is shown in Figure 2. Ensure a distribution with a peak height of 150-200 bp is achieved. Set up the machine and prepare the tape, samples and ladder following the manufacturer's instructions.*
15. 

16. **Figure 5: Analysis of sheared and repaired DNA using an Agilent D1000 ScreenTape assay. Size distribution with a peak between 230 to 260 bp (+/– 10%).**

    OPTIONAL STOPPING POINT: If the samples are not to be used immediately for the end repair step, store them at 4°C overnight or at –20°C for long-term storage.
17. Proceed to End repair and 3' end 'A' Tailing.

12 Post-shear purification (Covaris)

Estimated time: 40 min for 8 - 24 samples.

1. Use only room temperature AMPure XP beads.
2. Mix well so that the AMPure XP beads appear homogeneous and consistent in colour.
3. If shearing was performed in 130 μl add 180 μl of homogenous AMPure XP beads to each sheared DNA sample in 1.5 ml LoBind tubes. If performed in 50 μl add 70 μl of homogenous AMPure XP beads to each sheared DNA sample in 1.5 ml LoBind tubes
4. Mix well using a vortex mixer or by pipetting up and down at least 10 times and incubate at room temperature for 5 min.
5. Put the tube in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
6. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
7. Continue to keep the tube in the magnetic stand while you dispense 500 μl of 70% ethanol in each tube.
8. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
9. Repeat wash (step 7 and step 8).
10. Dry the samples on a 37°C heat block for 3–5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
    IMPORTANT: Do not over-dry as this will decrease yield.
    Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
11. Add 29 μl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
12. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
13. Remove 27 μl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
14. Optional: *The quality and quantity of DNA obtained after shearing should be assessed using the Agilent 2200 TapeStation, Agilent Bioanalyser or similar. An example of a good DNA trace is shown in Figure 4. Ensure a distribution with a peak height of 150-200 bp is achieved. Set up the machine and prepare the tape, samples and ladder following the manufacturer's instructions.*

IMPORTANT: If starting with 500 ng, assess the quality and quantity using High-Sensitivity kits.

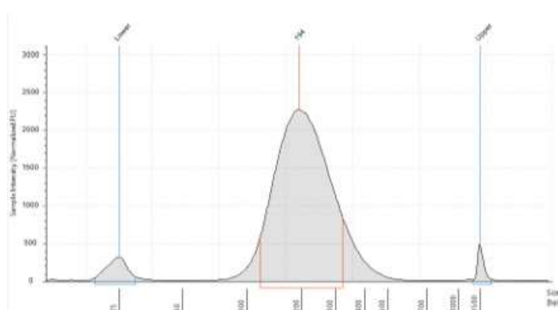
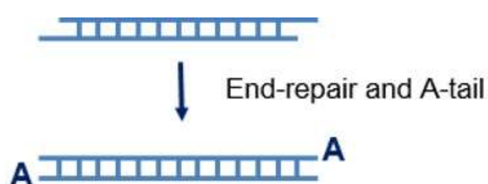


Figure 4: Analysis of sheared DNA using an Agilent High Sensitivity D1000 ScreenTape assay. Size distribution with a peak between 150 to 200 bp (+/– 10%).

OPTIONAL STOPPING POINT: If the samples are not to be used immediately for the end repair step, store them at 4°C overnight or at –20°C for long-term storage. If continuing proceed to End repair and 3' end 'A' Tailing

13 End repair and 3' end 'A' Tailing



The sheared dsDNA is repaired with enzymes in the Step 1: ER enzyme mix to create blunt ends. At the same time a 3' adenine overhang is created in preparation for adaptor ligation.

Estimated time: 45 min for 8 - 16 samples. Hands-on time: 15 min

13.1 Preparation

- Remove the Step 1 reagents (green cap) from storage (–15°C to –25°C). Place all Step 1 enzymes on ice and allow all the other Step 1 reagents to thaw to room temperature.

13.2 Prepare End Repair/'A' Tailing Master Mix

- To process multiple samples, prepare a master mix on ice. The volume of each reagent to add to the master mix for processing 16 samples (including excess) is shown below as an example.
- For multiple samples, prepare the reaction mix as shown in Table 7. Mix well on a vortex mixer.

Reagent	1x library (µl)	___ x library (µl)	16x library (µl)
DNA sample	25	-	-
Nuclease-free Water	9		153
Step 1 (green cap): ER Buffer	10		170
Step 1 (green cap): ER Enzyme Mix	6		102
TOTAL	**50**		**425**

Table 7 End Repair Mix/'A' Tailing Master Mix

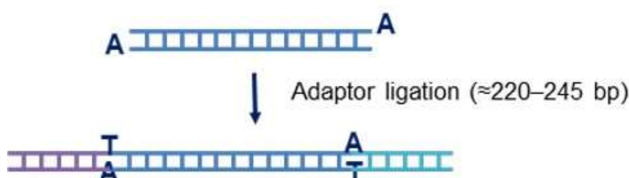
1. Add 25 µl of the reaction mix to each well or tube containing the sheared/fragmented DNA. Mix by pipetting 10 times. Change pipette tips between samples.
2. Set up the incubation using the profile and settings as shown in Table 8.

