Oncotarget NGS Kit

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Overview

Product Information

The ONCOTARGET panel has been developed to provide a fast and reliable response from all types of human samples in order to help the practitioner to guide the treatment of cancer patients. Its small size and the low sequencing depth required (>80X) allows low-cost analyses.

The capture solution used in the ONCOTARGET test is a combination of 1229 different probes distributed all over the coding sequences of 10 genes of interest linked to a marketing authorization, as well as the TP53 gene. SNPs (Single Nucleotide Polymorphism) present on 4 control genes are also covered by the design. This capture solution was the subject of a publication in 2022 (Chevrier et al., 2022 - Int J Mol Med) showing these performances on the analysis of nearly 3000 samples in diagnostic routine.

The ONCOTARGET kit therefore covers several areas of polymorphisms with different frequencies in the general population. Indeed, the EGFR, KDR, SLC28A1, TP53 genes as well as the coverage of the EIF1AY gene (present only on the Y chromosome) can allow the detection of inter-sample cross- contamination (Table 1).

This hybrid capture enrichment panel has been built to be usable both in individual capture and in pooled capture. However, to avoid preferential captures due to differences in quality between FFPE samples or liquid samples, an individual capture remains preferable to a pooled capture.

Table 1: List of the five SNPs integrated in the Oncotarget panel and their frequencies in Human Genome

Gene	Amino acid variation	Nucleotide variation	European population frequency (%)*	African population frequency (%)*
EGFR	p.(Gln797Gln)	c.2361G>A	40.59 / 59.41	59.18 / 40.82
EIF1AY	Present only on the Y chromosome			
KDR	p.(Gln472His)	c.1416A>T	76.03 / 23.97	88.19 / 11.81
SLC28A1	p.(Val189Ile)	c.565G>A	64.85 / 35.15	78.62 / 21.38
TP53	p.(Arg72Pro)	c.215G>C	26.34 / 73.66	68.54 / 31.46

*Data retrieved from dbSNP.

The present test uses an enzymatic fragmentation taking place at the same time as the end-repair and A-tailing steps, thus saving time and increasing workflow efficiency. In addition, the use of double indexing and the incorporation of UMI, makes possible to use this kit for the ultrasensitive analysis of rare events, such as may be the case in liquid samples. Finally, doing the indexing of samples early in

the preparation of the library, limits the risk of cross-contamination during the preparation of the libraries.

Applications

The ONCOTARGET panel can be used to detect mutations present on all the coding parts of 10 genes that are used to guide the therapy of 1st or 2nd metastatic line of several solid cancers, as well as the TP53 gene.

For this, the ONCOTARGET panel analyzes the entire coding sequence (coding exons + splicing zones) of 11 genes of interest (ALK, BRAF, EGFR, ERBB2 (HER2), KIT, KRAS, MAP2K1, MET, NRAS, PDGFRA,
TP53) for the study of solid lung tumors (non-small cell), colorectal tumors, melanomas and gastrointestinal stromal tumors (GIST). It also makes it possible to detect distant intronic mutations around the exon 14 of MET, which imply a constitutive activation of MET. (Table 2)

Table 2: Details of the analyzed genes and their use in diagnostic routine

Gene	*Tumor Type*	*Associated treatment or prognostic effect*	*Impact*
ALK	Non-small cell lung cancer	ALK TKI	Sensitivity of resistance to ALK inhibitor
BRAF	Colon cancer	No therapeutics	MSI Testing
	Non-small cell lung cancer	BRAF inhibitor	Sensitivity only for the p.(Val600Glu) variant
	Melanoma	BRAF inhibitor	Sensitivity only for p.(Val600X) variants
EGFR	Non-small cell lung cancer	EGFR TKI	Sensitivity or resistance (secondary mutations, insertions exon 20)
ERBB2	Non-small cell lung cancer	Mobocertinib	Sensitivity
KIT	GIST	Imatinib	Sensitivity or resistance (secondary mutations)
	Melanoma	Imatinib	Sensitivity
KRAS	Colon cancer	EGFR inhibitor	Resistance
	Non-small cell lung cancer	Sotorasib	Sensitivity only for the p.(Gly12Cys) variant
MAP2K1	Melanoma	BRAF inhibitor	Resistance
		MEK inhibitor	Sensitivity or resistance
MET	Non-small cell lung cancer	MET TKI	Sensitivity
NRAS	Colon cancer	EGFR inhibitor	Resistance
	Melanoma	Prognosis	/
PDGFRA	GIST	Imatinib	Sensitivity or resistance (secondary mutations)

GIST, gastrointestinal stromal tumor; MSI, microsatellite instability; TKI, tyrosine kinase inhibitor(s).

The ONCOTARGET panel has been developed and tested to work on any type of sample of human origin. It can be used on DNA extracted from fresh or frozen tumors, fixed and included in paraffin (FFPE) and on DNA from liquid samples (plasma samples, pleural fluids, cerebrospinal fluids). It can also be used on DNA extracted from human cell lines.

Other Applications

The ONCOTARGET panel can also be used for the analysis of the entire coding sequence of the 11 genes included in the panel for pathologies, which may present alterations on these genes (pancreas, low grade gynecological cancers ...), without having theranostic involvement.

In addition, the coverage of all the coding parts of the 11 genes can allow the development of copy number variation analyses on these 11 genes.

Due to the analytical sensitivity of the ONCOTARGET kit and the low sequencing coverage required (low sequencing cost) for the detection of nucleotide variations (coverage > 80X), it can also be used for more fundamental analyzes, in particular for the detection of the early appearance of cell line mutations under treatment pressure.

Compatible Sequencing Instruments

The ONCOTARGET kit uses the indexes which are compatible with all Illumina sequencing platforms.

