

oncoReveal® Multi-Cancer with CNV & RNA Fusion Panel

USER MANUAL



Revision History

Version 1.0 – 2.0	Initial release Minor edits for clarity
Version 3.0	Overhaul formatting, branding, and contents
Version 4.0	Added blood DNA as input in section 6.1 Updated CNV normal requirements in section 6.1 based on updated CNV calling algorithm Minor grammatical and formatting changes to improve clarity

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1. Product Introduction

The *oncoReveal® Multi-Cancer with CNV & RNA Fusion Panel* combines Pillar Biosciences' *oncoReveal® Multi-Cancer v4 with CNV* and *oncoReveal® Multi-Cancer RNA Fusion v2* panels into one parallel workflow. For more information on the panel specifications, see the *oncoReveal® Multi-Cancer with CNV & RNA Fusion Panel* product sheet (Doc. No.: MK-0024).

The **oncoReveal® Multi-Cancer v4 with CNV** (DNA) Panel is a robust NGS assay that targets numerous gene regions of interest for researchers looking to explore the genetic sequences of formalin-fixed paraffin-embedded (FFPE) DNA from solid tumor samples.

The **oncoReveal® Multi-Cancer RNA Fusion v2** Panel targets regions of interest for researchers looking to explore fusion events using cDNA generated from FFPE tissue, frozen tissue, or cell lines. Detection of common solid tumor fusion transcripts includes *ALK*, *FGFR3*, *MET*, *NRG1*, *NTRK1*, *NTRK2*, *NTRK3*, *RET*, and *ROS1*, among others. Additionally, this panel can be used to detect exon 14 skipping in *MET* and contains two housekeeping genes as internal controls.

2. Product Description

This DNA-RNA parallel workflow utilizes our SLIMamp® (stem-loop inhibition mediated amplification) technology with a single-tube multiplex PCR reaction for each nucleic acid type.

DNA Workflow: oncoReveal® Multi-Cancer v4 with CNV

Pairs of DNA oligos designed for each region of interest, or hotspot, are used in the first round of gene-specific PCR (GS-PCR). Excess primers are then digested, and the products are subsequently purified via size selection. After purification, a second round of PCR adds index adaptors and P5 & P7 sequences to each library for sample tracking and sequencing. The final libraries are further purified and can be sequenced on the Illumina sequencing platform.

RNA Workflow: oncoReveal® Multi-Cancer RNA Fusion v2

Primer and Amplicon Design

A gene-specific primer was designed for each major breakpoint in the fusion genes (e.g., *ALK*), and another primer was designed for each partner gene (e.g., *EML4*). Additionally, amplicons were designed for each side of the breakpoint for the major driver genes.

Library Preparation

Using cDNA as input, the chimeric transcripts and wildtype transcripts of the driver genes are targeted in the first round of PCR. In the absence of a fusion event, 5' and 3' balance amplicons amplify distal regions of the driver gene transcripts, and no fusion PCR product is formed. When a fusion event occurs, fusion primer binding sites are present on the same RNA transcript and allow for chimeric fusion amplicons to amplify.

After gene-specific PCR (GS-PCR), the primers undergo digestion, and the products are purified via size selection. After purification, a second round of PCR adds index adaptors and P5 & P7 sequences to each library for sample tracking and sequencing. The final libraries are further purified and can be sequenced on the Illumina sequencing platform.

The panel requires a dual barcode indexing kit, and the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of 150 (2x150). The workflow for this panel can be completed in less than two days. In addition, the protocol contains numerous stopping points for users who have time limitations.

3. General Laboratory Guidelines for DNA and RNA Preparation

The following steps are recommended to improve consistency and reduce contamination:

- **Work areas:** To reduce the risk of contamination from PCR amplicons, supplies should not be moved from one area to another. Separate storage areas (e.g., refrigerators, freezers) should also be designated for pre- and post-PCR products. Work areas for RNA should be cleaned regularly and should be free of dust. Bacteria and skin are common sources of RNase enzymes, therefore areas and equipment should be cleaned with a sterilizing solution (70% alcohol).
- **Handling:** Due to the prevalence of ribonucleases (RNase enzymes) in the environment, RNA should be handled with care to avoid sample degradation. RNA is susceptible to degradation, therefore when handling RNA, all components should be kept in benchtop coolers, and repeated freeze/thaw cycles should be avoided. Gloves should be worn to touch equipment used to process RNA samples.
- **Hygiene:** When handling RNA, aseptic techniques should be used. Gloves should be sprayed often with a laboratory cleaning solution of 70% alcohol, and gloves should be changed regularly. Touching items on the body or body parts such as the face, hands, or glasses should be avoided as they can be sources of RNases.
- **Equipment and consumables:** All consumables used to process RNA samples should be RNase-free. Equipment should be cleaned or treated to inactivate RNases.
- **Lab cleanliness:** To further reduce the possibility of contamination, work areas should be cleaned between experiments with laboratory cleaning solution (70% alcohol and/or freshly made 10% hypochlorite solution). A periodic cleaning of the floor is also recommended.
- **Floor:** Items that have fallen to the floor are assumed to be contaminated and should be discarded. If a sample tube or non-consumable item has fallen to the floor and remained sealed, the outer surface of the item should be thoroughly cleaned before use using 70% alcohol or freshly-made 10% hypochlorite solution. Gloves should be changed after handling a contaminated item.
- **Aliquot reagents:** Frozen reagents should be aliquoted into smaller volumes to reduce freeze/thaw cycles. To reduce the risk of stock contamination, it is recommended to aliquot from the stock and work from the aliquots. In cases of contamination, the use of aliquots can also help to determine the source more quickly and easily.
- **Multichannel pipettes:** Multichannel pipettes should be used to maintain consistency and efficiency across numerous samples.
- **Pipette tips:** Tips should be changed between each sample to prevent cross-contamination. Any tips that may have become contaminated due to contact with gloves, the lab bench, tube exteriors, etc., should be discarded.
- **Open containers and lids:** To prevent possible contamination from air, tubes should be kept closed when not directly in use, and plates, troughs, and similar reservoirs should be covered with seals or lint-free laboratory wipes. Additionally, reaching over open containers should be avoided.

