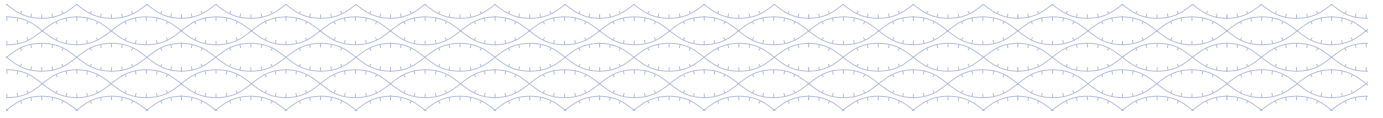


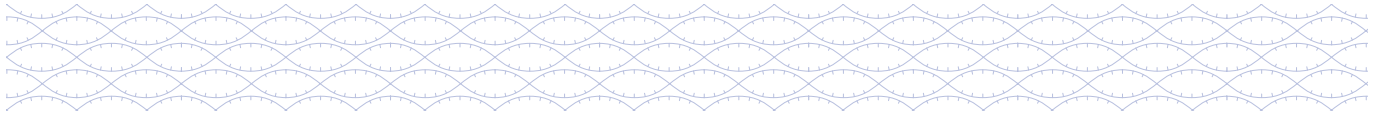
## OGT Handbook

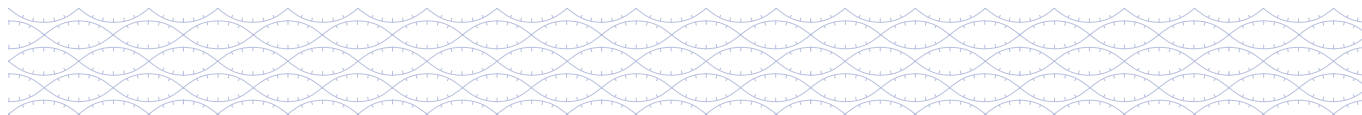
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# Universal NGS Workflow Solution V2



## Table of contents





## Introduction

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The Universal NGS Workflow Solution V2 has been developed and optimised for use with SureSeq™ and CytoSure® gene panel baits designed by Oxford Gene Technology (OGT) to deliver accurate detection of a wide range of variants.

The OGT next-generation sequencing (NGS) range is compatible with Illumina MiniSeq™, MiSeq™, NextSeq™ and NovaSeq™ chemistries.

There are sufficient reagents contained within the 24-reaction kit to process a set of 8 samples on three occasions. For the 96-reaction kit, there are sufficient reagents to process a set of 24 samples on four occasions.

## Reagents, consumables and equipment

### Reagents supplied by OGT

Contents	Shipping/storage conditions	Cat. No. (24 reactions)	Cat. No. (96 reactions)
Universal Library Preparation Kit	Shipped at -20°C, store at -20°C	770100-24	770100-96
Universal Index Adapters	Shipped at -20°C, store at -20°C	770200-24	770200-96
Universal Hybridisation & Wash Kit V2	Shipped at -20°C, store at -20°C	770410-24	770410-96
Pre-PCR Universal Bead Kit	Shipped at 4°C, store at 4°C	770310-24	770310-96
Post-PCR Universal Bead Kit	Shipped at 4°C, store at 4°C	770315-24	770315-96
<b>SureSeq CLL-CNV V3 Panel</b>			
SureSeq Reference DNA	Shipped at -20°C, store at 4°C	770600	-

Universal Library Preparation Kit  
770100-24 and 770100-96



Universal Index Adapters  
770200-24 and 770200-96



Universal Hybridisation & Wash Kit V2  
770410-24 and 770410-96



Pre-PCR Universal Bead Kit  
770310-24 and 770310-96

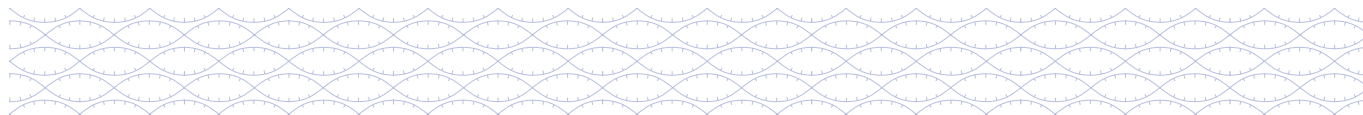


Post-PCR Universal Bead Kit  
770315-24 and 770315-96



SureSeq Reference DNA  
770600





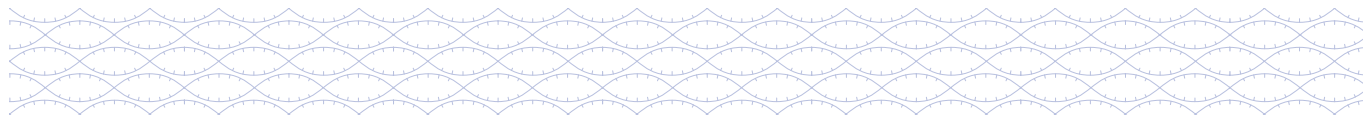
## Reagents, consumables and equipment

### Reagents supplied by user

Component	Suggested supplier	Cat. No.*
Molecular biology grade 100% ethanol	General laboratory supplier	–
Molecular biology grade water	General laboratory supplier	–
Molecular biology grade 5.0 M sodium hydroxide solution	General laboratory supplier	–
Qubit® dsDNA High Sensitivity (HS) Assay Kit	Thermo Fisher Scientific	Q32854
Qubit dsDNA Broad Range (BR) Assay Kit	Thermo Fisher Scientific	Q32853
D1000 ScreenTape	Agilent Technologies	5067-5582
D1000 Reagents	Agilent Technologies	5067-5583
High Sensitivity D1000 ScreenTape	Agilent Technologies	5067-5584
High Sensitivity D1000 Reagents	Agilent Technologies	5067-5585
MiSeq Reagent Kit V2 (300-cycles)	Illumina <sup>†</sup>	MS-102-2002
MiSeq Reagent Kit V3 (600-cycles)	Illumina <sup>†</sup>	MS-102-3003
NextSeq 500/550 Mid-Output Kit v2.5 (300 cycles)	Illumina <sup>†</sup>	20024905
NextSeq 500/550 High-Output Kit v2.5 (300 cycles)	Illumina <sup>†</sup>	20024908
<i>Optional: Genomic DNA ScreenTape</i>	<i>Agilent Technologies</i>	<i>5067-5365</i>
<i>Optional: Genomic DNA Reagents</i>	<i>Agilent Technologies</i>	<i>5067-5366</i>

\* Catalogue numbers are correct for the UK; they may vary in other territories. For more information contact support@ogt.com

<sup>†</sup> Depending on sequencing device utilised.



## Reagents, consumables and equipment

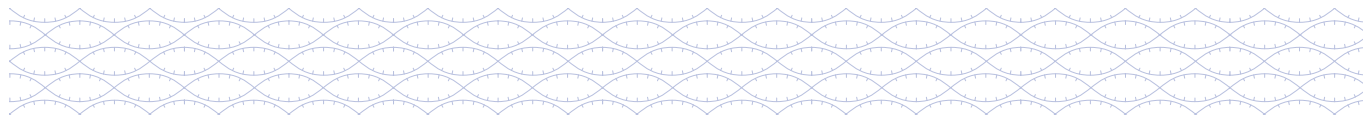
### Consumables supplied by user

Component	Suggested supplier	Cat. No.*
Qubit Assay Tubes	Thermo Fisher Scientific	Q32856
DNA 1.5 ml LoBind® Tubes	Eppendorf	22431021
PCR Strips with attached Caps	Starlab	A1402-3700
Aerosol-resistant sterile pipette tips with filters 2, 10, 20, 200, 1000 µl	General laboratory supplier	–
<i>Optional: 15 ml or 50 ml Tubes</i>	<i>General laboratory supplier</i>	–
<i>Optional: Disposable reagent reservoirs</i>	<i>General laboratory supplier</i>	–

### Equipment supplied by user

Component	Stage required	Suggested supplier	Cat. No.*
Agilent® 4200 TapeStation®	Post-PCR	Agilent Technologies	G2991BA
2 x thermal cyclers (96-well) with heated lid	Pre- and Post-PCR	General Laboratory Suppliers	–
Cold block for 0.2 ml tubes — OGT recommends Aluminium heating/cooling block	Pre- and Post-PCR	Sigma Aldrich	Z740270-1EA
Laboratory vortex mixer — OGT recommends IKA™ MS 3 Digital Vortex Mixer	Pre- and Post-PCR	IKA	IKA 0003319000
Plate adapter for vortex mixer — OGT recommends IKA MS 3.4 Microtiter Attachment	Post-PCR	IKA	IKA 0003426400
Microfuge for standard 1.5 ml tubes and 8-strip PCR tubes	Pre- and Post-PCR	General Laboratory Supplier	–
Magnet for 96-well microwell plate — OGT recommends DynaMag™-96 Side Magnet	Pre- and Post-PCR	Thermo Fisher Scientific	12331D
Magnet for 1.5 ml tubes — OGT recommends DynaMag-2 Magnet	Post-PCR	Thermo Fisher Scientific	12321D
Fluorometer (Qubit 4)	Pre- and Post-PCR	Thermo Fisher Scientific	Q33238
NanoDrop™ (One Microvolume UV-Vis Spectrophotometer)	Pre-PCR	Thermo Fisher Scientific	ND-ONE-W
20–200 µl and 1–10 µl 8-channel pipette	Pre- and Post-PCR	General Laboratory Supplier	–
Illumina MiniSeq, MiSeq, NextSeq or NovaSeq	Post-PCR	Illumina	–

\* Catalogue numbers are correct for the UK; they may vary in other territories. For more information contact support@ogt.com



## General guidelines

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### Recommended before you start

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For best results, OGT recommends all steps are performed in PCR strip-tubes with attached caps.

It is highly recommended to test the hybridisation conditions (thermal cycler and plasticware) to ensure minimal evaporation occurs during the overnight incubation:

- To test, add 17  $\mu$ l of Nuclease-free Water (without DNA) in each well that you might use and use the thermal cycler settings in Table 9.
- Check after overnight incubation that the evaporation does not exceed 1–2  $\mu$ l per tube.
- If required, adjust the setting of the thermal cycler lids and/or use spacers appropriate to the model of thermal cycler.

Use fresh solution of 80% ethanol throughout the workflow using molecular biology grade ethanol and molecular biology grade water.

Where appropriate, allow the Mag-Bind<sup>®</sup> TotalPure NGS beads and the Dynabeads<sup>™</sup> M-270 Streptavidin magnetic beads to equilibrate to room temperature by removing it from its storage conditions at least 30 min before use.

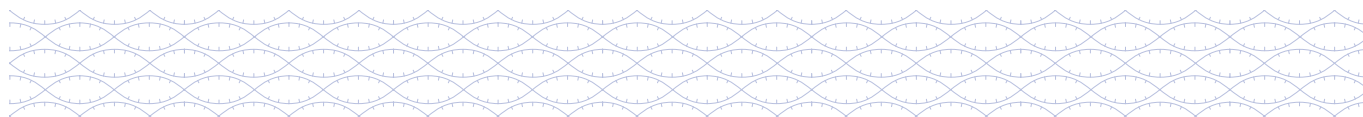
### Storage and handling

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The kit should be used before the expiry date indicated on the kit label.

The Universal Library Preparation Kit, Universal Index Adapters, SureSeq and NGS Constitutional Panels and Universal Hybridisation & Wash Kit V2 should be stored at  $-20^{\circ}\text{C}$ .

The SureSeq Reference DNA, Pre-PCR and Post-PCR Universal Bead Kits should be stored at  $4^{\circ}\text{C}$ .



## General guidelines







### Safety

Handling of the OGT 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 Universal Hybridisation & Wash Kit V2 contains chemicals that are potentially hazardous when mishandled. Particular care should be given to both Formamide and the Hybridisation Buffer.

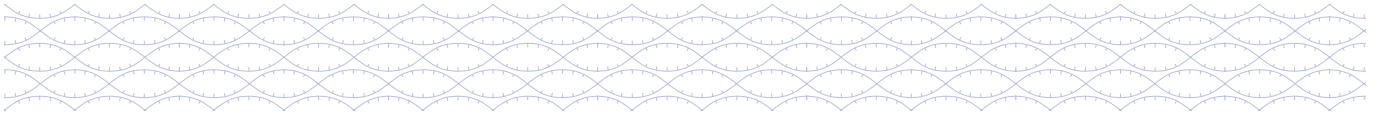
Ensure that all operators have familiarised themselves with the safety data sheets (SDS) and relevant risk assessments before proceeding with the protocol.

### Symbols key

Symbol	Definition
	Important information: These notes are particularly important to read, understand and follow precisely.
	Time-saving tip: Optional suggestion to increase protocol efficiency.
	Cold step: Keep all components on ice for these steps.
	On-magnet step: Keep all tubes on magnet during these steps.
	Sequencing-specific information: These notes are particularly important to read, understand and follow precisely to optimally load the sequencing run.
	Safe stopping point: Samples can be safely stored at this stage without impacting results.

## Intended use

These products are for Research Use Only assays. The Universal NGS Workflow Solution is designed to be used by suitably trained personnel using DNA extracted from a variety of tissues including blood and bone marrow.



## Interpret NGS Analysis Software

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

## Workflow overview

For ordering information about OGT products, visit [www.ogt.com](http://www.ogt.com).

The following section contains instructions for sample library production specific to the Illumina sequencing platform. For each sample, an individual library preparation is performed. Each sample is tagged with an index (barcode) sequence, as well as a unique molecular identifier (UMI) for error correction and increased accuracy during sequencing. Samples are amplified and then pooled into sets of eight. Each pool is hybridised to design specific baits, washed, captured and then amplified ready for sequencing.