Kit Contents

Name	Ref
Frag/AT buffer	OCT1
Frag/AT enzyme	OCT2
DNA Adaptor Plate	OCT3
Adaptor Dilution Buffer	OCT4
Ligation Master Mix	OCT5
Primer mix	OCT6
PCR Dilution Buffer	OCT7
Equinox Amplification Master Mix (2X)	OCT8
Premix Indexing Block	OCT9
Hybridization 1	OCT10
Hybridization 2	OCT11
Hybridization 3	OCT12
Hybridization 4	OCT13
RNase Block	OCT14
Capture probes	OCT15

Required Materials and Equipment Not Included

- Thermal cycler (with adjustable lid temperature if possible)
- Tapestation 4200 and screen tape D1000 (Agilent) or equivalent
- Magnetic rack
- Vacuum (Eppendorf brand or equivalent)
- 96-well plates
- Pipettors
- Mini-centrifuge
- Vortex
- Mixmate type plate stirrer Water bath
- Well plate centrifuge
- Filtered pipette tips
- Ice and ice box
- Qubit Fluorometer and dsDNA HS (high sensitivity) assay kit or equivalent
- Streptavidine T1 Dynabeads MyOne (Thermo Fisher Scientific)
- Agencourt AMPure XP kit Beads SPRI Beckman Coulter (B23318)
- 80% Ethanol (freshly prepared)
- Nuclease free water
- EB Buffer

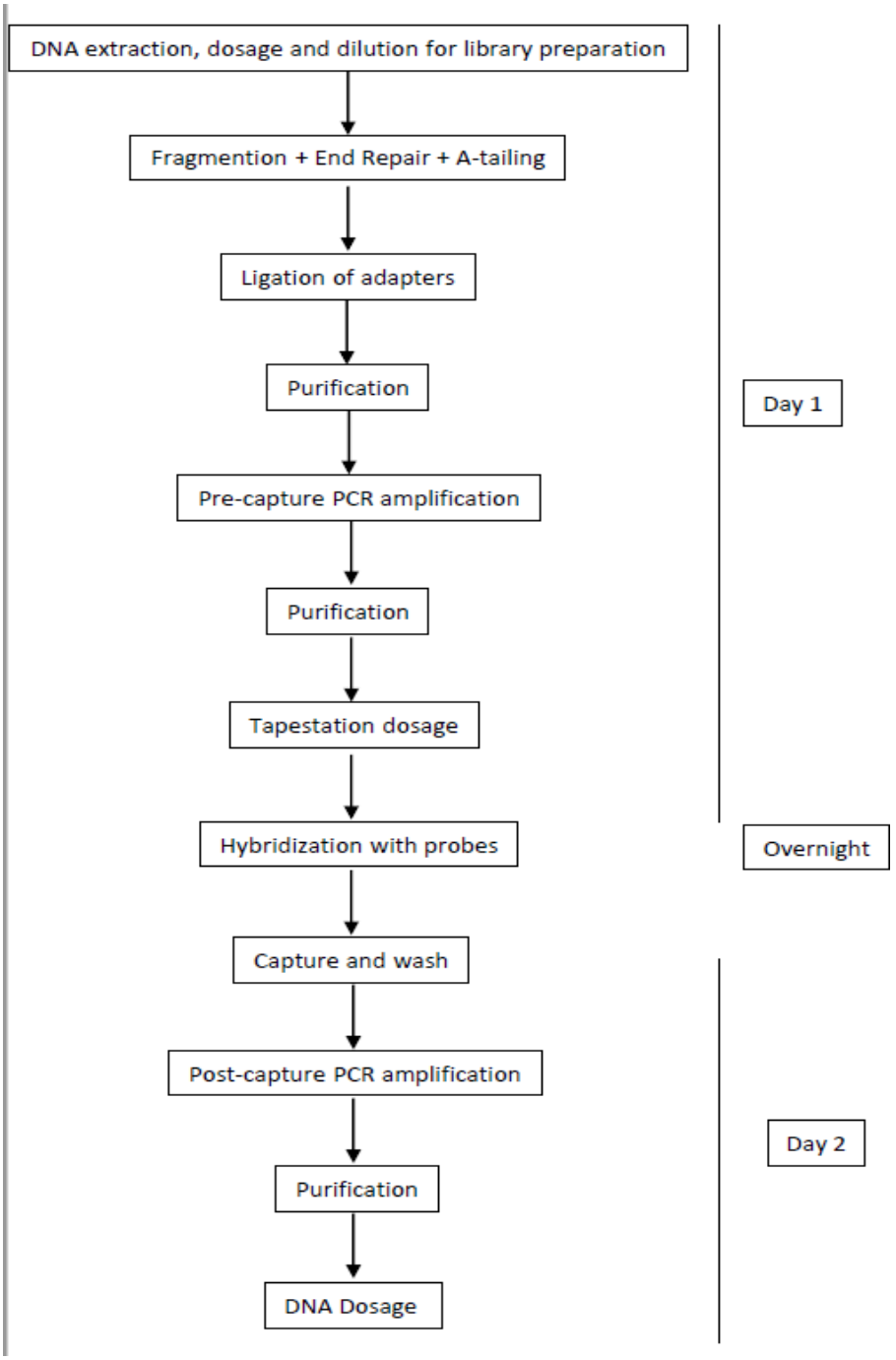
Storage and Handling

The Oncotarget kit is shipped frozen. Store all components at -20 ± 5°C upon receipt.

Keep all components and reaction mixes on ice or a cooled reagent block during routine use. Many components are viscous; therefore, take care to homogenize solutions thoroughly before use and during reaction setup. All buffers should be vortexed for 5 sec before use. The enzymes and Equinox Amplification Master Mix (2X) should be inverted ten times prior to reaction setup. The combined fragmentation/end-repair and A-tailing reaction must be set up on ice. The ligation and library amplification reactions may be set up at room temperature.

When stored and handled as indicated, the product will retain full performance until the expiry date printed on the kit box

Workflow


DNA extraction, dosage and dilution for library preparation
Fragmention + End Repair + A-tailing
Ligation of adapters
Purification
Pre-capture PCR amplification
Purification
Tapestation dosage
Hybridization with probes
Capture and wash
Post-capture PCR amplification
Purification
DNA Dosage
Day 1
Overnight
Day 2

Protocol

Best Practices

- When using the kit for the first time, briefly vortex and spin the tubes in the kit to bring the liquid to the bottom of the tubes. Store the tubes containing enzymes on ice during their respective procedures. All other components, including primer pools, may be thawed at room temperature, mixed thoroughly by vortexing and spun down before use.

- Use good laboratory practices to minimize cross-contamination. If possible, perform PCR setup in an isolated area or room to minimize cross-contamination between samples, or indexed PCR primers. Always change pipette tips between samples and change gloves frequently. Clean all workstations and tools with 10% bleach followed by water and alcohol at the end of each workday.

- Use a calibrated PCR thermal cycler as specified by the manufacturer's user guide. Generally, a thermal cycler set with the highest ramp speed, such as 5°C/second and higher, is not recommended. For thermal cyclers with adjustable ramp speed, we recommend 3°C/second up and 2°C/second down speed or use the default setting (no ramp adjustment).

