SureSeq

A Sysmex Group Company

OGT Handbook

SureSeq Myeloid MRD Ultra Low Workflow

HBK N-0002; 990377

Table of contents

Introduction

This handbook describes the SureSeq™ Myeloid MRD Ultra Low Workflow which has been developed and optimised for use with the SureSeq Myeloid MRD NGS Panel 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 NextSeq™ and NovaSeq™ chemistries.

There are sufficient reagents contained within the 96-reaction kit to process a total of 48 samples as a set of 8 samples on six occasions where 2 libraries are prepared per sample.

Reagents, consumables and equipment

Reagents supplied by OGT

Contents	Shipping/storage conditions	Cat. No. (96 reactions)
Universal Library Preparation Kit	Shipped at –20˚C, store at –20˚C	770100-96
Universal Index Adapters	Shipped at –20˚C, store at –20˚C	770200-96
Universal Hybridisation & Wash Kit V2	Shipped at –20˚C, store at –20˚C	770410-96
Pre-PCR Universal Bead Kit	Shipped at 4˚C, store at 4˚C	770310-96
Post-PCR Universal Bead Kit	Shipped at 4˚C, store at 4˚C	770315-96
SureSeq Myeloid MRD NGS Panel	Shipped at –20˚C, store at –20˚C	770026-48

Universal Library Preparation Kit
770100-96

Universal Index Adapters
770200-96

Universal Hybridisation & Wash Kit V2
770410-96

Pre-PCR Universal Bead Kit
770310-96

Post-PCR Universal Bead Kit
770315-96

SureSeq Myeloid MRD NGS Panel
770026-48

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
NextSeq 500/550 High-Output Kit v2.5 (300 cycles)	Illumina†	20024908
NextSeq 1000/2000 P2 Reagents (300 Cycles)	Illumina†	20046813
NovaSeq 6000 SP Reagent Kit v1.5 (300 cycles)	Illumina†	20028400
Optional: Genomic DNA ScreenTape	*Agilent Technologies*	*5067-5365*
Optional: Genomic DNA Reagents	*Agilent Technologies*	*5067-5366*

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

† 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	–
Optional: 15 ml or 50 ml Tubes	*General laboratory supplier*	–
Optional: Disposable reagent reservoirs	*General laboratory supplier*	–

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 supplier	–
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 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

Recommended before you start

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 µl of Nuclease-free Water (without DNA) in each well that you might use and use the thermal cycler settings in Table 8.
- Check after overnight incubation that the evaporation does not exceed 1–2 µl 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® TotalPure NGS beads and the Dynabeads™ M-270 Streptavidin magnetic beads to equilibrate to room temperature by removing them from their storage conditions at least 30 min before use.

Storage and handling

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

The Universal Library Preparation Kit, Universal Index Adapters, SureSeq Myeloid MRD NGS Panel and Universal Hybridisation & Wash Kit V2 should be stored at −20°C.

The Pre-PCR and Post-PCR Universal Bead Kits should be stored at 4°C.

General guidelines

Safety

Handling of the SureSeq Myeloid MRD NGS Panel 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. The SureSeq Myeloid MRD Ultra Low Workflow is designed to be used by suitably trained personnel using DNA extracted from whole blood and/or bone marrow.

Interpret NGS Analysis Software

Raw data FASTQ files with encoded unique molecular identifiers (UMI) 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. For more information on how to generate the UMI FASTQ files, or any other software queries, contact your local OGT Field Application Specialist (FAS).

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. In the SureSeq Myeloid MRD Ultra Low Workflow, each sample has two pre-hybridisation libraries prepared with the same index adapter. Each library pair is tagged with unique dual index (barcode) sequences, as well as UMIs for error correction and increased accuracy during analysis. Libraries are amplified and then pooled into sets of eight. Each pool is hybridised, washed, captured and then amplified ready for sequencing.

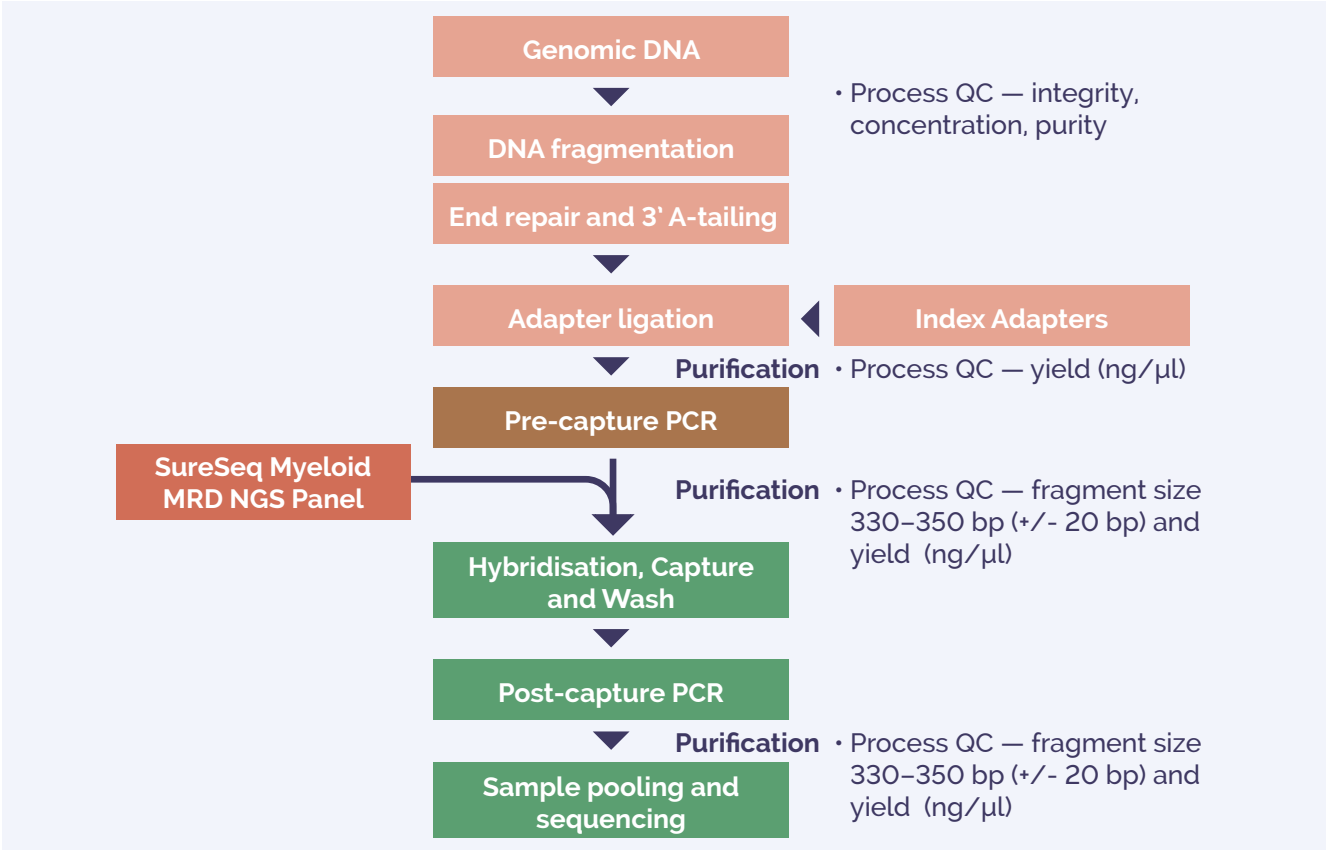


Figure 1: Workflow of sample library preparation indicating the expected DNA fragment size at each step of the procedure.