Step	Temperature (°C)	Time
1	20	30 min
2	72	30 min
3	4	Hold

Table 8 Incubation profile

IMPORTANT: Ensure the samples are held at 4°C for 5–10 min before proceeding immediately with Adaptor ligation

14 Adaptor ligation



Illumina compatible adaptor sequences are ligated onto the repaired dsDNA fragments with Step 2: Ligase, using the 3' overhang created during end repair/A tailing

Estimated time: 30 min for 8 - 16 samples. Hands-on time: 15 min.

14.1 Preparation

Remove the Step 2 reagents (yellow cap) from storage (–15°C to –25°C). Place the Step 2 enzyme on ice and allow all other Step 2 reagents to thaw to room temperature.

14.2 Prepare Ligation Master Mix

1. Prepare a master mix (on ice) based on the starting input DNA using the volumes shown in Table 9. Mix well on a vortex mixer.
2. Add 15 µl of the reaction mix to each well or tube on ice. Mix by pipetting 10 times. Change pipette tips between samples.
3. Incubate in a thermal cycler for 15 min at 20°C. **Do not use a heated lid.**

IMPORTANT: Proceed immediately to ligated library purification.

Starting DNA	**1x (1 µg)**	**1x (0.5–0.99 µg)**	**1x (0.499–0.25 µg)**	**1x (0.25-0.1 µg)**	**1x (<0.1 µg)**	**___ x library (µl)**
DNA sample	50 µl	50 µl	50 µl	50 µl	50 µl	-
Step 2 (yellow cap): Ligase Buffer	3 µl	3 µl	3 µl	3 µl	3 µl	
Step 2 (yellow cap): Adaptor A	5 µl	1.5 µl	1 µl	0.5 µl	0.25 µl	
Step 2 (yellow cap): Adaptor B	5 µl	1.5 µl	1 µl	0.5 µl	0.25 µl	
Step 2 (yellow cap): Ligase	2 µl	2 µl	2 µl	2 µl	2 µl	
Nuclease-free Water	0 µl	7 µl	8 µl	9 µl	9.5 µl	
TOTAL	65 µl	65 µl	65 µl	65 µl	65 µl	

Table 9 Ligation reaction mixes

14.3 Ligation library purification

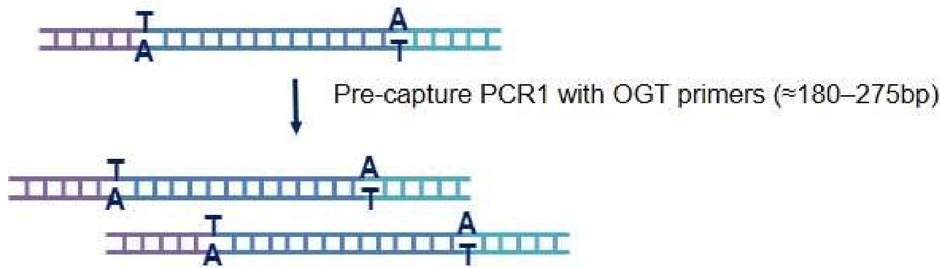
Estimated time: 40 min for 8 - 16 samples.

1. Use only room temperature AMPure XP beads.

2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 117 µl of homogenous AMPure XP beads to each DNA sample (in either 1.5 ml LoBind tubes or 0.2 ml tubes/96-well plate). Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand/rack whilst adding ♦ 500 µl (or • 200 µl) of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 32 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove 30 µl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
13. Assess yield using the Qubit BR Kit as per manufacturer's instructions.
    IMPORTANT: If starting with <500 ng and/or the DNA was fragmented enzymatically assess the yield using a Qubit High-Sensitivity kit.

OPTIONAL STOPPING POINT: If the samples are not to be used immediately, store at 4°C. If continuing proceed to Pre-capture PCR.

15 Pre-capture PCR

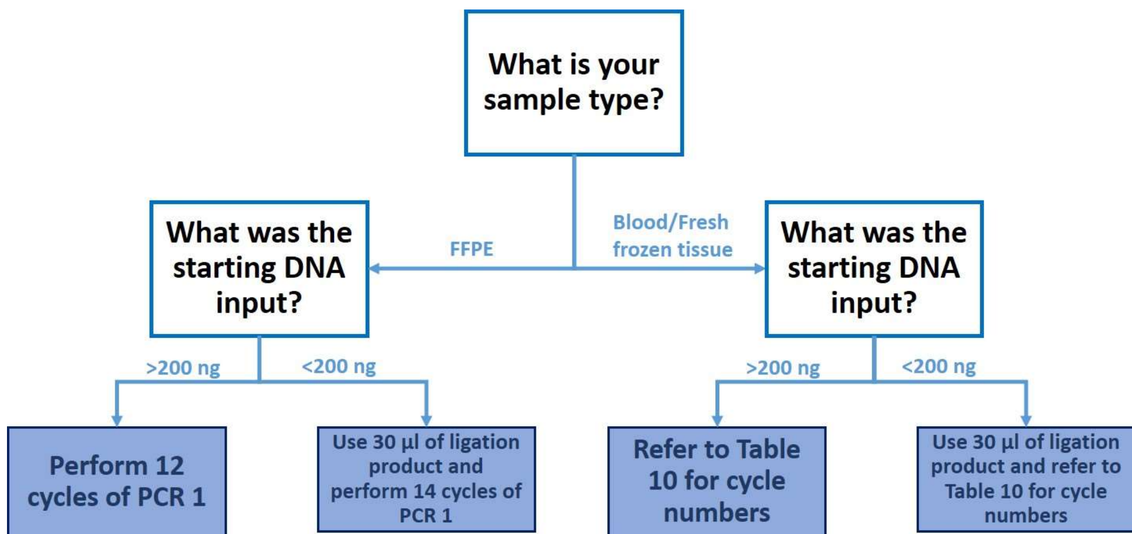


High fidelity PCR is used to amplify the DNA library prior to hybridisation and target capture. The number of PCR cycles is minimised to reduce amount of duplicate reads (PCR copies of the same original DNA fragment) in the sequence data.