- To ensure accurate assembly of reactions, withdraw viscous solution slowly from containers and dispense it slowly into the reaction mixtures. A good practice is to remove excess from outside of the tip and rinse it by pipetting up and down several times after dispensing any viscous solution into an aqueous mixture. Thoroughly pipette or vortex mix each mixture to ensure solutions are homogeneous prior to PCR and incubations. Remember to briefly spin the PCR tubes or 96-well PCR plate after mixing.

- When working with 96-well PCR plates, take extra care to ensure thorough mixing of all samples and proper sealing to avoid cross contamination between samples. Magnetic bead purification steps should be performed carefully to minimize residual supernatant and ethanol washes, and to minimize bead loss. Using a strong magnetic rack or plate specifically designed for manual handling of PCR tubes or 96-well PCR plates is critical for a successful bead purification.

- Always pre-warm thermal cyclers, water baths and heat blocks.

- Assign sample indexes to specific samples before starting the protocol.

Input DNA Requirements

An amount of 250 ng of DNA, according to a fluorimetric assay, is recommended for an optimal result. It is not advisable to put a higher amount because of the risk of inhibition of the various enzymatic reactions, including PCR. However, lower quantities can be used, in particular for liquid samples which have a very low amount of DNA (do not hesitate to use the DNA extracted from liquid samples even if it is OUT OF RANGE with the Qubit assay).

The maximum volume of DNA that can be used is 40 µl. The dilutions must be carried out in EB buffer.

In order not to overestimate the concentration of the DNA, a fluorimetry assay (Qubit) is strongly recommended. If only a measurement at 260 nm is possible, it must be considered that the actual concentration is 3 times lower than the concentration quantified by spectrophotometry.

Note: For DNA extracted from paraffinized tissues, it is advisable to carry out a purification of the extracted DNA with AMPure beads in order to eliminate potential inhibitors and chemicals that may impact the yield of the preparation of libraries.

The unused DNA must be stored at -20 ° C in screw tubes, allowing long-term preservation without loss of material (evaporation).

Set up and Preparations

- Bring the Magnetic bead solution to room temperature for at least 30 minutes before use.

- Freshly prepare 80% ethanol by combining 100% ethanol and nuclease-free water. Do not top off one liquid with the other in a volumetric container because the volumetric ratio will not be accurate. When water and ethanol are mixed, the final volume will be less than the sum of individual volumes. A lower concentration of ethanol will affect the final library yield.

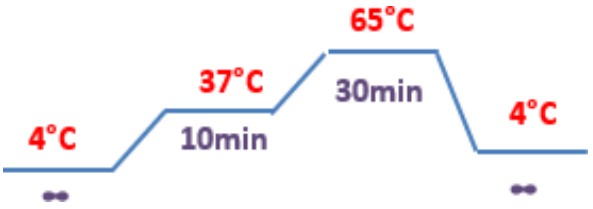
1) Fragmentation, End-repair, A-tailing

These three steps are carried out simultaneously.

It is advisable to work in 96-well plates and to seal the plates during each incubation step at temperatures other than room temperature. The use of tubes is possible but involves more frequent handling (caps to be closed and opened), which can increase the risk of contamination.

It is also advisable to add a positive control to validate the entire manipulation and a negative control to confirm the absence of contamination of the various solutions used.

Start by preparing the program below on the thermocycler.



Volume: 50µl

Lid Temperature = 105°C

Start the thermocycler (the step at +4 °C starts and will stop only when you intervene on the thermocycler). This will allow the immediate start of the step at 37°C.

i. Dilute the samples in EB buffer to a final volume of 40 µl of and

keep on ice.

ii. On ice, prepare the fragmentation/end-repair/A-tailing mix according to the following table:

	Reference	Volume (µl)
Frag/AT buffer	OCT1	4
Frag/AT enzyme	OCT2	6
Final Volume		10

Vortex and centrifuge the mix briefly.

iii. Immediately distribute 10 µl of this mix in each sample.

iv. Vortex and centrifuge briefly.

v. Put the samples in the thermocycler, skip the 1st step at +4°C in progress and run the program.

At the end of the program, keep the samples on ice, and proceed to step 2: ligation of adapters.

2) Ligation of adapters

For sample multiplexing : Please read **Supporting Information**

Please note the position of the DNA adaptors on the 96-well plate.
You can find the sequence of the DNA adaptors (Index) according to their location on the 96-well plate in: Supporting information > Index Sequences

Start by preparing the program below on the thermocycler.

20°C	20°C
∞	15min

Volume: 75µl

Lid temperature = off (no heating of the thermocycler cover)

Start the thermocycler (the step at 20 °C starts and will stop only when you intervene on the thermocycler). This will allow the immediate start of the step at 20 °C.

i. At room temperature, prepare the adapter ligation mix according to the following table:

	Reference	Volume (µl)
DNA Adaptor Plate	OCT3	2,5
Adaptor Dilution Buffer	OCT4	2,5
Ligation Master Mix	OCT5	20
Final volume		25

Vortex and centrifuge briefly

ii. Distribute 25 µl of this mix in each sample coming from step 1.

iii. Vortex and centrifuge briefly.

iv. Put the samples in the thermocycler, skip the 1st step at +20°C in progress and run the program.

At the end of the program, keep the samples at room temperature and proceed to step 3: purification.

3) Purification

Have the 80% ethanol solution freshly prepared and vortex the SPRI beads well (Beckman Coulter B23318) to put them back in suspension.

i. Add 60 µl of beads in each sample and mix by up and down 5-10 times.

ii. Incubate for 5 minutes at room temperature.

iii. Put the plate or tubes on the magnetic holder and leave it until the supernatant is translucent.

iv. Remove and eliminate the supernatant (the DNA remains trapped in the magnetic beads).

v. Keep the plate or tubes on the magnetic support and add 200 µl of 80% ethanol to the beads without re-suspending them.

vi. Remove the washing solution without touching the magnetic beads (put the tip of the pipette in the center of the well).

vii. Repeat steps **iv** to **vi** one more time to ensure the washing of the beads.

viii. Let the beads dry for exactly 3 minutes.

ix. Remove the plate or tubes from the magnetic support and add 22 µl of EB buffer to each well/tube. Mix by up and down 5-10 times.