Workflow overview

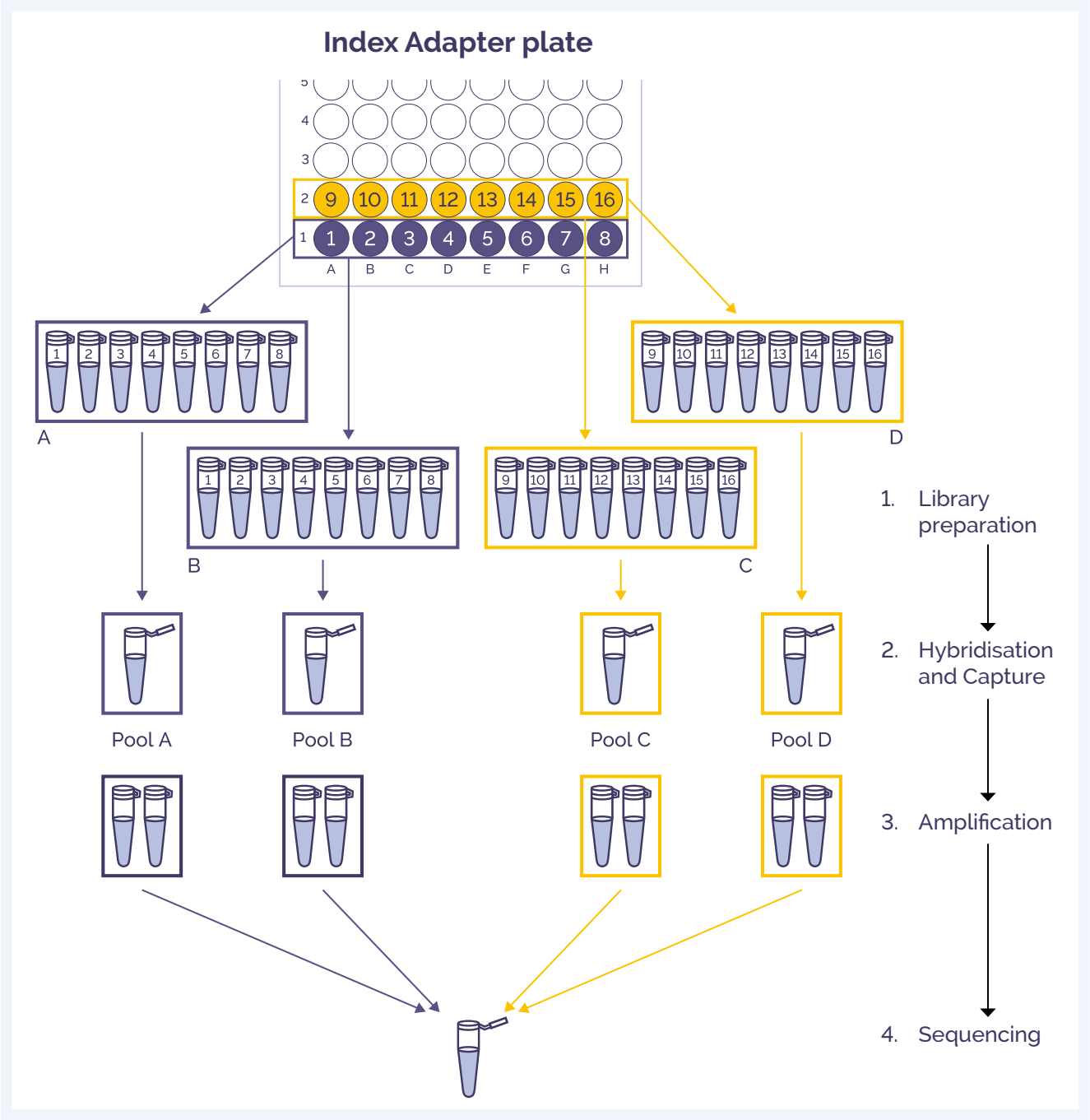


Figure 2: Outline of SureSeq Myeloid MRD Ultra Low Workflow for 16 samples.

1. Library preparation: 16 samples prepared in duplicate. Duplicates are ligated with the same Index Adapter.
2. Hybridisation: 4 pools, each consisting of one strip of eight libraries.
3. Post-capture PCR: 8 reactions prepared from duplicates of each hybridisation pool.
4. Sequencing: PCR reactions are pooled for sequencing.

Sample requirements

The protocol has been optimised for a total DNA input of 400 ng per sample, split into two 200 ng replicates.

Modifying the recommended DNA input will impact downstream sequencing results.

DNA — sample preparation

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

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.

Samples with an initial DNA concentration >100 ng/µl should be prediluted to 20–100 ng/µl 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

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

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

1. Prepare the ladder by mixing 10 µl of Genomic DNA Sample Buffer with 1 µl 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 µl of DNA sample to 10 µl 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".

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

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 µl of sample buffer or blanking solution.
4. Click "Measure blank".
5. Clean the pedestal with a lint-free wipe.
6. Load 1–2 µl of each sample onto the pedestal.
7. Click "Measure".
8. Record the readings for 260/230, 260/280 and the concentration (ng/µl).

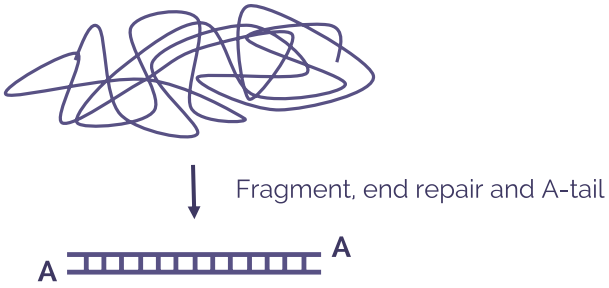
An OD 260/280 ratio of 1.8 to 2.0 and OD 260/230 ratio of 2.0 to 2.2 is recommended. Use of DNA samples with lower ratios may result in poor performance.

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.

It is recommended to process no more than 16 samples (32 libraries) in a single set up during the fragmentation step.

Before starting:

- Remove TE Buffer (blue lid; ●) from storage (−15°C to −25°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°C to −25°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.

Library preparation: Step 1

- Remove Step 1: Frag + ER Enzyme (orange lid; ●) from storage (−15°C to −25°C) and place on ice.
- Ensure DNA sample(s) are prediluted to 20–100 ng/µl with TE Buffer (blue lid; ●).

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

Estimated time: 1.25 hr for 8–16 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 **400 ng** of sample DNA with the chilled TE Buffer (blue lid; ●) provided to a total volume of **54 µl**. Mix on a vortex mixer for **3–5 sec**, pulse spin to collect the contents and place on ice.

Recommendation: prepare the samples as shown in Figure 3. This format will minimise pipetting steps during the Adapter ligation step.

