CytoSure™ NGS Library Preparation - Instructions for use

Oxford Gene Technology

Founded by Professor Sir Ed Southern, Oxford Gene Technology (OGT) provides world-class genetics research solutions. OGT has a strong reputation and increasing share in the large and growing genomic medicine market.

OGT is part of Sysmex Corporation's life science business, reinforcing Sysmex's initiatives towards personalised medicine.

- **CytoSure**™ — Class-leading products and services offering the complete array and NGS solutions for clinical genetics research.
- **Cytocell**® — High-quality fluorescence *in-situ* hybridisation (FISH) probes for the detection of gene rearrangements related to inherited genetic disease and cancer.
- **SureSeq**™ — Delivering comprehensive, high–quality targeted sequencing products to clinical and academic researchers.

For more information visit www.ogt.com.

Contents

Introduction 4
Storage 4
Safety 4
Intended use 4
Equipment and reagents required 5
Equipment required (not supplied) 5
Reagents and consumables required (supplied by OGT) 5
Reagents and consumables required (not supplied by OGT) 6
Interpret NGS Analysis Software 6
Sample preparation and experimental design 7
Sample requirements 8
DNA shearing using Covaris 10
End repair and 3’ end ‘A’ Tailing 14
Adaptor ligation 16
PCR 1 19
Hybridisation 23
Hybridisation Set-Up 23
Hybridisation Wash with CytoSure NGS Hybridisation & Wash Kit 25
Post-capture PCR 29
NextSeq sequencing 33
Preparing the sequencing pool 33
Preparing the NextSeq 34
Glossary of Symbols 36
Legal information 36
NGS library prep assay 36

Introduction

The CytoSure NGS library preparation system consists of reagents and software designed to detect Copy Number Variations (CNVs), Single Nucleotide Variations (SNVs) and Loss of Heterozygosity (LOH) regions; which may be implicated in intellectual disability and developmental delay.

The Constitutional NGS Panel enriches human genomic DNA using oligonucleotide baits. The baits have been designed to hybridise to the following genomic regions:

- Genes that are important in intellectual disability and development delay. These genes have been curated in collaboration with the ClinGen and Deciphering Developmental Disorders (DDD) consortiums.
- ‘Backbone’ regions spaced throughout the genome. The spacing is dependent on the importance of the region

In addition to the Constitutional NGS Panel, the CytoSure NGS Library Preparation Kit (LPK) and the CytoSure NGS Hybridisation & Wash Kit, provide the reagents required to prepare hybridisation-based enriched libraries. These libraries are then designed to be run on the Illumina NextSeq™ instrument using the High-Output kits.

The complete solution including Interpret NGS Analysis Software will provide the necessary components to perform analysis on 24 samples per batch – in a 3x8 plex hybridisation format.

Storage

The kits should be used before the expiry date indicated on the kit label. All kits should be stored at -15°C to –25°C.

Safety

Handling of the CytoSure NGS Protocol 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 CytoSure NGS Hyb & Wash Kit contains chemicals which are potentially hazardous when mishandled, particular care should be given to both Formamide and the Hybridisation Buffer. Any chemicals used are potentially hazardous. Please refer to the SDS for specific information.

Intended use

The CytoSure Constitutional NGS Panel is a *Research Use Only* assay.

Equipment and reagents required

Equipment required (not supplied)

Item	Supplier
Focused-ultrasonicator	Covaris®, cat. no. S2 or equivalent
2200 TapeStation	Agilent®, cat. no. G2965A or equivalent
SpeedVac®	Thermo Fisher scientific or equivalent
Thermal Cycler	Agilent® SureCycler 8800, cat. no. G8800A or equivalent
Laboratory vortex mixer + adapter for a standard microwell plate	IKA™ MS 3 Digital Vortex Mixer with attachments or equivalent
Hot block for 1.5 ml tubes	
Microfuge for standard 1.5 ml tubes and 8-Strip PCR tubes	
Magnetic rack for 96–well microwell plates and 1.5 ml tubes	Invitrogen DynaMag™- 96 Side Magnet, cat. no. 12331D or equivalent
Qubit® fluorometer	Life Technologies, cat. no. Q32857
Thermo Scientific NanoDrop™	Thermo Scientific or equivalent
20-200 µl and 1-10 µl 8-Channel pipette	
NextSeq 550 Sequencer	Illumina®, cat. no. SY-415-1002

Reagents and consumables required (supplied by OGT)

Item	Supplier
CytoSure NGS Library Preparation Kit With CytoSure NGS Index Kit included	OGT, cat. no. 502001 – 24 (24 reactions) OGT, cat. no. 502001 – 96 (96 reactions)
CytoSure NGS Hybridisation and Wash Kit	OGT, cat. no. 502002 – 24 (24 reactions) OGT, cat. no. 502002 – 96 (96 reactions)
CytoSure Constitutional NGS Panel	OGT, cat. no. 502003 – 24 (24 reactions) OGT, cat. no. 502003 – 96 (96 reactions)
Dynabeads® M-270 Streptavidin	OGT, cat. no. 500080
Agencourt AMPure® beads	OGT, cat. no. 500081

Reagents and consumables required (not supplied by OGT)