4. Reagents and Equipment

This section describes the necessary equipment, reagents, and consumables needed before performing the protocol. All reagents should be used in designated pre-PCR or post-PCR areas to prevent amplicon contamination. Each area designated for pre- and post-PCR should have dedicated equipment, reagents, and supplies (including gloves, lab coats, etc.) to prevent contamination.

4.1 Kit Components

oncoReveal® Multi-Cancer with CNV & RNA Fusion Panel Part No.: HNA-HS-1001-48

Reagent	Use	Area Use	Storage
Gene-Specific PCR 2x Master Mix	Gene-Specific PCR	Pre-PCR	-25° to -15°C
oncoReveal® Multi-Cancer with CNV v4 Oligo Pool	Gene-Specific PCR	Pre-PCR	-25° to -15°C
oncoReveal® Multi-Cancer RNA Fusion v2 (5x) Oligo Pool	Gene-Specific PCR	Pre-PCR	-25° to -15°C
Exonuclease I	Gene-Specific PCR	Post-PCR	-25° to -15°C
Indexing PCR 2x Master Mix	Indexing PCR	Pre-PCR	-25° to -15°C

4.2 Indexing Kits

Reagent	Part Number	Use	Area Use	Storage
Pillar Custom Indexing Primers Kit A , indices PI501-8, PI701-4 (32 combinations - 96 reactions)	IDX-PI-1001-96	Indexing PCR	Pre-PCR	-25° to -15°C
Pillar Custom Indexing Primers Kit D , indices PI501-8, PI701-12 (96 combinations - 192 reactions)	IDX-PI-1004-192	Indexing PCR	Pre-PCR	-25° to -15°C

Only one index kit is needed per assay. Multiple options are available to meet a variety of throughput needs.

4.3 User-Supplied Reagents

The SuperScript™ VILO™ cDNA master mix is **not** supplied with the panel components and must be purchased separately.

Reagent	Area Use	Supplier
SuperScript™ VILO™ Master Mix	cDNA Synthesis	ThermoFisher, Cat# 11755500
10N NaOH or 1N NaOH	Post-PCR	General lab supplier
AMPure XP Beads	Post-PCR	Beckman Coulter, Cat# A63881 or A63880
Ethanol, 200 proof for molecular biology	Post-PCR	General lab supplier
Nuclease-free water	Post-PCR	General lab supplier
Qubit dsDNA High Sensitivity Assay kit	Post-PCR	Invitrogen, Cat# Q32851 or Q32854
Qubit dsDNA Broad Range Assay kit ¹	Post-PCR	Invitrogen, Cat# Q32850 or Q32853
Qubit RNA High Sensitivity Assay kit ²	Post-PCR	Invitrogen, Cat# Q32852 or Q32855
Qubit RNA Broad Range Assay kit ²	Post-PCR	Invitrogen, Cat# Q10210 or Q10211
Agarose gel, 2% (optional) ¹	Post-PCR	General lab supplier
DNA molecular weight markers (optional) ¹	Post-PCR	General lab supplier
TapeStation High Sensitivity D1000 ScreenTape and Reagents (optional) ¹	Post-PCR	Agilent Technologies, Cat # 5067-5584 and 5067-5585
Uracil-DNA glycosylase (UDG) (optional)	Post-PCR	New England Biolabs, Cat# Mo280S or #Mo280L
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat# T7724
PhiX Control v3	Post-PCR	Illumina, Cat# FC-110-3001
200 mM Tris-HCl, pH 7.0 (optional) ³	Post-PCR	General lab supplier

¹ The Qubit dsDNA High Sensitivity Assay kit is the primary DNA quantitation assay used throughout this protocol. Additional DNA quantification can optionally be performed using the Qubit dsDNA Broad Range Assay kit, an agarose gel, or using the TapeStation High Sensitivity D1000 ScreenTape system.

² The RNA concentration can be determined using the Qubit RNA BR Assay kit or the Qubit RNA HS Assay kit.

³ The 200 mM Tris-HCl, pH 7.0 reagent is only required for denaturing libraries for sequencing on the Illumina NextSeq or MiniSeq. If sequencing on the MiSeq, this reagent is not needed.