Library preparation: Step 1

4. Label two new sets of PCR strip-tubes for each sample as shown in Figure 3 and place on ice.

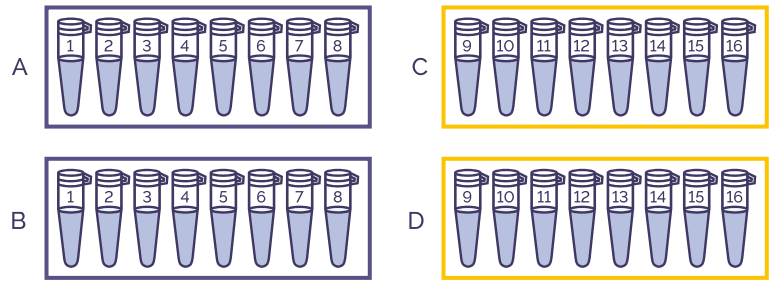


Figure 3: Recommended configuration for 16 samples. A/B and C/D indicate replicate strips and numbers indicate unique sample numbers.

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.

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 sample 16x library (µl) (includes 2 excess)	16x sample 32x library (µl) (includes 3 excess)
DNA sample	27	–	–
Step 1: Frag + ER Buffer (orange lid; ●)	7	126	245
Step 1: Frag + ER Enzyme (orange lid; ●)	1	18	35
TOTAL	**35**	**144**	**280**

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.

Library preparation: Step 1

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 minutes of the thermal cycler program.

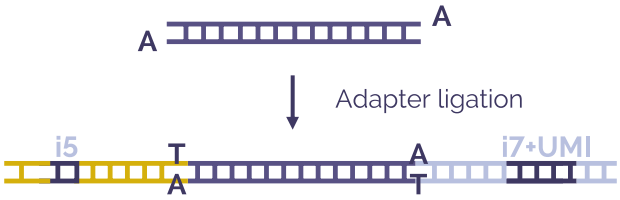
12. When the program is complete and the thermal cycler has reached 4°C, remove the samples and place them on ice until you are ready to proceed with Adapter ligation.

> 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



Illumina compatible Adapter sequences are ligated onto the repaired dsDNA fragments with DNA ligase, using the 3' overhang created during end repair/A-tailing. 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°C to −25°C) and thaw to room temperature. Ensure that all components in the Ligase Buffer are well dissolved. If necessary, incubate at 37°C until dissolved.
- Remove the Step 2: Ligase (yellow lid; ●) from storage (−15°C to −25°C) and place on ice.
- Remove the Universal Index Adapter plate from storage (−15°C to −25°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 library pair. See Figure 5 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 8–16 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.
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 sample 16x library (µl) (includes 2 excess)	16x sample 32x library (µl) (includes 3 excess)
DNA sample	35	–	–
Index Adapter	3.5	–	–
Step 2: Ligase Buffer (yellow lid; ●)	9	162	315
Step 2: Ligase (yellow lid; ●)	3	54	105
TOTAL	**50.5**	**216**	**420**

Table 4: Ligation Master Mix.

Library preparation: Step 2

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** Index Adapter to each DNA sample tube from Step 5. Ensure the same Index Adapter is used for both replicates of a sample (see Figure 4).
7. Add **12 µl** of the Ligation Master Mix to each chilled DNA sample tube containing the fragmented product(s).

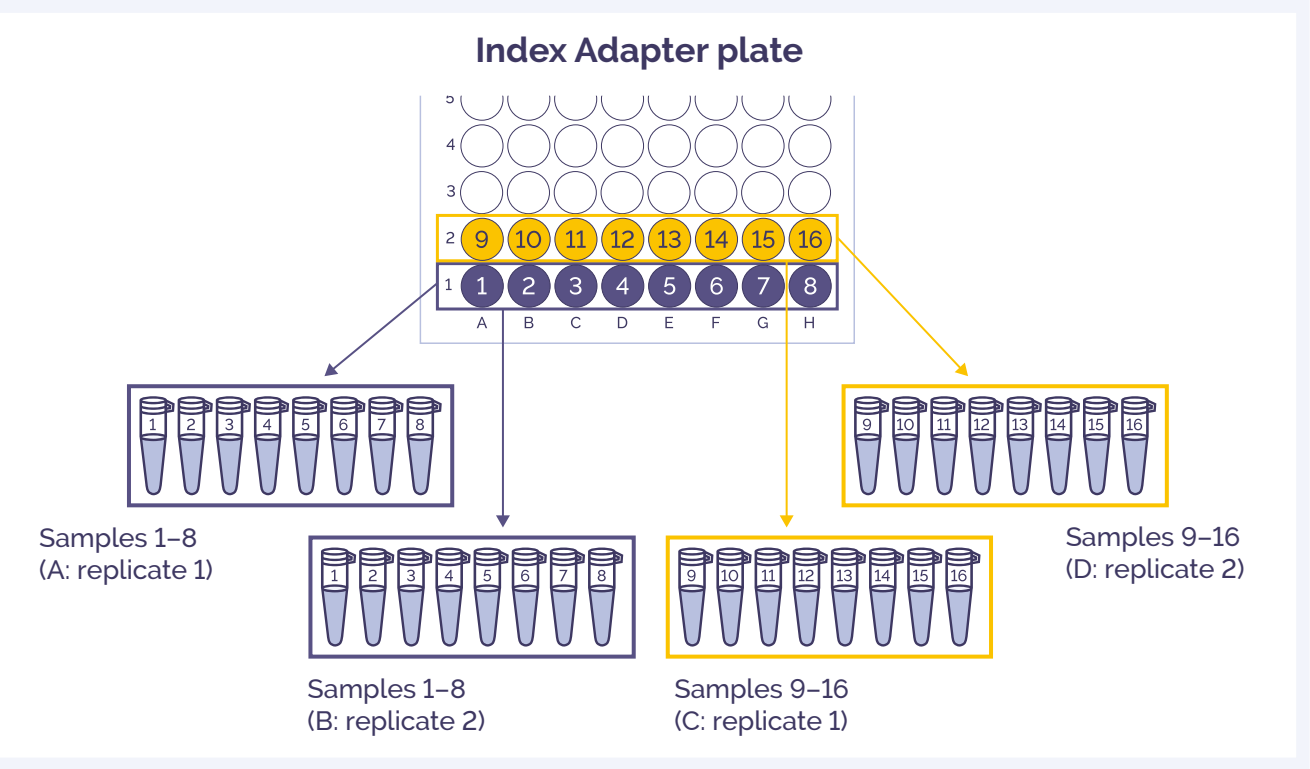


Figure 4: Pipetting scheme used when adding Index Adapter to DNA sample tubes. Duplicate strips are ligated with the same Index Adapter.

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

Perform Ligated library purification

Estimated hands on time: 50 min for 8–16 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 libraries.
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 7.

Library preparation: Step 2

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 second set of 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.
14. Repeat wash (Steps 12 and 13) 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.

Library preparation: Step 2

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.

