SureSeq

A Sysmex Group Company

OGT Handbook

SureSeq myPanel Myeloid Fusion MRD Panel

HBK N-0006 ; 990391

For Research Use Only; Not for Use in Diagnostic Procedures

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Introduction

The Universal NGS Complete Workflow Solution 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)
Double-Stranded cDNA Synthesis Kit	Shipped at –20˚C, store at –20˚C	880500-24	880500-96
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

Double-Stranded cDNA Synthesis Kit
880500-24 and 880500-96

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

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	–
RNase Decontamination Solution	General laboratory supplier	–
Qubit® RNA Broad Range (BR) Assay Kit	Thermo Fisher Scientific	Q10210
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 1000/2000 P2 Reagents (300 Cycles)	Illumina[ǂ]	20046813
Optional: RNA ScreenTape	*Agilent Technologies*	*5067-5576*
Optional: RNA Reagents	*Agilent Technologies*	*5067-5577* *5067-5578*

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

ǂ 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	Suggested location	Suggested supplier	Cat. No.*
UV PCR cabinet	Pre-PCR	General Laboratory Supplier	–
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

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

Reagents, consumables and equipment

Component	Suggested location	Suggested supplier	Cat. No.*
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	–

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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 thermocycler settings in Table 12.
- Check after overnight incubation that the evaporation does not exceed 1–2 µl per well.
- 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 it from its storage conditions at least 30 min before use.

General guidelines

Storage and handling

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

The Double-Stranded cDNA Synthesis Kit, Universal Library Preparation Kit, Universal Index Adapters, SureSeq myPanel Myeloid Fusion MRD 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.

Safety

Handling of the SureSeq myPanel Myeloid Fusion MRD 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.

General guidelines

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.
STOP	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 SureSeq myPanel Myeloid Fusion MRD Panel Workflow is designed to be used by suitably trained personnel using good quality RNA 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 (RNA)

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

The following section contains instructions for sample library production specific to the Illumina sequencing platform. For each sample, RNA is converted into double-stranded cDNA (ds cDNA) and an individual cDNA 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, washed, captured and then amplified ready for sequencing.

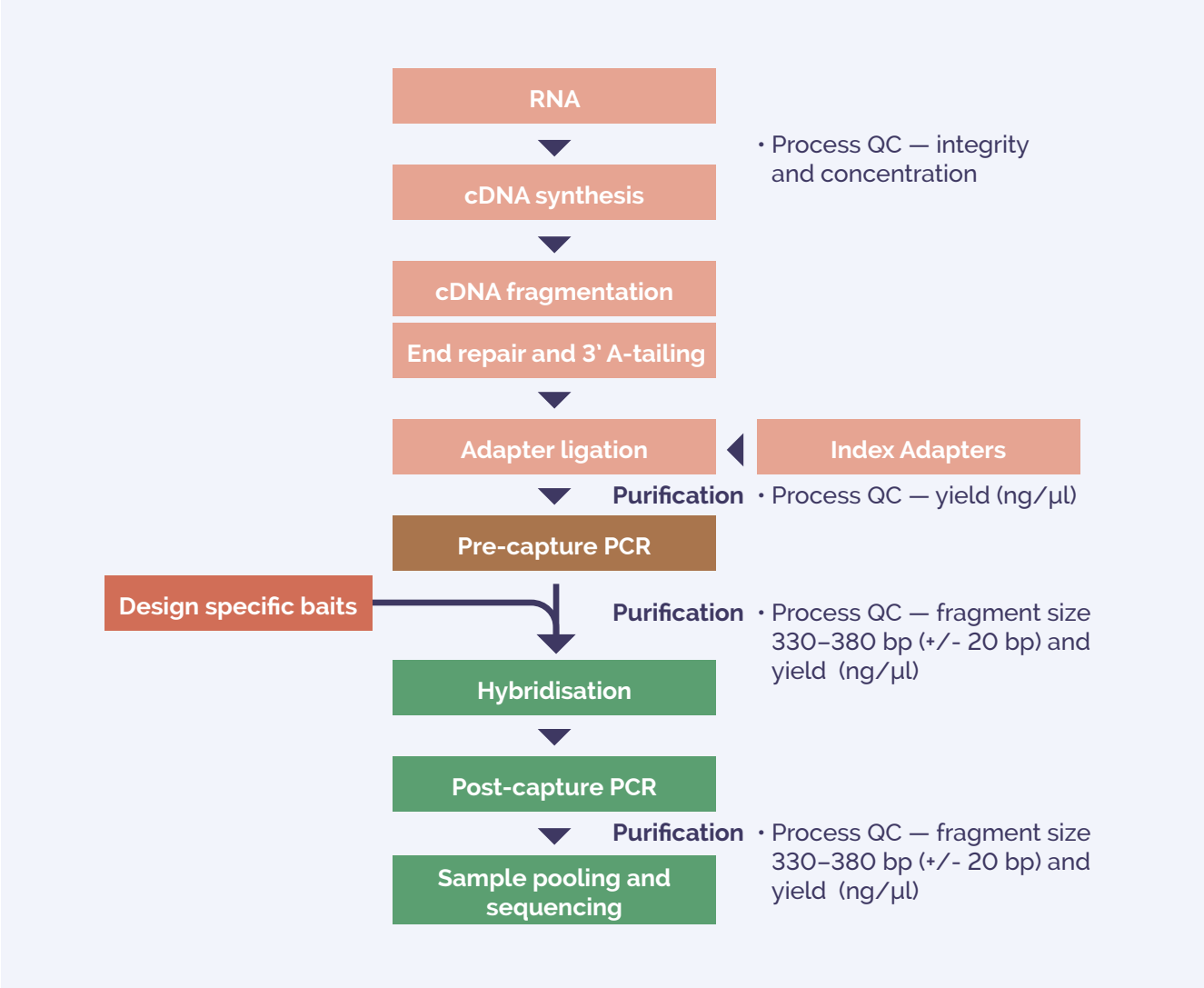


Figure 1: Workflow of cDNA synthesis and sample library preparation indicating the expected cDNA fragment size at each step of the procedure.

Sample requirements

Sample requirements

RNA: the protocol has been optimised for good quality RNA inputs of 500–1000 ng. However, it can be used for total RNA inputs ranging from 100 ng up to 1000 ng.

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

RNA — sample preparation

Determination of the concentration of total RNA sample is mandatory for all samples prior to starting the SureSeq myPanel Myeloid Fusion MRD Panel Workflow. In addition, we highly recommend that further quality control (QC) is carried out to assess RIN, as RNA quality impacts downstream yields.