4.4 Other Consumables

Compatible Sequencing Reagents

Sequencing Reagent Kit	Supplier	Catalog No.
MiniSeq™ Mid Output kit (300 cycles)	Illumina	FC-420-1004
MiniSeq™ High Output kit (300 cycles)	Illumina	FC-420-1003
MiSeq™ Reagent Nano kit v2 (300 cycles)	Illumina	MS-103-1001
MiSeq™ Reagent Micro kit v2 (300 cycles)	Illumina	MS-103-1002
MiSeq™ Reagent kit v2 (300 cycles)	Illumina	MS-102-2002
MiSeq™ Reagent kit v3 (600 cycles)	Illumina	MS-102-3003
NextSeq™ 500/550 Mid Output v2.5 kit (300 cycles)	Illumina	20024905
NextSeq™ 500/550 High Output v2.5 kit (300 cycles)	Illumina	20024908
NextSeq™ 1000/2000 P1 Reagents (300 cycles)	Illumina	20050264
NextSeq™ 1000/2000 P1 Reagents (600 cycles)	Illumina	20075294
NextSeq™ 1000/2000 P2 Reagents v3 (300 cycles)	Illumina	20046813
NextSeq™ 1000/2000 P2 300M Reagents (600 cycles)	Illumina	20075295
NextSeq™ 2000 P3 Reagents (300 cycles)	Illumina	20040561

General Laboratory Consumables

In addition to the consumables listed below, other general laboratory supplies needed to carry out the protocol include gloves, pre-chilled benchtop cooler, tube racks, etc.

Consumable	Area Use	Supplier
1.5 mL microcentrifuge tubes	Pre- and Post-PCR	General lab supplier
96-well PCR plates, 0.2 mL	Pre- and Post-PCR	Corning Life Sciences, Cat# 6551 or equivalent
Microplate sealing film	Pre- and Post-PCR	Corning Life Sciences, Cat# PCR-TS or equivalent
Conical tubes, 15 mL	Pre- and Post-PCR	General lab supplier
Conical tubes, 50 mL	Post-PCR	General lab supplier
Low retention, aerosol filter pipette tips	Pre- and Post-PCR	General lab supplier
Solution basin (trough or reservoir)	Pre- and Post-PCR	Fisher Scientific, Cat# 13-681-506 or equivalent
Qubit Assay tubes	Post-PCR	Invitrogen, Cat# Q32856

4.5 Equipment

Equipment	Area Use	Supplier
Centrifuge adapted for PCR plates, tabletop	Pre- and Post-PCR	General lab supplier
Gel electrophoresis apparatus (optional)*	Post-PCR	General lab supplier
4150 TapeStation system or equivalent (optional)*	Post-PCR	Agilent Technologies, Cat# G2992BA
Magnetic stand for 96 wells	Post-PCR	Life Technologies, Cat# 12331D or 12027
Microfuge	Pre- and Post-PCR	General lab supplier
Thermal cycler, heated lid capability	Post-PCR	General lab supplier
Pipettes, 0.5-1000 µL capabilities	Pre- and Post-PCR	General lab supplier
Qubit Fluorometer	Post-PCR	Invitrogen, Cat# Q33216/Q33218
Vortex mixer	Pre- and Post-PCR	General lab supplier

* The Qubit dsDNA High Sensitivity kit is the primary DNA quantitation assay used throughout this protocol. Additional DNA quantification can optionally be performed using the Qubit dsDNA Broad Range Assay kit, an agarose gel, or using the TapeStation High Sensitivity D1000 ScreenTape system.

5. Workflow

The following chart demonstrates the workflow for performing library preparation.