Item	Supplier
Covaris® microTUBE AFA Fiber Snap-Cap	Covaris®, cat. no. 520045
Molecular Biology Grade 100% Ethanol	Sigma Aldrich, cat. no. E7023 or equivalent
Molecular Biology Grade water	Sigma Aldrich, cat. no. W4502-1L or equivalent
Quant-iT™ dsDNA HS Assay Kit and Quant-iT™ dsDNA BR Assay Kit	Life Technologies, cat. no. Q32850, Q32854
Qubit Assay Tubes	Life Technologies, cat. no. Q32856
Agilent D1000 and High Sensitivity D1000 Reagents and ScreenTapes	Agilent cat. no. 5067-5582, 5067-5583, 5067-5584, 5067-5585
DNA LoBind™ Tubes	Eppendorf, cat. no. 022431021 or equivalent
96-well microwell plates and 8-Strip PCR tubes	Starlab, cat. no. E1403-0100, Starlab cat. no. A1402-3700 or equivalent
Sequencing reagents required for the Illumina NextSeq	Illumina NextSeq High Output 2x150 bp (300 cycles) v2.5 (Illumina Cat no. 20024908)
5.0 M Sodium hydroxide solution, molecular biology-grade	Sigma-Aldrich, cat. no. S8263 or equivalent
15 ml or 50 ml Falcon Tubes or similar	

Interpret NGS Analysis Software

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

Sample preparation and experimental design

This IFU includes the protocol for the CytoSure NGS Library Preparation Kit and CytoSure NGS Hybridisation & Wash Kit. For ordering information about OGT products please visit www.ogt.com.

The following section contains instructions for sample library production specific to the Illumina sequencing platform. For each sample, individual library preparations are performed. Each sample is tagged by PCR with an index (barcode) sequence and then pooled into groups of eight. Each pool is hybridised, captured, and then sequencing primers are added on ready for sequencing on the Illumina NextSeq.

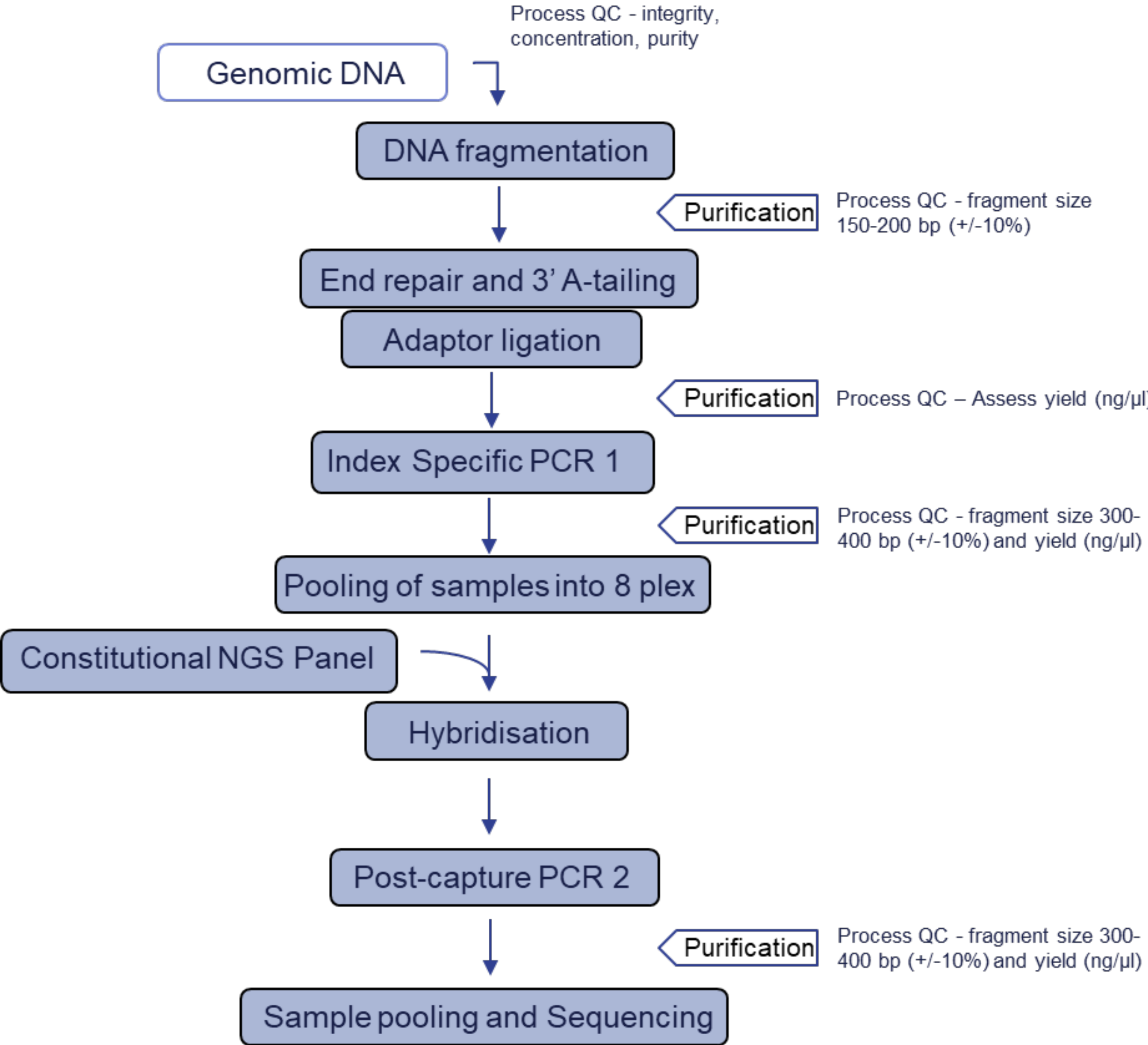


Figure 1: Workflow of sample library preparation indicating the expected DNA fragment size at each step of the procedure.

It is important to consider the data analysis before designing and running the experiment.