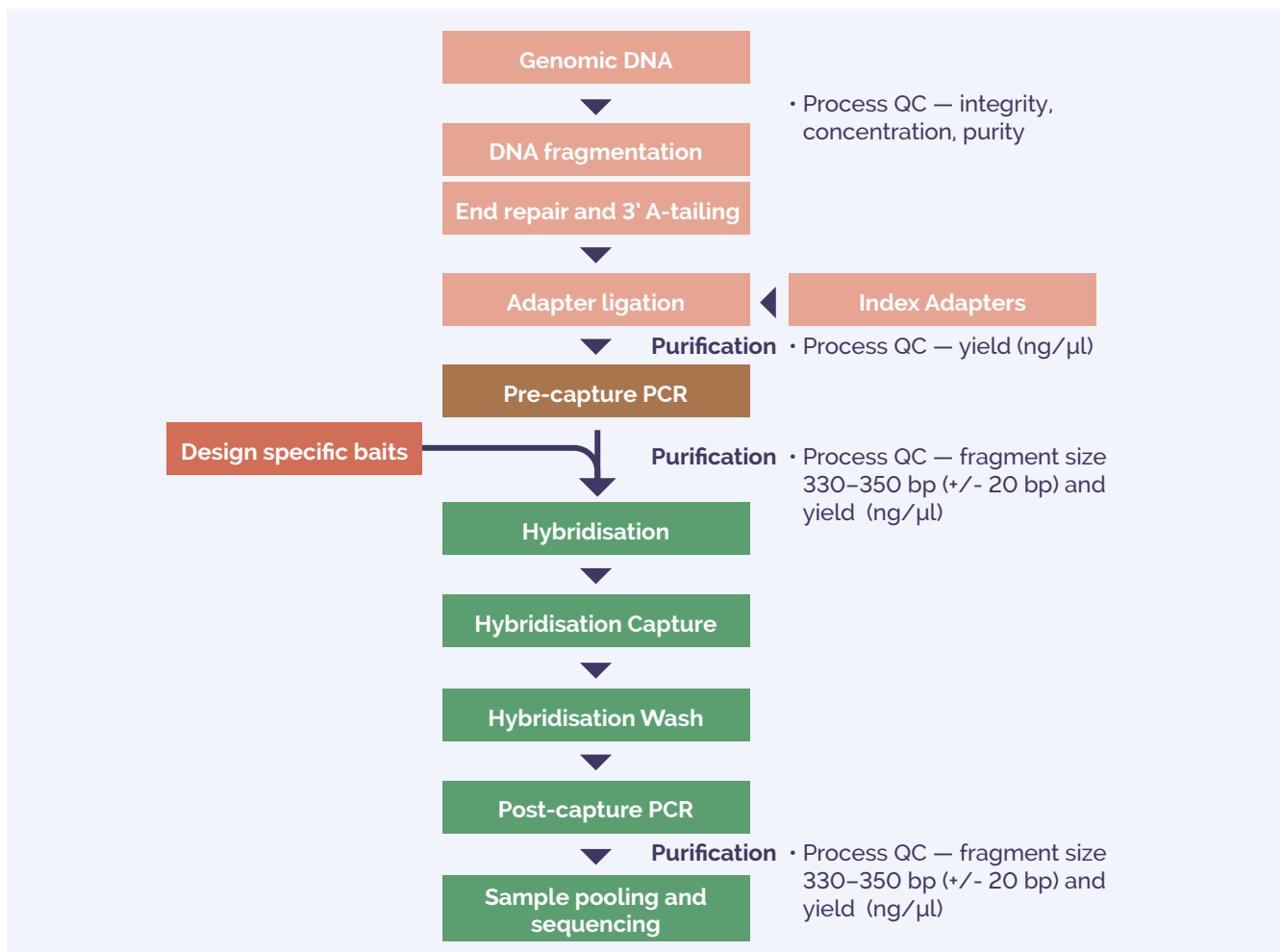
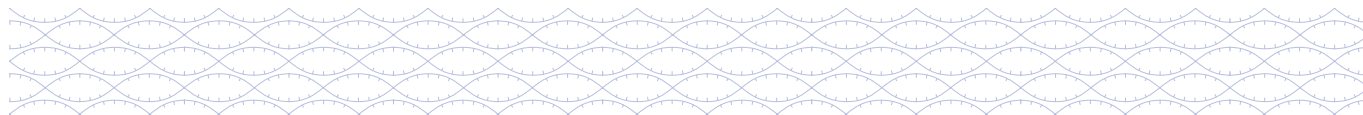


Figure 1: Workflow of sample library preparation indicating the expected DNA fragment size at each step of the procedure.



## Sample requirements

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### Sample requirements

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DNA: the protocol has been optimised for DNA inputs of 200–500 ng.

For SureSeq SureSeq CLL + CNV V3 Panel: include 500 ng of the SureSeq Reference Female and 500 ng of the SureSeq Reference Male from the SureSeq Reference DNA Kit. One reference sample is required per hybridisation pool with a total of two in a 16 sample MiSeq run.



Modifying the recommended DNA input will impact the downstream sequencing results.

### DNA — sample preparation

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Determination of the concentration of gDNA sample is mandatory for all samples prior to starting the protocol.

DNA integrity and purity assessment are optional but recommended.

We recommend the following assays to assess the sample integrity, concentration and purity:

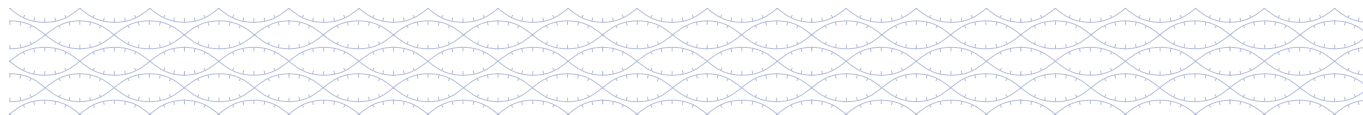
- Concentration: Thermo Fisher Scientific Qubit
- DNA integrity: Agilent 4200 TapeStation
- Purity: Thermo Scientific NanoDrop

### DNA concentration — Qubit dsDNA HS Assay Kit

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Refer to manufacturer's user guide for the Thermo Fisher Scientific Qubit. The key steps are described below:

1. Prepare the Qubit working solution by diluting the Qubit reagent 1:200 in Qubit buffer.
2. Aliquot 190 µl of Qubit working solution for the two standards.
3. Add 10 µl of each Qubit standard to the appropriate tube.
4. Aliquot 199 µl of Qubit working solution for each sample under assessment.
5. Add 1 µl of sample to the appropriate tube.



## Sample requirements

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Samples with an initial DNA concentration  $>100$  ng/ $\mu$ l should be prediluted to 20–100 ng/ $\mu$ l with TE Buffer (provided in the kit). Confirm the DNA concentration using the Qubit dsDNA HS Assay Kit. The precise quantification of DNA input is essential for reproducible fragmentation results.

6. Mix by vortexing for 2–3 sec, being careful not to generate bubbles.
7. Incubate the tubes at room temperature for 2 min.
8. Measure and record DNA concentrations following the onscreen prompts.

## DNA integrity — Genomic DNA ScreenTape

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**Optional:** This step is important to assess the level, if any, of DNA degradation.

Refer to manufacturer's user guide for the Agilent 4200 TapeStation. The key steps are described below:

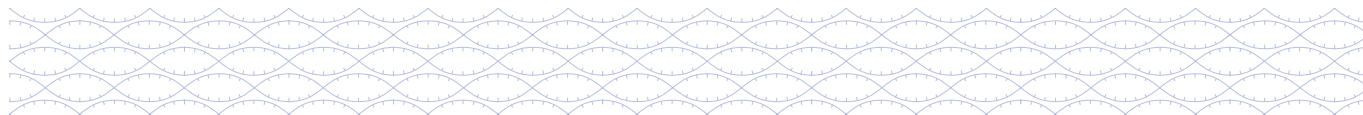
1. Prepare the ladder by mixing 10  $\mu$ l of Genomic DNA Sample Buffer with 1  $\mu$ l Genomic DNA ladder in the first tube/well of the strip tube or plate.

**Note:** A ladder is required for each run. No electronic ladder is available for the Genomic DNA assay

2. For each sample under assessment, add 1  $\mu$ l of DNA sample to 10  $\mu$ l of Sample Buffer.
3. Seal all the tubes/wells.
4. Vortex the tubes or plate for 1 min at 2000 rpm and then briefly centrifuge to collect liquid at the bottom.
5. Briefly spin down to collect the sample at the bottom of the tubes/wells.
6. Load the strip of tubes or plate into the Agilent 4200 TapeStation.

**Note:** If using strip tubes remember to take off the caps.

7. Highlight the required samples on the controller software and fill in the sample names in the sample sheet.
8. Provide a filename in the "Prefix" field of the controller software to save your results and select "Start".



## Sample requirements

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9. Check that the electropherogram shows that the integrity of the gDNA is intact with an even distribution and maximum peak size >5000 bp.
10. After DNA electrophoresis using the Agilent 4200 TapeStation, a DNA Integrity Number (DIN) is generated. A DIN >7 indicates the presence of intact DNA, while a DIN <7 indicates that the DNA is degraded. If a sample has a DIN <7 contact your local FAS.

## Purity — NanoDrop

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**Optional:** This step is important to assess the purity of the DNA sample.

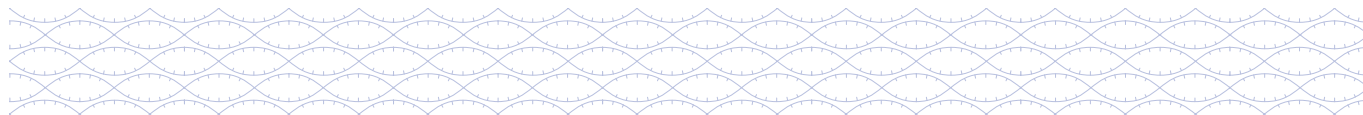
Refer to the manufacturer's user guide for the NanoDrop.

The key steps are described below:

1. Use the “Nucleic Acid” and “DNA-50” setting.
2. Clean the pedestal with Nuclease-free Water.
3. Load 1–2  $\mu$ l of sample buffer or blanking solution.
4. Click “Measure blank”.
5. Clean the pedestal with a lint-free wipe.
6. Load 1–2  $\mu$ l of each sample onto the pedestal.
7. Click “Measure”.
8. Record the readings for 260/230, 260/280 and the concentration (ng/ $\mu$ l).

An OD 260/280 ratio of 1.8 to 2.0 and OD 260/230 ratio of 2.0 to 2.2 is recommended. Use of DNA samples with lower ratios may result in poor performance.

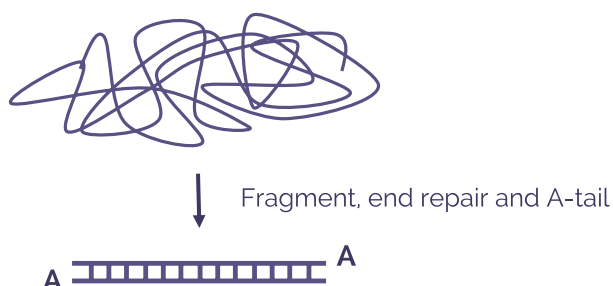
Contact your local OGT Field Application Specialist (FAS) if you require any advice as to the quality of your samples.



## Library preparation: Step 1

### DNA fragmentation, end repair and 3' end A-tailing

#### Genomic DNA



#### Overview

Genomic DNA is enzymatically fragmented. The fragmented dsDNA is repaired with enzymes in the Fragmentation and end repair (ER) mix to create blunt ends. At the same time, a 3' adenine overhang is created in preparation for adapter ligation.

#### Before starting:

- ❄ Remove TE Buffer (blue lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ). Allow to thaw to room temperature then place on ice.

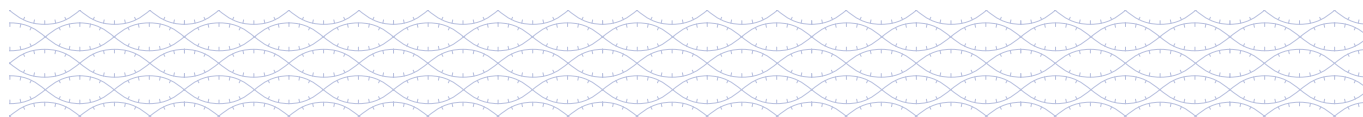


Use only the TE Buffer (blue lid; ●) provided in the kit for preparation of DNA samples (10 mM Tris, 1 mM EDTA). Use of other TE formulations (e.g., 0.1x TE) or water may affect fragmentation results.

- ❄ Remove Step 1: Frag + ER Buffer (orange lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ). Allow to thaw to room temperature and place on ice. Ensure that all components in the Buffer are dissolved.

It is not uncommon to see precipitation in the Buffer. If this occurs, pipette the Buffer several times to break up the precipitate, followed by a quick vortex to mix until dissolved. If precipitate remains, then avoid pipetting precipitates into the Master Mix.

- ❄ Remove Step 1: Frag + ER Enzyme (orange lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and place on ice.
- Ensure DNA sample(s) are prediluted to 20–100 ng/ $\mu\text{l}$  with TE Buffer (blue lid; ●).



## Library preparation: Step 1

### Perform step 1: DNA fragmentation, end repair and 3' end A-tailing

Estimated time: 1.25 hr for 8–24 samples. Hands-on time: 15 min.

1. Program the thermal cycler using the settings shown in Table 1. Save the program as “OGT Fragmentation”.

Step	Temperature (°C)	Time
1	37	20 min
2	65	30 min
3	4	Hold

Table 1: Incubation profile of program “OGT Fragmentation”.

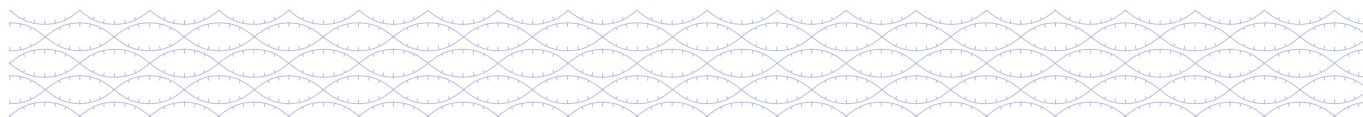
2. **Preheat** the thermal cycler to 37°C. Where possible, set the heated lid to 75°C, alternatively, have the pre-set heated lid activated.
- ❄️ 3. In PCR strip-tubes dilute **200–500 ng** of sample DNA with the chilled TE Buffer (blue lid; ●) provided to a total volume of **27 µl**. Mix on a vortex mixer for **3–5 sec**, pulse spin to collect the contents and place on ice.

**For SureSeq CLL + CNV V3:** Prepare 1 x 500 ng of Human Reference DNA Female and 1 x 500 ng of Human Reference DNA Male in a total volume of **27 µl** for each 16-sample run. Process the two reference samples in parallel with the 14 test samples.