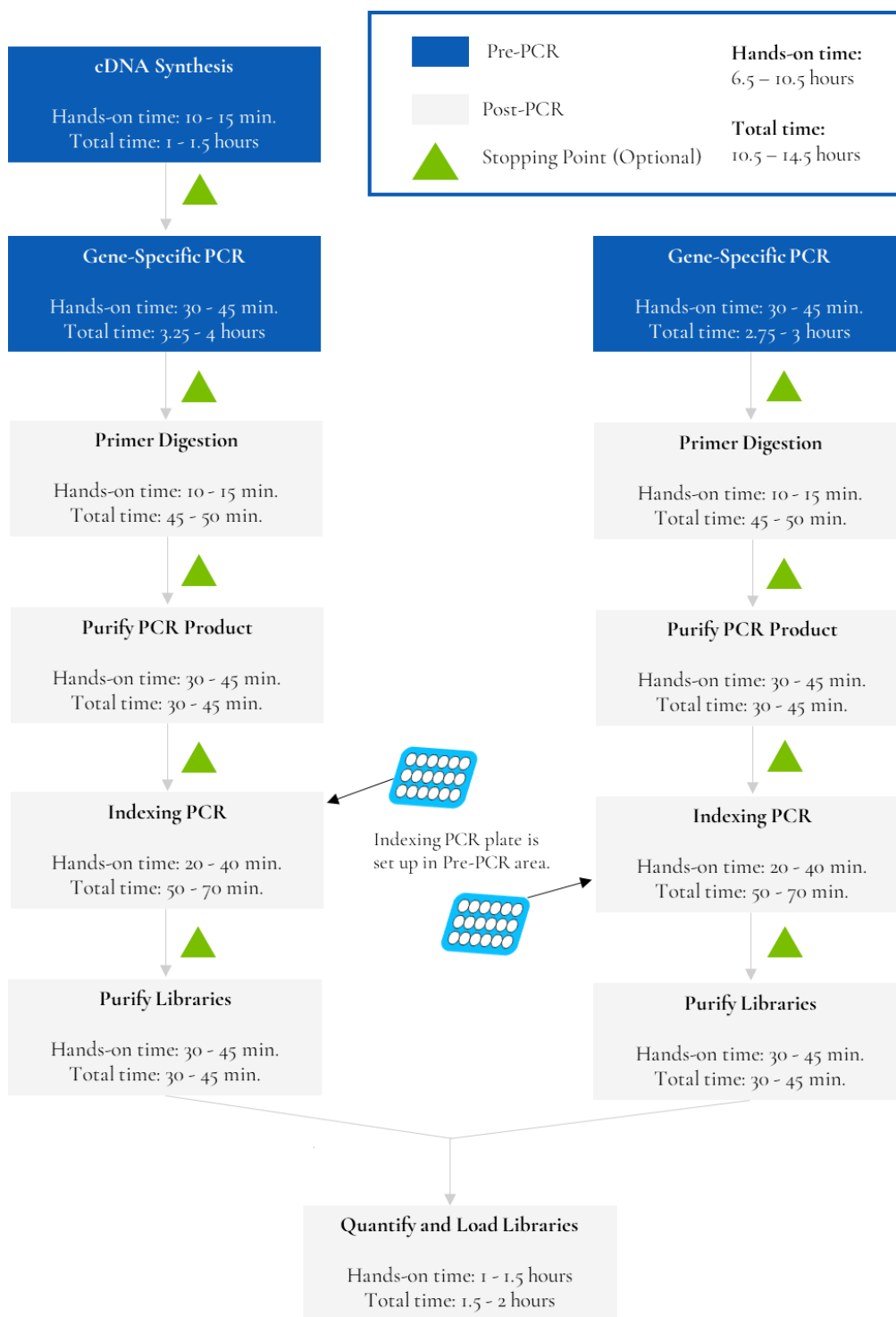





Figure 1. Library preparation workflow for oncoReveal® Multi-Cancer with CNV & RNA Fusion Panel. This workflow contains multiple optional stopping points for users with time constraints.

Symbol	Description
	Information: Information that follows this symbol is important and may require action.
	Optional Stopping Point: A point in the workflow at which work may be safely paused, and samples can be stored appropriately.
	Caution: Information that follows this symbol is critical to the workflow. Information following this symbol should not be skipped or ignored.

Symbols used throughout this protocol and their associated meaning.

6. Part I: DNA Library Preparation Protocol

Hands-on Time	3 – 5 hours
Total Time	6.5 – 8.5 hours

6.1 DNA Input Information

Inclusion of Normal Samples

Copy number variation (CNV) analysis is based on a Dirichlet process and utilizes at least 1 normal sample to establish the copy number baseline. CNVs are called at gene level. All amplicons contained in the panel are used for CNV analysis, either as a control or as CNV candidate.

For each CNV analysis run, 3 – 5 (**minimum 1**) in-run normal (negative; sample without any CNVs) samples should be provided. It is best if the negative control sample is derived from the same tissue type, handled with the same procedure, and prepared using the same DNA extraction method as the samples. CNV calling sensitivity increases with additional normal samples. The algorithm supports out-of-run normal samples, provided they were sequenced using the same chemistry lot. In the absence of normal samples, the CNV analysis will run on median normalization algorithm*, given the conditions listed below have been met. This sample setting configuration will be automatically detected by Pillar's secondary analysis pipeline, PiVAT®.

With the default PiVAT® settings, the following conditions are required to be met in the absence of normal references:

1. The minimum number of required samples is **10**.
2. The number of samples with the same sample type of CNV cannot exceed **30%** of all samples.
3. Final libraries pooled together for sequencing should have varied CNV signatures (e.g., CNVs with different exons, genes, or lengths).

** Support for median normalization algorithm is dependent on PiVAT® version. Please check the PiVAT® release notes or with your field application scientist to see if your version of PiVAT® supports this capability.*

DNA Input Information

The following protocol includes information for preparing libraries using genomic DNA from tissue or FFPE samples.

Recommended DNA input per sample:

- **Genomic DNA (from tissue, cell lines): 20 – 60 ng**
- **FFPE DNA: 20 – 80 ng**
- **Severely degraded FFPE DNA: 40 ng minimum**
- **Blood DNA: 20 ng minimum**

For severely degraded FFPE DNA, if the amount of DNA is limited, lower input may be used, but the sequencing yield, quality, and on-target alignment rate may be negatively affected. For best performance, normalize the DNAs and the

normal negative control DNAs to the same input amount and start with 20-30 ng of DNA per reaction. At 30 ng DNA input and above, CNV detection is robust within 30% input range variations (e.g., 30 ng \pm 10 ng).

For FFPE samples, it is recommended that Uracil-DNA glycosylase (UDG) be added before proceeding with gene-specific PCR. The deamination of cytosine to uracil is a common cause of the presence of artificial C>T (or G>A) variants. To reduce such artifacts which are present due to DNA damage in FFPE samples, UDG can be added to the reaction during the initial setup of gene-specific PCR.

6.2 Gene-Specific PCR (GS-PCR) and Purification

Hands-on Time	30 – 40 min.
Total Time	2.75 – 3 hours

Amplify Genomic DNA Targets

The following steps should be performed in a pre-PCR area.