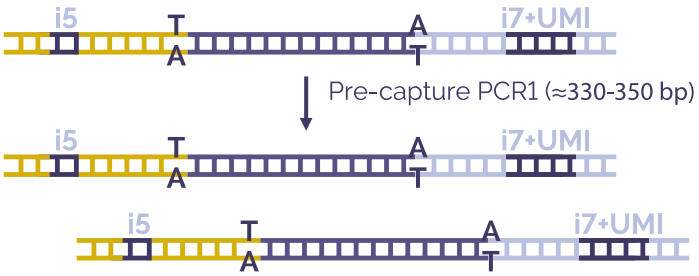
19. Label a new set of PCR strip-tubes for the libraries and set aside until required in Step 21.
20. Place the tubes on the magnetic stand and wait for the solution to clear (approx. 2–3 min).
21. Transfer **32 µl** of the eluate containing the purified ligated products to the tubes from Step 19. Tubes 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.

STOP **OPTIONAL STOPPING POINT:** If the samples are not to be used immediately, store at –20˚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 to be 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°C to −25°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°C until dissolved.
- Remove the Step 3: PCR Polymerase (red lid; ●) from storage (−15°C to −25°C) and place on ice.

Library preparation: Step 3

Perform step 3: Pre-capture PCR

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

1. Program the thermal cycler using the settings shown in Table 5. 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 for a total number of 6 cycles.	
6	72	10 min
7	4	Hold

Table 5: Incubation profile of program "OGT PCR1".

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 6 in a fresh 1.5 ml LoBind tube.

Reagent	1x library (µl)	8x sample 16x library (µl) (includes 2 excess)	16x sample 32x library (µl) (includes 3 excess)
Adapter-ligated DNA sample	31	–	–
Nuclease-free Water (clear lid ◯)	9.5	171	332.5
Step 3: PCR Buffer (red lid; 🔴)	5	90	175
Step 3: Primer Mix (red lid; 🔴)	2.5	45	87.5
Step 3: PCR Polymerase (red lid; 🔴)	**2**	**36**	**70**
TOTAL	**50**	**342**	**665**

Table 6: 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 8–16 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

15. Assess the size of the amplified product using the Agilent 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.

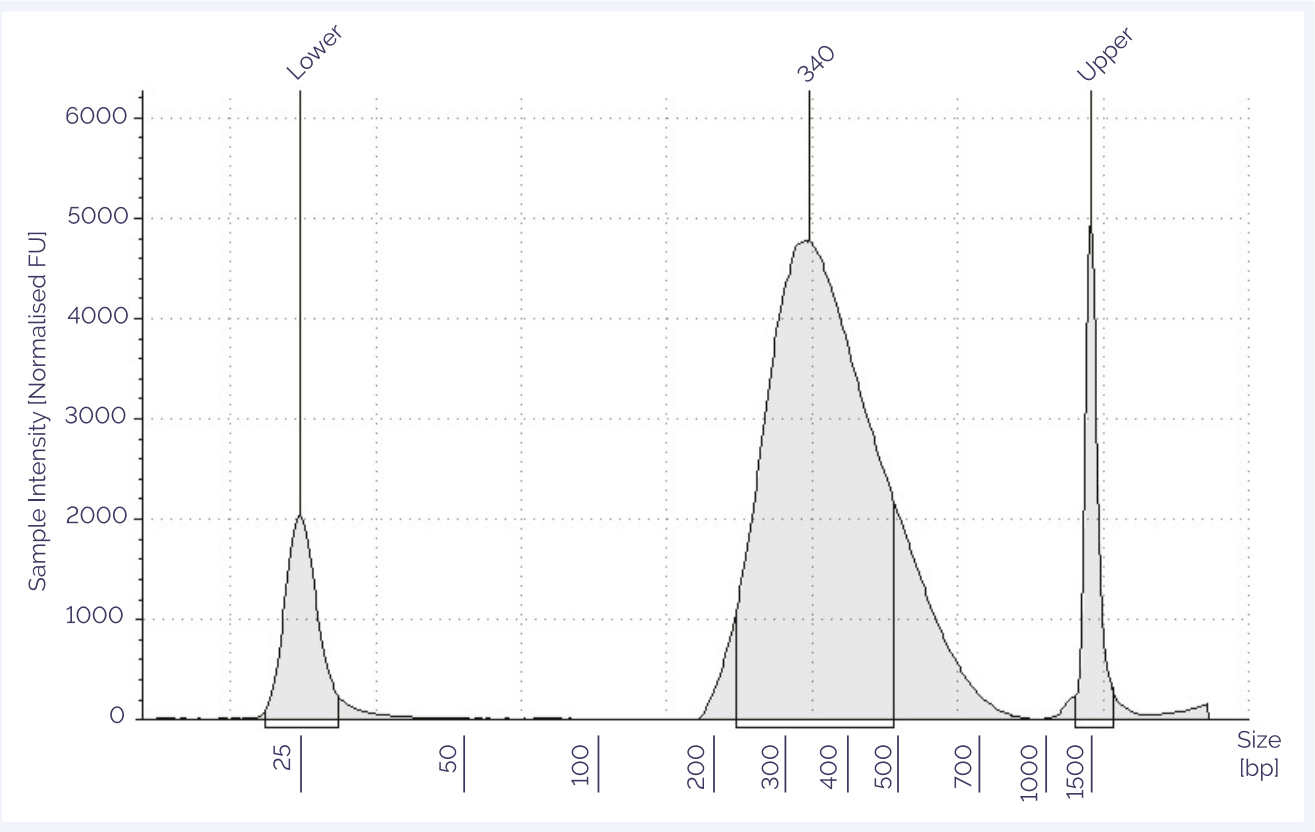


Figure 6: Electropherogram of purified Pre-capture PCR product generated using an Agilent D1000 ScreenTape assay. The electropherogram shows a maximum peak in the size range of approximately 330–350 bp (+/– 20 bp).

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

16. Assess yield using **1 µl** amplified product with the Qubit dsDNA HS Kit as per manufacturer's instructions.
The expected yield is >18 ng/µl (~400 ng per library).

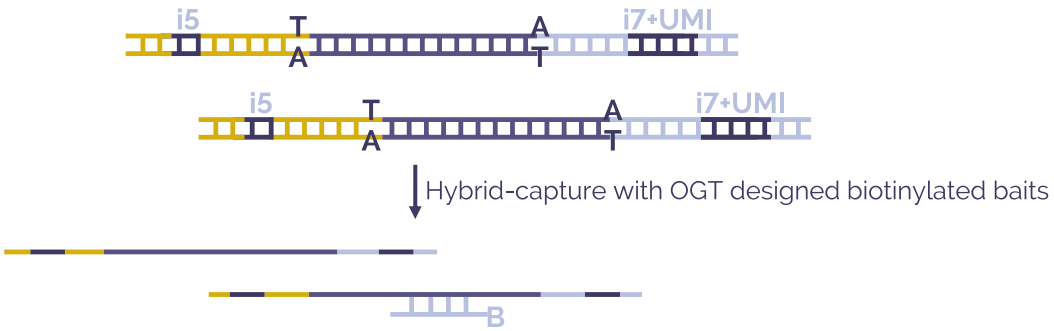
It is recommended to use a single channel pipette and ensure there is no excess liquid on the side of the tip to prevent inaccurate readings affecting pooling.

STOP **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 "Universal hybridisation".