- ❄️ 4. Label a new set of PCR strip-tubes for the sample and place on ice.
- ❄️ 5. Mix Step 1: Frag + ER Buffer and Step 1: Frag + ER Enzyme on a vortex mixer for **5–8 sec**. Pulse spin to collect the contents and place on ice.



It is essential to thoroughly mix the Frag + ER Enzyme for optimal performance.



## Library preparation: Step 1

- ❄️ 6. Prepare the Fragmentation and ER Master Mix according to Table 2 in a fresh 1.5 ml LoBind tube and place on ice.

Reagent	1x library (µl)	8x library (µl) (includes 2 excess)	16x library (µl) (includes 2 excess)	24x library (µl) (includes 3 excess)
DNA sample	27	–	–	–
Step 1: Frag + ER Buffer (orange lid; ●)	7	70	126	189
Step 1: Frag + ER Enzyme (orange lid; ●)	1	10	18	27
<b>TOTAL</b>	<b>35</b>	<b>80</b>	<b>144</b>	<b>216</b>

Table 2: Fragmentation and ER Master Mix.

- ❄️ 7. Mix the Fragmentation and ER Master Mix on a vortex mixer for **3–5 sec**, pulse spin to collect the contents and keep on ice.
- ❄️ 8. Add **8 µl** Fragmentation and ER Master Mix into each of the prepared empty tubes from step 4 and keep on ice.
- ❄️ 9. Using a multi-channel pipette, add **27 µl** of DNA sample from step 3 to the tubes from step 4.



Proceed in a timely manner to avoid over-fragmentation as the enzyme is active at room temperature. Keep samples on ice when not being vortexed or spun.

10. **Immediately** mix on a vortex mixer for **3 sec**.
11. Pulse spin to collect the contents and **immediately** transfer to the **preheated** thermal cycler. Start the program “OGT Fragmentation”.

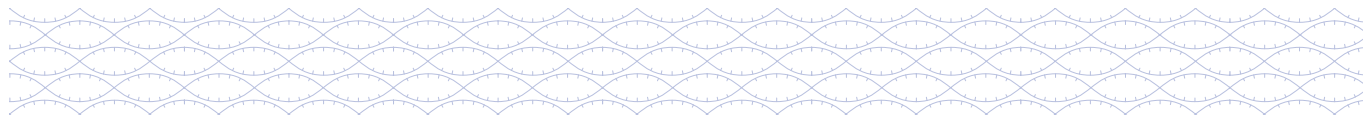


Recommendation: prepare the Ligation Master Mix in the final 10 min of the thermal cycler program.

12. When the program is complete and the thermal cycler has reached 4°C, remove the samples and place them on ice until you are ready to proceed with Adapter ligation.



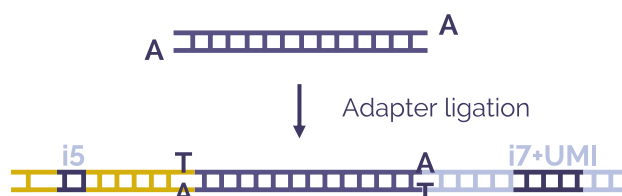
We recommend continuing with “Step 2: Adapter ligation and purification” immediately. If necessary, samples can be stored at –20°C; however, a loss in yield (~20%) may be observed.



## Library preparation: Step 2

### Adapter ligation and purification

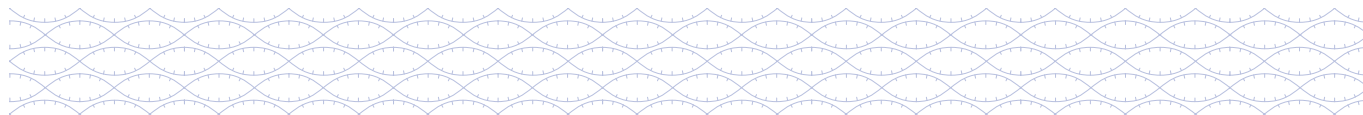
#### Overview



Using the 3' overhang created during end repair/A-tailing and DNA Ligase, Illumina compatible Adapter sequences are ligated onto dsDNA fragments. Adapters contain unique molecular identifier (UMI) sequences and unique sample indexes.

#### Before starting:

- Take the Mag-Bind TotalPure NGS beads from the Pre-PCR Universal Bead Kit out of the fridge at least **30 min before use** to allow them to warm to room temperature.
- Make up a fresh solution of 80% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Remove the Step 2: Ligation Buffer (yellow lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and thaw to room temperature. Ensure that all components in the Ligase Buffer are dissolved. If necessary, incubate at  $37^{\circ}\text{C}$  until dissolved.
- ❄ • Remove the Step 2: Ligase (yellow lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and place on ice.
- ❄ • Remove the Universal Index Adapter plate from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and allow to thaw on ice **5-10 min before use**. Pulse spin the Adapter plate in a centrifuge to collect the contents. Keep the plate on ice at all times. Do not heat above room temperature. Check Index Adapters are fully thawed before use.
- Index Adapters are for single use only. If only using part of the plate, cover the used Adapter wells to avoid spillage of excess Index Adapter. Unused Adapters can also be aliquoted into strip tubes and thawed immediately prior to use.



## Library preparation: Step 2

- Assign a different Index Adapter to each sample. See Figure 2 and Figure 3 for the location of the Index Adapters on the plate.
- OGT recommends processing in batches of eight samples, each pool will contain a complete column from the index plate.

### Perform Step 2: Ligation

Estimated time: 45 min for 8–24 samples. Hands-on time: 15 min.

1. Program the thermal cycler using the settings shown in Table 3. Save the program as “OGT Ligation”.

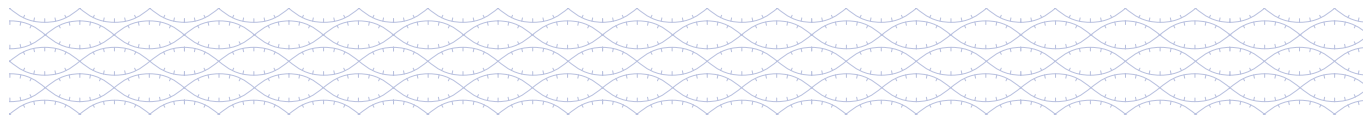


Do not use heated lid. If heated lid cannot be turned off, keep lid open.

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

Table 3: Incubation profile of program “OGT Ligation”.

2. Mix Step 2: Ligase Buffer and Index Adapters on a vortex mixer for **3–5 sec**, pulse spin to collect the contents and place on ice.
3. Flick mix Step 2: Ligase, pulse spin to collect the contents and place on ice.



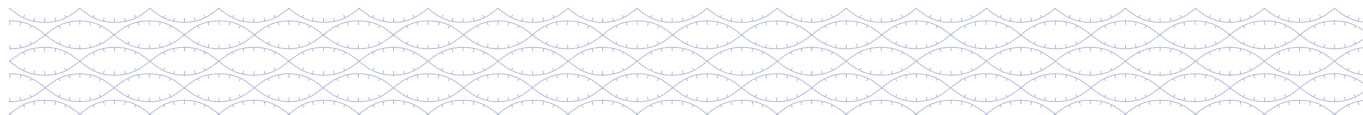
## Library preparation: Step 2

- ❄️ 4. Prepare the Ligation Master Mix according to Table 4 in a fresh 1.5 ml LoBind tube and place on ice.

Reagent	1x library (µl)	8x library (µl) (includes 2 excess)	16x library (µl) (includes 2 excess)	24x library (µl) (includes 3 excess)
DNA sample	35	–	–	–
Index Adapter	3.5	–	–	–
Step 2: Ligase Buffer (yellow lid; ●)	9	90	162	243
Step 2: Ligase (yellow lid; ●)	3	30	54	81
<b>TOTAL</b>	<b>50.5</b>	<b>120</b>	<b>216</b>	<b>324</b>

Table 4: Ligation Master Mix.

- ❄️ 5. Mix the Ligation Master Mix on a vortex mixer for **3–5 sec**, pulse spin to collect the contents and keep on ice.
- ❄️ 6. Add **3.5 µl** of Index Adapter to each DNA sample tube.
- ❄️ 7. Add **12 µl** of the Ligation Master Mix to each chilled DNA sample tube containing the fragmented product(s).
8. Mix on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
9. **Immediately** transfer to the thermal cycler and start the program “OGT Ligation”.
10. When the program is complete and the thermal cycler has reached 4°C, remove the samples and **proceed immediately** to “Ligated library purification”.



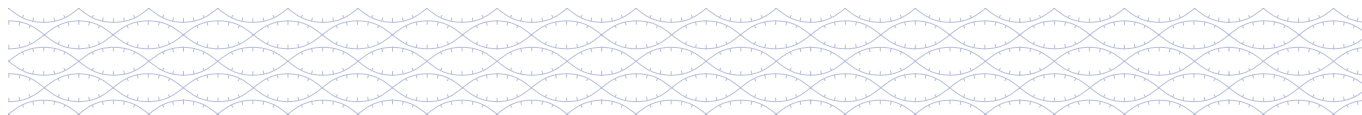
## Library preparation: Step 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	x	x	x	x	x	x	x	x	x
B	2	10	18	x	x	x	x	x	x	x	x	x
C	3	11	19	x	x	x	x	x	x	x	x	x
D	4	12	20	x	x	x	x	x	x	x	x	x
E	5	13	21	x	x	x	x	x	x	x	x	x
F	6	14	22	x	x	x	x	x	x	x	x	x
G	7	15	23	x	x	x	x	x	x	x	x	x
H	8	16	24	x	x	x	x	x	x	x	x	x

Figure 2: Layout of Universal Index Adapter Plate (1–24). X: empty well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Figure 3: Layout of Universal Index Adapter Plate (1–96).



## Library preparation: Step 2

### Perform Ligated library purification

Estimated hands on time: 50 min for 8–24 samples.



Prior to use, mix beads on a vortex mixer for at least 1 min or until the bead solution appears homogeneous and consistent in colour.

#### Before starting:

1. Prepare a **fresh** set of PCR strip-tubes for each strip of samples.
2. Add **58 µl** of homogeneous, room temperature Mag-Bind TotalPure NGS beads from the Pre-PCR Universal Bead Kit to each **empty** tube and set aside until required in step 6.

#### To the DNA sample tubes:

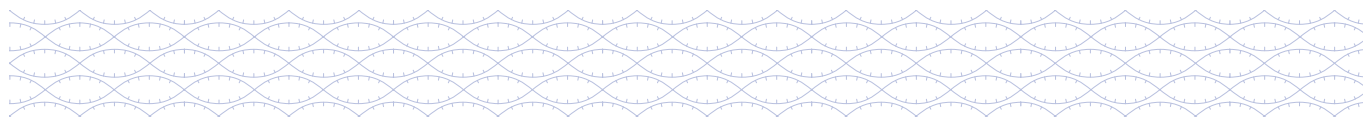
3. Add **49.5 µl** Nuclease-free Water to each DNA sample tube.
4. Add **22 µl** of homogeneous, room temperature Mag-Bind TotalPure NGS beads from the Pre-PCR Universal Bead Kit to each DNA sample tube. Mix on a vortex mixer and pulse spin to collect the contents.
5. Incubate at room temperature for **5 min**.




6. Place the tubes in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
7. Transfer **125 µl** of **cleared supernatant containing the DNA sample** to the tubes containing beads prepared in step 2. The used bead pellets can be discarded.
8. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.
9. Incubate at room temperature for **5 min**.



10. Place the tubes in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
11. Avoiding the bead pellet, remove and discard the cleared supernatant (**~180 µl**). **Keep the beads containing the DNA sample.**
12. Add **200 µl** of 80% ethanol to each tube without resuspending the bead pellet.
13. Incubate for **30 sec**, then remove the ethanol.



## Library preparation: Step 2

-  14. Repeat wash (steps 11 and 12) once, for a total of **two** washes.
- 15. Seal the tubes and pulse spin to collect the residual ethanol. Return the tubes to the magnetic stand. Remove the residual ethanol with a P20 pipette.
- 16. Dry the bead pellets at room temperature for **1–2 min**.




Do not over-dry as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte. Over-drying results in cracks in the bead pellet.

- 17. Remove from the magnetic stand and add **34 µl** of Nuclease-free Water directly to the bead pellet to elute the DNA sample. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.
- 18. Incubate for **5 min** at room temperature.



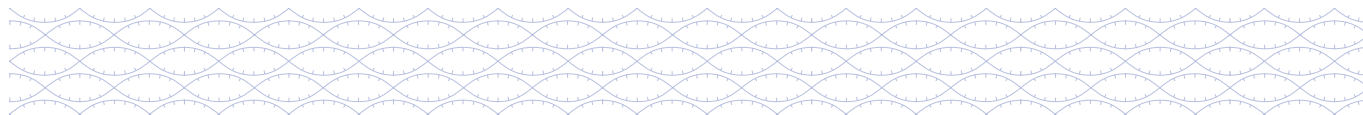
Recommendation: If proceeding with Step 3: Pre-capture PCR immediately, the Step 3: Primer Mix and Step 3: PCR Buffer can be removed from storage to thaw to room temperature now.

- 19. Label a new set of PCR strip-tubes for the samples and set aside until required in step 20.

-  20. Place the tubes on the magnetic stand and wait for the solution to clear (approx. 2–3 min).
- 21. Transfer **32 µl** of the eluate containing the purified ligated products to the tubes from step 18. Tubes containing beads can be discarded at this time.
- 22. Assess yield using **1 µl** ligated product with the Qubit dsDNA HS Kit as per manufacturer's instructions. The expected yield is **>2 ng/µl**.



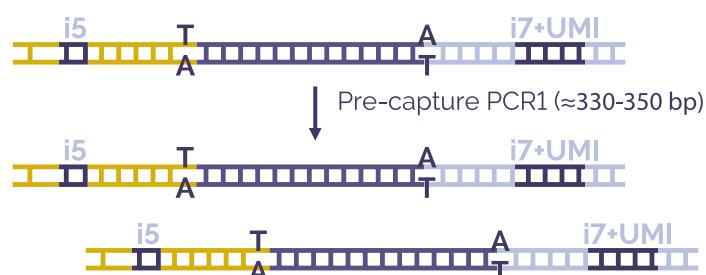
**OPTIONAL STOPPING POINT:** If the samples are not to be used immediately, store at  $-20^{\circ}\text{C}$ . If continuing, proceed to "Pre-capture PCR".