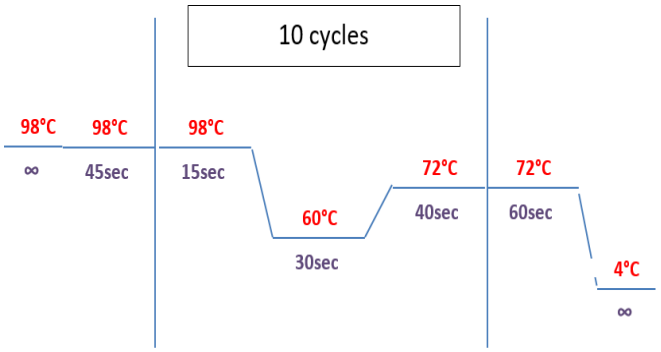
x. Incubate for 2 minutes outside the magnetic support.

xi. Put the plate or tubes back on a magnetic support until the supernatant is translucent.

While keeping the plate or tubes on the magnetic support, transfer 20 µl of the eluate into a new well/tube and proceed to step 4: Pre-capture PCR

4) Pre-capture PCR

Start by preparing the program below on the thermocycler.



Volume: 50 µl

Lid Temperature = 105°C

Start the thermocycler (the step at 98°C starts and will stop only when you intervene on the thermocycler). This will allow the immediate start of the step at 98 °C.

i. On ice, prepare reaction PCR mix according to the following table:

	Reference	Volume (µl)
Primer Mix	OCT6	2.5
PCR Dilution Buffer	OCT7	2.5
Equinox Amplification Master Mix (2X)	OCT8	25
Final Volume		30

Be careful, do not vortex the pure enzyme solution.

Vortex and centrifuge the mix briefly.

ii. Distribute 30 µl of this mix in each sample coming from step 3.

Vortex and centrifuge briefly

iii. Put the samples in the thermocycler, skip the 1st step at +98°C in progress and run the program.

At the end of the PCR, take out the samples, keep them at room temperature for 5 minutes and proceed to step 5: purification of the pre- capture PCR products.

5) Purification of the pre-capture PCR products

Have the 80% ethanol solution freshly prepared and vortex the SPRI beads well (Beckman Coulter B23318) to put them back in suspension.

i. Add 50 µl of beads in each sample and mix by up and down 5-10 times.

ii. Follow the steps **ii** to **viii** from section 3 (Purification).

iii. Without removing the plate from the magnetic support, add 22 µl of nuclease free water to each well/tube.

iv. Remove the plate from the magnetic support and resuspend by up and down 5 to 10 times.

v. Incubate for 2 minutes outside the magnetic support.

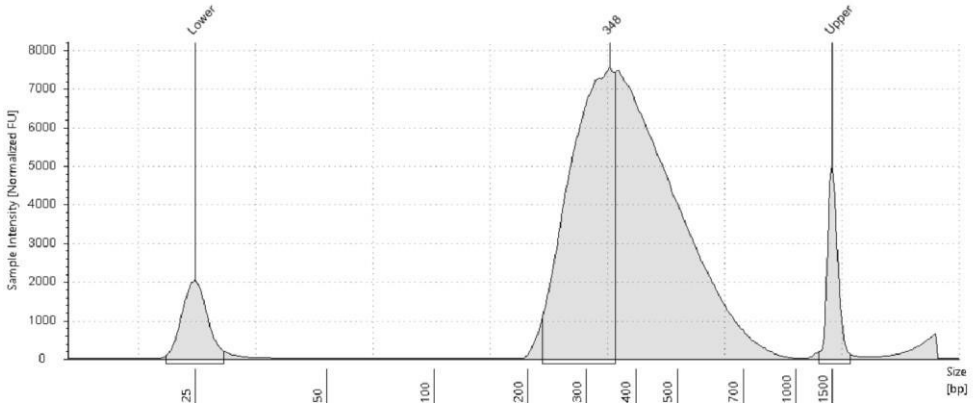
vi. Put the plate or tubes back on a magnetic support until the supernatant is translucent.

While keeping the tubes/plate on the magnetic support, transfer 20 µl of the eluate into a new tube/plate well and proceed to step 6: Pre-capture quality check.

6) Pre-capture quality check

Check the quality of the pre-capture samples by migration on a ScreenTape D1000 assay in a TapeStation system (Agilent).

The observed graphic profile must be close to the one below:



The average size of the library must be around 350 bp and a quantity detectable by the system. A smaller size may be a sign of too much fragmentation. This can lead to a loss of coverage during sequencing. If the size is larger, this is a sign of less fragmentation, but this will have little effect on the coverage.

If these criteria are met, proceed to step 7: Evaporation of the samples.

7) Evaporation of samples

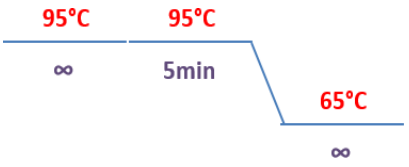
Evaporate all the samples in the vacuum at room temperature for 20 minutes (about 1 µl/ minute).

Add 4.2µl of nuclease free water to each evaporated well. Seal the plate, vortex it and proceed to step 8: Hybridization of the probes.

8) Hybridization of probes

Start by taking out all the hybridization reagents and letting them return to room temperature.

Meanwhile, prepare the program below on the thermocycler.



Volume: 30µl

Lid Temperature = 105°C

Start the thermocycler (the step at 95°C starts and will stop only when you intervene on the thermocycler). This will allow the immediate start of the step at 95°C.

i. Vortex and centrifuge the Premix Indexing Block (OCT9) briefly. Distribute 5.6µl of this mix in each sample coming from step 7.

ii. Mix by up and down 5-10 times and centrifuge the samples briefly.

iii. Transfer the samples to the thermocycler, skip the 1st step at +95°C in progress and run the program.

iv. When the step at 65°C begins, leave the samples for at least 5 minutes at this temperature.

v. Meanwhile, prepare the hybridization mix at room temperature according to the following table:

	Reference	Volume (µl)
Hybridization 1	OCT10	6,63
Hybridization 2	OCT11	0,27
Hybridization 3	OCT12	2,65
Hybridization 4	OCT13	3,45
DNAse/RNAse free water		5,9
RNase Block	OCT14	0,5
Capture probes	OCT15	0,66
Final volume		20

Vortex and centrifuge the mix briefly.

vi. Once the 5 minutes at 65°C have been completed, open the lid of the thermocycler. Without removing the samples from the thermocycler, unseal the plate/open the tubes and distribute 20 µl of the hybridization mix in each sample while keeping the samples in the block at 65 °C.

vii. Mix by suction / discharge 5-10 times.

viii. Seal the plate / reseal the tubes, close the lid of the thermal cycler and incubate at 65°C overnight.