Estimated time: 40 - 90 mins for 8 - 16 samples. Hands-on time: 15 min.

15.1 Preparation

Remove the Step 3 (orange cap) reagents from storage (–15°C to –25°C), place the Step 3 enzyme on ice and allow all other Step 3 reagents to thaw to room temperature.
The number of cycles (from step 2 to step 4) required can be determined using the guidelines in Table 10 and the decision chart below.



DNA concentration (ng/µl) post adaptor ligation	Cycle numbers	Average expected duplication following sequencing*
>9 ng	6	≤10%
2–8 ng	8	10–30%
1–1.9 ng	10	30–40%
>1 ng	12 Always use 30 µl of ligation product	40+%

DNA concentration (ng/µl) post adaptor ligation	Cycle numbers	Average expected duplication following sequencing*
FFPE	12 or 14	variable

Table 10 Cycle numbers — based on results from the Qubit HS/BR Kit post adaptor ligation purification. If starting material is from FFPE tissue then carry out 12 or 14 cycles of PCR according to decision chart above.

* These values are very approximate and can vary significantly dependent on the size and type of bait library used. These percentage duplication values will be higher for poor quality samples.

15.2 Prepare PCR Master Mix

1. For multiple samples, prepare the reaction mixes as shown in Table 11 on ice and mix well on a vortex mixer.
2. Add 15 or 30 µl of each ligated DNA sample to the relevant well or tube.

Reagent	1x library (µl)		___ x library (µl)	16x library (µl)
Ligated Library*	15	30	-	-
Nuclease-free Water*	23	8		391/136
Step 3 (orange cap): PCR Buffer	5			85
Step 3 (orange cap): Primer Mix	5			85
Step 3 (orange cap): DNA Polymerase	2			34
TOTAL	**50**			**595**

Table 11 Components for PCR reaction mix

*See decision chart above and Table 10 for further information on the volume of ligated library and Nuclease-free Water to use.

3. Add 35 or 20 µl of the master mix to each well or tube on ice. Mix by pipetting 10 times. Change pipette tips between samples.
4. Set up PCR using the profile and settings as shown in Table 12.
5. Place the tubes in a thermal cycler and run the PCR programme.

Step	Temperature (°C)	Time
1	98	3 min
2	98	30 sec
3	65	30 sec
4	72	1 min
5	Repeat Step 2 to Step 4 (for cycle numbers see Table 10)	
6	72	10 min
7	4	Hold

Table 12 PCR Profile

15.3 PCR 1 purification

Estimated time: 40 min for 8 - 16 samples

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 90 µl of homogenous AMPure XP beads to each DNA sample (in either 1.5 ml LoBind tubes or 0.2 ml tubes/96-well plate). Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand/rack whilst adding ♦ 500 µl (or • 200 µl) of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 32 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove 30 µl of the supernatant and transfer to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
13. Optional: *Assess the quality of the DNA with an Agilent DNA1000 TapeStation and check that the electropherogram shows a distribution with a peak height of 180-275 bp (+/– 10%) (Figure 4). Setup the instrument and prepare the tape, samples and ladder following manufacturer's instructions.*
14. Assess the quantity using Qubit BR kit. The expected yield is between 40 – 80 ng/µl.
15. Calculate the amount of sample required for 500 or 1000 ng (panel and starting input of gDNA dependant) to proceed to hybridisation.

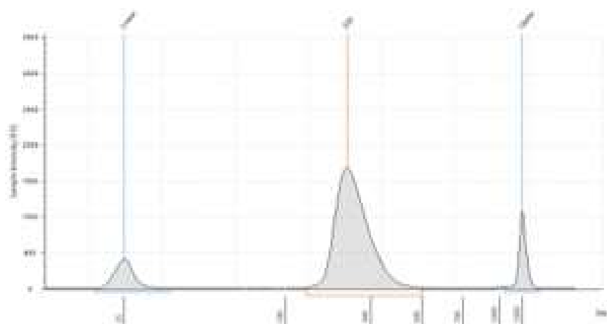
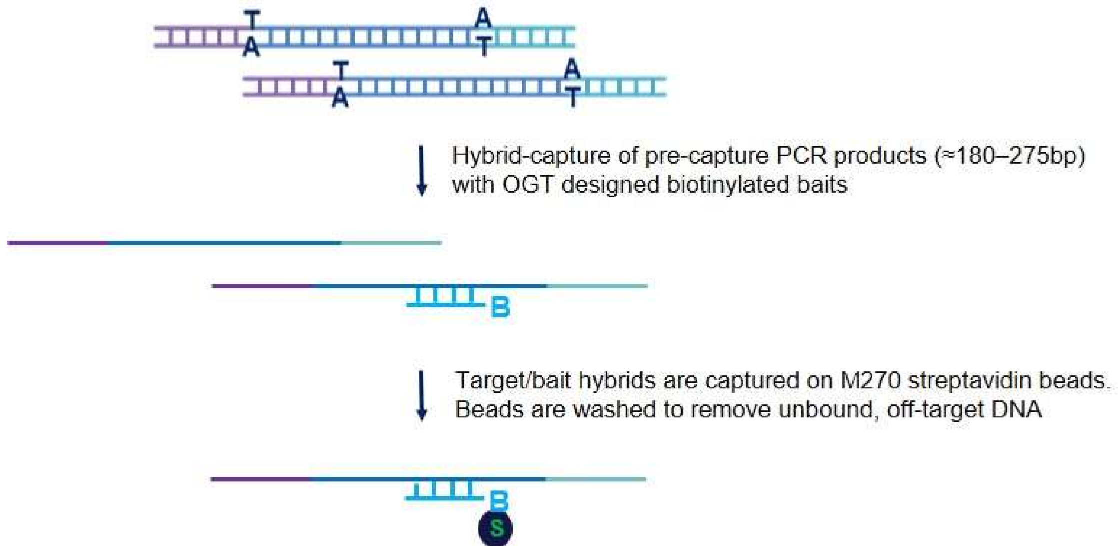