## Library preparation: Step 3

### Pre-capture PCR

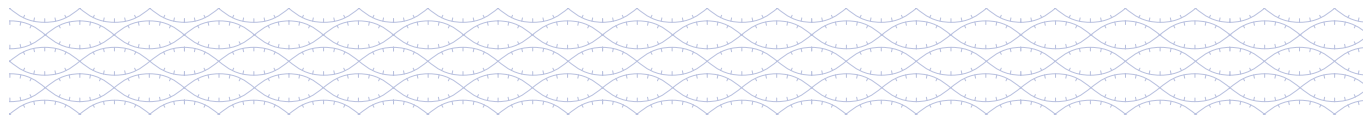
#### Overview



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

#### Before starting:

- Take the Mag-Bind TotalPure NGS beads from the Post-PCR Universal Bead Kit out of the fridge at least **30 min before use** to allow them to warm to room temperature.
- Remove the Step 3: Primer Mix (red lid; ●) and Step 3: PCR Buffer (red lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and allow to thaw at room temperature.
- Ensure that all components in the PCR Buffer are well dissolved. If necessary, vortex mix and/or incubate at  $37^{\circ}\text{C}$  until dissolved.
- ❄ • Remove the Step 3: PCR Polymerase (red lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and place on ice.



## Library preparation: Step 3

### Perform step 3: Pre-capture PCR

Estimated time: 45 min for 8–24 samples. Hands-on time: 15 min.

1. Program the thermal cycler using the settings shown in Table 5 and Table 6. Save the program as “OGT PCR1”. Where possible, set the heated lid to 105°C, alternatively have the pre-set heated lid activated.

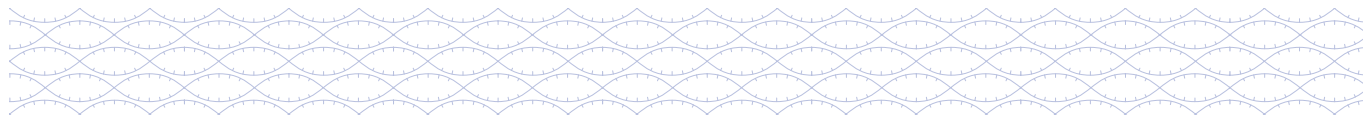
Step	Temperature (°C)	Time
1	98	3 min
2	98	30 sec
3	65	30 sec
4	72	1 min
5	Repeat Step 2 to Step 4 according to number of cycles indicated in Table 6	
6	72	10 min
7	4	Hold

Table 5: Incubation profile of program “OGT PCR1”.

DNA input	Total number of PCR cycles
200–350 ng	6
351–500 ng	5

Table 6: Total number of PCR cycles for recommended range of DNA inputs.

2. Vortex Step 3: Primer Mix and Step 3: PCR Buffer on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
- ❄️ 3. Flick mix Step 3: PCR Polymerase, pulse spin to collect the contents and keep on ice.



## Library preparation: Step 3

4. Prepare the Pre-capture PCR Master Mix according to Table 7 in a fresh 1.5 ml LoBind tube.

Reagent	1x library (µl)	8x library (µl) (includes 2 excess)	16x library (µl) (includes 2 excess)	24x library (µl) (includes 3 excess)
Adapter-ligated DNA sample	31	-	-	-
Nuclease-free Water (clear lid; ○)	9.5	95	171	256.5
Step 3: PCR Buffer (red lid; ●)	5	50	90	135
Step 3: Primer Mix (red lid; ●)	2.5	25	45	67.5
Step 3: PCR Polymerase (red lid; ●)	2	20	36	54
<b>TOTAL</b>	<b>50</b>	<b>190</b>	<b>342</b>	<b>513</b>

Table 7: Pre-capture PCR Master Mix.

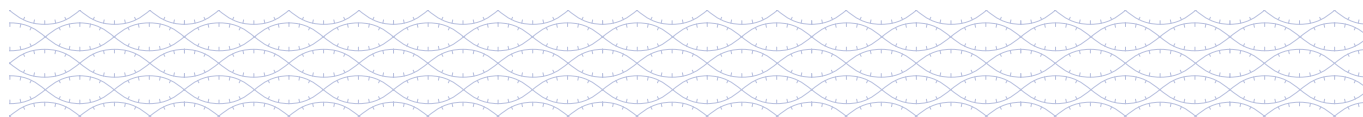
5. Mix the Pre-capture PCR Master Mix on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
6. Add **19 µl** of the Pre-capture PCR Master Mix to each DNA sample tube containing the ligated products.
7. Mix on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
8. Transfer to the thermal cycler and start the program “OGT PCR1”.

### Perform pre-capture PCR purification

Estimated hands-on time: 40 min for 8–24 samples.




Prior to use, mix beads on a vortex mixer for at least 1 min or until the bead solution appears homogeneous and consistent in colour.



## Library preparation: Step 3

### To the DNA sample tubes:

1. Add **45 µl** of homogeneous, room temperature Mag-Bind TotalPure NGS beads from the Post-PCR Universal Bead Kit to each DNA sample tube. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.
  2. Incubate at room temperature for **5 min**.
  3. Place the tubes in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
  4. Avoiding the bead pellet, remove and discard the cleared supernatant (**~90 µl**). **Keep the beads containing the DNA sample.**
  5. Add **200 µl** of 80% ethanol to each tube without resuspending the bead pellet.
  6. Incubate for **30 sec**, then remove the ethanol.
  7. Repeat wash (step 5 and step 6) once, for a total of two washes.
  8. Seal the tubes and pulse spin to collect the residual ethanol. Return the tubes to the magnetic stand. Remove the residual ethanol with a P20 pipette.
  9. Dry the bead pellets at room temperature for **1–2 min**.
-  Do not over-dry as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte. Over-drying results in cracks in the bead pellet.
10. Remove from the magnetic stand and add **25 µl** of Nuclease-free Water directly to the bead pellet to elute the DNA sample. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.
  11. Incubate for **5 min** at room temperature.
  12. Label a new set of PCR strip-tubes for the samples and set aside until required in step 14.
  13. Place the tubes on the magnetic stand and wait for the solution to clear (approx. 2–3 min).
  14. Transfer **24 µl** of the eluate containing the purified amplified products to the tubes from step 12. Tubes containing beads can be discarded at this time.

## Library preparation: Step 3

- Assess the size of the amplified product using the Agilent D1000 ScreenTape System. The electropherogram should show a peak size of 330–350 bp ( $\pm 20$  bp) (Figure 4). Set up the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.

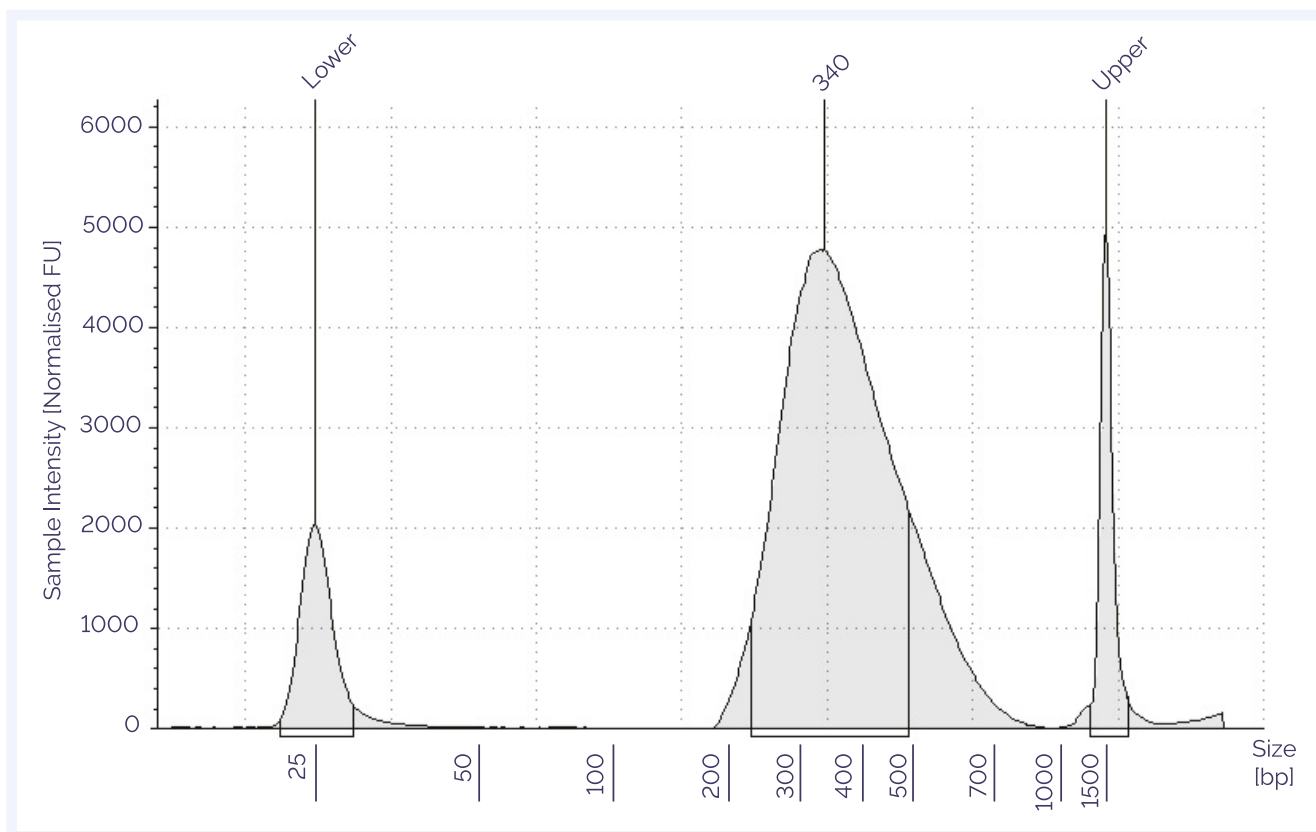


Figure 4: Electropherogram of purified Pre-capture PCR product generated using an Agilent D1000 ScreenTape assay. The electropherogram shows a maximum peak in the size range of approximately 330–350 bp ( $\pm 20$  bp).



Fragment sizes outside of this range may reduce sequence data quality. Contact your local Field Application Specialist (FAS) if you require further advice.

- Assess yield using **1  $\mu$ l** amplified product with the Qubit dsDNA HS Kit as per manufacturer's instructions.

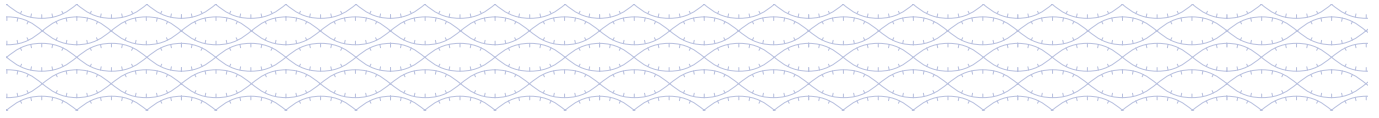
The expected yield is  $>18$  ng/ $\mu$ l ( $\sim 400$  ng per library).



It is recommended to use a single channel pipette and ensure there is no excess liquid on the side of the tip to prevent inaccurate readings which may affect pooling.



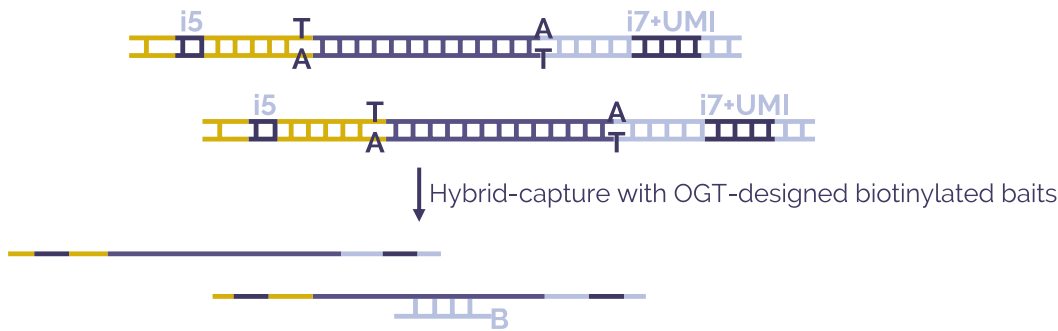
**OPTIONAL STOPPING POINT:** If the samples are not to be used immediately, store at 4°C overnight or at  $-20^{\circ}\text{C}$  for long-term storage. If continuing, proceed to "Universal hybridisation".



## Library preparation: Universal hybridisation

### Universal hybridisation

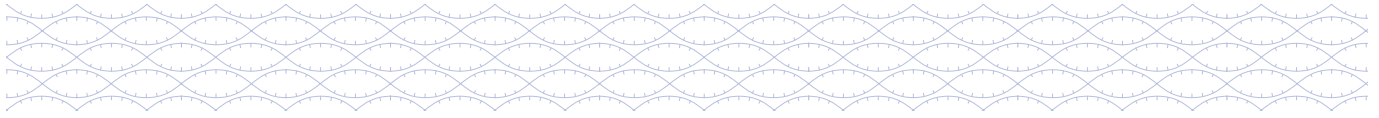
#### Overview



The amplified library is denatured and captured by SureSeq or CytoSure NGS (biotinylated) baits.

#### Before starting:

- Take the Mag-Bind TotalPure NGS beads from the Post-PCR Universal Bead Kit out of the fridge at least **30 min before use** to allow them to warm to room temperature.
- Make up a fresh solution of 80% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Remove the Hybridisation Buffer (red lid; ●), Formamide (yellow lid; ●), Cot Human DNA (green lid; ●), Index Blockers (blue lid; ●) and Nuclease-free Water (clear lid; ○) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and allow to thaw to room temperature.
- Remove the SureSeq or CytoSure baits from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and allow to thaw to room temperature.
- Ensure that all components in the Hybridisation Buffer are well dissolved. If necessary, incubate at  $37^{\circ}\text{C}$  until dissolved.



## Library preparation: Universal hybridisation

### Pool samples and hybridise to capture baits

Estimated time: 45 min for 8–24 samples.