For this portion of the protocol, prepare a pre-chilled benchtop cooler and keep the gene-specific PCR Master Mix (GS-PCR MMX) and the custom oligo pool should be kept in the cooler until needed.

See recommended DNA input quantities in section 6.1 “DNA Input Information.”

- Prepare Gene-Specific PCR Mix 1:** Vortex and centrifuge the GS-PCR MMX and oligo pool before use. For each PCR reaction, the volume of each component is listed below.

i Important: The gene-specific PCR master mix is viscous. Ensure the mix is fully homogenized before adding other reaction components. Vortexing is recommended and will not adversely affect enzyme activity.

GS-PCR Mix 1		
Reagent	Volume (µL) without UDG	Volume (µL) with UDG
Gene-Specific PCR 2x Master Mix	12.5	12.5
oncoReveal® Multi-Cancer v4 with CNV Oligo Pool	5.0	5.0
UDG (5 units/µL)	0.0	1.0
Subtotal	17.5	18.5

- Transfer:** Transfer 17.5 µL (or 18.5 µL if using UDG) of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.
- Add input DNA:** Add 7.5 µL (6.5 µL DNA if using UDG) of DNA* (diluted if necessary) to the GS-PCR plate. Add 7.5 µL (or 6.5 µL if using UDG) of nuclease-free water to the no-template control well.

Final GS-PCR Mix		
Reagent	Volume (µL) without UDG	Volume (µL) with UDG
GS-PCR Mix 1	17.5	18.5
DNA (or water)	7.5	6.5
Total	25.0	25.0

* The DNA concentration can be determined by the Qubit dsDNA HS Assay Kit or the Qubit dsDNA BR Assay Kit.

- Seal and mix:** Carefully seal the reactions and vortex for 10 – 15 seconds.
- Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.

6. **Perform GS-PCR:** Perform the following program with the heated lid on:

GS-PCR Program			
Temperature	Time	Number of Cycles (without UDG)	Number of Cycles (with UDG)
37°C	10 min	0	1
95°C	15 min	1	1
97°C	1 min		
58°C	1 min		
60°C	2 min	5	5
64°C	30 sec		
72°C	1 min		
95°C	30 sec	18	18
66°C	3 min		
8°C	Hold	1	1

NOTE: If multiple sample types are being processed (non-FFPE and FFPE DNA), reactions with and without UDG can be run simultaneously with the same PCR conditions. The 37°C incubation will not adversely affect the PCR reaction.



Do not leave reactions on the thermocycler overnight at 8°C. Once cycling is complete the reactions can be stored at -20°C.



Optional Stopping Point: The gene-specific PCR reactions may be stored at -20°C after cycling is complete.

Gene-Specific Primer Digestion

Hands-on Time	10 – 15 min.
Total Time	45 – 50 min.

The following steps should be performed in a post-PCR area.

For this portion of the protocol, have a pre-chilled benchtop cooler prepared. The exonuclease should be kept in the cooler until it is needed. Keep the sample reactions at ambient temperature.

- Briefly centrifuge the gene-specific PCR product to remove droplets from the side walls. Carefully remove the seal or caps.
- Dilute Exonuclease:** Invert the Exonuclease I to mix and centrifuge briefly to remove any droplets from the lid. Prepare the Exonuclease Dilution as indicated below, pipette to mix and place in the cooler.

Exonuclease Dilution (per reaction)	
Reagent	Volume (µL)
Exonuclease I	3.0
Exonuclease I Buffer (or nuclease-free water)	2.0
Total	5.0



Important: The exonuclease solution is viscous and requires careful attention when pipetting. Ensure the Exonuclease Dilution is mixed thoroughly.

3. **Add Exonuclease:** Add 5 µL of the Exonuclease Dilution to each well containing gene-specific PCR product. Pipette up and down to mix.
4. **Seal and mix:** Carefully seal the reactions. Pulse vortex the reactions on a medium setting for 5 – 10 seconds.
5. **Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.
6. **Perform the digestion:** Perform the following program with the heated lid on:

GS Primer Digestion Program		
Temperature	Time	Number of Cycles
37°C	20 min	1
80°C	10 min	1
8°C	Hold	1



Do not leave reactions on the thermocycler overnight at 8°C. Once cycling is complete the reactions can be stored at -20°C.



Optional Stopping Point: The reactions may be stored at -20°C after primer digestion is complete.

Gel Image After Gene-Specific PCR

The following image is an example of samples after gene-specific PCR and **before** primer digestion on a 2% agarose gel.