Figure 5: Analysis of amplified prepared library DNA using an Agilent D1000 ScreenTape assay. The electropherogram shows a single peak in the size range of 180 to 275 bp (+/– 10%). FFPE samples will also give a single peak but in a smaller size range of 160 to 200 bp (+/– 10%).

OPTIONAL STOPPING POINT: If the samples are not to be used immediately, store at 4°C overnight or at –20°C for long-term storage. If continuing proceed to Hybridisation

16 Hybridisation



The amplified library is denatured and captured by SureSeq (biotinylated) baits. The hybridised targets are then bound to streptavidin beads and washed to remove any off-target DNA.

16.1 Before you begin

- It is highly recommended to test the hybridisation conditions (thermal cycler and plasticware) to ensure minimal evaporation occurs during the 4 hr incubation.
- To test, add 15 µl of OGT Hybridisation Buffer (without DNA) in each well that you might use and incubate at 65°C for 4 hr. Check after 4 hr that the evaporation does not exceed 1–2 µl per well.
- The minimum required hybridisation time is 30 min for CoreMPN or 4 hr for all other SureSeq panels; however, for more flexibility this can be increased up to 17 hr. Please check before you begin.

What is your choice of sample concentration?

Vacuum concentration - SpeedVac or similar

AMPure XP bead-based approach

17 Alternative to vacuum concentration

17.1 Hybridise the library to the baits with alternative to vacuum concentration

Pre-capture library is prepared for hybridisation using an AMPure XP bead-based approach

Estimated time: 4.5 hr for 8 - 16 samples. Hands-on time: 30 min.

17.1.1 Preparation

- Take the AMPure XP beads out of the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Remove the OGT Hybridisation Buffer, Formamide, Cot Human DNA, OGT Blocking Oligos and Nuclease-free Water from storage and allow to come to room temperature. Incubate further at 37°C for 5-10 min to resuspend any precipitates in the OGT Hybridisation Buffer.
- For each DNA sample prepared, carry out one hybridisation capture. The hybridisation reaction requires 500 or 1000 ng of pre-capture library depending on the panel used and the starting amount of gDNA - contact your FAS or support@ogt.com for further details.

17.2 Protocol

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Aliquot 500 or 1000 ng of each prepared library in 0.2 ml tubes.
4. Add 50 µl of AMPure XP beads to the above mixture. Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
5. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
6. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
7. Continue to keep the tube in the magnetic stand/rack whilst adding 200 µl of 70% ethanol to each tube.
8. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matt.
10. While the beads are drying prepare the hybridisation mix at room temperature by combining the reagents detailed in Table 13.

Reagent	1x library (µl)	___ x library (µl)	16 x library (µl)
OGT Hybridisation Buffer	7.5		127.5

Reagent	1x library (µl)	___ x library (µl)	16 x library (µl)
Formamide	3		51
Cot Human DNA	2.5		42.5
OGT Blocking Oligos	0.6		10.2
Nuclease-free Water	2.5		42.5
Total	**16.1**		**273.7**

Table 13 Hybridisation Mix

11. Add 16.1 µl of the Hybridisation Mix (Table 13) directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
12. Place the tube on the magnetic stand and leave for 3–5 min until the solution is clear.
13. Remove 16.0 µl of supernatant and place in a fresh tube containing 2 µl of SureSeq panel-specific baits.
14. Cap the tubes, mix well on a vortex mixer and spin down. The final volume should be 18.0 µl.
15. Place the tubes into the thermal cycler and run the following programme:
    a. 95°C for 5 min
    b. 65°C Hold

    Note: Set the heated lid of the thermal cycler to 105°C to hold the temperature of the plate at 65°C.
16. Make sure all caps are on tightly and all wells are sealed.
17. Incubate the hybridisation mixture for a minimum of 4 hours at 65°C with a heated lid at 105°C.
    Note: The alternative to vacuum concentration method is not compatible with short 30 min hybridisations. A minimum of 4 hours is required.
18. Continue to Hybridisation wash

18 Hybridisation with SureSeq NGS Hyb & Wash Kit

Estimated time: 4.25 hr for 8–16 samples (excluding drying-down time). Hands-on time: 15 min.