Library preparation: Universal hybridisation

Universal hybridisation

Overview



The amplified library is denatured and captured by SureSeq Biotinylated (B) 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°C to −25°C) and allow to thaw to room temperature.
- Remove the SureSeq Myeloid MRD NGS Panel from storage (−15°C to −25°C) and allow to thaw to room temperature.
- Ensure that all components in the Hybridisation Buffer are well dissolved. If necessary, incubate at 37°C until dissolved.

Library preparation: Universal hybridisation

Pool samples and hybridise to capture baits

Estimated time: 40 min for 8–16 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, add the maximum possible input and keep other samples at 500 ng.

1. Use the OGT MRD Library Preparation worksheet provided to 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 support@ogt.com for further help.

2. Aliquot 300 ng–500 ng of each of the eight libraries in a strip into a single 1.5 ml LoBind tube (see Figure 7).

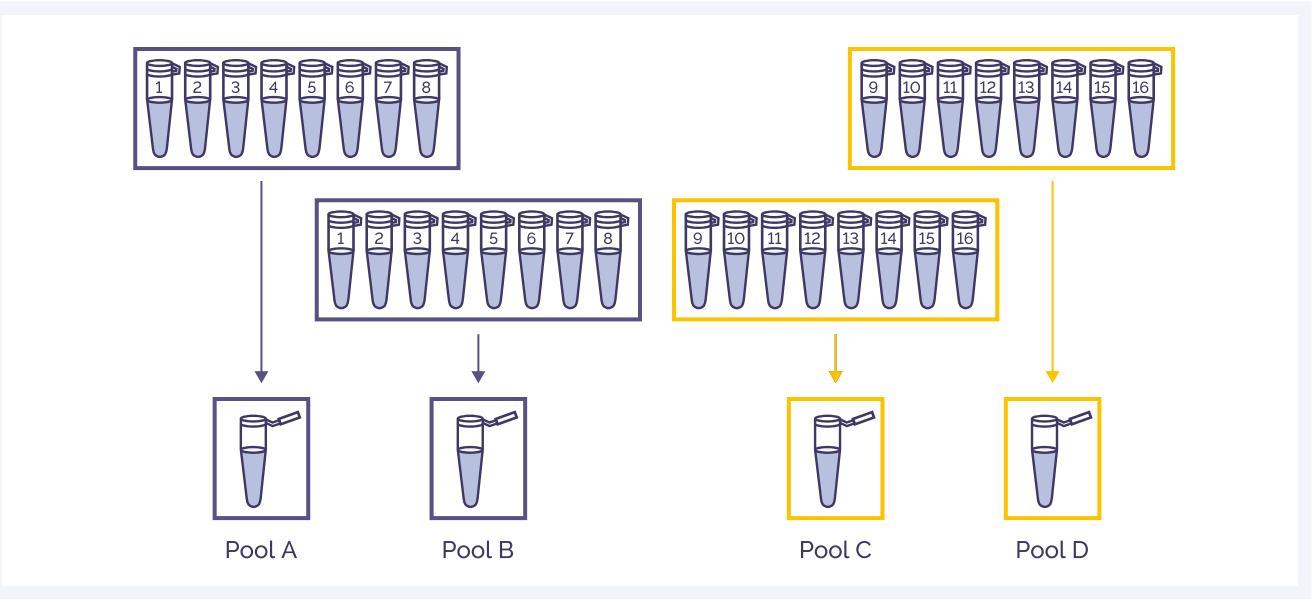


Figure 7: Pooling guide to pool 16 samples in four pools, each consisting of one strip of eight libraries.

3. Mix Hybridisation Buffer, Formamide, Cot Human DNA, Myeloid MRD Panel and Index Blockers on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.

Library preparation: Universal hybridisation

4. Prepare the Hybridisation Master Mix according to Table 7 in a fresh 1.5 ml LoBind tube, vortex mix and set aside until required in Step 15.

Reagent	1x Pool (µl)	2x Pool (µl) (includes 0.5 excess)	4x Pool (µl) (includes 0.5 excess)
Nuclease-free Water (clear lid; ◯)	2.5	6.25	11.25
Hybridisation Buffer (red lid; ●)	7.5	18.75	33.75
Formamide (yellow lid; ●)	3.5	8.75	15.75
TOTAL	**13.5**	**33.75**	**60.75**

Table 7: Hybridisation Master Mix.

5. Add **10 µl** of 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 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 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. **Keep the beads containing the DNA sample.**

Library preparation: Universal hybridisation

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 bead 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 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.

16. Incubate for **5 min** at room temperature.
17. Label a new set of PCR strip-tubes for the pools and set aside until required in Step 19.

Recommendation: Program the thermal cycler using the settings shown in Table 8. Save the program as "OGT Hybridisation".

18. Place the tubes 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 15. Tubes containing beads can be discarded at this time.
20. Add **2 µl** of Index Blockers (blue lid; ●) to the pools.
21. Add **2 µl** of SureSeq Myeloid MRD NGS Panel to the pools.
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.

Library preparation: Universal hybridisation

24. Place the tubes into the thermal cycler and run the program "OGT Hybridisation" shown in Table 8. 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

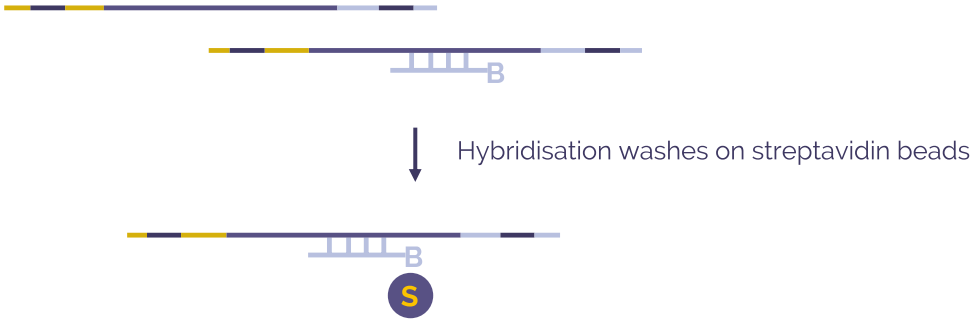
Table 8: Incubation profile of program "OGT Hybridisation".

25. Incubate the hybridisation mixture overnight (**16–20 hr**) at 65°C.
26. Continue to "Universal capture and wash".

Remove the Hyb Wash Buffer (blue dot; ●) and Bead Priming Buffer (orange 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.

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 8–16 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 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 sequence capture and bead wash buffers

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

> Incubate at 37˚C for 5–10 min to resuspend any precipitates. It is possible to defrost these buffers at room temperature during your overnight incubation.

2. Aliquot **6 x 200 µl** of Hyb Wash Buffer per hybridisation pool into PCR strip-tubes as shown in Figure 8 for one hybridisation pool.
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 / pool
- 3 x 200 µl at 35°C / pool

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

Figure 8: Set-up of Hyb Wash Buffer aliquots for one hybridisation pool.

Prepare magnetic beads

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

> 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.

2. Immediately before use, resuspend the room temperature Dynabeads M-270 Streptavidin magnetic beads using a **200 µl** pipette set to **100 µl** 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 1x the original volume of Bead Priming Buffer (i.e., for 75 µl of beads add 75 µl of 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 contents.
12. Place the tubes 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 sample (**~17 µl** 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 tubes to the thermal cycler still running program "OGT Hybridisation" at 65°C 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 tubes back in a thermal cycler running program "OGT Hybridisation".
5. After the 45 min incubation, remove the tubes 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.