RNA integrity and purity assessment are optional.

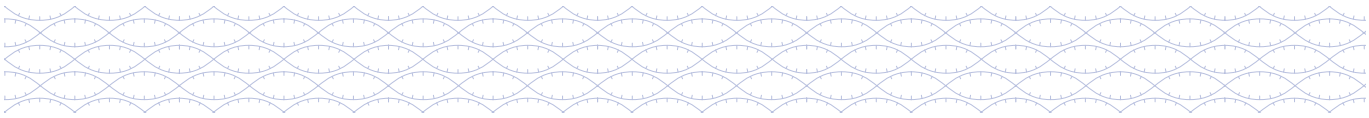
We recommend the following assays to assess the sample integrity, concentration and purity:

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

RNA concentration — Qubit RNA BR 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.



Sample requirements

4. Aliquot 199 µl of Qubit working solution for each sample under assessment.
5. Add 1 µl of sample to the appropriate tube.
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 RNA concentrations following the onscreen prompts.

RNA integrity — RNA ScreenTape

Optional: This step is important if degraded RNA is used. Refer to manufacturer's user guide for the Agilent 4200 TapeStation. The key steps are described below:

1. Prepare the ladder by mixing 5 µl of RNA sample buffer with 1 µl of RNA ladder in the first tube/well of the strip-tubes or plate.
2. For each sample under assessment, add 1 µl of RNA sample to 5 µl of RNA 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. Denature the ladder and the samples by heating at 72°C for 3 min. Following this, place the ladder and samples on ice for 2 min.
7. Following denaturation, load the strip of tubes or plate into the Agilent 4200 TapeStation.

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

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

Sample requirements

10. Following RNA electrophoresis using the Agilent TapeStation, RNA Integrity Number (RIN) is generated.

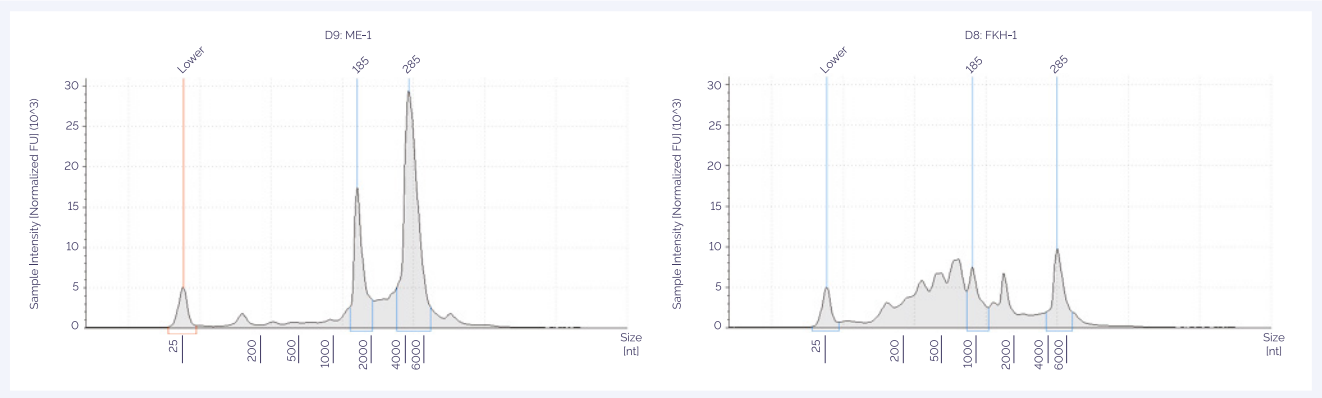


Figure 2: Assessment of RNA integrity using RNA Broad Range ScreenTape. The electropherogram trace on left shows a high-quality sample with a RIN of 10.0, while the trace on the right shows a degraded RNA sample with a RIN of 2.9.

Purity — NanoDrop

Optional: This step is important if high-quality RNA is not used.

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

1. Use the "Nucleic Acid" and "RNA-40" 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).

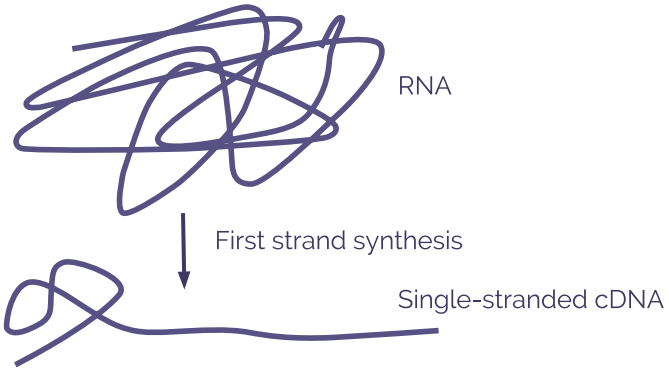
An OD 260/280 ratio of 2.0 and OD 260/230 ratio of 2.0 to 2.2 is recommended.
Use of RNA samples with lower ratios may result in poor performance.

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

Double-stranded cDNA synthesis: Step 1

The following section describes cDNA synthesis from RNA. cDNA synthesis should be carried out in a PCR hood sterilised with RNaseZap™ RNase Decontamination Solution and UV or in a dedicated area free of DNA contamination. This is to avoid cross contamination with DNA.

First strand (FS) cDNA synthesis

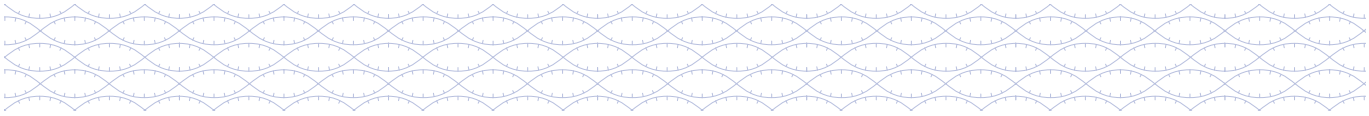


Overview

Single-stranded cDNA is synthesised from total RNA using random primers.

Before starting:

- Remove the Step 1: FS Buffer (red lid; ●), Step 1: dNTP Mix 100 mM (red lid; ●), Step 1: Primer mix (red lid; ●) and Nuclease-free Water (clear lid; ○) from storage (−15°C to −25°C) and allow to thaw to room temperature then place on ice.
- Remove the Step 1: FS Enzyme (red lid; ●) from storage (−15°C to −25°C) and place on ice.