18.1 Preparation

- Remove the OGT Bead Wash Buffer, OGT Hybridisation Buffer, OGT Stringency Buffer from storage (–15°C to –25°C), allow to come to room temperature and incubate further at 37°C for 5-10 min to resuspend any precipitates in the buffers (OGT Hybridisation Buffer and OGT Stringency Buffer).
- Remove the Cot Human DNA, OGT Blocking Oligos, Formamide and Nuclease-free Water from storage (–15°C to –25°C) and allow to come to room temperature.
- For each DNA sample prepared, carry out one hybridisation capture.

18.2 Protocol

1. For each library combine the components detailed in Table 14 in a 0.2 ml tube.

Reagent	1 x library (µl)	x library (µl)	16 x library (µl)
500 or 1000 ng DNA library*	-	-	-
Cot Human DNA	5		85
OGT Blocking Oligos	0.6		10.2

Table 14 Pre-hybridisation mix

*The hybridisation reaction requires 500 or 1000 ng of pre-capture library depending on the panel used and the starting amount of gDNA - contact your FAS or support@ogt.com for further details.

2. Dry down the contents of prepared tube(s) completely using a SpeedVac or simliar evaporation device.
3. Resuspend each tube with 2.5 µl nuclease-free water.
Note: For optimal recovery, incubate the sample at 37°C for 20 min and mix occasionally on a vortex or store at 4°C overnight.
4. Prepare the components detailed in Table 15 at room temperature.

Reagent	1 x library (**µl**)	x library (**µl**)	16 x library (**µl**)
OGT Hybridisation Buffer	7.5		127.5
Formamide	3		51
SureSeq Baits	2		34
Total	**12.5**		**212.5**

Table 15 Hybridisation mix

5. Add 12.5 µl of the Hybridisation Mix (Table 15). Cap the tubes, mix well on a vortex mixer and spin down. The final volume should be 15 µl.
6. Place the tubes into the thermal cycler and run the following thermal cycler programme:
    a. 95°C for 5 min
    b. 65°C Hold
7. Make sure all caps are on tightly and all wells are sealed.
8. Incubate the hybridisation mixture for 30 min (coreMPN) or 4+ hours (all other SureSeq panels) at 65°C with a heated lid at 105°C.
9. Continue to Hybridisation Wash

19 Hybridisation Wash with SureSeq NGS Hyb & Wash Kit

Estimated time: 1.25 hr for 8–16 samples. Hands-on time: 15 min.

19.1 Preparation

- Pre-warm a thermal cycler to 65°C for at least 30 min before use.
- Pre-warm a thermal cycler to 35°C for at least 30 min before use.

Note: It is important to maintain the correct temperature; it is recommended that you verify the temperature by using a calibrated thermometer.

- Allow the M270 Streptavidin magnetic beads to equilibrate to room temperature 30 min before use.

19.2 Prepare sequence capture and bead wash buffers

1. Ensure the OGT Bead Wash Buffer and OGT Stringency Buffer are fully thawed.
   Note: Incubate at 37°C for 5-10 min to resuspend any precipitates
2. Aliquot 6 x 200 µl of OGT Stringency Buffer per sample into 0.2 ml strip tubes.
3. Pre-warm the aliquots to the following temperatures in a heat block for a minimum of 30 min before use:

- 3 x 200 µl at 65°C / sample
- 3 x 200 µl at 35°C / sample

19.3 Prepare magnetic beads

1. Vigorously resuspend the Dynabeads M270 Streptavidin magnetic beads on a vortex mixer.
2. Add 100 µl M270 magnetic beads to a 1.5 ml microfuge tube for each hybridisation performed (up to 400 µl of beads can be washed in a single 1.5 ml microfuge tube).
    a. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
    b. Add 200 µl of 1x Bead Wash Buffer per 100 µl beads. Mix briefly on a vortex mixer and place back on the magnetic rack.
    c. Allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
    d. Repeat steps b-c for a total of two washes.
    e. Resuspend the beads in 1x the original volume of 1x OGT Bead Wash Buffer (i.e., for 100 µl bead add 100 µl buffer) and mix on a vortex mixer.
    f. Transfer the beads into a new 0.2 ml tube for each capture reaction (100 µl/sample).
    g. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.

Note: Proceed immediately to the next step. Small amounts of residual bead wash buffer will not interfere with the downstream binding of the DNA to Dynabeads. Do not allow the beads to dry out.

19.4 Hybrid capture

1. After the 30 min (CoreMPN) or 4 hour (all other SureSeq panels) incubation transfer the hybridised samples (15 µl volume) to the prepared streptavidin beads.
2. Mix thoroughly using a vortex and ensure that all the beads are resupended. Pulse spin to collect the contents.
3. Return the tubes to the thermal cycler set to 65°C for 15 or 45 min (see Table 16 for details). Ensure that the heated lid is on (set at 105°C).
4. Mix the tubes well on a vortex mixer for 3 sec with a brief spin every 5 or 15 min to ensure the beads remain in solution (see Table 16 for details). Place back in the thermal cycler set to 65°C until the incubation time is complete.

Note: Proceed immediately to the next step.