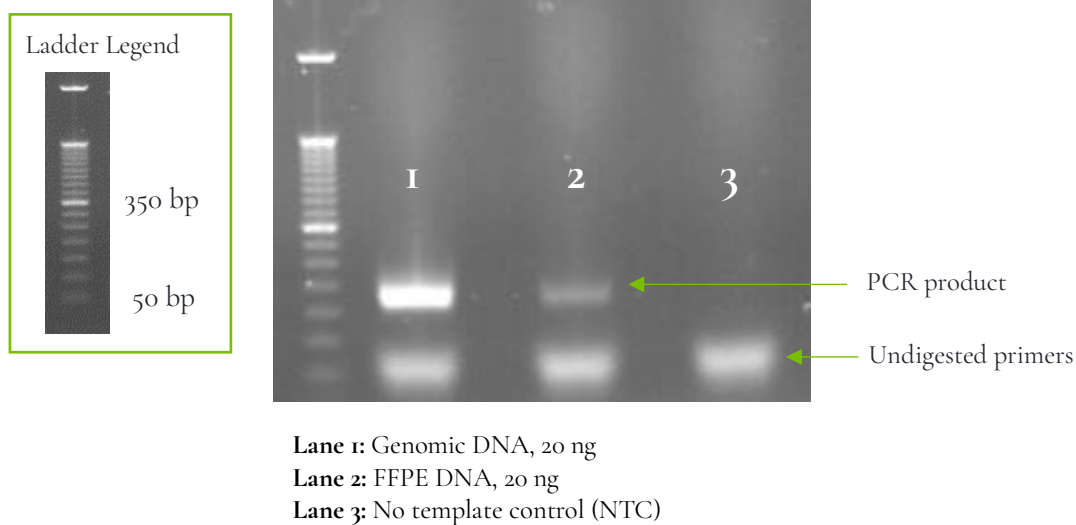



Figure 2. Analysis of GS-PCR product on 2% agarose gel.

Purify the Gene-Specific PCR Product

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

The following steps should be performed in a post-PCR area.


Before beginning the purification:

- Remove AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.
-  **Caution:** Ensure that the AMPure bead solution reaches room temperature before performing the purification. The temperature of the bead solution can have adverse effects on the purification process.
- If gene-specific PCR products were stored at -20°C, remove from the freezer to thaw at room temperature and then proceed immediately to purification.
- Prepare 70% ethanol by mixing three parts of water with seven parts of absolute ethanol in a conical tube, invert tube a few times to mix, and dispense sufficient volume to a disposable trough for convenient dispensing using a multichannel pipette.



Caution: Fresh 70% ethanol is required for optimal results.

Gene-Specific Product Purification:

- If condensation has formed or if reactions were stored at -20°C, briefly centrifuge the samples upon thawing. Carefully remove the seal.
- Mix Beads:** Vortex AMPure XP beads thoroughly until all beads are well-dispersed.
-  **Caution:** Ensure that the AMPure bead solution is homogenous. A non-uniform distribution can have unpredictable effects on the purification process.
- Add water to sample:** Add **20 µL** of nuclease-free water to each well containing GS-PCR product to bring the reaction volume to 50 µL.
- Add beads:** Add **60 µL** of AMPure beads (equivalent to a 1.2x bead ratio) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge the samples and mix again.
- Bind GS-PCR product to beads:** Incubate for 5 minutes at room temperature.
- Separate beads containing GS-PCR product:** Place the PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
- Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.
- Wash beads:** With the PCR plate still on the magnetic rack, add **150 µL** of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds and then remove the supernatant from each well.



Important: Do not allow the ethanol mixture to remain open to the air. The ethanol concentration will change over time, affecting the washing of the beads. Pour only enough solution for each wash.

9. **Second wash:** Repeat step 8 for a second 70% ethanol wash. Then remove the supernatant from each well. Put aside any remaining ethanol solution. The unused ethanol solution can be used to purify the libraries after the indexing PCR is completed.
10. **Remove remaining ethanol wash:** Centrifuge for 10–15 seconds, place the PCR plate back on the magnetic rack, and use a 10 or 20 μL tip to remove any trace amounts of ethanol from each well.
11. **Resuspend beads:** Remove the PCR plate from the magnetic rack, and **immediately resuspend** the dried beads in each well using **64 μL** of nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.

TIP: After resuspending the beads, cover the reactions and prepare for the indexing PCR step using the Indexing PCR Master Mix. The Indexing PCR Master Mix should be handled in the pre-PCR area. Alternatively, the purified gene-specific PCR product may be stored at -20°C after elution.

12. **Incubate and Elute:** Incubate the elution for 5 minutes at room temperature.
13. **Separate GS-PCR product from beads:** Place the reactions on a magnetic rack at room temperature until the solution appears clear. Transfer **62 μL** of clear supernatant from each well to a new PCR plate.



Optional Stopping Point: The purified GS-PCR products can be stored at -20°C .

6.3 Index PCR and Purification

Indexing PCR: Amplify the Libraries

Hands-on Time	20 – 40 min.
Total Time	50 – 70 min.

The following steps should be performed in a pre-PCR area.

For this portion of the protocol, prepare a pre-chilled benchtop cooler. The Indexing PCR Master Mix should be kept in the cooler until needed.

- Prepare Indexing Primer Mix:** In a new PCR plate, add **4 μ L** each of the appropriate forward and reverse indexing primers to each sample well that will be used.

Indexing Primer Mix (per reaction)	
Reagent	Volume (μ L)
Pi700 Pillar Index	4.0
Pi500 Pillar Index	4.0
Subtotal	8.0

- Prepare Indexing PCR Mix 1:** Briefly vortex and centrifuge the Indexing PCR Master Mix before use. To prepare the Indexing PCR Mix 1, combine the Indexing PCR Master Mix and water with sufficient overage.

Indexing PCR Mix 1 (per reaction)	
Reagent	Volume (μ L)
Indexing PCR 2x Master Mix	25.0
Nuclease-free water	11.0
Subtotal	36.0

- Add Indexing PCR Mix 1 to Indexing Primer Mix:** Transfer **36 μ L** of the Indexing PCR Mix 1 to each sample well of the PCR plate in step 1 that contains Indexing Primer Mix. To prevent cross-contamination of indices, be sure to change tips between each well.