Double-Stranded cDNA Synthesis: Step 1

Perform step 1: FS cDNA synthesis

Estimated time: 2.75 hr for 16 samples. Hands-on time: 30 min.

1. Program the thermal cycler using the settings shown in Table 1. Save the program as "OGT FS cDNA Synthesis".

Step	Temperature (°C)	Time
1	25	10 min
2	37	2 hours
3	85	5 min
4	4	Hold

Table 1: Incubation profile of program "OGT FS cDNA Synthesis".

2. Preheat the thermal cycler to 25°C. Where possible, set the heated lid to 105°C, alternatively have the pre-set heated lid activated.
3. In PCR strip-tubes dilute **500-1000 ng** of sample RNA with the Nuclease-free Water (clear lid; ○) provided to a total volume of 10 µl. Mix on a vortex mixer for **3–5 sec**, pulse spin to collect the contents and place on ice.
4. Mix the Step 1: FS Buffer, Step 1: dNTP Mix 100mM and Step 1: Primer Mix on a vortex mixer for 2-3 sec, pulse spin to collect the contents and keep on ice.
5. Flick mix Step 1: FS Enzyme, pulse spin to collect the contents and keep on ice.
6. Prepare the FS Master Mix according to Table 2 in a fresh 1.5 ml LoBind tube and place on ice.
7. Mix the FS Master Mix on a vortex mixer for **2–3 sec**, pulse spin to collect the contents and keep on ice.

Double-Stranded cDNA Synthesis: Step 1

Reagent	1x library (µl)	8x library (µl) (includes 2 excess)	16x library (µl) (includes 2 excess)	24x library (µl) (includes 2 excess)
RNA sample	10	–	–	–
Step 1: FS Buffer (red lid; ●)	2	20	36	52
Step 1: dNTP Mix (100mM) (red lid; ●)	0.8	8	14.4	20.8
Step 1: Primer Mix (red lid; ●)	2	20	36	52
Step 1: FS Enzyme (red lid; ●)	1	10	18	26
Nuclease-free Water (clear lid; ◯)	4.2	42	75.6	109.2
TOTAL	**20**	**100**	**180**	**260**

Table 2: FS Master Mix.

8. Add **10 µl** FS Master Mix into each of the PCR tubes containing prepared RNA from step 3. Keep on ice.

Proceed in a timely manner to avoid RNA degradation, as RNA will degrade quickly even on ice, if contaminated with RNases. Keep samples on ice when not being vortexed or spun.

9. **Immediately** mix on a vortex mixer for **2–3 sec**.
10. Pulse spin to collect the contents and **immediately** transfer to the **preheated** thermal cycler. Start the program "OGT FS cDNA Synthesis".

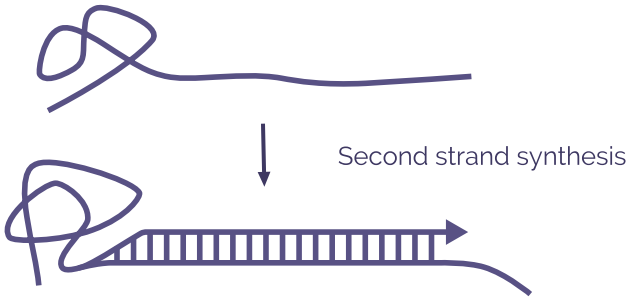
Recommendation: prepare the SS Master Mix in the final 10 minutes of the thermal cycler program.

11. 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 Second Strand cDNA Synthesis.

STOP **OPTIONAL STOPPING POINT:** If the samples are not to be used immediately, store at –20 °C. If continuing, proceed to "Second strand (SS) cDNA synthesis".

Double-stranded cDNA synthesis: Step 2

Second strand (SS) cDNA synthesis



Overview

DNA polymerase synthesises a complementary strand to the single-stranded cDNA. This yields double-stranded cDNA which can then be taken through the SureSeq workflow.

Before starting

- Remove the Step 2: SS Buffer (green lid; ●), Step 2: dNTP Mix (green lid; ●) and Nuclease-free Water (clear lid; ○) from storage (−15°C to −25°C) and allow to thaw to room temperature then place on ice.
- Remove the Step 2: RNase (green lid; ●) and Step 2: SS Enzyme (green lid; ●) from storage (−15°C to −25°C) and place on ice.
- 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 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 eluting ds cDNA from the beads (10 mM Tris, 1 mM EDTA). Use of other TE formulations (e.g., 0.1x TE) or water may affect downstream results.

Double-stranded cDNA synthesis: Step 2

Perform step 2: SS cDNA synthesis

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

1. Program the thermal cycler using the settings shown in Table 3. Save the program as "OGT SS cDNA Synthesis".

Step	Temperature (°C)	Time
1	16	1 hour
2	4	Hold

Table 3: Incubation profile of program "OGT SS cDNA Synthesis".

2. Preheat the thermal cycler to 16°C. Where possible, set the heated lid to 105°C, alternatively have the pre-set heated lid activated.
3. Mix Step 2: SS Buffer and Step 2: dNTP Mix on a vortex mixer for 3-5 sec, pulse spin to collect the contents and place on ice.
4. Flick mix Step 2: RNase and Step 2: SS Enzyme, pulse spin to collect the contents and keep on ice.
5. Prepare the SS 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 2 excess)
First Strand cDNA	20	-	-	-
Step 2: SS Buffer (green lid; ●)	1.5	15	27	39
Step 2: dNTP Mix (10mM) (green lid; ●)	1	10	18	26
Step 2: RNase (green lid; ●)	0.2	2	3.6	5.2
Step 2: SS Enzyme (green lid; ●)	1	10	18	26
Nuclease-free Water (clear lid; ○)	1.3	13	23.4	33.8
TOTAL	**25**	**50**	**90**	**130**

Table 4: SS Master Mix.

Double-stranded cDNA synthesis: Step 2

6. Mix the SS Master Mix on a vortex mixer for 2–3 sec. Pulse spin to collect the contents and keep on ice.
7. Add **5 µl** SS Master Mix into each of the PCR tubes containing prepared RNA from Step 1: FS cDNA synthesis. Keep on ice.

Proceed in a timely manner. Keep samples on ice when not being vortexed or spun.