After overnight incubation, leave the thermocycler at 65°C and proceed to step 9: Capture and wash.

9) Capture and wash

Pre-heat a mixmate stirrer and a water bath to 65°c

i. Start by washing the Streptavidine T1 Dynabeads MyOne (Thermo Fisher). To do this, mix 50 µl of beads with 200 µl of Binding Buffer (Thermo Fisher) in the wells of a plate or tubes intended to receive each sample.

ii. Mix by up and down 5-10 times.

iii. Place the plate/tubes on a magnetic support. When the supernatant is translucent, remove the supernatant.

iv. Remove the plate from the magnetic support and take up the beads with 200 µl of Binding Buffer (Thermo Fisher).

v. Repeat the washing with 200 µl of Binding Buffer (Thermo Fisher) two more times.

vi. Transfer one by one the samples from the plate still present in the thermocycler at 65°C

into the wells/tubes containing the Streptavidine T1 Dynabeads MyOne (Thermo Fisher).

vii. Mix immediately by suction/discharge 5-10 times.

viii. Seal the plate and transfer it to a Mixmate type stirrer. Incubate for 30 min at 65º C with stirring at 1800 rpm.
Meanwhile, put the Wash 2 buffer (Thermo Fisher) in a water bath at 65°C.

ix. Centrifuge the plate briefly and transfer it to a magnetic support. When the solution becomes translucent, remove the supernatant without separating the beads.

x. Remove the plate from the magnetic support and return the beads to suspension in 200 µl of Wash 1 buffer (Thermo Fisher) by suction and discharge 5-10 times.

xi. Incubate for 15 minutes at room temperature.

xii. Centrifuge the plate briefly and transfer it to a magnetic support. When the solution becomes translucent, remove the supernatant without separating the beads.

xiii. Remove the plate from the magnetic support and return the beads to suspension in 200 µl of Wash 2 buffer (Thermo Fisher) pre-warmed at 65°C by suction and discharge 5-10 times.

xiv. Incubate the sealed plate for 10 minutes at 65°C.

xv. Centrifuge the plate briefly and transfer it to a magnetic support. When the solution becomes translucent, remove the supernatant without separating the beads.

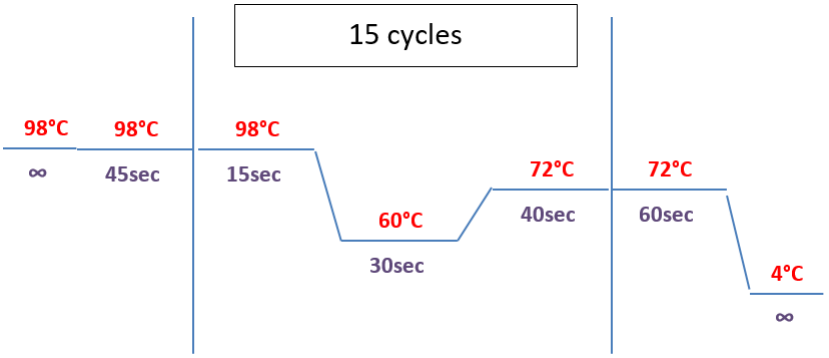
xvi. Repeat the washing with 200 µl of Wash 2 buffer (Thermo Fisher) two more times.

xvii. Remove the plate from the magnetic support and put the beads back in suspension with 20 µl of nuclease free water. Mix by suction and discharge 5-10 times.

Proceed to step 10: Post-capture PCR.

10)Post-capture PCR

Start by preparing the program below on the thermocycler.



Volume: 50 µl

Lid Temperature = 105°C

Start the thermocycler (the step at 98°C starts and will stop only when you intervene on the thermocycler). This will allow the immediate start of the step at 98°C.

i. On ice, prepare the PCR mix according to the following table:

	Reference	Volume (µl)
Primer mix	OCT6	2.5
PCR Dilution Buffer	OCT7	2.5
Equinox Amplification Master Mix (2X)	OCT8	25
Final Volume		30

Vortex and centrifuge the mix briefly.

ii. Distribute 30 µl of this mix in each sample coming from step 9.

iii. Seal the plate, vortex and centrifuge briefly.

iv. Put the samples in the thermocycler, skip the 1st step at +98°C in progress and run the program.

Proceed to step 11: Purification of the post-capture PCR products.

11) Purification of the post-capture PCR products

Have the 80% Ethanol solution freshly prepared and vortex the SPRI beads well (Beckman Coulter B23318) to put them back in suspension.

Remove the samples from the thermocycler and let them come back to room temperature for a few minutes.

i. Add 90 µl of beads in each sample and mix by up and down 5 times.

ii. Follow the steps **ii** to **viii** from section 3 (Purification).

iii. Without removing the plate from the magnetic support, add 30 µl of EB buffer in each well.

iv. Remove the plate from the magnetic support and resuspend by up and down 5-10 times.

v. Incubate for 2 minutes outside the magnetic support.

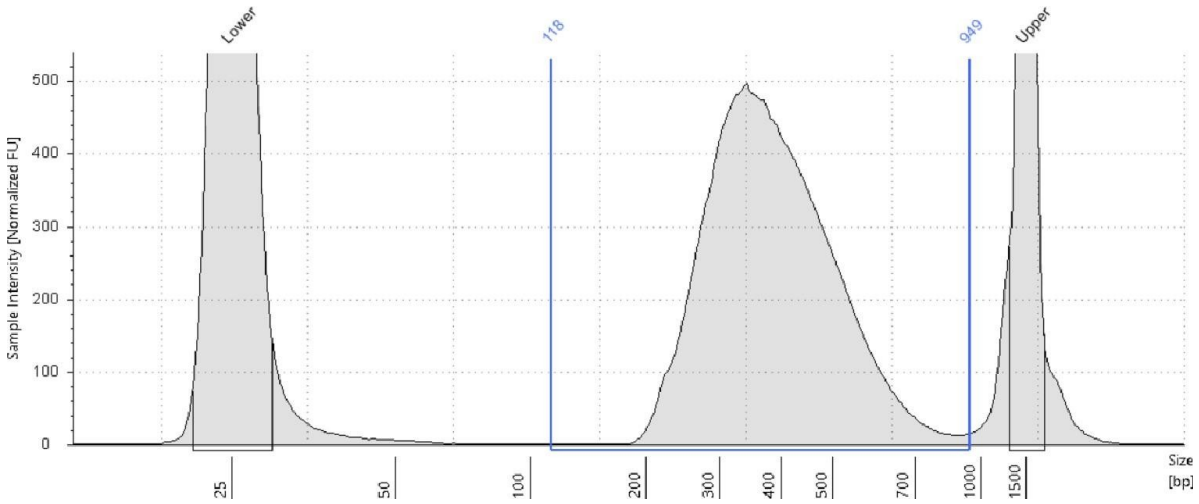
vi. Put the plate back on a magnetic support until the supernatant is translucent.

vii. While keeping the tubes/plate on the magnetic support, transfer the 30 µl of the supernatant into a new tube/ well.