SureSeq panel	Hybridisation time	Bead incubation time (min)	Mixing frequency
CoreMPN	30 min	15	Every 5 min
All other SureSeq panels	4 hr to overnight	45	Every 15 min

Table 16 Capture times and mixing frequency for SureSeq panels

19.5 Wash Streptavidin beads to remove unbound DNA

Note: Work quickly to ensure the temperature does not drop much below 65°C. To achieve this, we recommend performing all washes in 0.2 ml capped strip-tubes and using a multichannel pipette.

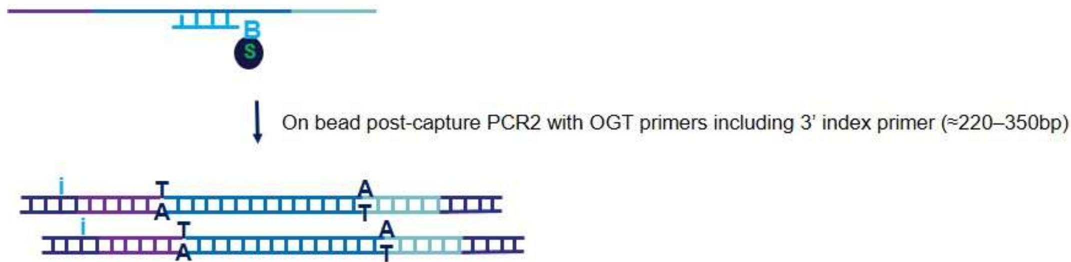
Note: After the addition of fresh buffer ensure the pellet has been fully resuspended by a brief mix on a vortex mixer followed by visual inspection. DO NOT use multichannel pipette for mixing.

1. Place the tubes on a magnetic rack, allow the beads to separate from the supernatant.
2. Carefully remove and discard the supernatant containing unbound DNA.
   Caution: The hybridisation mix contains Formamide.
3. Remove the tubes from the magnetic rack.
4. Add 200 µl of pre-warmed (to 65°C) OGT Stringency Buffer to the capture beads plus bound DNA.
5. Mix briefly on a vortex mixer to disrupt the bead pellet.
6. Place the tubes in a plate vortex mixer set at approximately 2000 RPM and mix for 1 min at room temperature. Briefly spin in a centrifuge to collect the liquid.
7. Return the samples to the thermal cycler set to 65°C for 5 min. (Ensure that the lid is heated above 65°C).
8. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
   Caution: The supernatant may contain trace amounts of Formamide.
9. Remove the tubes from the magnetic rack.
10. Repeat steps 4-9 for a total of three 65°C washes.
11. Add 200 µl of pre-warmed (to 35°C) OGT Stringency Buffer and mix briefly on a vortex mixer to disrupt the bead pellet. Ensure the mixture is homogenous.
12. Place the tubes in a plate vortex mixer and mix vigorously on a plate vortex mixer for 2 min.
    Note: If using a IKA MS 3 Digital Vortex Mixer, set mixing speed to 2000 RPM
13. Briefly spin in a centrifuge to collect the liquid.

14. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
15. Add 200 µl of pre-warmed (to 35°C) OGT Stringency Buffer and mix briefly on a vortex mixer to disrupt the bead pellet. Ensure the mixture is homogenous.
16. Place the tubes in a plate vortex mixer and mix vigorously on a plate vortex mixer for 1 min.
17. Briefly spin in a centrifuge to collect the liquid.
18. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
19. Add 200 µl of pre-warmed (to 35°C) OGT Stringency Buffer and mix briefly on a vortex mixer to disrupt the bead pellet. Ensure the mixture is homogenous.
20. Place the tubes in a plate vortex mixer and mix vigorously on a plate vortex mixer for 30 sec.
    Note: do not mix for more than 30 sec.
21. Briefly spin in a centrifuge to collect the liquid.
22. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
23. Remove from the magnetic rack and resuspend the beads in 30 µl of nuclease-free water, mix thoroughly on a plate vortex mixer.

OPTIONAL STOPPING POINT: If the samples are not to be used immediately, store the bead slurry at 4°C. Do not freeze the bead slurry. If continuing proceed to Post-capture PCR

20 Addition of indexes by post-capture PCR



After capture of target sequences, indexes are added using PCR. The index sequences identify the sample source of each sequence in the sequencing run. The dsDNA PCR products then contain both adaptor sequences and index sequences.

Estimated time: 1.75 hr for 8 - 16 samples. Hands-on time: 15 min.

20.1 Preparation

- Remove the Step 4 reagents (purple cap) from storage (–15°C to –25°C). Place the Step 4 enzyme on ice and allow all other Step 4 reagents to thaw to room temperature.
- Briefly spin a centrifuge the index tube(s)/plate to collect the liquid. To avoid cross-contamination take care when opening the index tube(s)/plate.

20.2 Prepare PCR reaction mixes

For multiple samples, prepare the reaction mix as shown in Table 17, on ice. Mix well on a vortex mixer.

Reagent	1x library (µl)	___x library (µl)	16x library (µl)
Captured DNA and Bead Slurry	14	-	-
Index (1-48)	5	-	-
Nuclease-free H_2O	23		391
Step 4 (purple cap): PCR Buffer	5		85
Step 4 (purple cap): Primer	1		17
Step 4 (purple cap): DNA Polymerase	2		34
TOTAL	**50 µl**		**527**

Table 17 Components for PCR

1. Add 31 µl of the reaction mix to each well or tube.
2. Add 5 µl of the appropriate index PCR Primer to each well and mix by pipetting remembering to use a different index primer for each sample to be sequenced in the same lane.
   Note: If the final sequencing pool is to contain less than eight samples ensure the colour balance for each base on the index read is maintained. See Low Multiplexing Guidelines for further details.
3. Add 14 µl of each DNA sample and bead slurry to the relevant well or tube. Mix by pipetting remembering to change pipette tips between samples to avoid cross-contamination.
4. Set up PCR using the profile and settings as shown in Table 18.