Indexing PCR Mix 2 (per reaction)	
Reagent	Volume (μ L)
Indexing Primer Mix	8.0
Indexing PCR Mix 1	36.0
Subtotal	44.0

The following steps should be performed in a post-PCR area. Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area.

If the purified GS-PCR products were stored at -20°C after the GS-PCR purification, ensure that they have been thawed at room temperature before proceeding.

4. **Add purified GS-PCR product:** Aliquot 6 µL of purified GS-PCR product into the appropriate wells containing Indexing PCR Mix 2.

Index PCR Final Mix (per reaction)	
Reagent	Volume (µL)
Indexing PCR Mix 2	44.0
Purified Gene-Specific PCR product	6.0
Total	50.0

5. **Seal and Mix:** Carefully seal the reactions and vortex for 10-15 seconds.
6. **Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.
7. **Perform Indexing PCR:** Perform the following program with the heated lid on:

Indexing PCR Program		
Temperature	Time	Number of Cycles
95°C	2 min	1
95°C	30 sec	6*
66°C	30 sec	
72°C	1 min	
72°C	5 min	1
8°C	Hold	1

* Additional indexing PCR cycles can be performed if final library yield is low or initial DNA input is below the recommended minimum.



Optional Stopping Point: The indexed libraries can be stored at -20°C after cycling is complete.

Indexing PCR: Purify the Libraries

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

Before beginning the purification:

- Keep AMPure XP beads at room temperature while the Indexing PCR is being performed unless reactions will be stored at -20°C after the program is complete.



Caution: Ensure that the AMPure bead solution reaches room temperature before performing the purification. The temperature of the bead solution can have adverse effects on the purification process.

- If the indexed libraries were stored at -20°C, remove them from the freezer to thaw thoroughly to ambient temperature before purification. After samples have thawed, briefly centrifuge the samples to remove any droplets from the side walls.

Library Purification

The following steps should be performed in a post-PCR area.

1. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.



Caution: Ensure that the AMPure bead solution is homogenous. A non-uniform distribution can have unpredictable effects on the purification process.

2. **Add beads:** Add **50 µL** beads (equivalent to a 1.0x bead ratio) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.
3. **Bind libraries to beads:** Incubate for 5 minutes at room temperature.
4. **Separate libraries on beads:** Place the PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
5. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.
6. **Wash beads:** With the PCR plate still on the magnetic rack add **150 µL** of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove the supernatant from each well.



Caution: Do not allow the ethanol mixture to remain open to the air. The ethanol concentration will change over time, affecting the washing of the beads. Pour only enough solution for each wash.

7. **Second wash:** Repeat step 6 for a second 70% ethanol wash. Then remove the supernatant from each well.
8. **Remove remaining ethanol wash:** Centrifuge for 10–15 seconds, place the PCR plate back on the magnetic rack, and use a 10 or 20 µL tip to remove any trace amounts of ethanol from each well.
9. **Dry beads:** Let the beads air dry at room temperature for 2–5 minutes.



Important: To mitigate product loss, do not allow the beads to over-dry. The beads have sufficiently dried when the bead mass has small cracks in the middle. If large cracks have appeared throughout the entire bead ring or if the beads are flaky, they are over-dried. Beads that are too dry may be difficult to resuspend.

10. **Resuspend beads:** Remove the PCR plate from the magnetic rack and resuspend the dried beads in each well using **32 µL** of nuclease-free water. Gently pipette the beads suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.
11. **Elute libraries:** Incubate at room temperature for 5 minutes.

12. **Separate libraries from beads:** Place the bead suspensions on the magnetic rack at room temperature until the solution appears clear. Transfer **30 µL** of clear supernatant from each well to a new plate.

TIP: During the incubation and magnetic separation of the beads, cover the samples with microplate sealing film and prepare the solutions needed for quantitation performed in the next section.

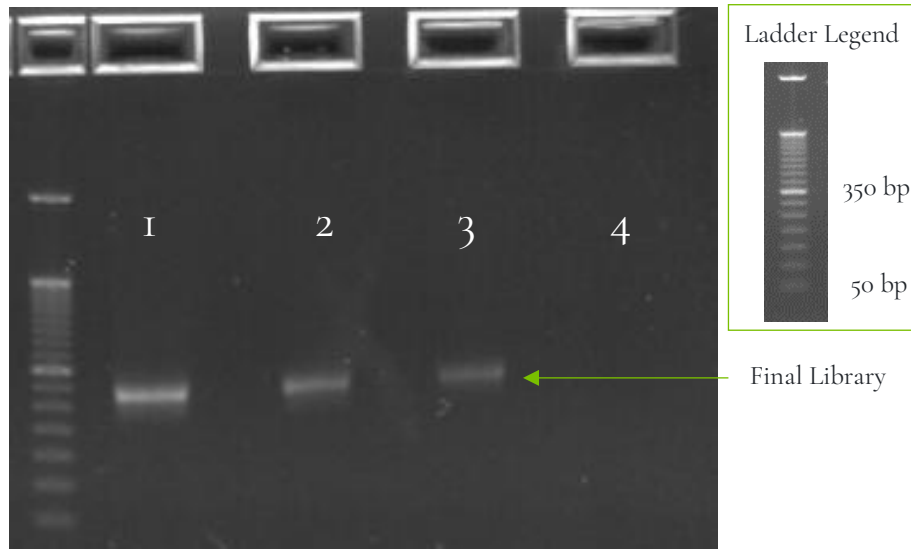
13. **Quantification:** Analyze an aliquot of each library per the instructions in the next section.