The CNV detection relies on comparing the sample data to a set of data generated from reference DNA (with CNVs). The Interpret software will do this comparison and is flexible as to how the reference data is generated. There are several options for generating reference data:

- All samples loaded on the same NGS run are used as a reference for the samples to be analysed.
- Multiple controls loaded in the same NGS run with the samples to be analysed.
- Multiple controls loaded in a different NGS run from the samples to be analysed.
- A reference database built up from multiple different NGS runs.

OGT has found that the optimal CNV detection is when all samples in the same run are used as the reference. If this is the case then it is important to:

- Use 24 samples per NextSeq High Output run.
- Only samples that have adequate metrics, as shown in the QC metric report, should be included as references.
- It is better if the samples are sex matched to the references.
- The aberrations within the samples are unlikely to all be in the same place. For example, multiple Trisomy 21 samples should not be run together.

The LOH and SNV detection is not dependent on references.

Sample requirements

The recommended amount of input DNA is 1 µg.

Sample QC

We highly recommend that quality control (QC) assessments are carried out for all samples prior to starting the CytoSure NGS Library Preparation protocol to assess the DNA integrity, concentration and purity.

Testing sample integrity, concentration and purity

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

DNA integrity — Genomic DNA TapeStation

Assessing DNA integrity

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

Concentration — Qubit

Assessing DNA concentration

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

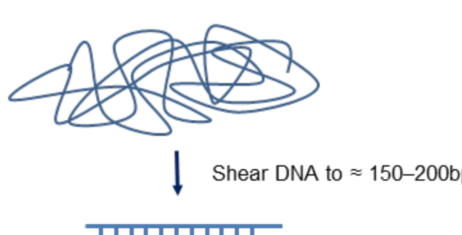
Purity — NanoDrop

Assessing DNA purity

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

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

DNA shearing using Covaris



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

Estimated time: 6 min shearing per sample.

Preparation

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

Shear the DNA

1. Dilute 1 µg Genomic DNA in 130 µl TE Buffer.
2. Put a Covaris microTUBE into the loading and unloading station. Keep the cap on the tube.
3. Use a tapered pipette tip to slowly transfer the 130 µl Genomic DNA through the pre-split septum. Be careful not to introduce a bubble into the bottom of the tube.
4. Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 1. The target peak for base pair size is 175-200 bp.

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

Table 1: Shear settings for DNA samples.

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

5. Place the microTUBE into an appropriately sized tube adapter and spin in a picofuge for 5 seconds to collect all liquid in the bottom of the microTUBE.
6. Return the Covaris microTUBE into the loading and unloading station.
7. Keeping the snap-cap on, insert a tapered pipette tip through the pre-split septa, and then slowly remove the sheared DNA.
8. Transfer the sheared DNA into a new 1.5 ml LoBind tube.

NOTE: If required, it is possible to shear in 50 µl volume, follow the manufacturers settings for shearing to the target peak size. Before continuing to purification, ensure that you top up the sample volume to 130 µl with TE Buffer.

Post-shear purification

Estimated time: 45 min for 24 samples.

1. Use only room temperature AMPure XP beads.
2. Mix well so that the AMPure XP beads appear homogeneous and consistent in colour.
3. Add 180 µl of homogenous AMPure XP beads to each sheared DNA sample in 1.5 ml LoBind tubes. Mix well using a vortex mixer and incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (approx. 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µl of 70% ethanol in each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Return the tube to the magnetic stand and wait 1 min. Remove any remaining ethanol using a P20 pipette and tip being careful not to touch the bead pellet.
10. Dry the samples on a 37°C heat block for 3–5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.

    IMPORTANT: Do not over-dry as this will decrease yield.
    Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
11. Add 27 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer and incubate for 5 min at room temperature.
12. Spin the tube briefly and place on the magnetic stand and leave for 2–3 min until the solution is clear.
13. Remove 27 µl of the supernatant to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.
14. Optional: *The quality and quantity of DNA obtained after shearing should be assessed using the Agilent 2200 TapeStation, Agilent Bioanalyser or similar. An example of a good DNA trace is shown in Figure 2. Ensure a distribution with a peak height between 150 and 200 nucleotides is achieved. Set up the machine and prepare the tape, samples and ladder following the manufacturer's instructions.*

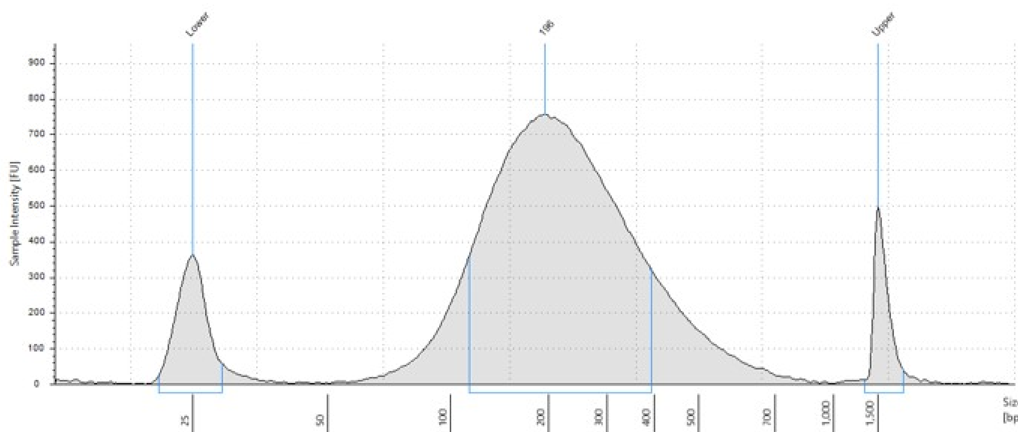
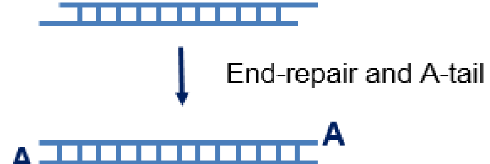


Figure 2: Analysis of sheared DNA using an Agilent High Sensitivity D1000 ScreenTape assay. Size distribution with a peak between 150-200 bp (+/– 10%).