Step	Temperature (°C)	Time
1	98	3 min
2	98	30 sec
3	65	30 sec
4	72	1 min
5	Repeat Step 2 to Step 4 for a total of 20 cycles	
6	72	10 min
7	4	Hold

Table 18 PCR programme

5. Place the tubes in a thermal cycler and run the PCR programme.

20.3 Post-capture PCR purification

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 90 µl of homogenous AMPure XP beads to each DNA sample (in either 1.5 ml LoBind tubes or 0.2 ml tubes/96-well plate). Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand/rack whilst adding ♦ 500 µl (or • 200 µl) of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 32 µl nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove approximately 30 µl of the supernatant to a fresh 1.5 ml LoBind tube or 0.2 ml tubes/plate. The beads can be discarded at this time.
13. Analyse amplified product size using the Agilent TapeStation (High Sensitivity Kit) or similar, to determine the peak size for each DNA sample. The electropherogram should show a peak height of 250-350 bp (+/– 10%) (Figure 5). Set up the instrument and prepare the chip, samples and ladder following manufacturer's instructions.
14. Assess the PCR yield using High Sensitivity dsDNA Qubit assay. The expect yield is panel dependent and between 1 – 35 ng/µl - contact your FAS for further details.

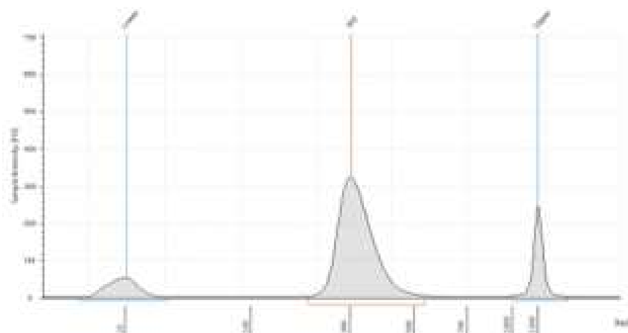


Figure 6: Analysis of amplified capture DNA using an Agilent High Sensitivity D1000 ScreenTape assay. The electropherogram shows a peak in the size range of approximately 220–350 bp (+/– 10%).

OPTIONAL STOPPING POINT: If the samples are not to be used immediately, store them at 4°C overnight or at –20°C for long-term storage.

21 MiSeq Sequencing

The DNA samples prepared in the previous section (Addition of indexes by post-capture PCR) need to be combined such that each index-tagged sample is present in equimolar amounts in the final pool. This requires both accurate determination of peak size (bp), provided by Agilent TapeStation (High-Sensitivity Kit), and accurate determination of sample concentration (ng/µl), provided by Life Technologies Qubit (High-Sensitivity) assay.

21.1 Preparing the sequencing pool

1. To determine the volume (µl) of each indexed DNA sample required generate the 4 nM Sequencing Pool, use the MiSeq pooling template file provided by OGT (or the formulae below).
2. Complete the “Pool Parameter” and “Samples” tables in the “Pool” tab of the MiSeq pooling template. Cells in green should be modified as required; parameters marked with * must be completed.
   Note: This protocol has been validated with 150-base paired-end reads using the MiSeq Reagent Kit v2 300 cycle (cat. no. MS-102-2002).
3. Add the appropriate volume of each indexed DNA sample to a fresh 1.5 ml LoBind tube labelled “4 nM Sequencing Pool”; these can be found in column C of “Volumes to pipette” tab.
4. Adjust the final volume of the sequencing pool with nuclease free water to the desired final concentration (4 nM). This can be found in column C of “Volumes to pipette” tab.
   Optional: *If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C.*
5. Validation of Sequencing Pool concentration — Analyse the Sequencing Pool using an Agilent TapeStation (High Sensitivity Kit) to determine peak height and determine the concentration (ng/µl) yield using High Sensitivity Qubit assay. Complete the “Pool validation and dilution” tab to determine the molar concentration of the Sequencing Pool.
6. The Sequencing Pool can now be prepared for loading on to the MiSeq. Refer to the appropriate Illumina protocol.

As cluster density can vary between machines we recommend loading a final concentration of 8-12 pM if using a V2 300 cycle kit.

21.1.1 Formula 1 – nM of each sample

$$nM = \frac{[\text{Sample concentration (ng/µl)}] \times 10^6}{([\text{Sample size in bp}] \times 660) + 157.9}$$

21.1.2 Formula 2 — Volume of each Indexed DNA Sample

$$\text{Volume of each indexed sample} = \frac{\text{Sequencing Pool volume (µl)} \times \text{pool concentration (4nM)}}{\text{Number of samples in the pool} \times \text{nM concentration of the sample}}$$

22 Preparing the MiSeq Sample Sheet

The MiSeq Sample sheet can be created in Excel using the MiSeq pooling template provided.

1. Open the completed MiSeq pooling sheet (from “Preparing the sequencing pool”) and click on the “Sample sheet” tab
2. Highlight all cells with text as shown in Figure 7. Adjust the number of rows highlighted as appropriate.