Proceed to step 12: Post-capture quality control and quantification.

12) Post-capture quality check and quantification

Check the quality of the post-capture samples by migration on a ScreenTape D1000 assay in a TapeStation system (Agilent).
The observed graphic profile must be close to the one below:



The average size of the library must be around 350 bp. A smaller size may be a sign of too much fragmentation. This can lead to a loss of coverage during sequencing. If the size is larger, this is a sign of less fragmentation, but this will have little effect on the coverage.

Perform the Qubit assay to determine the final concentration of the library.

13) Injection on the sequencer

Refer to the instructions of the sequencer manufacturer to find out the amount of library to be injected. Indeed, this quantity is specific to the sequencer model or even to the sequencer itself. We advise you to do several tests to know the specifics of your machine in order to obtain an optimal result.

Recommended Sequencing Length and Depth

For the sequencing conditions, depending on your machine, you can perform a paired end sequencing, 2*100, 2*110, 2*120 or 2*150. Long sequencing (2*150) does not necessarily generate more data than in 2*100 or 2*120 sequencing. Indeed, depending on the size of your insert, a long sequencing may lead to the sequencing of the adapters and not the inserts.

Since the indexing used is a dual indexing with the presence of UMI, refer to the instructions below to know the index sequencing conditions:

- Regarding the minimum reading depth, a minimum depth of 80X is possible for a sensitivity of 5% (Chevrier et al., 2022 - Int J Mol Med).
- Depending on the desired sensitivity, the reading depth can be increased by decreasing the sample multiplexing.

- For the use of UMI, a very high depth is necessary. This depth is to be determined in accordance with the bioinformatics support that will be in charge of analyzing the results.

Supporting Information

Dual-Indexed PCR Primers for Illumina

Pool equimolar amounts of libraries for sequencing on Illumina platforms using the cycle settings shown in the table below.

Run Segment	Read Lenght
Read 1	X defined by users
Index 1 (i7)	20
Index 2 (i5)	8
Read 2	X defined by user

Note: Our kit is available for pooling up to 384 samples. Each of the sets allows pooling of up to 96 samples and, when combined, they allow for a maximum of 384 samples to be pooled. The following sections give guidance to pool fewer than eight samples.

Index Pooling Guidelines Within Each Set

PLEX	WELL POSITION

< 4	Not recommended
4	A6, B6, C6, D6 A12, B12, C12, D12 B6, C6, D6, E6 B12, C12, D12, E12 C1, D1, E1, F1 C7, D7, E7, F7 E4, F4, G4, H4 E10, F10, G10, H10
5	A6, B6, C6, D6 A12, B12, C12, D12 B6, C6, D6, E6 B12, C12, D12, E12 C1, D1, E1, F1
6-7	A6, B6, C6, D6 A12, B12, C12, D12 B6, C6, D6, E6 B12, C12, D12, E12 C1, D1, E1, F1 C7, D7, E7, F7 E4, F4, G4, H4 E10, F10, G10, H10
8	Any column

For all HiSeq®/MiSeq® sequencers, Illumina uses a red laser/LED to sequence bases A and C and a green laser/LED to sequence bases G and T. For each cycle, both the red and the green channel need to be read to ensure proper image registration (A or C must be in each cycle, and G or T must be in each cycle). If this color balance is not maintained, sequencing the index read could fail. The table lists some valid combinations (up to 8-plex) that can be sequenced together. For combinations > 8 choose any column and add any plex combinations as needed.

For the NovaSeq®/NextSeq®/MiniSeq®, which utilize 2 color chemistry, valid index combinations must include some indices that do not start with GG in the first two cycles. Use Tables for some suggested combinations for each of the sets.

Good and Bad Examples for Pooling and Color Balancing

Following table: Examples of "good" and "bad" index combinations based on DNA Set and HiSeq/MiSeq (4 color) guidelines.

Each index sequence is color coded to correspond to the red/green channel. For combinations of valid indices, ensure that you will have signal in both the red and green channels in each cycle.

GOOD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									Forward Strand Workflow								Reverse Complement Workflow								
C1	T	T	C	C	A	G	G	T	C	A	G	T	G	C	T	T	A	A	G	C	A	C	G	G	
D1	T	A	C	G	G	T	C	T	T	C	C	A	T	T	G	C	G	C	A	A	T	G	G	A	
E1	A	A	G	A	C	C	G	T	G	T	C	G	A	T	T	G	C	A	A	T	C	G	A	C	
F1	C	A	G	G	T	T	C	A	A	T	A	A	C	G	C	C	G	G	C	G	T	T	A	T	
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
A12	C	G	G	C	A	T	T	A	G	T	C	A	G	T	C	A	T	G	A	C	T	G	C	C	
B12	C	A	C	G	C	A	A	T	C	C	T	T	C	C	A	T	A	T	G	G	A	A	G	G	
C12	G	G	A	A	T	G	T	C	A	G	G	A	A	C	A	C	G	T	G	T	T	C	C	T	
D12	T	G	G	T	G	A	A	G	C	T	T	A	C	A	G	C	G	C	T	G	T	A	A	G	
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	

BAD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									Forward Strand Workflow								Reverse Complement Workflow								
E8	T	A	T	G	G	C	A	C	T	T	G	C	G	A	G	A	T	C	T	C	G	C	A	A	
F8	G	A	A	T	C	A	C	C	G	A	A	C	G	A	A	G	C	T	T	C	G	T	T	C	
G8	G	T	A	A	G	G	T	G	C	G	A	A	T	T	G	C	G	C	A	A	T	T	C	G	
H8	C	G	A	G	A	G	A	A	G	G	A	A	G	A	G	A	T	C	T	C	T	T	C	C	
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓	
A1	T	T	A	C	C	G	A	C	C	G	A	A	T	A	C	G	C	G	T	A	T	T	G	G	
B1	T	C	G	T	C	T	G	A	G	T	C	C	T	T	G	A	T	C	A	A	G	G	A	C	
C1	T	T	C	C	A	G	G	T	C	A	G	T	G	C	T	T	A	A	G	C	A	C	T	G	
D1	T	A	C	G	G	T	C	T	T	C	C	A	T	T	G	C	G	C	A	A	T	G	G	A	
	X	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	

Index Sequences

Lists each index sequence color coded to correspond to the red/green channel. For combinations of valid indices, ensure that you will have signal in both the red and green channels in each cycle.