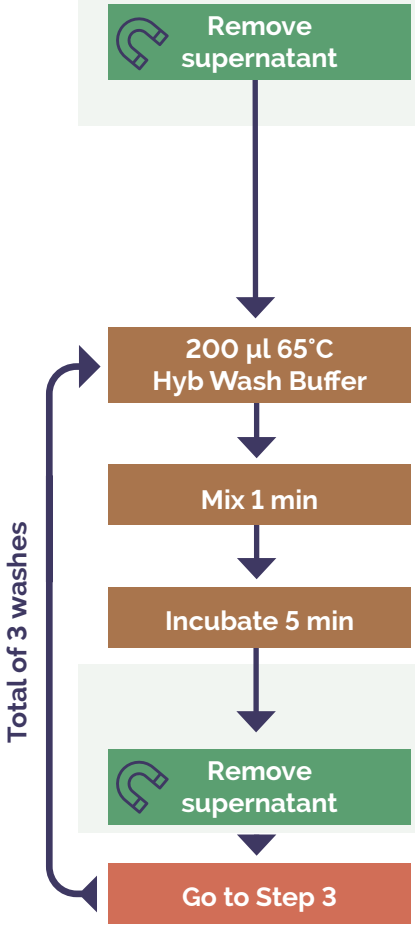
Wash streptavidin beads to remove unbound DNA

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



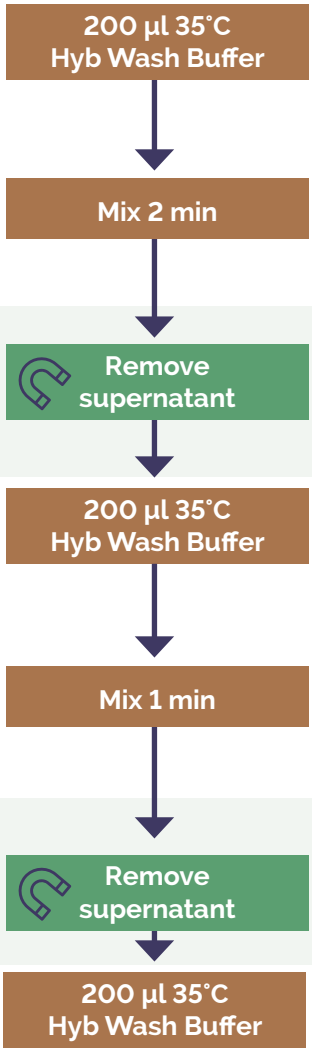
1. Place the tubes in the magnetic stand and wait for the solution to clear (approx. 10 sec).
2. Avoiding the bead pellet, remove and discard the supernatant (~**17 µl**) containing unbound DNA.

⚠ The hybridisation mix contains Formamide.

3. Remove the tubes from the magnetic stand.
4. Add **200 µl** of pre-warmed (to 65˚C) Hyb Wash Buffer to the capture beads (with bound DNA).
5. Mix on a vortex mixer to disrupt the bead pellet. Check the bead pellet has been fully resuspended.
6. Place the tubes in a plate vortex mixer and mix vigorously at approx. 2000 RPM for **1 min**.
7. Return the samples to the thermal cycler running program "OGT Hybridisation" for **5 min**.
8. Pulse spin and place the tubes in the magnetic stand and wait for the solution to clear (approx. 10 sec).
9. Avoiding the bead pellet, remove and discard the supernatant (~**200 µl**) containing unbound DNA.
10. Repeat steps 3–9 twice, for a total of three 65˚C washes.
11. Remove the tubes from the magnetic stand.
12. **Proceed immediately** to the next step "Shaking 35˚C washes".

Library preparation: Universal capture and wash

Shaking 35˚C washes



1. Add **200 µl** 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 tubes 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 tubes 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 µl**).
7. Add **200 µl** 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 tubes 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 tubes 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 µl**).
13. Add **200 µl** of pre-warmed (to 35˚C) Hyb Wash Buffer to the capture beads.

Library preparation: Universal capture and wash

Shaking 35˚C washes (continued)

Mix 30 sec

14. Mix on a vortex mixer to disrupt the bead pellet. Check the bead pellet has been fully resuspended.
15. Place the tubes in a plate vortex mixer and mix vigorously at approx. 2000 RPM for **30 sec**.

> Do not mix for more than 30 sec.

16. Pulse spin to collect the contents.

Remove supernatant

17. Place the tubes in the magnetic stand and wait for the solution to clear (approx. 10 sec).
18. Avoiding the bead pellet, remove and discard the supernatant (~**200 µl**).
19. Pulse spin to collect the residual wash buffer. Return the tubes to the magnetic stand and remove the residual wash buffer with a P20 pipette.

Add 30 µl NFW and mix

20. Remove the tubes from the magnetic stand and add **30 µl** of Nuclease-free Water to resuspend the bead pellet.
21. Mix on a vortex mixer to form bead slurry.

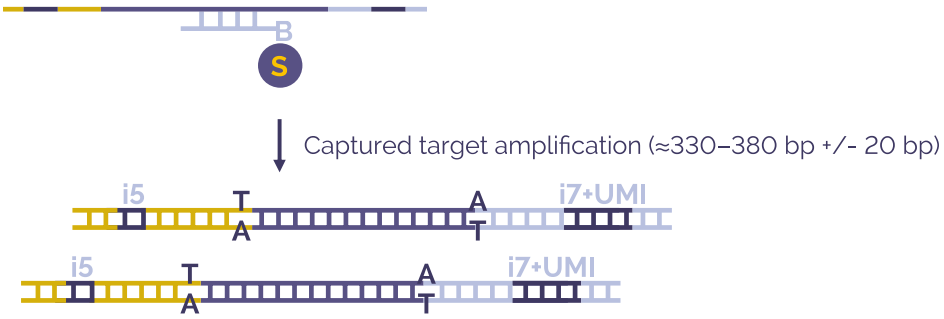
Recommendation: If proceeding with Step 4: Post-capture PCR immediately, the Step 4: Primer Mix and Step 4: PCR Buffer can be removed from storage to thaw to room temperature now.

STOP **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 sequences, ssDNA bound to the Streptavidin beads (S) 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°C to −25°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°C until dissolved.
- Remove the Step 4: PCR Polymerase (purple lid; ●) from storage (−15°C to −25°C) and place on ice.

Library preparation: Step 4

Perform step 4: Post-capture PCR

To preserve sample complexity use all of the bead slurry. Two PCRs are performed on each pool.

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

1. Program the thermal cycler using the settings shown in Table 9. 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 20 cycles	
6	72	10 min
7	4	Hold

Table 9: Incubation profile of program "OGT PCR2".

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.
4. For multiple pools, prepare the Post-capture PCR Master Mix according to Table 10 in a fresh 1.5 ml LoBind tube. There will be two PCRs prepared for each hybridisation capture pool.