8. **Immediately** mix on a vortex mixer for **2–3 sec**.
9. Pulse spin to collect the contents and **immediately** transfer to the **preheated** thermal cycler. Start the program "OGT SS cDNA Synthesis".
10. When the program is complete and the thermal cycler has reached 4°C, **proceed immediately** with Double-stranded cDNA purification.

Proceed immediately to "Post-Synthesis Purification". The quality and quantity of ds cDNA will be adversely affected if samples are not purified immediately.

Double-stranded cDNA synthesis: Step 2

Perform double-stranded cDNA 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.

1. Prepare a **fresh** set of PCR strip-tubes for each sample.

To the cDNA sample tubes:

2. Add **45 µl** of homogeneous, room temperature Mag-Bind TotalPure NGS beads from the Pre-PCR Universal Bead Kit to each ds cDNA sample tube.
3. Mix on a vortex mixer to resuspend the beads and pulse spin to collect the contents.
4. Incubate at room temperature for **5 min**.
5. Place the tubes in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
6. Avoiding the bead pellet, remove and discard the cleared supernatant (**~70 µl**). **Keep the beads containing the cDNA sample**.
7. Add **200 µl** of 80% ethanol to each tube without resuspending the bead pellet.
8. Incubate for **30 sec**, then remove the ethanol.
9. Repeat wash (steps 8 and 9) once, for a total of two washes.
10. 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.
11. 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.

Double-stranded CDNA synthesis: Step 2

12. Remove from the magnetic stand and add **30 µl** of TE (blue lid; ●) 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.

Use only the TE Buffer (blue lid; ●) provided in the kit for eluting ds cDNA from the beads (10 mM Tris, 1 mM EDTA). Use of other TE formulations (e.g., 0.1x TE) or water may affect downstream results.

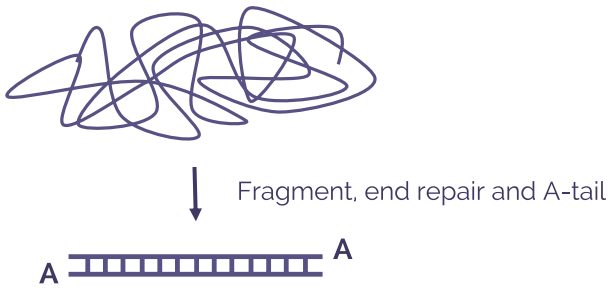
13. Incubate for **5 min** at room temperature.
14. Place the tubes on the magnetic stand and wait for the solution to clear (approx. 2–3 min).
15. Transfer **29 µl** of the eluate containing the purified ds cDNA to the fresh tubes from step 1 and keep on ice until use. Tubes containing beads can be discarded at this time.
16. Assess yield using **1 µl** ds cDNA with the Qubit dsDNA HS Kit as per manufacturer's instructions. The expected yield is >1 ng/µl.

STOP **OPTIONAL STOPPING POINT:** If the samples are not to be used immediately, store at –20˚C. If continuing, proceed to "cDNA fragmentation, end repair and 3' end A-tailing".

cDNA library preparation: Step 1

cDNA fragmentation, end repair and 3' end A-tailing

cDNA



Overview

cDNA is enzymatically fragmented. The fragmented cDNA 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 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 well 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°C to −25°C) and place on ice.

cDNA library preparation: Step 1

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

Estimated time: 1 hr for 8–24 samples. Hands-on time: 15 min.

1. Program the thermal cycler using the settings shown in Table 5. Save the program as "OGT cDNA Fragmentation".

Step	Temperature (°C)	Time
1	37	12 min
2	65	30 min
3	4	Hold

Table 5: Incubation profile of program "OGT cDNA 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. Keep the cDNA samples from step 15 of "Perform double-stranded cDNA purification" on ice.
4. Label a new set of PCR strip-tubes for each 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.

cDNA library preparation: Step 1

6. Prepare the cDNA Fragmentation and ER Master Mix according to Table 6 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)
cDNA sample	27	–	–	–
Step 1: Frag + ER Buffer (orange lid; ●)	7	70	126	189
Step 1: Frag + ER Enzyme (orange lid; ●)	1	10	18	27
TOTAL	**35**	**80**	**144**	**216**

Table 6: 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. Keep on ice.
9. Using a multi-channel pipette, add **27 µl** of cDNA sample to each tube containing the Fragmentation and ER Master Mix.

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 cDNA Fragmentation".

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

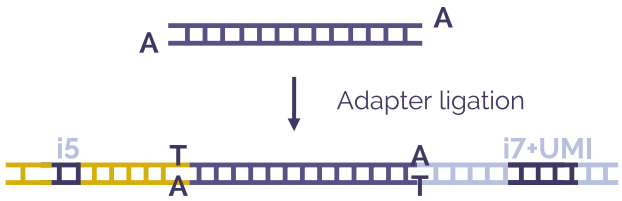
12. When the program is complete and the thermal cycler has reached 4°C, remove the samples and place them on ice until you are ready to proceed with Adapter ligation.

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.

cDNA 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 a part 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.

cDNA library preparation: Step 2

- Assign a different Index Adapter to each sample. See Figure 3 and Figure 4 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 7. 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 7: 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 8 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
TOTAL	**50.5**	**120**	**216**	**324**

Table 8: Ligation Master Mix.

cDNA 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 6.
7. Add **12 µl** of the Ligation Master Mix to each 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".

cDNA 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 3: 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 4: Layout of Universal Index Adapter Plate (1–96).

cDNA 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 sample.

To the DNA sample tubes:

1. Add 40.4 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 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.

cDNA library preparation: Step 2

10. 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.
11. 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.

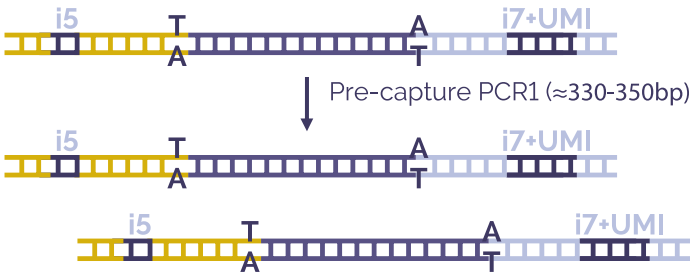
12. Label a new set of PCR strip-tubes for the samples and set aside until required in Step 14.
13. Place the tubes on the magnetic stand and wait for the solution to clear (approx. 2–3 min).
14. Transfer **32 µl** of the eluate containing the purified ligated products to the tubes from step 12. Tubes containing beads can be discarded at this time.
15. Assess yield using **1 µl** ligated product with the Qubit dsDNA HS Kit as per manufacturer's instructions. The expected yield is ≥ 1 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".

cDNA 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 number of duplicate reads (PCR copies of the same original cDNA fragment) in the sequence 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.

cDNA 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 9.
   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 of 6 cycles	
6	72	10 min
7	4	Hold

Table 9: Incubation profile of program "OGT PCR1".