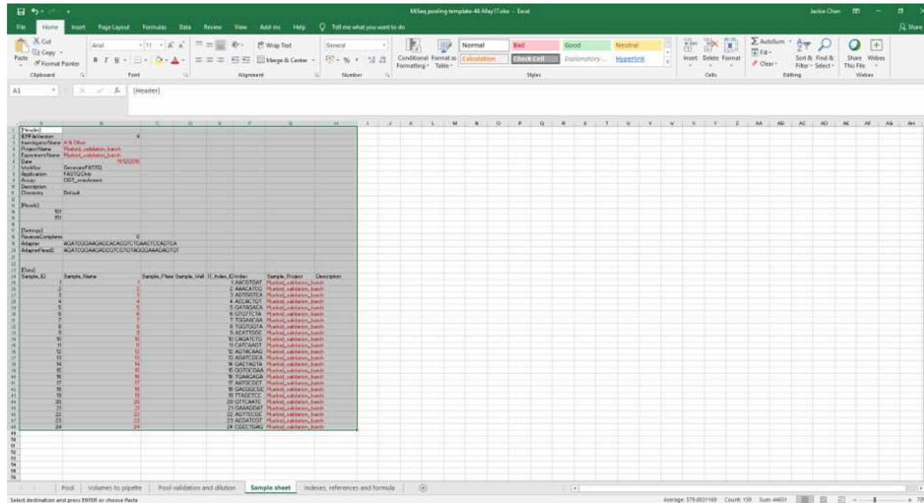


Figure 7. Example SampleSheet on the MiSeq pooling template file.

3. Copy highlighted cells and paste into a new Excel file.
   Note: All text in red is for user and sample specific information. All text in black is required to ensure that the MiSeq will recognise the file.
4. Save the new sheet as a CSV (comma delimited) file.
5. The file needs to be saved using the MiSeq Reagent Tray ID, which begins with MS then has 7 numbers followed by 300V2 (when running the version 2 MiSeq chemistry), e.g. MS2016935-300V2.
6. The MiSeq sample sheet can now be uploaded to the MiSeq.

23 Low Multiplexing Guidelines

Illumina MiSeq and HiSeq use a red laser to sequence A/C and a green laser to sequence G/T.

To ensure accurate registration of the index read, both a red and green signal must be present at each cycle.

It is also important to maintain colour balance where possible.

If pooling less than eight samples in the final sequencing pool we suggest using the following index combinations.

Number of samples in pool	Index
1	Any index
2	2 & 6
3	**Combination A:** 4, 6 & 7
	Combination B: 1, 11 & 16
4	**Combination A:** 2, 6, 10 & 14
	Combination B: 9, 12, 15 & 16
6	**Combination A:** 1, 2, 4, 6, 7 & 8
	Combination B: 2, 8, 9, 12, 15 & 16
8	**Combination A:** 1-8
	Combination B: 9-16

Table 19 Index combination guideline

24 Ordering information

Product Name	Product Description	Cat. No.
SureSeq NGS Hyb & Wash Kit (16)	Hybridisation buffer, Wash buffer, Cot and blocking oligos. Sufficient for 16 samples	500075
SureSeq NGS Hyb & Wash Kit (48)	Bundle of 3x SureSeq NGS Hyb & Wash Kit (16), containing Hybridisation buffer, Wash buffer, Cot and blocking oligos. Sufficient for 48 samples	500086
SureSeq NGS Library Preparation and Hyb & Wash Kit (16)	Bundle of 1x SureSeq NGS Library Preparation Kit (16), containing adaptors, PCR primers and enzymes, 1x SureSeq NGS Index Kit – Collection A and 1x SureSeq Hyb & Wash Kit (16). Sufficient for 16 samples	500082
SureSeq NGS Library Preparation and Hyb & Wash Kit (48)	Bundle of 3x SureSeq NGS Library Preparation Kit (16), containing adaptors, PCR primers and enzymes, 1x SureSeq NGS Index Kit – Collection B and 3x SureSeq Hyb & Wash kit (16). Sufficient for 48 samples	500083
SureSeq NGS Library Preparation Complete Solution (16)	Bundle of 1x SureSeq library preparation kit (16), containing adaptors, PCR primers and enzymes, 1x SureSeq NGS Index Kit – Collection A, 1x SureSeq Hyb & Wash Kit (16), 1x Dynabeads M270 Streptavidin (2ml) and 1x AMPure XP beads (10ml). Sufficient for 16 samples	500084
SureSeq NGS Library Preparation Complete Solution (48)	Bundle of 3x SureSeq NGS Library Preparation Kit (16), containing adaptors, PCR primers and enzymes, 1x SureSeq NGS Index Kit – Collection B, 3x SureSeq NGS Hyb & Wash Kit (16), 3x Dynabeads M270 Streptavidin (2ml) and 3x AMPure XP beads (10ml). Sufficient for 48 samples	500085
Dynabeads M270 Streptavidin, 2ml	Sample capture beads, sufficient for 20 samples	500080*
AMPure XP beads, 10ml	Sample purification beads, sufficient for 16 samples	500081*

Table 20 Ordering information

* only for use with SureSeq NGS panels

For an up-to-date product list and the latest product information, visit www.ogt.com

25 Legal information

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25.1 NGS library prep assay

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25.2 Trademarks

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26 Contact information

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