Library preparation: Step 4

Reagent	1x PCR (µl)	2x Pool (µl) 4 PCRs (includes 1 excess)	4x Pool (µl) 8 PCRs (includes 1 excess)
Captured DNA in bead slurry	14	–	–
Nuclease-free Water	26.5	132.5	238.5
Step 4: PCR Buffer (purple lid; ●)	5	25	45
Step 4: Primer Mix (purple lid; ●)	2.5	12.5	22.5
Step 4: PCR Polymerase (purple lid; ●)	2	10	18
TOTAL	**50**	**180**	**324**

Table 10: Post-capture PCR Master Mix.

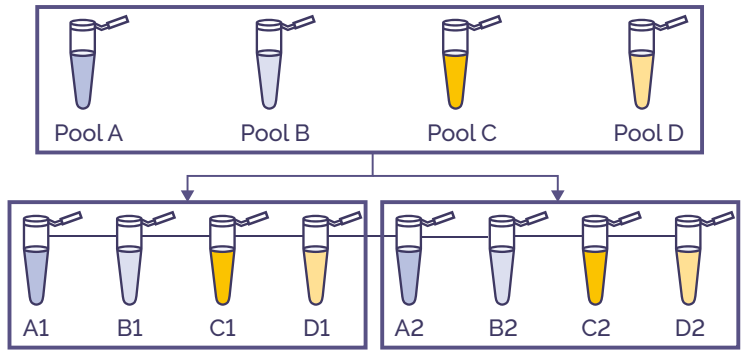


Figure 9: Post-capture PCR guide for 16 samples (4 pools). Duplicate reactions set up for each capture pool for a total of 8 PCRs.

5. Mix the Post-capture PCR Master Mix on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
6. Label two new PCR tubes for each hybridisation capture.
7. Add **36 µl** of the Post-capture PCR Master Mix into the tubes from Step 6.
8. 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.
9. Ensure two PCRs are performed for each pool (see Figure 9).
10. Pipette mix at least 10 times.
11. 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 8–16 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 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 (**~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 tubes and pulse spin to collect the residual ethanol. Return the tubes to the magnetic stand for **30 sec**. 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 **22 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-tubes for the eluates 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 **20 µl** of the eluate containing the purified post-capture PCR products to the tubes from Step 12. The tubes 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 maximum peak size of 330–350 bp (+/– 20 bp) (Figure 10). Set up the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.

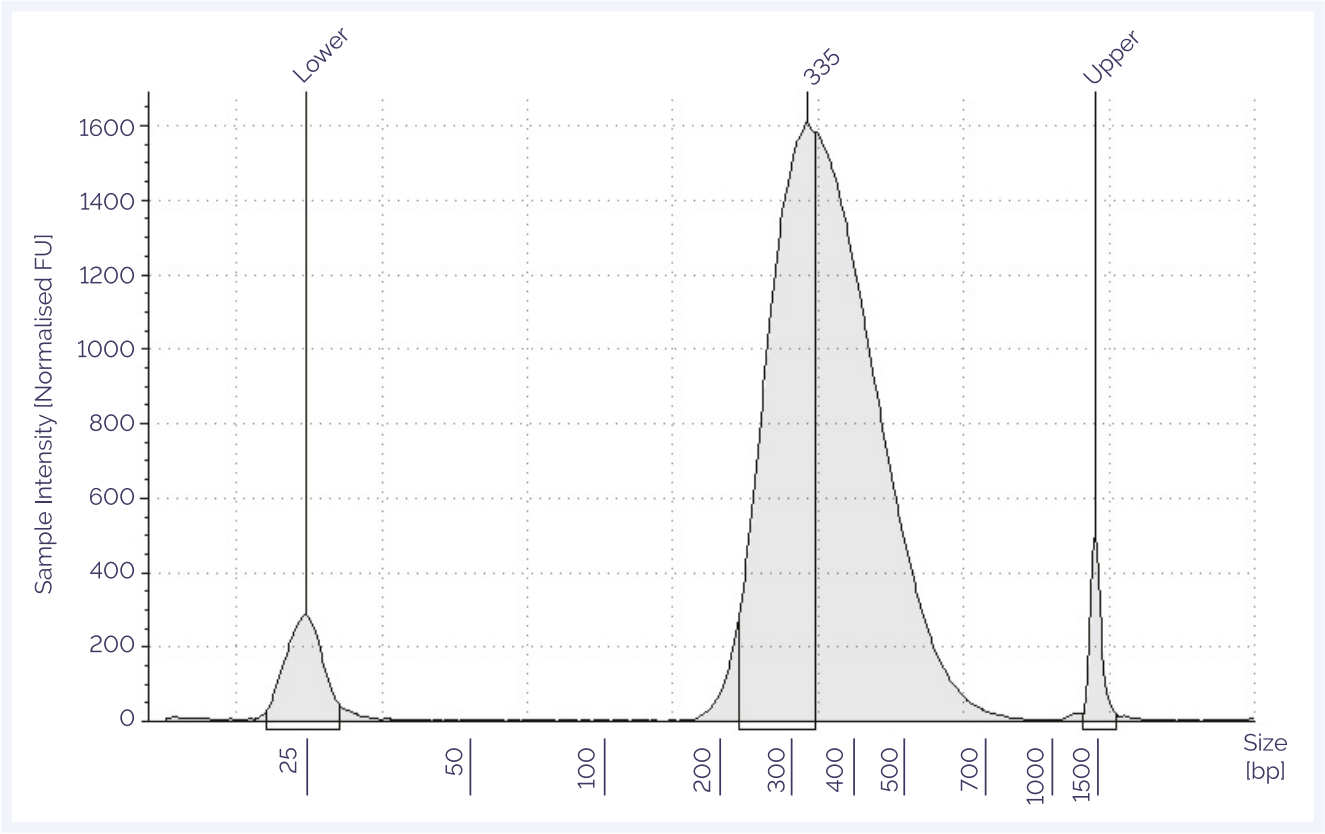


Figure 10: Electropherogram of purified Post-capture PCR product generated using an Agilent High Sensitivity D1000 ScreenTape assay. The electropherogram shows a maximum peak in the size range of approximately 330–350 bp (+/– 20 bp).

Library preparation: Step 4

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

16. Assess yield using **1 µl** amplified product with the Qubit dsDNA HS Kit as per manufacturer's instructions. The expected yield is 10–30 ng/µl.

STOP **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/µl), 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.

1. Use your worksheet described above or the formulae below to determine the volume (µl) 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 NextSeq 550 High-Output Kit 2x150bp (Illumina cat. no. 20024908).

Sequencing

2. 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.
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.

STOP **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

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

Formula 2 — volume of each indexed DNA sample

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

Sequencing

Preparing the Sample Sheet

A NextSeq Sample Sheet can be created using the NextSeq 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 11. Adjust the number of rows highlighted as appropriate.