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 Index ID		i5 Index ID	Forward Strand Workflow*	Reverse Strand Workflow*
A1	S762	TTACCGAC	S512	CGAATACG	CGTATTCG
B1	S713	TCGTCTGA	S586	GTCCTTGA	TCAAGGAC
C1	S736	TTCCAGGT	S543	CAGTGCTT	AAGCACTG
D1	S709	TACGGTCT	S575	TCCATTGC	GCAATGGA
E1	S732	AAGACCGT	S550	GTCGATTG	CAATCGAC
F1	S774	CAGGTTCA	S506	ATAACGCC	GGCGTTAT
G1	S747	TAGGAGCT	S524	GCCTTAAC	GTTAAGGC
H1	S794	TACTCCAG	S590	GGTATAGG	CCTATACC
A2	S729	AGTGACCT	S591	TCTAGGAG	CTCCTAGA
B2	S777	AGCCTATC	S526	TGCGTAAC	GTTACGCA
C2	S772	TCATCTCC	S567	CTTGCTAG	CTAGCAAG
D2	S725	CCAGTATC	S538	AGCGAGAT	ATCTCGCT
E2	S755	TTGCGAGA	S566	TATGGCAC	GTGCCATA
F2	S760	GAACGAAG	S511	GAATCACC	GGTGATTC
G2	S716	CGAATTGC	S559	GTAAGGTG	CACCTTAC
H2	S708	GGAAGAGA	S521	CGAGAGAA	TTCTCTCG
A3	S702	TCGGATTC	S523	CGCAACTA	TAGTTGCG
B3	S796	CTGTACCA	S507	CACAGACT	AGTCTGTG
C3	S757	GAGAGTAC	S545	TGGAAGCA	TGCTTCCA
D3	S783	TCTACGCA	S546	CAATAGCC	GGCTATTG
E3	S722	GCAATTCC	S578	CTCGAACA	TGTTCGAG
F3	S710	CTCAGAAG	S581	GGCAAGTT	AACTTGCC
G3	S770	GTCCTAAG	S540	AGCTACCA	TGGTAGCT
H3	S734	GCGTTAGA	S592	CAGCATAC	GTATGCTG
A4	S763	CAAGGTAC	S505	CGTATCTC	GAGATACG
B4	S797	AGACCTTG	S501	TTACGTGC	GCACGTAA
C4	S735	GTCGTTAC	S554	AGCTAAGC	GCTTAGCT
D4	S727	GTAACCGA	S598	AAGACACC	GGTGTCTT
E4	S742	GAATCCGT	S551	CAACTCCA	TGGAGTTG
F4	S795	CATGAGCA	S517	GATCTTGC	GCAAGATC
G4	S749	CTTAGGAC	S565	CTTCACTG	CAGTGAAG
H4	S773	ATCTGACC	S593	CTCGACTT	AAGTCGAG
A5	S769	TCCTCATG	S519	GTACACCT	AGGTGTAC
B5	S752	AGGATAGC	S544	CCAAGGTT	AACCTTGG
C5	S704	GGAGGAAT	S585	GAACGGTT	AACCGTTC
D5	S715	GACGTCAT	S518	CCAGTTGA	TCAACTGG
E5	S753	CCGCTTAA	S548	GTCATCGT	ACGATGAC
F5	S758	GACGAACT	S568	CAATGCGA	TCGCATTG
G5	S784	TCCACGTT	S541	GGTTGAAC	GTTCAACC
H5	S714	AACCAGAG	S520	CTTCGGTT	AACCGAAG

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 Index ID		i5 Index ID	Forward Strand Workflow*	Reverse Strand Workflow*
A6	S771	GTCAGTCA	S531	CGGCATTA	TAATGCCG
B6	S779	CCTTCCAT	S589	CACGCAAT	ATTGCGTG
C6	S788	AGGAACAC	S587	GGAATGTC	GACATTCC
D6	S739	CTTACAGC	S503	TGGTGAAG	CTTCACCA
E6	S737	TACCTGCA	S576	GGACATCA	TGATGTCC
F6	S728	AGACGCTA	S582	GGTGTACA	TGTACACC
G6	S780	CAACACAG	S530	GATAGCCA	TGGCTATC
H6	S761	GTACCACA	S533	CCACAACA	TGTTGTGG
A7	S712	CGAATACG	S562	TTACCGAC	GTCGGTAA
B7	S786	GTCCTTGA	S513	TCGTCTGA	TCAGACGA
C7	S743	CAGTGCTT	S536	TTCCAGGT	ACCTGGAA
D7	S775	TCCATTGC	S509	TACGGTCT	AGACCGTA
E7	S750	GTCGATTG	S532	AAGACCGT	ACGGTCTT
F7	S706	ATAACGCC	S574	CAGGTTCA	TGAACCTG
G7	S724	GCCTTAAC	S547	TAGGAGCT	AGCTCCTA
H7	S790	GGTATAGG	S594	TACTCCAG	CTGGAGTA
A8	S791	TCTAGGAG	S529	AGTGACCT	AGGTCACT
B8	S726	TGCGTAAC	S577	AGCCTATC	GATAGGCT
C8	S767	CTTGCTAG	S572	TCATCTCC	GGAGATGA
D8	S738	AGCGAGAT	S525	CCAGTATC	GATACTGG
E8	S766	TATGGCAC	S555	TTGCGAGA	TCTCGCAA
F8	S711	GAATCACC	S560	GAACGAAG	CTTCGTTC
G8	S759	GTAAGGTG	S516	CGAATTGC	GCAATTCG
H8	S721	CGAGAGAA	S508	GGAAGAGA	TCTCTTCC
A9	S723	CGCAACTA	S502	TCGGATTC	GAATCCGA
B9	S707	CACAGACT	S596	CTGTACCA	TGGTACAG
C9	S745	TGGAAGCA	S557	GAGAGTAC	GTACTCTC
D9	S746	CAATAGCC	S583	TCTACGCA	TGCGTAGA
E9	S778	CTCGAACA	S522	GCAATTCC	GGAATTGC
F9	S781	GGCAAGTT	S510	CTCAGAAG	CTTCTGAG
G9	S740	AGCTACCA	S570	GTCCTAAG	CTTAGGAC
H9	S792	CAGCATAC	S534	GCGTTAGA	TCTAACGC
A10	S705	CGTATCTC	S563	CAAGGTAC	GTACCTTG
B10	S701	TTACGTGC	S597	AGACCTTG	CAAGGTCT
C10	S754	AGCTAAGC	S535	GTCGTTAC	GTAACGAC
D10	S798	AAGACACC	S527	GTAACCGA	TCGGTTAC
E10	S751	CAACTCCA	S542	GAATCCGT	ACGGATTC
F10	S717	GATCTTGC	S595	CATGAGCA	TGCTCATG
G10	S765	CTTCACTG	S549	CTTAGGAC	GTCCTAAG
H10	S793	CTCGACTT	S573	ATCTGACC	GGTCAGAT