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

End repair and 3' end 'A' Tailing



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

Estimated time: 1 h 15 min for 24 samples. Hands-on time: 15 min.

Preparation

- Remove the Step 1 reagents (Green Cap) from storage (–15°C to –25°C). Place Step 1: Enzyme Mix on ice and allow the Step 1: ER Buffer to thaw to room temperature.
- If required, incubate the Step 1: ER Buffer further at 37°C for 5-10 min to resuspend any precipitates.

Prepare End Repair/ 'A' Tailing Master Mix

1. For multiple samples, prepare the reaction mix as shown in Table 2 (on ice). Mix well on a vortex mixer.

Reagent	1x library (µl)	25.5 x library (µl)
DNA sample	25	-
Nuclease-free H_2O	9	229.5
Step 1: ER Buffer (Green Cap)	10	255
Step 1: ER Enzyme Mix (Green Cap):	6	153
TOTAL	**50**	**637.5**

Table 2: Components for End Repair/ 'A' Tailing Master Mix

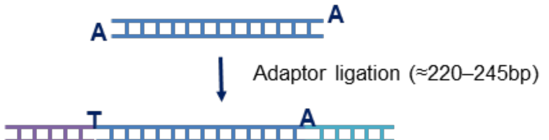
2. Add 25 µl of the master mix to each well or tube containing the sheared DNA. Mix by pipetting 10 times. Change pipette tips between samples.
3. Set up the incubation using the profile and settings as shown in Table 3, with heated lid at 105°C.

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

Table 3: Incubation profile

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

Adaptor ligation



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

Estimated time: 30 min for 24 samples. Hands-on time: 15 min.

Preparation

- Remove the Step 2 reagents (Yellow Cap) from storage (–15°C to –25°C). Place the Step 2: Ligase on ice and allow all other Step 2 reagents to thaw to room temperature.
- Remove AMPure XP beads from the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.

Prepare Ligation Master Mix

1. Prepare the ligation master mix (on ice) using the volumes shown in Table 4. Mix well on a vortex mixer.

Reagent	1x library (µl)	25.5 x library (µl)
DNA sample	50	-
Step 2: Ligase Buffer (Yellow Cap)	3	76.5
Step 2: Adaptor A (Yellow Cap)	5	127.5
Step 2: Adaptor B (Yellow Cap)	5	127.5
Step 2: Ligase (Yellow Cap)	2	51
TOTAL	**65**	**382.5**

Table 4: Components for Ligation Master Mix

2. Add 15 µl of the master mix to each well or tube on ice. Mix by pipetting 10 times. Change pipette tips between samples.
3. Incubate in a thermal cycler for 15 min at 20°C. Do not use a heated lid.

Ligated library purification

Estimated time: 45 min for 24 samples.

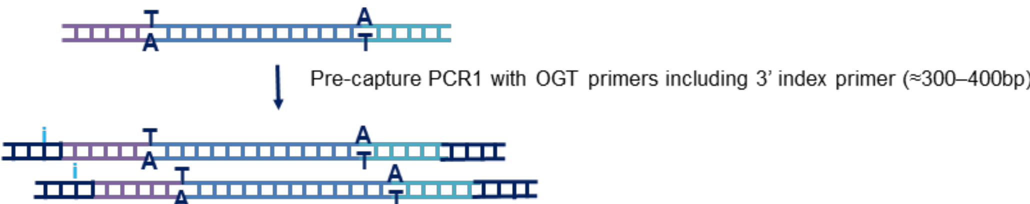
1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 117 µl of homogenous AMPure XP beads to each DNA sample. Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand whilst adding 200 µl of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.

   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 32 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove 30 µl of the supernatant to a fresh 0.2 ml tube. The beads can be discarded at this time.
13. Assess yield using the Qubit BR Kit as per manufacturer's instructions. The expected yield is between 15-25 ng/µl.

    Optional: Assess the quality of the DNA with an Agilent DNA1000 TapeStation and check that the electropherogram shows a distribution with a peak height around 250 bp (+/– 10%). Setup the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.

    STOPPING POINT: If the samples are not to be used immediately store at 4°C.

PCR 1



High fidelity PCR is used with as few PCR cycles as possible to amplify and index the DNA library prior to hybridisation and target capture.

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

Preparation

- Remove the Step 3: Primer (Red Cap) and PCR Buffer (Blue Cap) from storage (–15°C to –25°C) and allow to thaw at room temperature. Remove the DNA Polymerase (blue cap) from storage (–15°C to –25°C) and place on ice.
- If required, incubate the PCR Buffer further at 37°C for 5-10 min to resuspend any precipitates in the buffer.
- Remove the Index Plate from storage (–15°C to –25°C) and allow to thaw at room temperature.
- Briefly spin the Index Plate in a centrifuge to collect the liquid. To avoid cross-contamination, take care when opening the Index Plate.
- **IMPORTANT**: Assign a different index primer for each sample to be sequenced on the same lane.
- For simplicity, it is suggested that each pool will contain a complete column from the Index Plate, e.g. 1-8 form one pool, 9-16 form another pool and 17-24 form a third pool.
- Take the AMPure XP beads out of the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.