2. Mix 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.
4. Prepare the Pre-capture PCR Master Mix according to Table 10 in a fresh 1.5 ml LoBind tube.

cDNA library preparation: Step 3

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
TOTAL	**50**	**190**	**342**	**513**

Table 10: 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.

cDNA library preparation: Step 3

To the DNA sample tubes:

1. Add **40 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 ligated products to the tubes from step 12. Tubes containing beads can be discarded at this time.

cDNA library preparation: Step 3

15. Assess the size of the amplified product using the Agilent DNA1000 ScreenTape System. The electropherogram should show a maximum peak size of 330–380 bp (+/– 20 bp) (Figure 5). Set up the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.

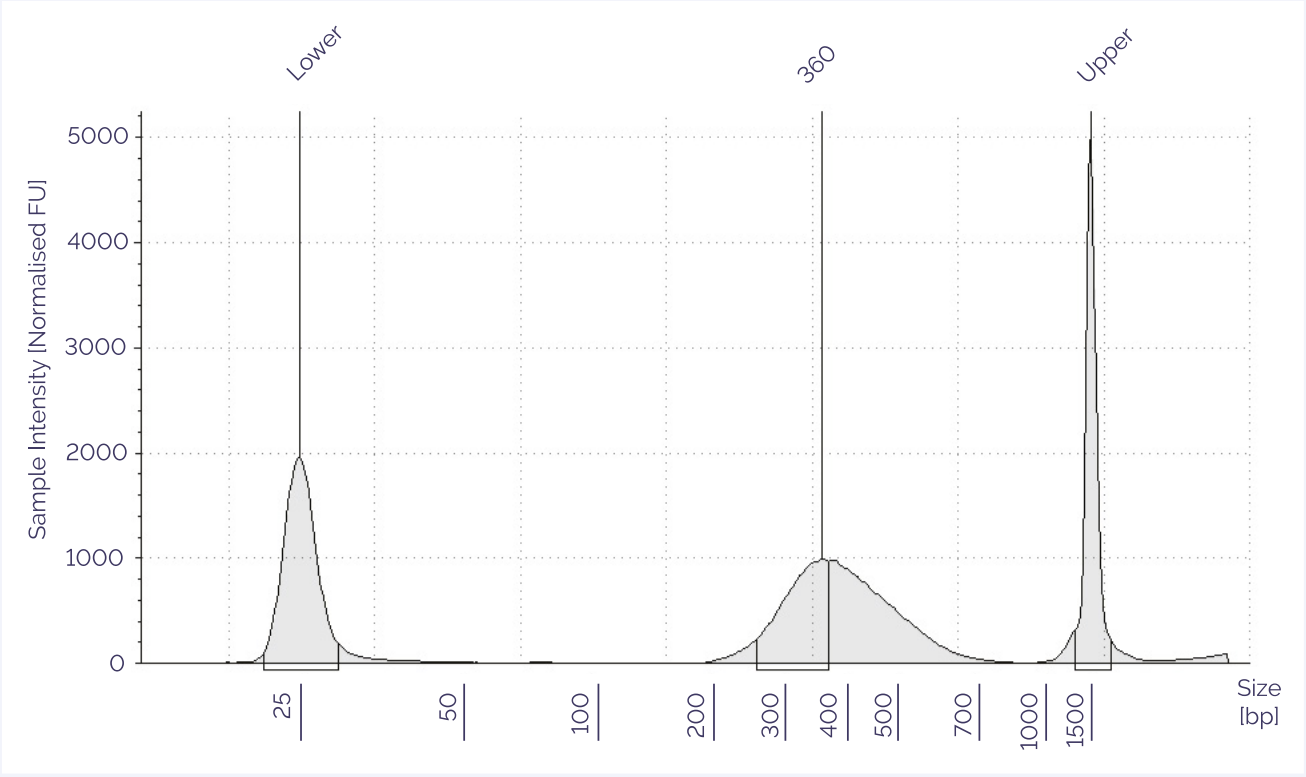


Figure 5: Electropherogram of purified Pre-capture PCR product generated using an Agilent D1000 ScreenTape assay. The electropherogram shows a maximum peak in the size range of approximately 330–380 bp (+/– 20 bp).

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 >11 ng/µl (~250 ng per library).

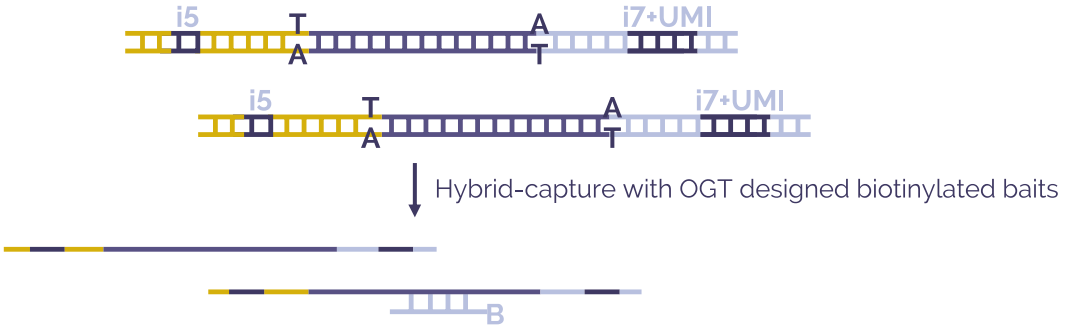
It is recommended to use a single channel pipette and ensure there is no excess liquid on the side of the tip to prevent inaccurate readings affecting pooling.

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".

cDNA library preparation: Universal hybridisation

Universal hybridisation

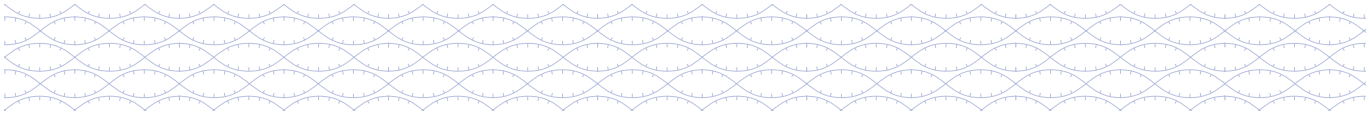
Overview



The amplified library is denatured and captured by SureSeq myPanel Myeloid Fusion MRD Panel 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 myPanel Myeloid Fusion MRD 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.



cDNA 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, add the maximum possible input and keep other samples at 500 ng.