The hybridisation reaction requires equal DNA inputs from each sample to be combined in an 8-sample pool. For each pool, carry out one hybridisation capture. The hybridisation is best performed with the maximum possible input per sample, up to 500 ng. For library yields below 500 ng, adjust the input of all samples down to the lowest yield in the hybridisation pool.

1. Calculate the volumes of Pre-capture PCR product required to combine eight libraries to form a hybridisation pool.



Do not use inputs less than 300 ng per sample; contact [OGT Support](#) for further help.

2. Aliquot equal amount of purified amplified product from step 3 (**300 ng–500 ng**) of each of the eight libraries in a pool into a 1.5 ml LoBind tube.

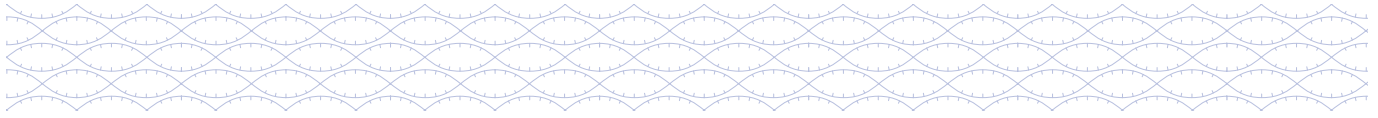
See the SureSeq Myeloid MRD Plus Ultra Low Workflow for reference.

**For SureSeq CLL+CNV V3 Panel:** Include 1 x SureSeq Reference library per pool, along with 7 other libraries. 1 x SureSeq Reference Female and 1 x SureSeq Reference Male — a total of 2 reference samples should be included in a 16-sample sequencing run.

3. Vortex mix the Hybridisation Buffer, Formamide, Cot Human DNA, Panel-specific Baits and Index Blockers on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.

Reagent	1x Pool (µl)	2x Pool (µl) (includes 0.5 excess)	3x Pool (µl) (includes 0.5 excess)
Nuclease-free Water (clear lid; ○)	2.5	6.25	8.75
Hybridisation Buffer (red lid; ●)	7.5	18.75	26.25
Formamide (yellow lid; ●)	3.5	8.75	12.25
<b>TOTAL</b>	<b>13.5</b>	<b>33.75</b>	<b>47.25</b>

Table 8: Hybridisation Master Mix.



## Library preparation: Universal hybridisation

4. Prepare the Hybridisation Master Mix according to Table 8 in a fresh 1.5 ml LoBind tube, vortex mix and set aside until required in Step 15.
5. Add **10 µl** Cot Human DNA (green lid; ●) to each pool.
6. Mix on a vortex mixer and pulse spin to collect the contents.



Prior to use, mix beads on a vortex mixer for at least 1 min or until the bead solution appears homogeneous and consistent in colour.

7. Add **2 x** volume Mag-Bind TotalPure NGS beads from the Post-PCR Universal Bead Kit to each pool.  
Example: To a 60 µl hybridisation pool (+ 10 µl Cot Human DNA) add 140 µl beads.
8. Mix on a vortex mixer and pulse spin to collect the contents.
9. Incubate at room temperature for **5 min**.



10. Place the tube(s) in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
11. Avoiding the bead pellet, remove and discard the cleared supernatant.  
**Keep the beads containing the DNA sample.**
12. Add **500 µl** of 80% ethanol to each tube without resuspending the bead pellet.
13. Incubate for **30 sec**, then remove the ethanol.
14. Dry the pellet for **approx. 5 min** or until the residual ethanol completely evaporates.

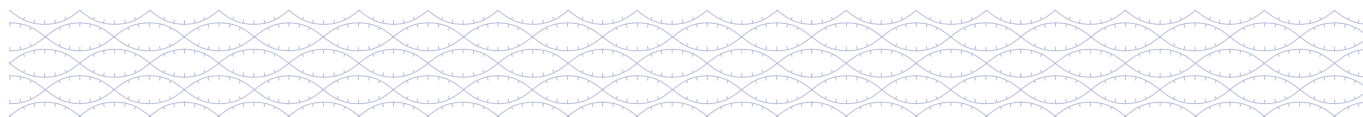


Do not over-dry as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte. Over-drying results in cracks in the bead pellet.

15. Remove the tube(s) from the magnetic stand and add **13.5 µl** of the Hybridisation Master Mix directly to the bead pellet to elute the pooled DNA libraries. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.



Make sure beads are resuspended well. Larger volumes of beads might have to be vortexed longer to ensure complete resuspension.



## Library preparation: Universal hybridisation

16. Incubate for **5 min** at room temperature.
17. Label a new set of PCR strip-tubes for the pool(s) and set aside until required in step 19.



Recommendation: Program the thermal cycler using the settings shown in Table 9. Save the program as "OGT Hybridisation".



18. Place the tube(s) on the magnetic stand and wait for the solution to clear (approx. 2–3 min).
19. Transfer **13 µl** of the eluate to the empty tubes from step 17. Tubes containing beads can be discarded at this time.
20. Add **2 µl** Index Blockers (blue lid; ●) to the pool(s).
21. Add **2 µl** of panel-specific baits to the pool(s).
22. Seal the tubes, mix on a vortex mixer and pulse spin to collect the contents. The final volume should be **17 µl**.
23. Make sure all caps are tightly sealed.
24. Place the tubes into the thermal cycler and run the program "OGT Hybridisation" shown in Table 9. Where possible, set the heated lid to 105°C, alternatively, have the pre-set heated lid activated.

Step	Temperature (°C)	Time
1	95	5 min
2	65	Hold

Table 9: Incubation profile of program "OGT Hybridisation".

25. Incubate the hybridisation mixture overnight (**16–20 hr**) at 65°C.

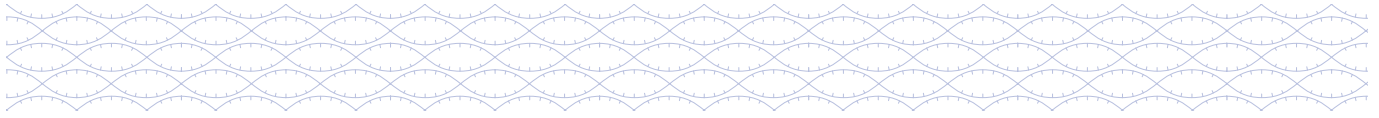


Avoid going beyond 24 hours as it increases the risk of evaporation.

26. Continue to "Universal capture and wash".



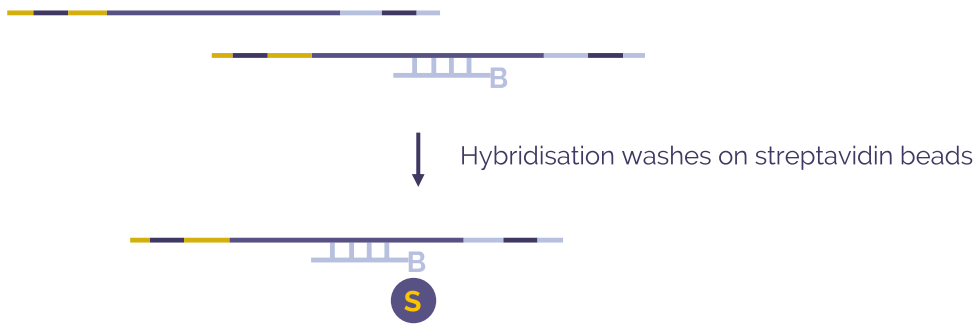
Remove the Hyb Wash Buffer (blue dot; ●) and Bead Priming Buffer (orange lid/dot; ●) from storage (–15°C to –25°C) and allow to thaw to room temperature. These can be left on the bench overnight to defrost.



## Library preparation: Universal capture and wash

### Universal capture and wash

#### Overview



The hybridised targets are bound to streptavidin beads and washed to remove any off-target DNA.

Estimated time: 1.25 hr for 8–24 samples. Hands-on time: 45 min.

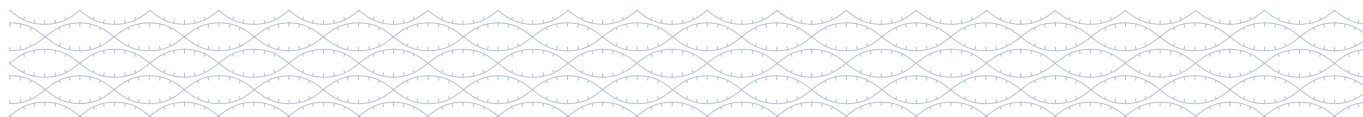
#### Before starting:

- 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**.



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

- Take the Dynabeads M-270 Streptavidin magnetic beads from the Post-PCR Universal Bead Kit out of the fridge at least **30 min before use** to allow them to warm to room temperature.
- Remove the Hyb Wash Buffer (blue dot; ●) and Bead Priming Buffer (orange lid/dot; ●) from storage (–15°C to –25°C) and allow to thaw to room temperature. These can be left on the bench overnight to defrost.



## Library preparation: Universal capture and wash

### Prepare Hyb Wash Buffer for use with CytoSure Constitutional NGS Panel only

- The CytoSure Constitutional NGS Panel requires the addition of Component A (brown lid; ●) to the Hyb Wash Buffer bottles.



Component A is **not** required for SureSeq Cancer Panels

- Remove Component A (brown lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and allow to thaw to room temperature.
- Follow Table 10 for the volume required for the kit size.

Kit size	Volume of Component A to add to Hyb Wash Buffer bottle ( $\mu\text{l}$ )
24 reaction	100
96 reaction	180 (per Hyb Wash Buffer bottle)

Table 10: Volume of Component A to add to the Hyb Wash Buffer bottle.

- Label the bottle to indicate that Component A has been added.
- Invert the bottle 10 times after Component A has been added.

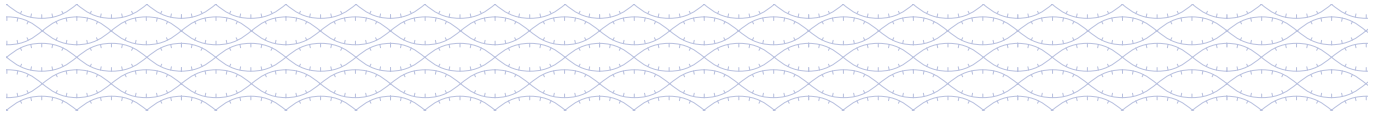
### Prepare sequence capture and bead wash buffers

- Ensure the Hyb Wash Buffer and Bead Priming Buffer are fully thawed.



Incubate at  $37^{\circ}\text{C}$  for 5–10 min to resuspend any precipitates. It is possible to defrost these buffers at room temperature during your overnight incubation.

- Aliquot **6 x 200  $\mu\text{l}$**  of Hyb Wash Buffer per hybridisation pool into PCR strip-tubes as shown in Figure 5 for one hybridisation pool.



## Library preparation: Universal capture and wash

- Pre-warm the aliquots to the following temperatures in a heat block for a minimum of **30 min before use**:
  - 3 x 200  $\mu$ l at 65°C / pool
  - 3 x 200  $\mu$ l at 35°C / pool

Pool 1	Pool 1
W1 ●	W1 ●
W2 ●	W2 ●
W3 ●	W3 ●
65°C washes	35°C washes

Figure 5: Set-up of Hyb Wash Buffer aliquots for one hybridisation pool.

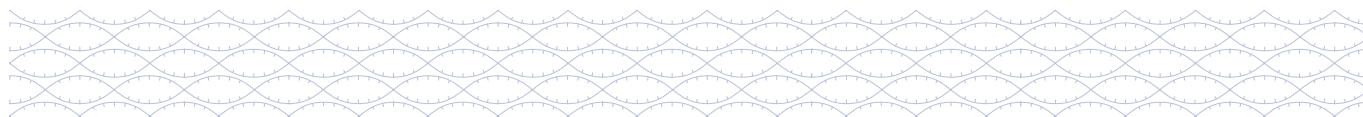
### Prepare magnetic beads

- Vortex the Dynabeads M-270 Streptavidin magnetic beads well for **1 min**, changing the orientation of the tube **every 15 sec**.



Ensure the bead cake is completely free from the bottom or sides of the tube as addition of excess of beads will negatively impact downstream processes.  
Do not pulse spin the magnetic beads after mixing.

- Immediately before use, resuspend the room temperature Dynabeads M-270 Streptavidin magnetic beads using a **200  $\mu$ l** pipette set to **100  $\mu$ l** and pipette mixing up and down at least **10 times**.



## Library preparation: Universal capture and wash

3. Add **75 µl** of the Dynabeads M-270 Streptavidin magnetic beads to a new PCR tube; one per hybridisation pool.



Alternatively: Up to 300 µl of beads (for four hybridisation pools) can be washed at once in a single 1.5 ml LoBind tube.



4. Place the tube(s) on a magnetic stand and wait for the solution to clear (approx. 10 sec).

5. Avoiding the bead pellet, remove and discard the cleared supernatant (~75 µl).

6. Add **150 µl** of 1x Bead Priming Buffer per **75 µl** beads. Mix on a vortex mixer and pulse spin to collect the contents.



7. Place the tube(s) back on the magnetic stand and wait for the solution to clear (approx. 10 sec).

8. Avoiding the bead pellet, remove and discard the cleared supernatant (~150 µl).

9. Repeat steps 6–8 once, for a total of **two** washes.

10. Remove from the magnetic stand and add the original volume of Bead Priming Buffer (i.e., for 75 µl of beads add 75 µl Bead Priming Buffer), mix on a vortex mixer and pulse spin to collect the contents.

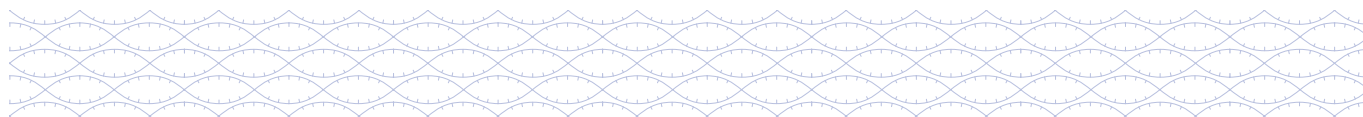
11. Label a new PCR strip-tube for each pool, pipette mix and transfer **75 µl** beads into each tube and pulse spin to collect the contents.