Prepare PCR 1 Master Mix

1. Prepare the reaction mixes as shown in Table 5 on ice and mix well on a vortex mixer.

Reagent	1x library (µl)	25.5 x library (µl)
Ligated Library	15	-
Nuclease-free H_2O	22	561
PCR Buffer (Blue Cap)	5	127.5
Step 3: Primer (Red Cap)	1	25.5
DNA Polymerase (Blue Cap)	2	51
Index (1-48)	5	-
TOTAL	**50**	**765**

Table 5: Components for PCR 1 Master Mix

2. Add 30 µl of the master mix to each well or tube.
3. Add 5 µl of the appropriate Index PCR Primer (1–48) to each well and mix by pipetting remembering to use a different index primer for each sample to be sequenced in the same lane.
4. Add 15 µl of each DNA samples to the relevant well or tube. Mix by pipetting up and down, remembering to change pipette tips between samples to avoid cross-contamination.
5. Place the tubes in a thermal cycler and run the PCR programme in Table 6.

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 6: PCR 1 Profile

PCR 1 purification

Estimated time: 45 min for 24 samples.

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 90 µl of homogenous AMPure XP beads to each DNA sample. Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand whilst adding of 200 µl 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.

   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 32 µl of nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove 30 µl of the supernatant and transfer to a fresh 0.2 ml tube or 96-well plate. The beads can be discarded at this time.

    Optional*: Assess the quality of the DNA with an Agilent DNA1000 TapeStation and check that the electropherogram shows a distribution with a peak height around 300 bp (+/– 10%) (Figure 3). Setup the instrument and prepare the tape, samples and ladder following the manufacturer's instructions.*
13. Assess the quantity using Qubit BR kit. The expected yield is between 15-40 ng/ul.

    Note: It is important to ensure accurate quantification of your samples to make certain that the pooling is uniform across all your samples.

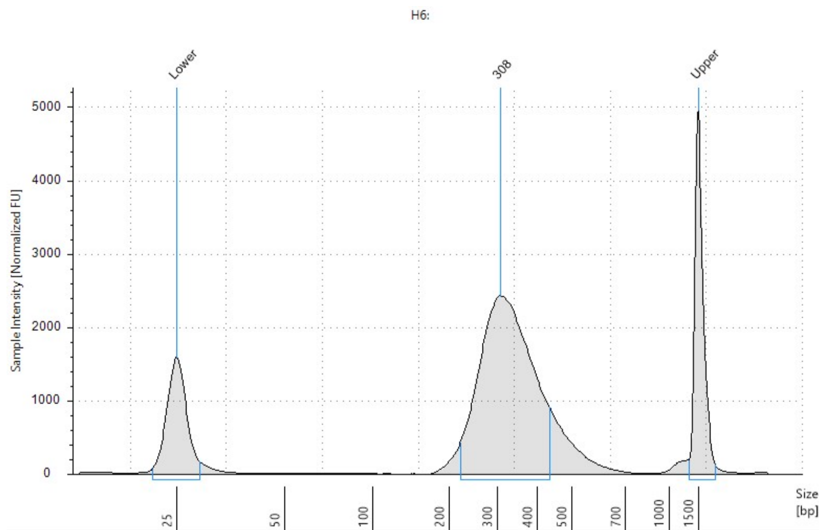
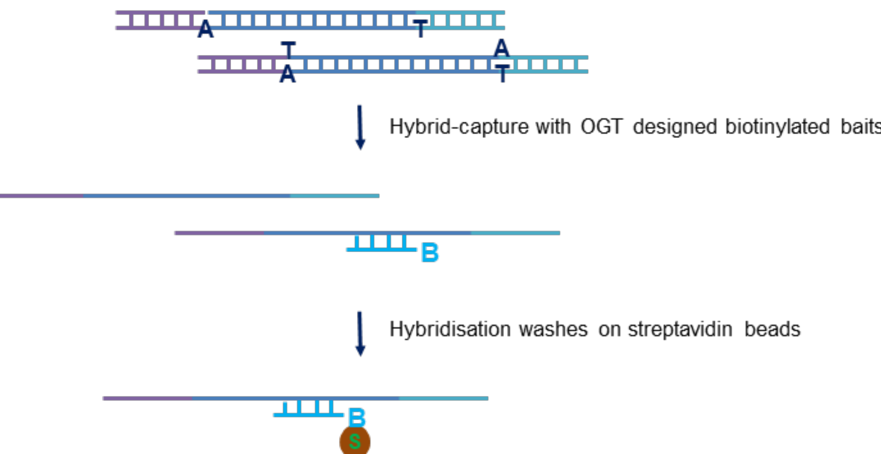


Figure 3: Analysis of amplified prepared library DNA using an Agilent D1000 ScreenTape assay. The electropherogram shows a single peak in the size range of ~300 bp (+/– 10%).

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

Hybridisation



The amplified library is denatured and captured by the CytoSure Constitutional NGS Panel which contains biotinylated baits. The hybridised targets are then bound to streptavidin beads and washed to remove any off-target DNA.

Before you begin

- 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 15 µl of Hybridisation Buffer (without DNA) to each well that you might use and incubate at 65°C overnight. Check after the overnight incubation that the evaporation does not exceed 1–2 µl per well.

Hybridisation Set-Up

Estimated time: ~2 hr (including drying-down time). Hands-on time: 15 min.