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 250 ng per sample; contact **OGT Support** for further help.

2. Aliquot **250 ng–500 ng** of each of the eight libraries in a pool into a 1.5 ml LoBind tube.
3. Mix the Hybridisation Buffer, Formamide, Cot Human DNA, SureSeq myPanel Myeloid Fusion MRD panel and Index Blockers on a vortex mixer for **3–5 sec** and pulse spin to collect the contents.
4. Prepare the Hybridisation Master Mix according to Table 11 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)	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
TOTAL	**13.5**	**33.75**	**47.25**

Table 11: Hybridisation Master Mix.

cDNA library preparation: Universal hybridisation

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 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 samples.**
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.

16. Incubate for **5 min** at room temperature.

cDNA library preparation: Universal hybridisation

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 12. 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 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 SureSeq myPanel Myeloid Fusion MRD Panel 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 12. 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 12: Incubation profile of program "OGT Hybridisation".

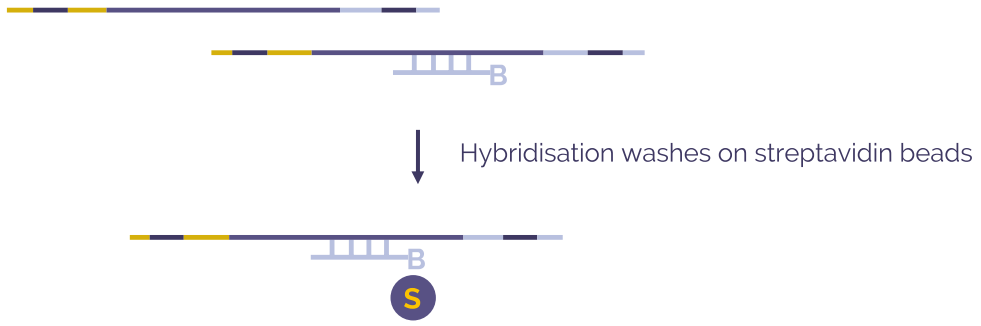
25. Incubate the hybridisation mixture overnight (**16–20 hr**) at 65°C.
26. Continue to "Universal capture and wash".

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

cDNA 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.

cDNA 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 6 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 6: Set-up of 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**.

cDNA library preparation: Universal capture and wash

3. Add **75 µl** of the Dynabeads M-270 Streptavidin magnetic beads to a 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.

cDNA 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 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.

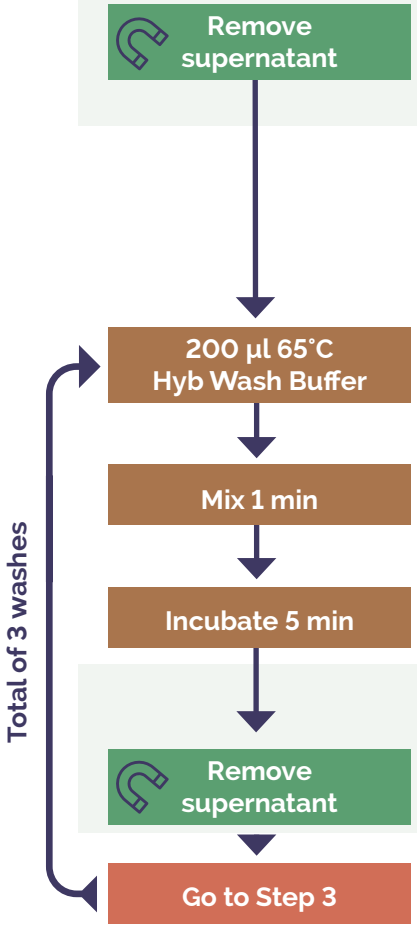
Wash streptavidin beads to remove unbound DNA

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.

cDNA library preparation: Universal capture and wash

Hot 65˚C washes



1. Place the tube(s) 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 tube(s) 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 tube(s) 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 tube(s) 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 tube(s) from the magnetic stand.
12. **Proceed immediately** to the next step "Shaking 35˚C washes".

cDNA library preparation: Universal capture and wash

Shaking 35˚C washes

200 µl 35˚C Hyb Wash Buffer

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.

Mix 2 min

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.

Remove supernatant

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 µl**).

200 µl 35˚C Hyb Wash Buffer

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.

Mix 1 min

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.

Remove supernatant

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 µl**).

200 µl 35˚C Hyb Wash Buffer

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

cDNA 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 tube(s) 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 tube(s) 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 tube(s) to the magnetic stand and remove the residual wash buffer with a P20 pipette.

Add 30 µl NFW and mix

20. Remove the tube(s) 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.

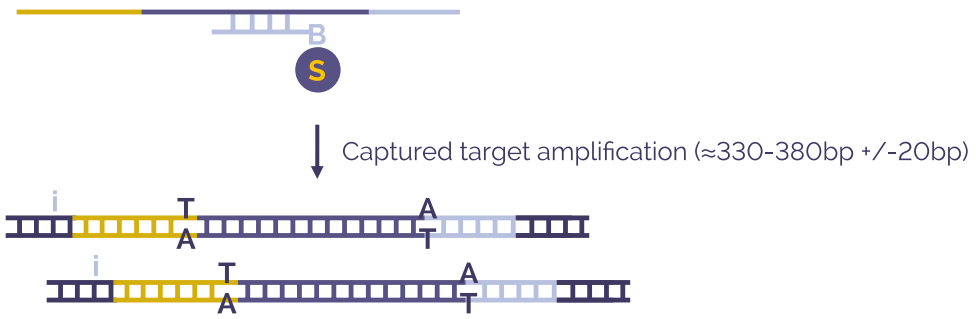
Recommendation: If proceeding with Step 4: Post-capture PCR immediately, the Step 4: Primer Mix and Step 4: PCR Buffer can be removed from storage to thaw to room temperature now.

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".

cDNA library preparation: Step 4

Post-capture PCR

Overview



After capture of target sequences, 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 the Universal Hybridisation & Wash Kit (stored at −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 and/or incubate at 37°C until dissolved.
- Remove the Step 4: PCR Polymerase (purple lid; ●) from the Universal Hybridisation & Wash Kit stored at (−15°C to −25°C) and place on ice.