12. Place the tube(s) on a magnetic stand, allow the beads to separate from the supernatant, then carefully remove and discard the supernatant.



Proceed immediately to "Perform hybrid capture". Do not allow the beads to dry out. Small amounts of residual Bead Priming Buffer will not interfere with the downstream binding of the DNA to Dynabeads M-270 Streptavidin magnetic beads.



## Library preparation: Universal capture and wash

### Perform hybrid capture

1. After the overnight incubation, keep the hybridised samples on the thermal cycler and transfer all the hybridised samples (~17  $\mu$ l volume) to the prepared streptavidin beads.
2. Mix thoroughly on a vortex mixer for **3–5 sec** and ensure that the beads are resuspended. Pulse spin to collect the contents.
3. Return the tube(s) to the thermal cycler still running program “OGT Hybridisation” for **45 min**.
4. **Every 15 min**, mix on a vortex mixer for **3 sec** followed by a brief pulse spin to collect the contents. This ensures the beads remain in suspension. Place the tube(s) back in a thermal cycler running program “OGT Hybridisation”.
5. After the 45 min incubation, remove the tube(s) from the thermal cycler and pulse spin to collect the contents. **Proceed immediately** to the next step “Wash streptavidin beads to remove unbound DNA”.



Keep the thermal cycler program “OGT Hybridisation” running.

### Wash streptavidin beads to remove unbound DNA



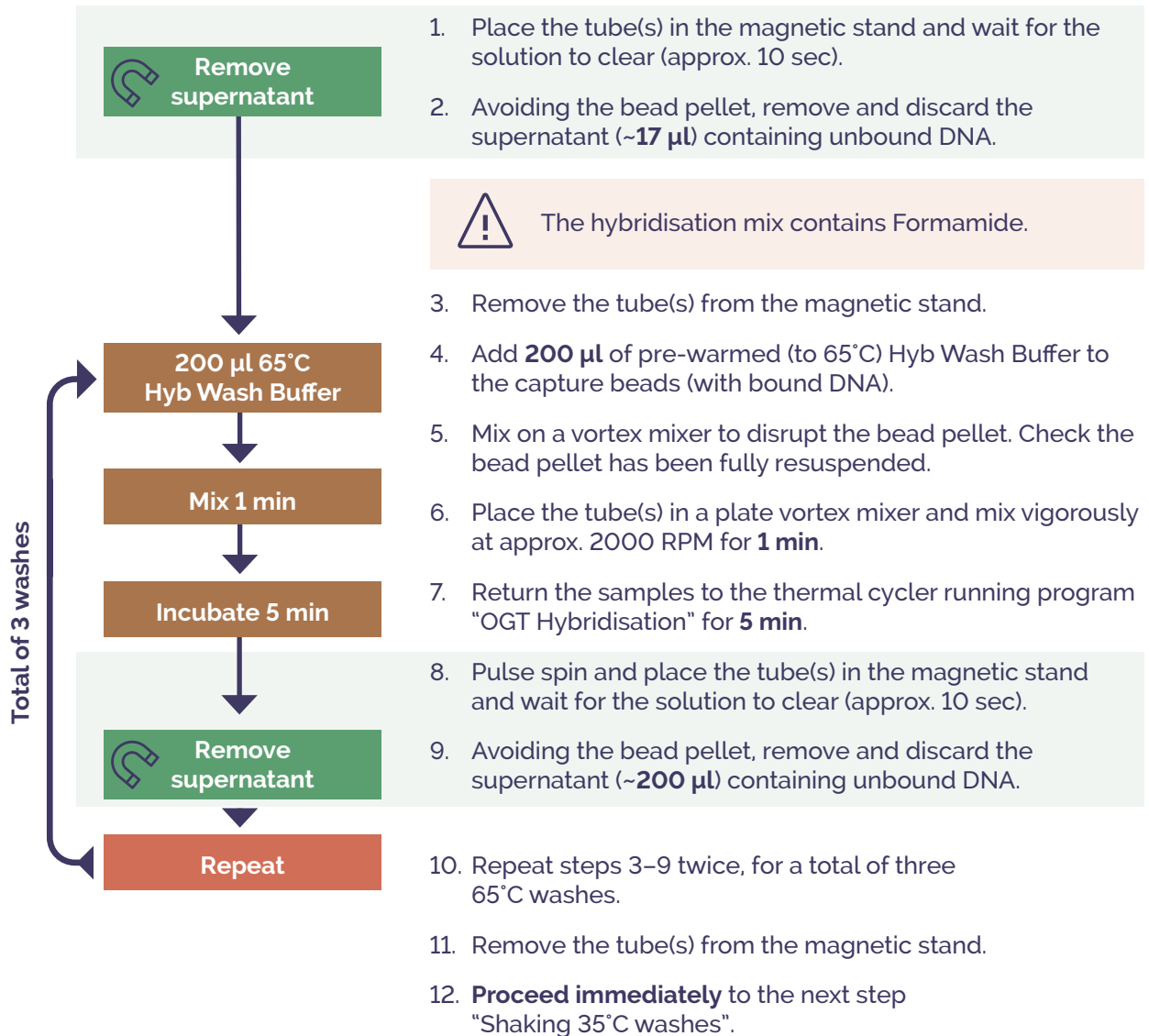
Work quickly to ensure the temperature does not drop much below 65°C. To achieve this, we recommend performing all washes in PCR strip-tubes and using a multichannel pipette.



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 a pipette for mixing.

## Library preparation: Universal capture and wash

## Hot 65°C washes



## Library preparation: Universal capture and wash

### Shaking 35°C washes

200  $\mu$ l 35°C  
Hyb Wash Buffer

Mix 2 min



Remove  
supernatant

200  $\mu$ l 35°C  
Hyb Wash Buffer

Mix 1 min



Remove  
supernatant

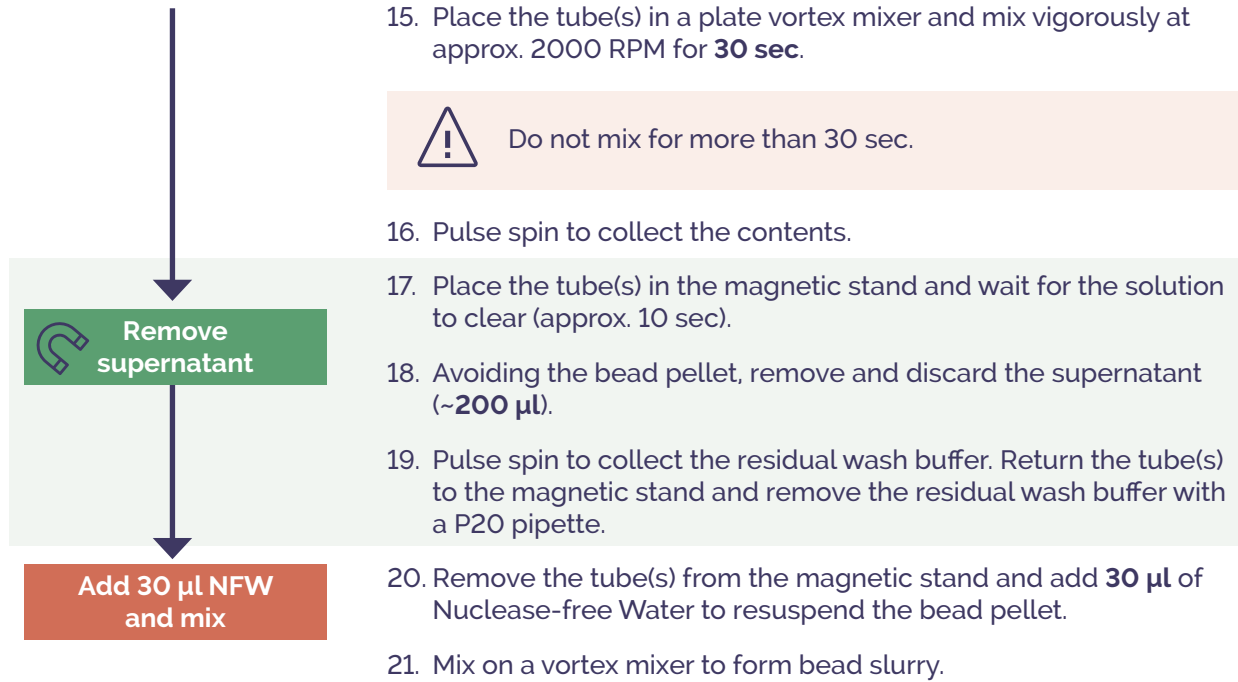
200  $\mu$ l 35°C  
Hyb Wash Buffer

Mix 30 sec

1. Add **200  $\mu$ l** of pre-warmed (to 35°C) Hyb Wash Buffer to the capture beads.
2. Mix on a vortex mixer to disrupt the bead pellet. Check the bead pellet has been fully resuspended.
3. Place the tube(s) in a plate vortex mixer and mix vigorously at approx. 2000 RPM for **2 min**.
4. Pulse spin to collect the contents.
5. Place the tube(s) in the magnetic stand and wait for the solution to clear (approx. 10 sec).
6. Avoiding the bead pellet, remove and discard the supernatant containing unbound DNA (~**200  $\mu$ l**).
7. Add **200  $\mu$ l** of pre-warmed (to 35°C) Hyb Wash Buffer to the capture beads.
8. Mix on a vortex mixer to disrupt the bead pellet. Check the bead pellet has been fully resuspended.
9. Place the tube(s) in a plate vortex mixer and mix vigorously at approx. 2000 RPM for **1 min**.
10. Pulse spin to collect the contents.
11. Place the tube(s) in the magnetic stand and wait for the solution to clear (approx. 10 sec).
12. Avoiding the bead pellet, remove and discard the supernatant containing unbound DNA (~**200  $\mu$ l**).
13. Add **200  $\mu$ l** of pre-warmed (to 35°C) Hyb Wash Buffer to the capture beads.
14. Mix on a vortex mixer to disrupt the bead pellet. Check the bead pellet has been fully resuspended.

## Library preparation: Universal capture and wash

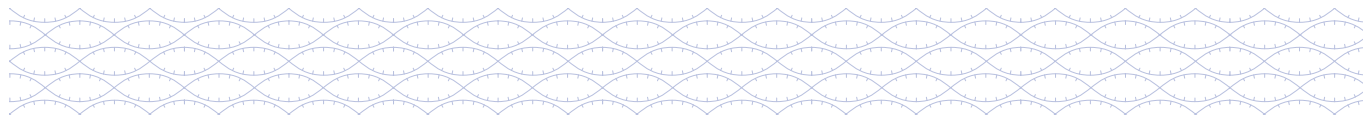
## Shaking 35°C washes (continued)



Recommendation: If proceeding with Step 4: Post-capture PCR immediately, the Step 4: Primer Mix and Step 4: PCR Buffer can be removed from storage to thaw to room temperature now.



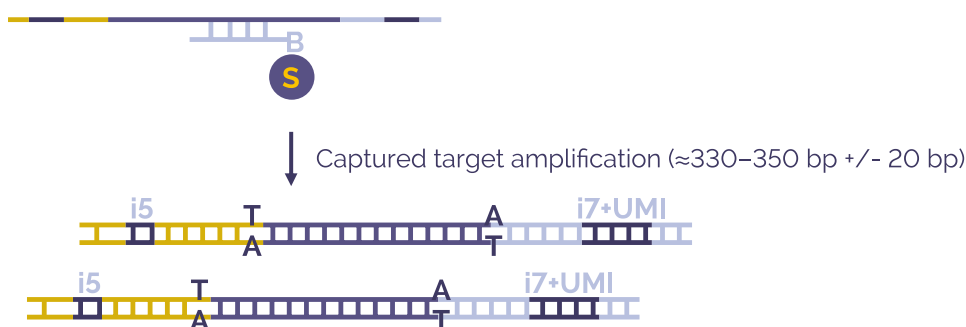
**OPTIONAL STOPPING POINT:** If the samples are not to be used immediately, store the bead slurry at 4°C. Do not freeze the bead slurry. If continuing, proceed to "Post-capture PCR".



## Library preparation: Step 4

### Post-capture PCR

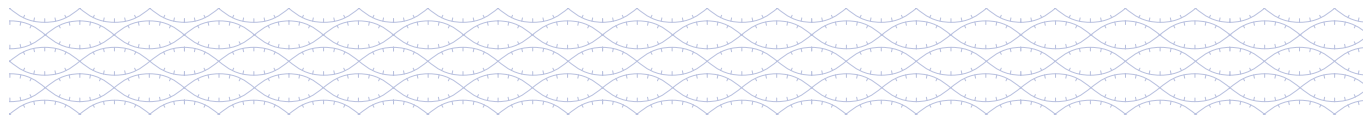
#### Overview



After capture of target fragments, ssDNA bound to the Streptavidin beads are amplified.

#### Before starting:

- Take the Mag-Bind TotalPure NGS beads from the Post-PCR Universal Bead Kit out of the fridge at least **30 min before use** to allow them to warm to room temperature.
- Make up a fresh solution of 80% ethanol using molecular biology grade ethanol and molecular biology grade water.
- Remove the Step 4: Primer Mix (purple lid; ●) and Step 4: PCR Buffer (purple lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and allow to thaw at room temperature.
- Ensure that all components in the PCR Buffer are dissolved. If necessary, vortex mix and/or incubate at  $37^{\circ}\text{C}$  until dissolved.
- ❄ • Remove the Step 4: PCR Polymerase (purple lid; ●) from storage ( $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ) and place on ice.



## Library preparation: Step 4

### Perform step 4: Post-capture PCR

Estimated time: 1.25 hr for 8–24 samples. Hands-on time: 15 min.


1. Program the thermal cycler using the settings shown in Table 11 and Table 12. Save the program as “OGT PCR2”. Where possible, set the heated lid to 105°C, alternatively have the pre-set heated lid activated.

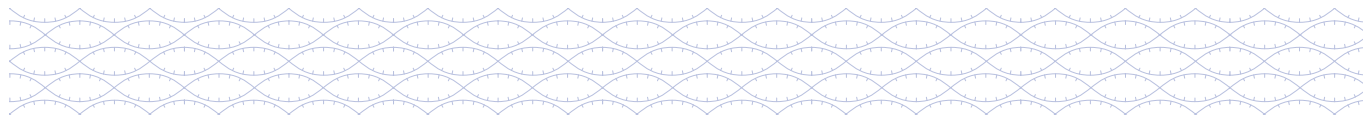
Step	Temperature (°C)	Time
1	98	3 min
2	98	30 sec
3	65	30 sec
4	72	1 min
5	Repeat Step 2 to Step 4 for a total number of cycles as specified in Table 12	
6	72	10 min
7	4	Hold

Table 11: Incubation profile of program “OGT PCR2”.