Preparation

- Remove the Bead Priming Buffer (Orange Cap), Hybridisation Buffer (Red Cap) and Wash Buffer from storage (–15°C to –25°C). Bring to room temperature and incubate further at 37°C for 5-30 min to resuspend any precipitates in the buffers (Hybridisation Buffer and Wash Buffer).
- Remove the Cot Human DNA (Green Cap), Blockers (Purple Cap), Formamide (Yellow Cap) and Nuclease-free Water from storage (–15°C to –25°C) and allow to reach room temperature.

- Per 8 DNA samples prepared, carry out one hybridisation capture. The hybridisation reaction requires 187.5 ng of each DNA library, for a total of 1500 ng for each 8-sample pool

Prepare for Hybridisation

1. For each library combine the components detailed in Table 7 in a 0.2 ml tube.

Reagent	1x Hybridisation capture (µl)
187.5ng Library (per sample)	-
Cot Human DNA (Green Cap)	5
Blockers (Purple Cap)	15

Table 7: Pre-hybridisation mix

2. Dry down the contents of prepared tube(s) completely using a SpeedVac or similar evaporation device.
3. Resuspend each tube with 2.5 µl nuclease-free water.
   Note: For optimal recovery, incubate the sample at 37°C for 10 min and mix occasionally on a vortex or store at 4°C overnight.
4. Add the components in Table 8 to each tube, changing tips with each addition to avoid cross-contamination. Cap the tubes, mix well on a vortex mixer and spin down. The final volume should be 15 µl.

Reagent	1 x Hybridisation Capture (µl)
Hybridisation Buffer (Red Cap)	7.5
Formamide (Yellow Cap)	3
CytoSure Constitutional NGS Panel	2

Table 8: Hybridisation mix

5. Place the tubes into the thermal cycler and set up the incubation using the profile and settings as shown in Table 9, with heated lid at 105ºC.

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

Table 9: Hybridisation Incubation profile

6. Make sure all caps are on tightly and all wells are sealed.
7. Incubate the hybridisation mixture overnight at 65°C with a heated lid at 105°C.
8. Continue to Hybridisation Wash.

Hybridisation Wash with CytoSure NGS Hybridisation & Wash Kit

Estimated time: 1.25 hr. Hands-on time: 15 min.

Preparation

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

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

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

Prepare sequence capture and bead wash buffers

1. Ensure the Bead Priming Buffer and Wash Buffer are fully thawed.
   Note: Incubate at 37°C for 5-10 min to resuspend any precipitates.
2. Aliquot 6 x 200 µl of Wash Buffer per hybridisation into 0.2 ml strip tubes.
3. Pre-warm the aliquots to the following temperatures in a heat block for a minimum of 30 min before use:
   - 3 x 200 µl at 65°C / hybridisation
   - 3 x 200 µl at 35°C / hybridisation

Prepare magnetic beads

1. Vigorously resuspend the Dynabeads M270 Streptavidin magnetic beads on a vortex mixer.
2. Add 100 µl M270 magnetic beads to a 1.5 ml microfuge tube for each hybridisation performed (up to 300 µl of beads can be washed in a single 1.5 ml microfuge tube).
3. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
4. Add 200 µl of 1x Bead Priming Buffer per 100 µl beads (600 µl for 300 µl beads). Mix briefly on a vortex mixer and place back on the magnetic rack.
5. Allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
6. Repeat steps 3-6 for a total of two washes.
7. Resuspend the beads in 1x the original volume of 1x Bead Priming Buffer (i.e., for 300 µl beads add 300 µl buffer) and mix on a vortex mixer.
8. Transfer the beads into a new 0.2 ml tube for each capture reaction (100 µl per hybridisation).
9. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.

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

Hybrid capture

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

   Note: Proceed immediately to the next Wash Streptavidin beads to remove unbound DNA

Wash Streptavidin beads to remove unbound DNA

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

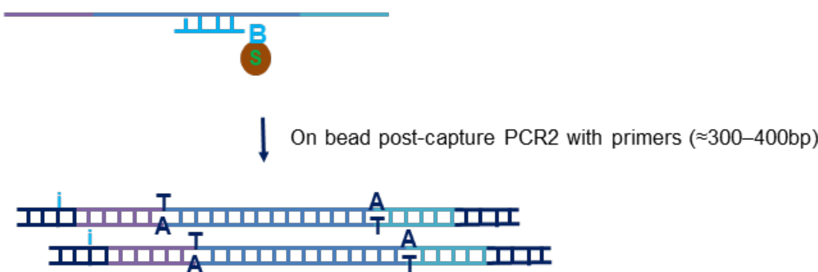
Note: After the addition of fresh buffer ensure the pellet has been fully resuspended by a brief mix on a vortex mixer followed by visual inspection. DO NOT use a multichannel pipette for mixing.

1. Place the tubes on a magnetic rack, allow the beads to separate from the supernatant.
2. Carefully remove and discard the supernatant containing unbound DNA.
   Caution: The hybridisation mix contains Formamide.
3. Remove the tubes from the magnetic rack.
4. Add 200 µl of pre-warmed (to 65°C) Wash Buffer to the capture beads plus bound DNA.
5. Mix briefly on a vortex mixer to disrupt the bead pellet.
6. Place the tubes in a plate vortex mixer set at approximately 2000 RPM and mix for 1 min at room temperature. Briefly spin in a centrifuge to collect the liquid.
7. Return the samples to the thermal cycler set to 65°C for 5 min (ensure that the lid is heated is set at 105°C).
8. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
   Caution: The supernatant may contain trace amounts of Formamide.
9. Remove the tubes from the magnetic rack.
10. Repeat steps 4-9 for a total of three 65°C washes.
11. Add 200 µl of pre-warmed (to 35°C) Wash Buffer and mix briefly on a vortex mixer to disrupt the bead pellet. Ensure the mixture is homogenous.
12. Place the tubes in a plate vortex mixer and mix for 2 min at 2000 RPM.
13. Briefly spin in a centrifuge to collect the liquid.
14. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
15. Add 200 µl of pre-warmed (to 35°C) Wash Buffer and mix briefly on a vortex mixer to disrupt the bead pellet. Ensure the mixture is homogenous.
16. Place the tubes in a plate vortex mixer and mix for 1 min at 2000 RPM
17. Briefly spin in a centrifuge to collect the liquid.
18. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.