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 13.
   Save the program as "OGT PCR2". Where possible, set the heated lid to 105°C, alternatively have the pre-set heated lid activated.

cDNA library preparation: Step 4

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 15 cycles	
6	72	10 min
7	4	Hold

Table 13: 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 14 in a fresh 1.5 ml LoBind tube. There will be two PCRs prepared for each hybridisation capture pool.

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 14: Post-capture PCR Master Mix.

cDNA library preparation: Step 4

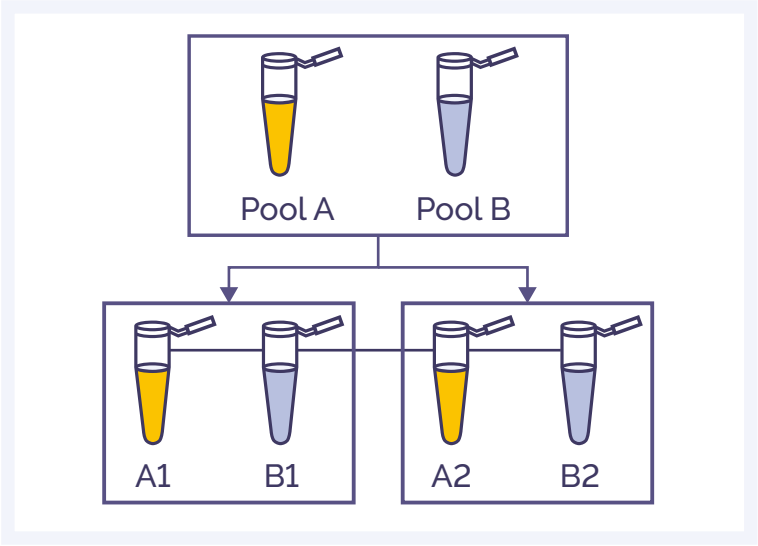


Figure 7: Post-capture PCR guide for 16 samples (2 pools). Duplicate reactions set up for each capture pool for a total of 4 PCRs.

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 pool.
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. Ensure two PCRs are performed for each pool (see Figure 7).
9. Pipette mix at least 10 times.
10. Transfer to the thermal cycler and start the program "OGT PCR2".

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.

cDNA library preparation: Step 4

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 **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.
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).

cDNA library preparation: Step 4

14. Transfer **30 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 330–380 bp (+/– 20 bp) (Figure 8). Set up the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.

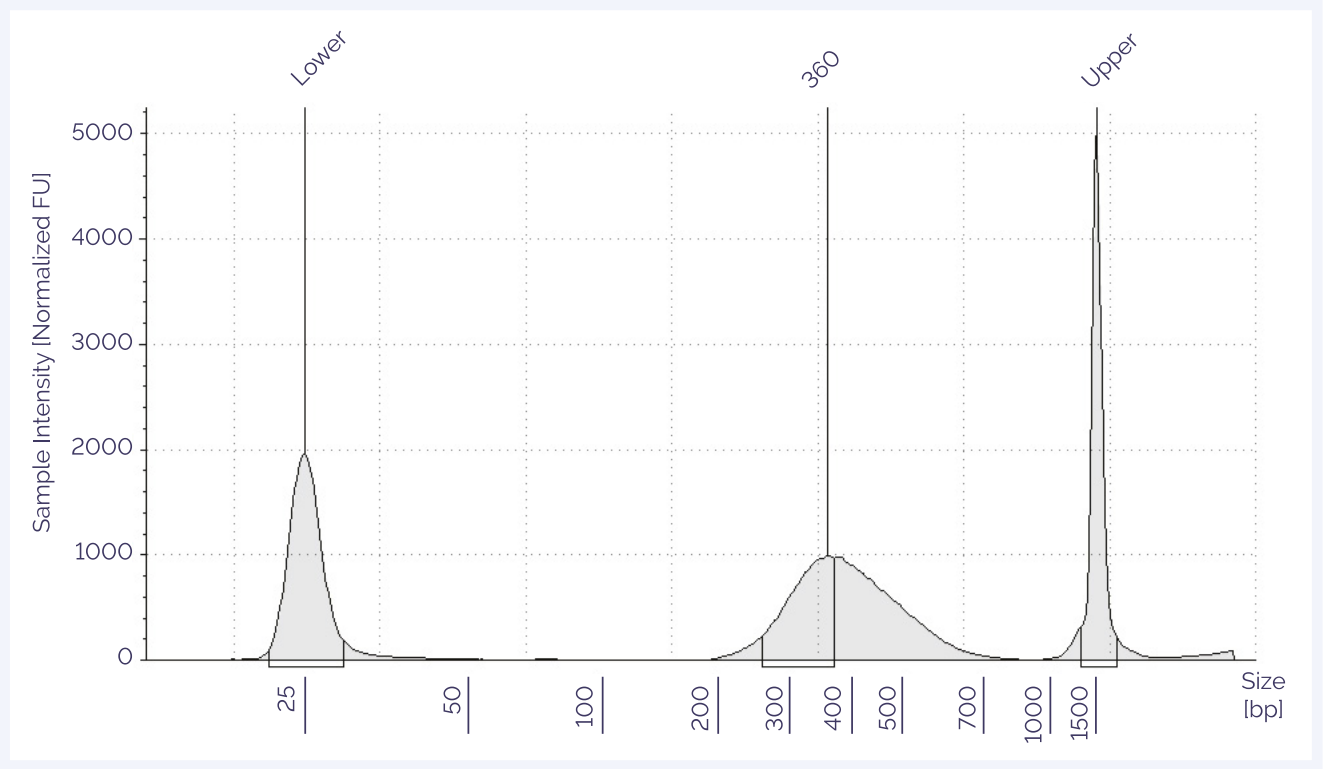


Figure 8: Electropherogram of purified post-capture PCR product generated using an Agilent High Sensitivity D1000 ScreenTape assay. The electropherogram shows a maximum peak in the size range of approximately 330–380 bp (+/– 20 bp).

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 panel dependent and between 1–15 ng/µl. Contact your local Field Application Specialist (FAS) for further details.

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 the formulae below to determine the volume (µl) of each DNA capture pool required to generate the 4 nM sequencing pool by diluting with Nuclease-free water.
2. 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.
3. 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.