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 Index ID		i5 Index ID	Forward Strand Workflow*	Reverse Strand Workflow*
A11	S719	GTACACCT	S569	TCCTCATG	CATGAGGA
B11	S744	CCAAGGTT	S552	AGGATAGC	GCTATCCT
C11	S785	GAACGGTT	S504	GGAGGAAT	ATTCCTCC
D11	S718	CCAGTTGA	S515	GACGTCAT	ATGACGTC
E11	S748	GTCATCGT	S553	CCGCTTAA	TTAAGCGG
F11	S768	CAATGCGA	S558	GACGAACT	AGTTCGTC
G11	S741	GGTTGAAC	S584	TCCACGTT	AACGTGGA
H11	S720	CTTCGGTT	S514	AACCAGAG	CTCTGGTT
A12	S731	CGGCATTA	S571	GTCAGTCA	TGACTGAC
B12	S789	CACGCAAT	S579	CCTTCCAT	ATGGAAGG
C12	S787	GGAATGTC	S588	AGGAACAC	GTGTTCCT
D12	S703	TGGTGAAG	S539	CTTACAGC	GCTGTAAG
E12	S776	GGACATCA	S537	TACCTGCA	TGCAGGTA
F12	S782	GGTGTACA	S528	AGACGCTA	TAGCGTCT
G12	S730	GATAGCCA	S580	CAACACAG	CTGTGTTG
H12	S733	CCACAACA	S561	GTACCACA	TGTGGTAC

Troubleshooting Guide

1. If recovery of gDNA from samples is low

Using excess tissue for gDNA isolation can reduce yield. Use only the amount of each specific tissue type recommended by the gDNA isolation protocol.

Tissue sample lysis may not have been optimal during gDNA isolation. Monitor the extent of sample lysis during the Proteinase K digestion at 56°C by gently pipetting the digestion reaction every 20– 30 minutes, visually inspecting the solution for the presence of tissue clumps. If clumps are still present after the 1- hour incubation at 56°C, add another 10 µl of Proteinase K and continue incubating at 56°C, with periodic mixing and visual inspections, for up to two additional hours. When the sample no longer contains clumps of tissue, move the sample to room temperature until lysis is complete for the remaining samples. Do not over- digest. Individual samples may be kept at room temperature for up to 2 hours before continuing the protocol. Do not exceed 3 hours incubation at 56°C for any sample.

2. If concentration of FFPE DNA samples is too low for enzymatic fragmentation

The standard enzymatic fragmentation protocol requires 10–250 ng input DNA in a volume of 7 µl, and uses a final fragmentation reaction volume of 10 µl. To dilute FFPE samples, enzymatic fragmentation may be performed .

3. If yield of pre-capture libraries is low

- The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- Ensure that the ligation master mix is kept at room temperature for 30–45 minutes before use.

- PCR cycle number may require optimization. Repeat the library preparation for the sample increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat the library preparation for the sample decreasing the pre- capture PCR cycle number by 1 to 3 cycles.

- DNA isolated from degraded samples, including FFPE tissue samples, may be over- fragmented or have modifications that adversely affect library preparation processes.

- Performance of the solid- phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly- prepared 80% ethanol for each SPRI procedure.

- DNA elution during SPRI purification steps may be incomplete. Ensure that the AMPure XP beads are not overdried just prior to sample elution.

4. If solids observed in the End Repair-A Tailing Buffer

Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

5. If pre-capture library fragment size is larger than expected in electropherograms

- Shearing may not be optimal. For intact, high- quality DNA samples, ensure that shearing is completed using the two- round shearing protocol provided, including all spinning and vortexing steps.
- Any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

6. If pre-capture library fragment size is different than expected in electropherograms

- FFPE DNA pre- capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the input DNA that are smaller than the target DNA fragment size.

- DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for pre- capture purification.

7. If low molecular weight adaptor-dimer peak is present in pre-capture library electropherograms

- The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor- dimers are observed in the electropherogram at low abundance. The presence of excessive adaptor- dimers in the samples may be associated with reduced yield of pre- capture libraries. If excessive adaptor- dimers are observed, verify that the adaptor ligation protocol is being performed as directed on protocol. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the Adaptor Oligo Mix to the mixture. Do not add the Ligation master mix and the Adaptor Oligo Mix to the sample in a single step.

8. If yield of post-capture libraries is low

- PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post- capture PCR cycle number by 1 to 2 cycles.
- The RNA probe used for hybridization may have been compromised. Verify the expiration date on the Probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Probe Hybridization Mix is prepared immediately before use and that solutions containing the Probe are not held at room temperature for extended periods.

9. If post-capture library fragment size is different than expected in electropherograms

DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for post- capture purification.

10. If low % on-target is observed in library sequencing results

- Stringency of post-hybridization washes may have been lower than required. Complete the wash steps as directed, paying special attention to the details of washes listed below:
- Samples are maintained at 65°C during washes.
-Bead suspensions are mixed thoroughly during washes by pipetting up and down and vortexing Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate the vortex, plate spinner and centrifuge close to the thermal cycler to retain the 65°C sample temperature during mixing and transfer steps.

11. If low uniformity of coverage with high AT-dropout is observed in library sequencing results

High AT- dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT- rich targets. Repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature from 65°C to 62.5°C or 60°C.

Technical Support

For any question, please contact tech@neobiotech.com

Chemical Safety

Wear appropriate personal protective equipment (PPE) when working in the laboratory.

For hazard information, please refer to the Safety Data Sheet (SDS), which is available upon request.