19. Add 200 µl of pre-warmed (to 35°C) Wash Buffer and mix briefly on a vortex mixer to disrupt the bead pellet. Ensure the mixture is homogenous.
20. Place the tubes in a plate vortex mixer and mix for 30 sec at 2000 RPM
    Note: Do not mix for more than 30 sec.
21. Briefly spin in a centrifuge to collect the liquid.
22. Place on a magnetic rack, allow the beads to separate from the supernatant then carefully remove and discard the supernatant.
23. Remove from the magnetic rack and resuspend the beads in 30 µl of nuclease-free water, mix thoroughly on a plate vortex mixer.

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

Post-capture PCR



After capture of target sequences, primers are added to amplify the NGS libraries for compatibility for Illumina platforms.

Estimated time: 1.75 hr. Hands-on time: 15 min.

Preparation

- Remove the Step 4 Primer (Brown Cap) and PCR Buffer (Blue Cap) from storage (–15°C to –25°C). Place the DNA Polymerase (Blue Cap) on ice and allow all other reagents to thaw to room temperature.
- If required, incubate the buffer further at 37°C for 5-10 min to resuspend any precipitates in the buffer
- Take the AMPure XP beads out of the fridge at least 30 min before use to allow them to warm to room temperature.
- Make up fresh solution of 70% ethanol using molecular biology grade ethanol and molecular biology grade water.

Prepare Post-Capture PCR Master Mix

1. For multiple samples, prepare the reaction mix as shown in Table 10, on ice. Mix well on a vortex mixer.

Reagent	1x library (µl)	3.5 x library (µl)
Captured DNA and Bead Slurry	14	-
Nuclease-free H_2O	26.5	92.75
PCR Buffer (Blue Cap)	5	17.5
Step 4: Primers (Brown Cap)	2.5	8.75
DNA Polymerase (Blue Cap)	2	7
TOTAL	**50 µl**	**126**

Table 10: Components for Post-Capture PCR Master Mix

2. Add 36 µl of the master mix to each tube.
3. Pipette mix the bead slurry to ensure the beads are homogeneous. Transfer 14 µl of each DNA bead slurry to the relevant well or tube. Mix by pipetting remembering to change pipette tips between samples to avoid cross-contamination.
4. Set up PCR using the profile and settings as shown in Table 11.

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 10 cycles	
6	72	10 min
7	4	Hold

Table 11: Post-Capture PCR profile

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

Post-Capture PCR purification

Estimated time: 45 min for 24 samples.

1. Use only room temperature AMPure XP beads.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in colour.
3. Add 90 µl of homogenous AMPure XP beads to each DNA sample. Mix well using a vortex mixer or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.
4. Put the tube in the magnetic stand and wait for the solution to clear (which should take approximately 3–5 min).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand whilst adding 200 µl of 70% ethanol to each tube.
7. Let the tube sit for 1 min to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat wash (step 6 and step 7).
9. Dry the samples on a 37°C heat block for 3-5 min or until the residual ethanol completely evaporates. Ensure you check the pellet frequently to prevent loss of yield.
   IMPORTANT: Do not over-dry as this will decrease yield.
   Note: The bead pellet is dry when the appearance of the surface changes from shiny to matte.
10. Add 32 µl nuclease-free water directly to the bead pellet, mix well on a vortex mixer, spin briefly and incubate for 5 min at room temperature.
11. Place the tube on the magnetic stand and leave for 2–3 min until the solution is clear.
12. Remove approximately 30 µl of the supernatant to a fresh 0.2 ml tube~~s~~/plate. The beads can be discarded at this time.
13. Analyse amplified product size using the Agilent TapeStation (High Sensitivity Kit) or similar, to determine the peak size for each DNA sample. The electropherogram should show a peak height of 300-400 bp (+/– 10%) (Figure 4). Set up the instrument and prepare the tape, samples and ladder following manufacturer's instructions.
14. Assess the PCR yield using High Sensitivity dsDNA Qubit assay. The expected yield should be between 1 – 20 ng/µl.

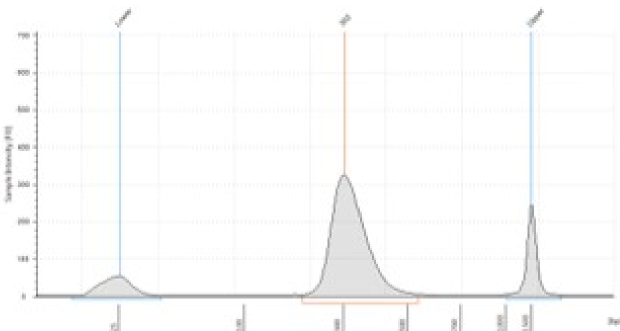


Figure 4: Analysis of amplified capture DNA using an Agilent High Sensitivity D1000 ScreenTape assay. The electropherogram shows a peak in the size range of approximately 300-400 bp (+/– 10%).