Formula 1 — nM of each sample

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

Formula 2 — volume of each indexed DNA sample

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

Appendix

Adapter sequences

Adapter	Sequence
1	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
2	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Table 15: Adapter sequences – forward configuration. Please note some Illumina sequencing instruments require the reverse complement of the Index 2 (i5) Adapter sequences.

Index sequences

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

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
880002 - SureSeq myPanel Myeloid Fusion MRD Panel	NextSeq 1000 P2 Cartridge	16	2 x 8-plex	1 x 24 reactions	1.5 runs
				1 x 96 reactions	6 runs

Table 16: Recommended sequencing guidelines

Appendix

Reagent tube locations

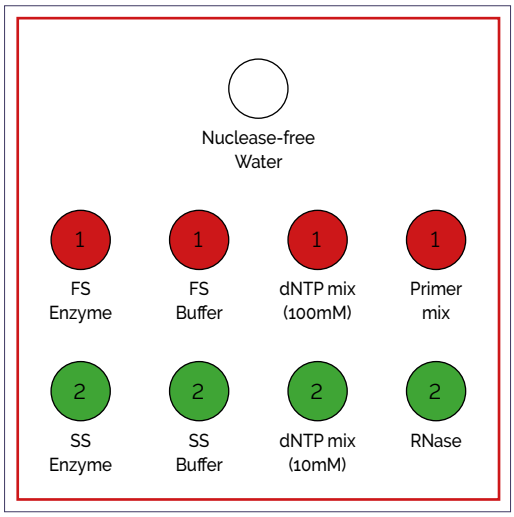


Figure 9: Location of tubes in the 24 and 96 Double-Stranded cDNA Synthesis Kit (880500-24 and 880500-96).

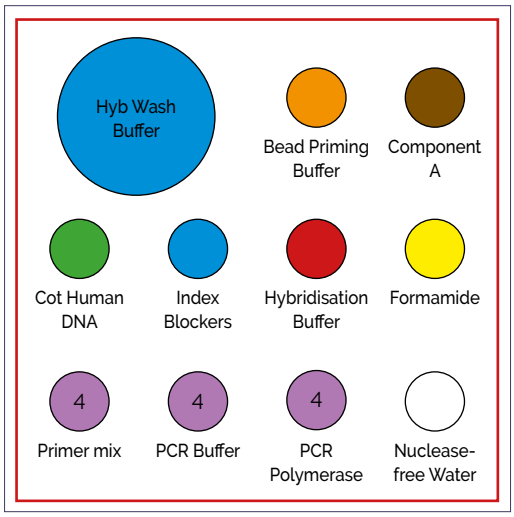


Figure 10: Location of tubes in 24 reaction Hybridisation & Wash Kit V2 (770410-24).

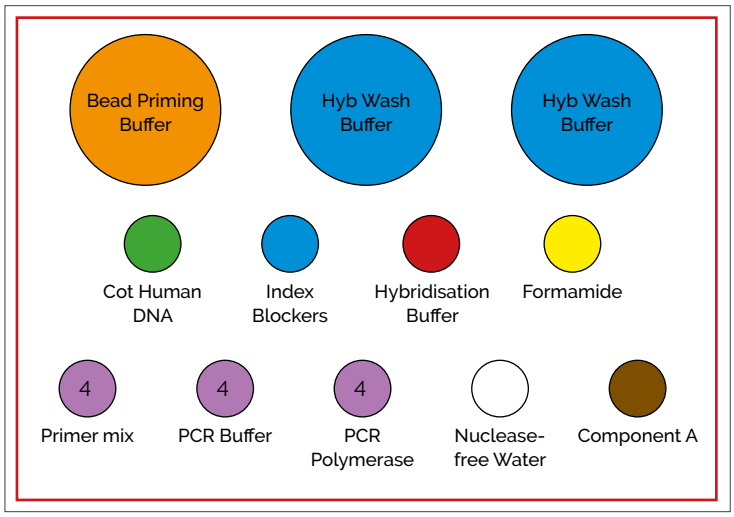


Figure 11: Location of tubes in 96 reaction Hybridisation & Wash Kit V2 (770410-96).

Appendix

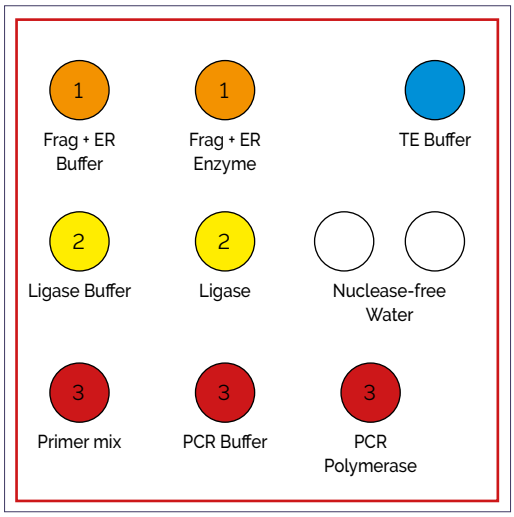


Figure 12: Location of tubes in 24 reaction Library Preparation Kit (770100-24).

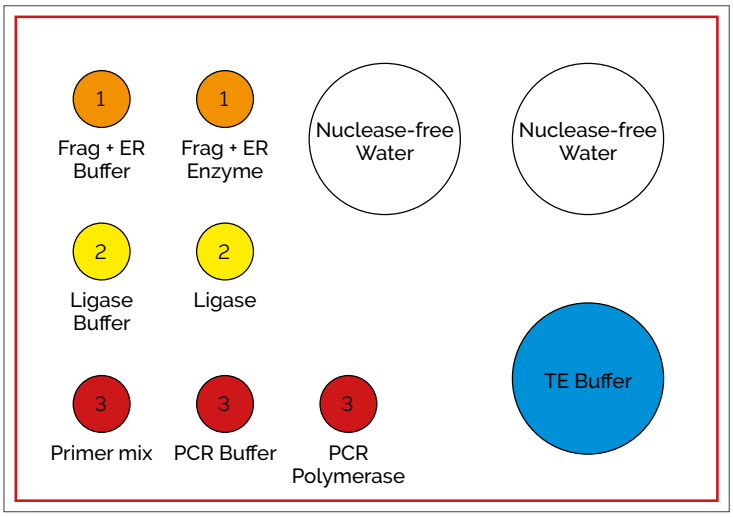


Figure 13: Location of tubes in 96 reaction Library Preparation Kit (770100-96).

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