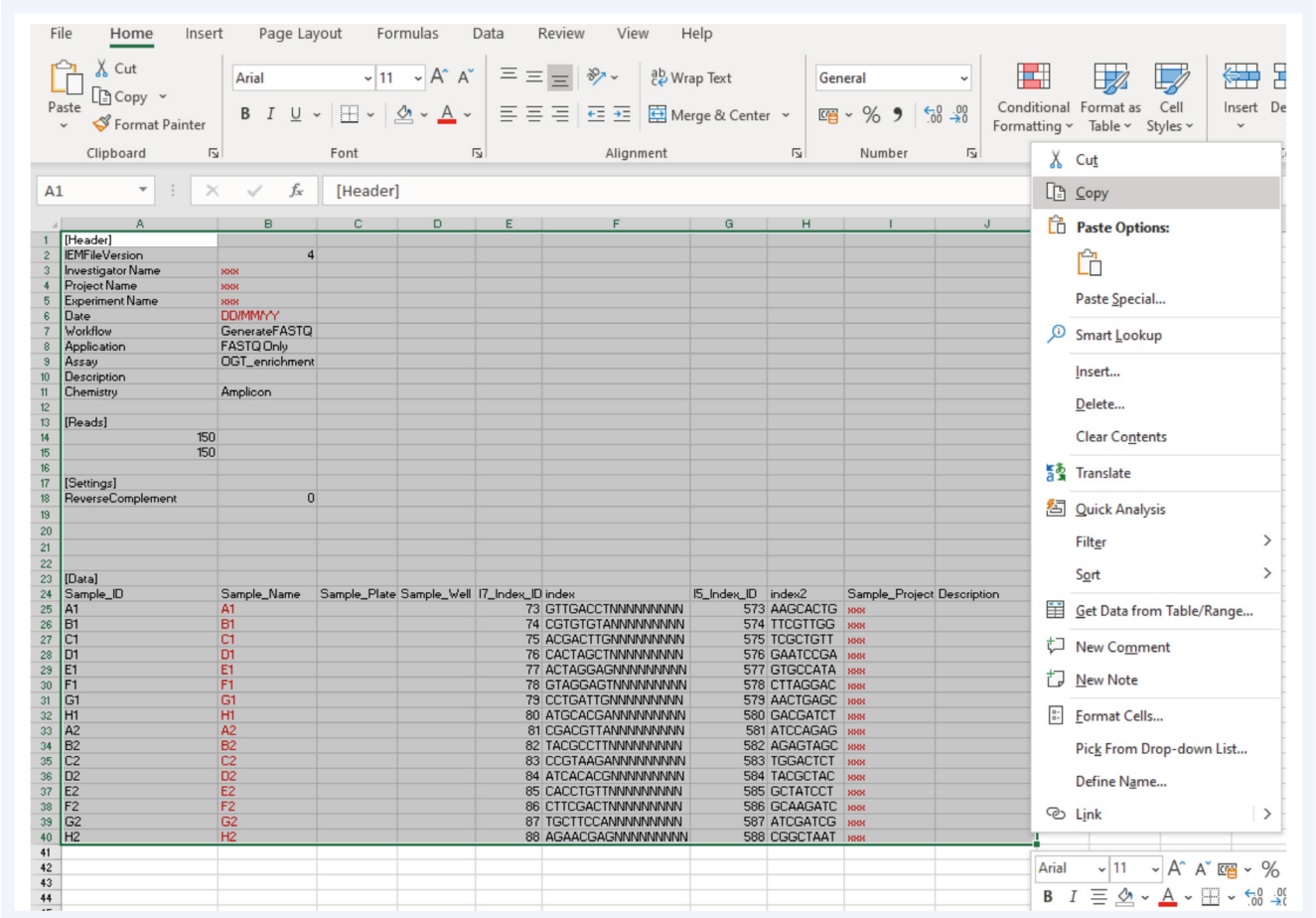


Figure 11: Example Sample Sheet on the NextSeq pooling template.

Sequencing

3. 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.

4. Save the new sheet as a CSV (comma delimited) file.
5. The Sample Sheet can now be uploaded to the sequencer.

STOP **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 "Denaturing and loading the sequencing pool" or refer to the appropriate Illumina protocol.

Denaturing and loading the sequencing pool

1. 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 a 1.4 pM Loading pool on a NextSeq, continue with Step 2.

2. Make the volume of the pool up to 1 ml with ice-cold HT1 to dilute your denatured pool to ~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 ~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.

3. 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 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 11: 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

For sequencers not listed in Table 12, for example NovaSeq platforms, ensure each sample is assigned 50 million paired-end reads.

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
770026-48 SureSeq Myeloid MRD NGS Panel	NextSeq 500/550 (High Output) or NextSeq 1000/2000 (P2)	16	4 x 8-plex	1 x 96 reactions	3 runs

Table 12: Recommended sequencing guidelines.

Reagent tube locations

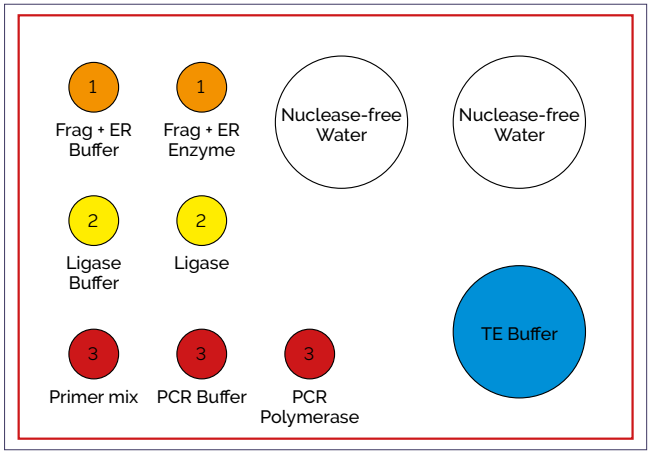


Figure 12: Location of tubes in 96 reaction Library Preparation Kit (770100-96).

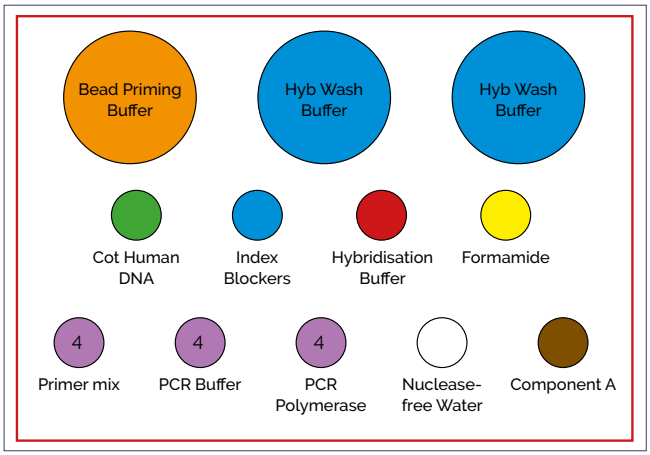


Figure 13: Location of tubes in 96 reaction Hybridisation & Wash Kit V2 (770410-96).

Legal information

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Ordering information

Product	Contents	Cat. No.
SureSeq Myeloid MRD Complete NGS Workflow Solution V2	Bundle of 1 x Universal Library Preparation Kit (96) containing PCR primers and enzymes. 1 x Universal Hybridisation & Wash Kit V2 (96). 1 x Pre-PCR Universal Bead Kit (96). 1 x Post-PCR Universal Bead Kit (96). 1 x Universal Index Adapter Kit (96).	780126-48
SureSeq Myeloid MRD NGS Panel	1 x SureSeq Myeloid MRD NGS Panel (48)	770026-48

Table 13: Ordering information.

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

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