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

NextSeq sequencing

The DNA samples prepared in the previous section need to be combined such that each index-tagged sample is present in equimolar amounts in the final pool. This requires both accurate determination of peak size (bp) and accurate determination of sample concentration (ng/µl).

Preparing the sequencing pool

To determine the volume (µl) of each indexed DNA sample required to generate the 4 nM Sequencing Pool, use the NextSeq pooling template file provided by OGT (or the formulae below).

1. Complete the “Pool Parameter” and “Samples” tables in the “Pool” tab of the NextSeq pooling template. Cells in green should be modified as required; parameters marked with * must be completed.

   Note: This protocol has been validated with 150-base paired-end reads using the NextSeq High-Output Kit 2x150bp (Illumina cat. no. 20024908).
2. Each indexed sample should be entered in separately.
3. In the “Volumes to pipette” tab, column D contains the values of each 8-plex pool that should be pipetted. Pipette this volume of each hybridisation pool into a fresh 1.5 ml LoBind tube labelled “4 nM Sequencing Pool”; remembering to change tips to avoid cross-contamination.
4. Adjust the final volume of the sequencing pool with nuclease free water to the desired final concentration (4 nM). This can also be found in column D of “Volumes to pipette” tab.

   Optional: If you store the library before sequencing, add Tween 20 at 0.1% v/v and store at -20°C.
5. **Validation of Sequencing Pool concentration** — Analyse the Sequencing Pool using an Agilent TapeStation (High Sensitivity Kit) to determine peak height and determine the concentration (ng/µl) yield using High Sensitivity Qubit assay. Complete the “Pool validation and dilution” tab to determine the molar concentration of the Sequencing Pool.
6. The Sequencing Pool can now be prepared for loading on to the NextSeq. Refer to the appropriate Illumina protocol for your NextSeq kit.

NOTE: As cluster density can vary between machines, please use the recommended final loading concentration for your machine. We have used a final loading concentration of 1.4pM when using a 2x150bp High Output kit.

Formula 1 – Concentration (nM) of each sample

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

Formula 2 — Volume of each Indexed DNA Sample

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

Preparing the NextSeq

1. The NextSeq should be prepared according to manufacturer’s protocols.
2. The sequencing pool should be made to a final concentration of 1.4 pM with the HT1 Buffer
3. The sequencing pool should be combined with denatured PhiX before running.
4. When using BaseSpace, select **+ Custom Library Prep Kit** in the Library Prep Kit dropdown menu.
5. Use the information in Tables 12 and 13 to create the CytoSure NGS Library prep kit.
6. The CytoSure NGS Library prep kit will now be available to use for the sequencing run.

Adapter	Sequence
1	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
2	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Table 12: Adapter Sequences

Index Number	Sequence	Index Number	Sequence	Index Number	Sequence
1	AACGTGAT	17	AATGCGCT	33	ACTGTAAT
2	AAACATCG	18	GACGGCGC	34	ATTCTACT
3	AGTGGTCA	19	TTAGCTCC	35	TGACCTGA
4	ACCACTGT	20	GTTCAATC	36	GCCTAAGG
5	GATAGACA	21	GAAAGGAT	37	ACTCGCTT
6	GTGTTCTA	22	AGTTCCGC	38	GCGGCGCA
7	TGGAACAA	23	ACGATCGT	39	GGAGCGAC
8	TGGTGGTA	24	CGCCTGAG	40	AGCATGTA
9	ACATTGGC	25	TTACCCTG	41	ACGGGGAA
10	CAGATCTG	26	TTCGACGA	42	ACGATGAT
11	CATCAAGT	27	TCTAGTAC	43	TAGCAGCT
12	AGTACAAG	28	AGTCGGAA	44	TGCGAGCC
13	AGATCGCA	29	ATTAGCGC	45	GGCATATC
14	GACTAGTA	30	GACTACTT	46	CTTGAATC
15	GGTGCGAA	31	ACGTATAT	47	CGCTCAGT
16	TGAAGAGA	32	AAAGTTCT	48	GAACTCGT

Table 13: Index sequences

FASTQ and data analysis

1. Interpret is a powerful and easy-to-use next generation sequencing analysis solution. The software is able to process FASTQ files and analyse the data to investigate SNVs, CNVs and LOH – all in a data rich, user friendly interface. Please refer to the installation guide and Interpret user manual for detailed instructions.

2. The Interpret analysis expects only two files per sample, a forward and reverse file. If more than two FASTQ files are generated per sample then the forward and reverse files should be concatenated to produce the two files.

   Please contact OGT for assistance if required: support@ogt.com

Glossary of Symbols

A description of the symbols found on the CytoSure NGS Kit.

Symbol	Title of Symbol
LOT	Batch code
REF	Catalogue number
	Use-by date
	Temperature limit
	Consult instructions for use

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NGS library prep assay

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Contact information

Oxford Gene Technology, Begbroke Hill, Woodstock Road, Begbroke, Oxfordshire, OX5 1PF, UK.

Oxford Gene Technology (Operations) Ltd. Registered in England No: 03845432 Begbroke Hill, Woodstock Road, Begbroke, Oxfordshire, OX5 1PF.

Tel: +44(0)1865 856800 (US: 914-467-5285)

Email: products@ogt.com Technical support email: support@ogt.com

Web: www.ogt.com

Contact us

Oxford Gene Technology,
Begbroke Hill,
Woodstock Road,
Begbroke,
Oxfordshire
OX5 1PF, UK

T: +44(0)1865 856800; (US: 914-467-5285)
E: products@ogt.com; W: www.ogt.com

Technical Support: E: support@ogt.com
T: +44(0)1865 856800

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