Total number of PCR cycles	Panel
18	770001 – Core MPN
16	770002 – Myeloid plus
	770027 – CLL + CNV V3
	770005 – Germline Breast Cancer + CNV
	770007 – Comprehensive FH
15	770003 – Pan-Myeloid
10	502003 – CytoSure Constitutional NGS Panel

Table 12: Total number of PCR cycles for various OGT NGS Panels. Note: if using a custom MyPanel, then please contact your FAS for the number of PCR cycles.

2. Mix Step 4: Primer Mix and Step 4: PCR Buffer on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
3.  Flick mix Step 4: PCR Polymerase, pulse spin to collect the contents and keep on ice.



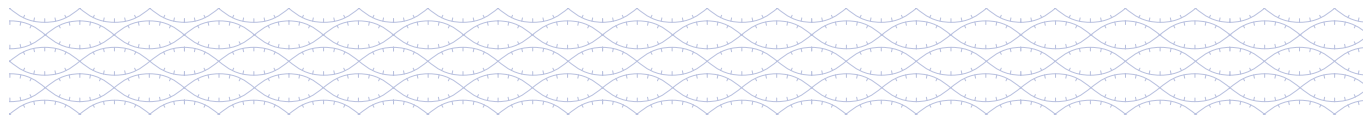
## Library preparation: Step 4

- For multiple pools, prepare the Post-capture PCR Master Mix according to Table 13 in a fresh 1.5 ml LoBind tube.

Reagent	1x Pool (µl)	2x Pool (µl) (includes 0.5 excess)	3x Pool (µl) (includes 0.5 excess)
Captured DNA in bead slurry	14	–	–
Nuclease-free Water (clear lid; ○)	26.5	66.25	92.75
Step 4: PCR Buffer (purple lid; ●)	5	12.5	17.5
Step 4: Primer Mix (purple lid; ●)	2.5	6.25	8.75
Step 4: PCR Polymerase (purple lid; ●)	2	5	7
<b>TOTAL</b>	<b>50</b>	<b>90</b>	<b>126</b>

Table 13: Post-capture PCR Master Mix.

- Mix the Post-capture PCR Master Mix on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
- Label a new set of PCR strip-tube(s) for each PCR reaction.
- Add **36 µl** of the Post-capture PCR Master Mix into the tube(s) from step 6.
- Resuspend the bead slurry by pipette mixing up and down at least 10 times to ensure the beads are homogeneous. **Immediately** add **14 µl** of well-mixed bead slurry to each of the tubes prepared in step 6.
- Pipette mix at least 10 times.
- Transfer to the thermal cycler and start the program “OGT PCR2”.



## Library preparation: Step 4


### Perform post-capture PCR purification

Estimated time: 40 min for 8–24 samples.



Prior to use, mix beads on a vortex mixer for at least 1 min or until the bead solution appears homogeneous and consistent in colour.

#### To the DNA sample tubes:

1. Add **45 µl** of homogeneous, room temperature Mag-Bind TotalPure NGS beads from the Post-PCR Universal Bead Kit to each DNA sample tube. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.
2. Incubate at room temperature for **5 min**.
-  3. Place the tube(s) in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
4. Avoiding the bead pellet, remove and discard the cleared supernatant (**~95 µl**). **Keep the beads containing the DNA sample.**
5. Add **200 µl** of 80% ethanol to each tube without resuspending the bead pellet.
6. Incubate for **30 sec**, then remove the ethanol.
7. Repeat wash (steps 5 and 6) once, for a total of two washes.
8. Seal the tube(s) and pulse spin to collect the residual ethanol. Return the tube(s) to the magnetic stand for **30 sec**. Remove the residual ethanol with a P20 pipette.
9. Dry the bead pellet(s) at room temperature for **1–2 min**.



Do not over-dry as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte. Over-drying results in cracks in the bead pellet.

10. Remove from the magnetic stand and add **32 µl** of Nuclease-free Water directly to the bead pellet to elute the DNA sample. Mix on a vortex mixer and pulse spin to collect the contents.
11. Incubate for **5 min** at room temperature.

## Library preparation: Step 4

12. Label a new set of PCR strip-tube(s) for the eluate(s) and set aside until required in step 14.



13. Place the tube(s) on the magnetic stand and wait for the solution to clear (approx. 2–3 min).

14. Transfer **30 µl** of the eluate containing the purified post-capture PCR product(s) to the tube(s) from step 12. The tube(s) containing beads can be discarded at this time.

15. Assess the size of the amplified product using the Agilent High Sensitivity D1000 ScreenTape System. The electropherogram should show a peak size of 330–350 bp (+/- 20 bp) (Figure 6). Set up the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.

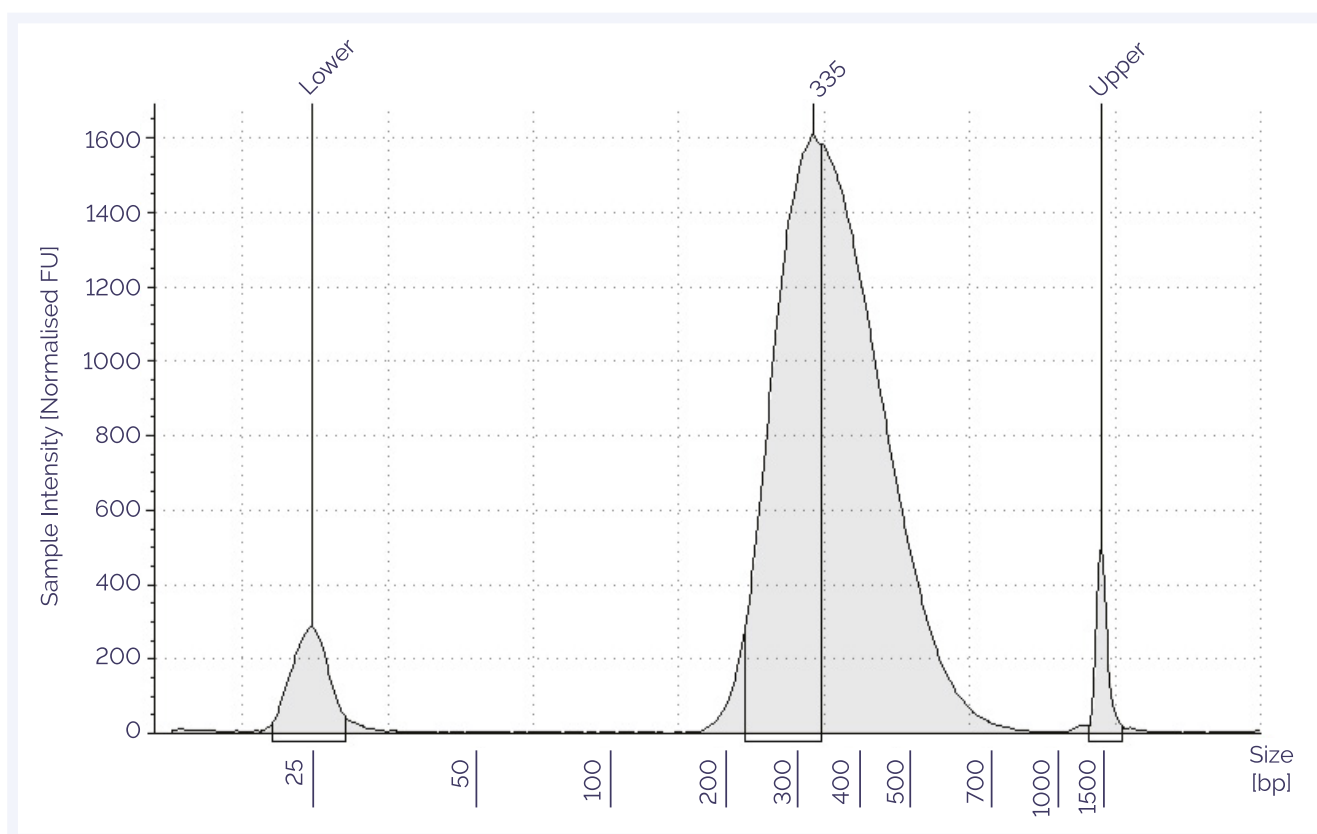
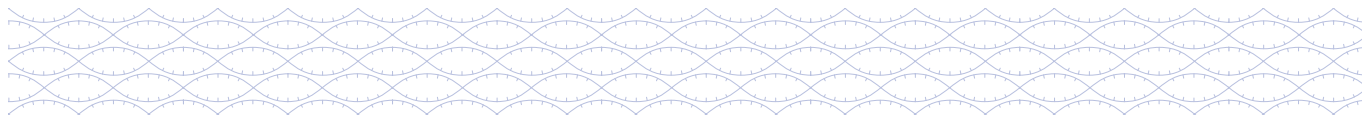


Figure 6: Electropherogram of purified Post-capture PCR product generated using an Agilent High Sensitivity D1000 ScreenTape assay. The electropherogram shows a maximum peak in the size range of approximately 330–350 bp (+/- 20 bp).



Fragment sizes outside of this range may reduce sequence data quality. Contact your local Field Application Specialist (FAS) for further details.



## Library preparation: Step 4

- Assess yield using **1  $\mu$ l** amplified product with the Qubit dsDNA HS Kit as per manufacturer's instructions. The expected yield is panel dependent and between 1–10 ng/ $\mu$ l. Contact your local Field Application Specialist (FAS) for further details.



**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. If continuing, proceed to "Sequencing".

## Sequencing

### Sequencing

#### Overview

The DNA capture pools prepared in the previous section (Post-capture PCR) need to be combined such that each pool is present in equimolar amounts when loaded onto the sequencer. This requires both accurate determination of peak size (bp), provided by Agilent TapeStation (High Sensitivity Kit), and accurate determination of sample concentration (ng/ $\mu$ l), provided by Thermo Fisher Scientific Qubit (High Sensitivity) assay.

#### Preparing the sequencing pool



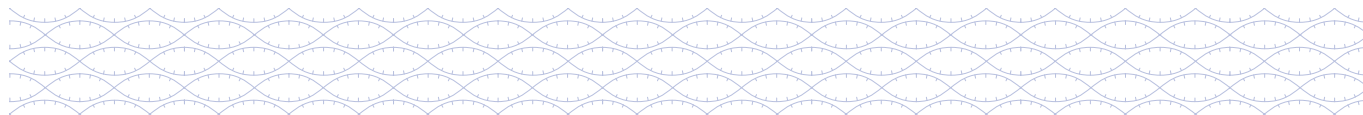
A worksheet can be created using the "OGT\_ULPK\_Worksheet" template provided by OGT. Alternatively use the formulae below.

- Use your worksheet described above or the formulae below to determine the volume ( $\mu$ l) of each DNA capture pool required to generate the 4 nM sequencing pool.



This protocol has been validated with 150-base paired-end reads using the MiSeq Reagent Kit v2 300 cycle (Illumina cat. no. MS-102-2002) and NextSeq 550 High-Output Kit 2 x 150 bp (Illumina cat. no. 20024908).

- Complete the "Sequencing Pool Parameters" and "Samples" tables in the "PCR2" tab of the pooling template. Cells in green should be manually modified as required; parameters marked with \* must be provided.



## Sequencing

3. Add the appropriate volume of each indexed sequencing pool to a fresh 1.5 ml LoBind tube labelled “4 nM Sequencing Pool”; the volumes can be found in the column labelled “Volume of PCR2 product to pipette” in the “Volumes to pipette” tabs.
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 B of the “Volumes to pipette” tabs next to “Volume of Nuclease-free water to pipette”.
5. Validation of sequencing pool concentration: Assess peak size distribution of the sequencing pool using the Agilent High Sensitivity D1000 ScreenTape System; and assess yield using the Qubit dsDNA HS Kit. Complete the “Pool validation and dilution” tab to determine the molar concentration of the sequencing pool.
6. The sequencing pool is ready for loading on to the sequencer.



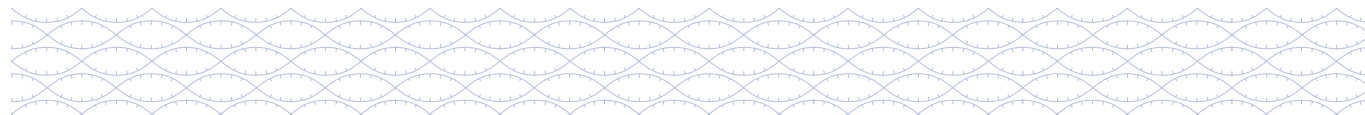
**OPTIONAL STOPPING POINT:** If the sequencing pool is not to be used immediately, store at -20°C for long-term storage. If continuing, proceed to “Preparing the Sample Sheet”.

### Formula 1 — nM of each sample

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

### Formula 2 — volume of each indexed DNA sample

$$\text{Volume of indexed sample} = \frac{\text{Sequencing Pool } (\mu\text{l}) \times \text{Pool concentration (4 nM)}}{\text{Number of samples in Pool} \times \text{nM concentration of the sample}}$$



## Sequencing

### Preparing the Sample Sheet



A MiSeq Sample Sheet can be created using the MiSeq pooling guide provided by OGT.

1. Open the completed Worksheet and click on the relevant Sample Sheet tab.

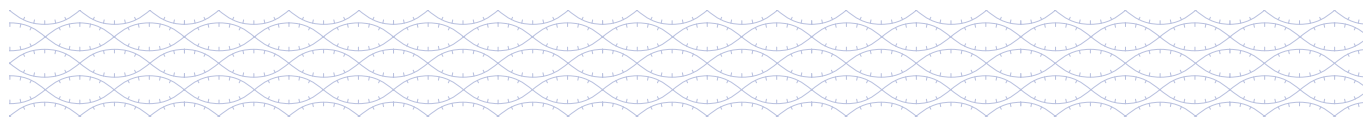


This sheet will be automatically filled with the parameters and sample data entered into the "PCR1" and "PCR2" sheets.

2. Highlight all cells with text as shown in Figure 7. Adjust the number of rows highlighted as appropriate.

[Header]										
1	[Header]									
2	IEMFileVersion								4	
3	Investigator Name								0	
4	Project Name	Example Samplesheet								
5	Experiment Name	Example Samplesheet								
6	Date	19/12/2023								
7	Workflow	GenerateFASTQ								
8	Application	FASTQ Only								
9	Assay	OGT_enrichment								
10	Description									
11	Chemistry	Amplicon								
12										
[Reads]										
14		150								
15		150								
16										
[Settings]										
18	ReverseComplement								0	
19										
20										
21										
22										
[Data]										
24	Sample_ID	Sample_Name	Sample_Plate	Sample_Well	I7_Index_ID	index	I5_Index_ID	index2	Sample_Project	Description
25	Sample01	Sample01			1	CTGATCGTNNNNNNNN	501	ATATGCGC	Example Samplesheet	
26	Sample02	Sample02			2	ACTCTCGANNNNNNNN	502	TGTTACAG	Example Samplesheet	
27	Sample03	Sample03			3	TGAGCTAGNNNNNNNN	503	AACCGTTC	Example Samplesheet	
28	Sample04	Sample04			4	GAGACGATNNNNNNNN	504	TAACCGGT	Example Samplesheet	
29	Sample05	Sample05			5	CTTGTGANNNNNNNN	505	GAACATCG	Example Samplesheet	
30	Sample06	Sample06			6	TTCCAAGNNNNNNNN	506	CCTGTGAG	Example Samplesheet	
31	Sample07	Sample07			7	CGCATGATNNNNNNNN	507	TCAGGCTT	Example Samplesheet	
32	Sample08	Sample08			8	ACGGACANNNNNNNN	508	GTTCTCGT	Example Samplesheet	
33	Sample09	Sample09			9	CGGCTAATNNNNNNNN	509	AGAACGAG	Example Samplesheet	
34	Sample10	Sample10			10	ATCGATCGNNNNNNNN	510	TGCTTCCA	Example Samplesheet	
35	Sample11	Sample11			11	GCAAGATCNNNNNNNN	511	CTTCGACT	Example Samplesheet	
36	Sample12	Sample12			12	GCTACTCTNNNNNNNN	512	CACCTGTT	Example Samplesheet	
37	Sample13	Sample13			13	TACGGTACNNNNNNNN	513	ATCACACG	Example Samplesheet	
38	Sample14	Sample14			14	TGGACTCTNNNNNNNN	514	CCGTAAGA	Example Samplesheet	
39	Sample15	Sample15			15	AGAGTAGCNNNNNNNN	515	TACGCCTT	Example Samplesheet	
40	Sample16	Sample16			16	ATCCAGAGNNNNNNNN	516	CGACGTTA	Example Samplesheet	

Figure 7: Example Sample Sheet on the MiSeq pooling template.



## Sequencing

- Copy highlighted cells and paste into a new Excel file.



All text in red is for user and sample specific information. All text in black is required to ensure that the sequencer will recognise the file.

- Save the new sheet as a CSV (comma delimited) file.
- The Sample Sheet can now be uploaded to the sequencer by “Manual sample sheet upload”.



**OPTIONAL STOPPING POINT:** If the sequencing pool is not to be used immediately, store at  $-20^{\circ}\text{C}$  for long-term storage. If continuing, proceed to “Denaturing and loading the sequencing pool” or refer to the appropriate Illumina protocol.

## Denaturing and loading the sequencing pool

- Follow the appropriate Illumina protocol for denaturing the sequencing pool.



If the sequencer has only one dilution step, follow the Illumina protocol for loading. If the sequencer requires a secondary dilution step, for example the dilution of the 20 pM Denatured pool to the 12 pM Loading pool on a MiSeq, continue with step 2.



- After denaturation make the volume of the pool up to 1 ml with ice-cold HT1 to dilute your denatured pool to  $\sim 20$  pM and keep on ice.

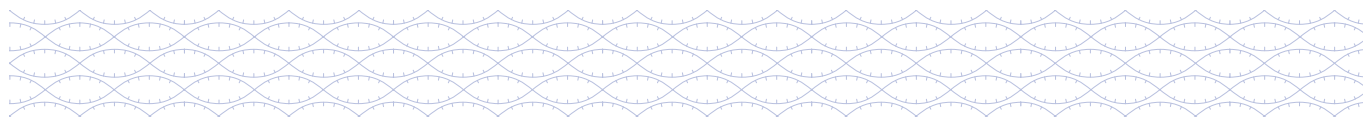


Volumes of HT1 required will vary depending on the volume of Denatured pool, dictated by the sequencer type. The actual concentration of the  $\sim 20$  pM pool can be found on the “Pool validation and dilution” tab in the Worksheet and will depend on the concentration of the sequencing pool.

- Enter the required sequencer loading concentration into the “Pool validation and dilution” tab in your worksheet.



Cluster density can vary between machines. We recommend loading a final concentration of 8–12 pM if using a MiSeq V2 300 cycle kit and 1.4 pM if using a NextSeq 500/550 High-Output Kit.



## Sequencing

4. Dilute the denatured 20 pM pool to this loading concentration by pipetting the volumes stated in the “Pool validation and dilution” tab in your worksheet.



If running a NextSeq, the sequencing pool should be combined with 5% denatured PhiX before running, according to the Illumina protocol.

5. Pipette the loading pool into the sequencer cartridge and set up the sequencing run according to the Illumina protocol.

## Appendix

### Adapter sequences

Adapter	Sequence
1	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
2	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Table 14: Adapter sequences – forward configuration. Please note some Illumina sequencing instruments require the reverse complement of the Index 2 (i5) Adapter sequence.

### Index sequences

Index sequences can be found by scanning the QC code or by visiting <https://www.ogt.com/products/sureseq-ngs/sureseq-ngs-index-sequences/>

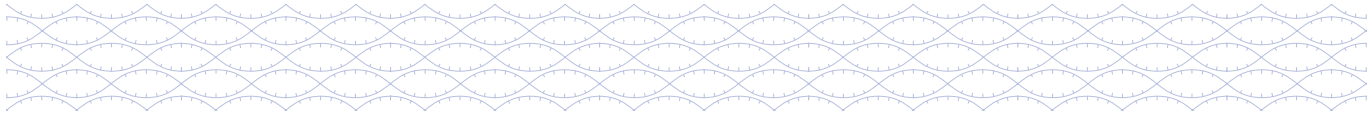


## Appendix

## Recommended sequencing guidelines

Panel	Recommended sequencing platform	Number of samples per sequencing run	Pool size proposal	Recommended kit size purchase	Number of runs based on kit size purchase
770001 – CoreMPN	MiSeq 300 V2	48	6 x 8-plex	2 x 24 reactions	1 run
				1 x 96 reactions	2 runs
770002 – Myeloid Plus	MiSeq 300 V2	16	2 x 8-plex	2 x 24 reactions	3 runs
				1 x 96 reactions	6 runs
770027 – CLL+CNV v3	MiSeq 300 V2	16	2 x 8-plex	2 x 24 reactions	3 runs
				1 x 96 reactions	6 runs
770003 – Pan Myeloid	MiSeq 300 V2	8	1 x 8-plex	1 x 24 reactions	3 runs
				MiSeq 600 V3	16
	NextSeq Mid-Output	48	6 x 8-plex	1 x 96 reactions	6 runs
				2 x 24 reactions	1 run
502003 – CytoSure Constitutional NGS	NextSeq High-Output	24	3 x 8-plex	1 x 24 reactions	1 run
				1 x 96 reactions	2 runs
770007 - CytoSure Comprehensive Familial Hypercholesterolaemia (FH) NGS	MiSeq 300 V2	16	3 x 8-plex	2 x 24 reactions and 1 x 96 reactions	3 and 6 runs

Table 16: Recommended sequencing guidelines.



# Appendix

## Reagent tube locations

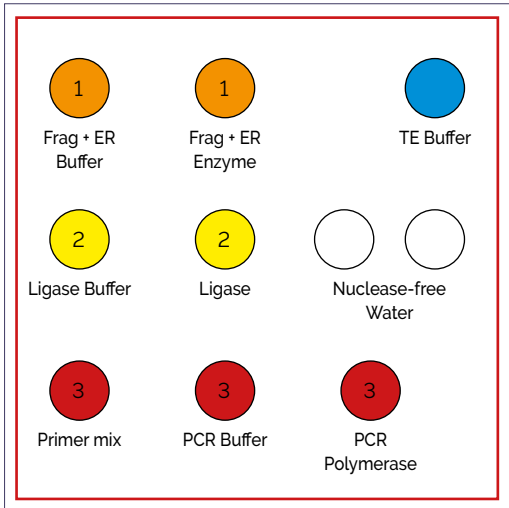


Figure 8: Location of tubes in 24 reaction Library Preparation Kit (770100-24).

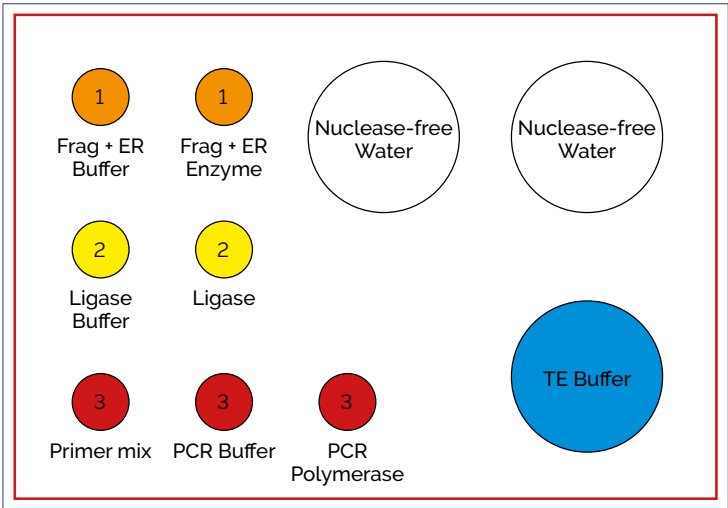


Figure 10: Location of tubes in 96 reaction Library Preparation Kit (770100-96).

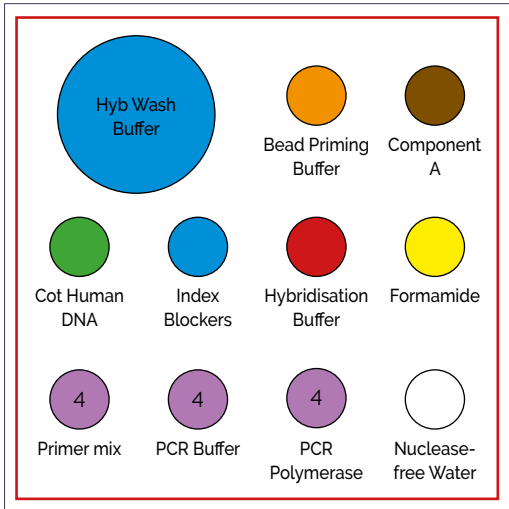


Figure 9: Location of tubes in 24 reaction Hybridisation & Wash Kit V2 (770410-24).

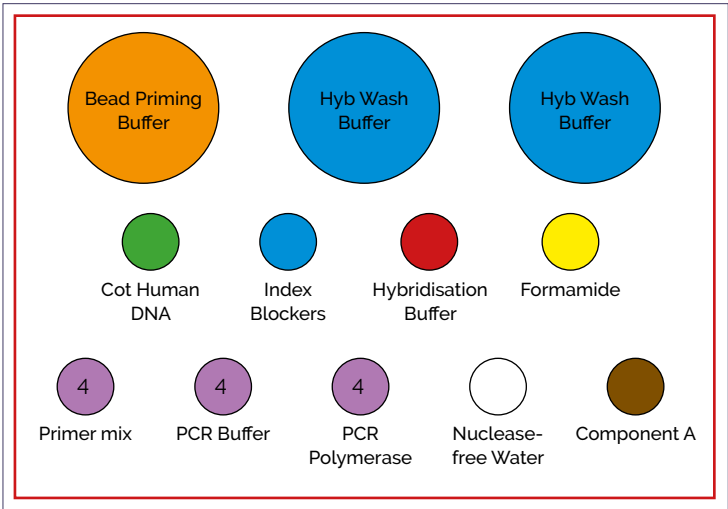
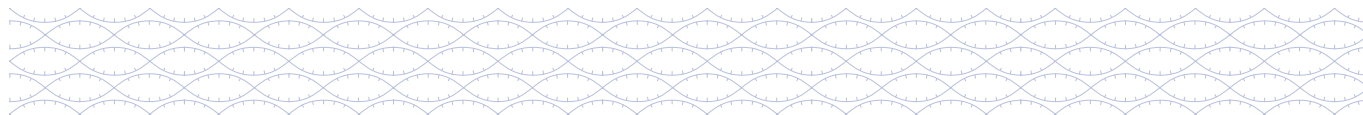


Figure 11: Location of tubes in 96 reaction Hybridisation & Wash Kit V2 (770410-96).



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## NGS Library Preparation assay

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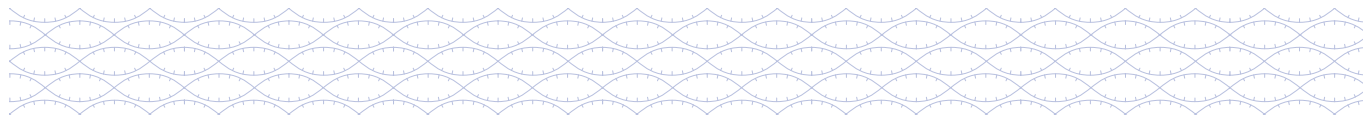
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## Ordering information

Product	Contents	Cat. No.
Universal NGS Workflow Solution V2	Bundle of 1 x Universal Library Preparation Kit, 1 x Universal Index Adapters, 1 x Universal Hybridisation & Wash Kit, 1 x Universal Bead Kit	770510-24 770510-96

Table 17: Ordering information.

**For an up-to-date product list and the latest product information, visit [ogt.com](http://ogt.com)**

### Contact information

**UK +44 (0) 1865 856826**

**US +1 914 467 5285**

**Technical support: [support@ogt.com](mailto:support@ogt.com)**

**[contact@ogt.com](mailto:contact@ogt.com)**

**[ogt.com](http://ogt.com)**

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990370 02/26

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