Optional Stopping Point: The purified libraries can be stored at 4°C for up to 3 days, or at -20°C for longer-term storage.

Final Library Gel Image

The following image is an example of final libraries after both rounds of PCR and purification on a 2% agarose gel.



Lane 1-2: Genomic DNA, 20 ng
Lane 3: FFPE DNA, 20 ng
Lane 4: No template control (NTC)

Figure 3. Gel analysis of final library material.

7. Part 2: RNA Library Preparation Protocol

Hands-on Time	3 – 5 hours
Total Time	6.25 – 8.75 hours

7.1 RNA Input Information

The following protocol includes information for preparing libraries using RNA isolated from FFPE samples.

Recommended minimum RNA input per sample:

- **FFPE RNA: 25 ng**
- **Degraded FFPE RNA: 75 – 100 ng**
- **cfRNA: 20 ng; for poor quality samples it may be necessary to increase the amount of input RNA**

NOTE: The concentration of cfRNA is assumed to be equal to the DNA concentration of the total circulating nucleic acid sample. The DNA concentration can be quantified using the Qubit dsDNA HS Assay Kit.

7.2 cDNA Synthesis

Hands-on Time	10 – 15 min.
Total Time	1 – 1.5 hours

Before performing the library preparation, prepare cDNA from total RNA extracted from FFPE samples, tissue, or cell lines. cDNA may also be prepared from total circulating nucleic acid extracted from blood.

For the preparation from RNA, the **cDNA should be prepared using random primers, not exclusively oligo d(T)s**. Using only oligo d(T)s may result in low or no coverage of the 5' end of transcripts.

Up to **15 µL** of undiluted cDNA reaction can be added to the Gene-Specific PCR without inhibiting the reaction. Alternatively, with a higher RNA input, the cDNA reaction can be diluted with low TE or nuclease-free water.

Two **examples** of the cDNA synthesis reaction, adapted from the recommended SuperScript VILO Master Mix protocol, are presented. The actual protocol may vary based on the cDNA master mix used – see manufacturer's instructions for details.

See recommended RNA input quantities in section 7.1: "RNA Input Information."

cDNA Synthesis Example Reaction 1: 20 µL or 10 µL cDNA reaction mix

Set up the reaction in a benchtop cooler and keep all components chilled.

1. **Dilute RNA:** Dilute RNA* in nuclease-free water to a final volume of **16 µL** (for a 20 µL reaction) or **8 µL** (for a 10 µL reaction) to each sample well in the PCR plate, strip tube, or PCR tube. Add nuclease-free water to the no-template control well.

** The RNA concentration can be determined by the Qubit RNA BR Assay kit or Qubit RNA HS Assay kit.*

2. **Add cDNA Synthesis Mix:** Add cDNA Master Mix to each sample well containing diluted RNA and add cDNA Master Mix to the well containing the no-template control.

Example 1: cDNA Synthesis Mix		
Reagent	Volume (µL) for 20 µL reaction	Volume (µL) for 10 µL reaction
cDNA Master Mix	4.0	2.0
Diluted RNA (or water)	16.0	8.0
Total	20.0	10.0

3. **Synthesize cDNA:** Perform the reverse transcription in a thermal cycler with the heated lid on:

Example 1: cDNA Synthesis Program	
Temperature	Time
25°C	10 min
42°C	30 min
85°C	5 min
8°C	Hold



Optional Stopping Point: After the completion of the cDNA synthesis, the cDNA can be diluted with nuclease-free water or low TE and stored at -20°C if necessary.

cDNA Synthesis Example Reaction 2: 5 µL cDNA reaction mix

A 5 µL cDNA reaction may be performed according to the example below for FFPE RNA. After cDNA synthesis is complete, the master mix for the gene-specific PCR reaction may be added directly to the cDNA synthesis plate before proceeding with thermal cycling for gene-specific PCR.

Set up the reaction in a benchtop cooler and keep all components chilled.

1. **Dilute RNA:** Dilute RNA* in nuclease-free water to a final volume of 4 µL to each sample well in PCR plate, strip tube, or PCR tube. Add nuclease-free water to the no-template control well.

* *The RNA concentration can be determined by the Qubit RNA BR Assay kit or Qubit RNA HS Assay kit.*

2. **Add cDNA Synthesis Mix:** Add cDNA Master Mix to each sample well containing diluted RNA and add cDNA Master Mix to the well containing the no-template control.

Example 2: cDNA Synthesis Mix	
Reagent	Volume (µL)
cDNA Master Mix	1.0
Diluted RNA (or water)	4.0
Total	5.0

3. **Synthesize cDNA:** Perform the reverse transcription in a thermal cycler with the heated lid:

Example 2: cDNA Synthesis Program	
Temperature	Time
25°C	10 min
50°C	10 min
85°C	5 min
8°C	Hold



Optional Stopping Point: After the completion of the cDNA synthesis, the cDNA can be diluted with nuclease-free water or low TE and stored at -20°C if necessary.

7.3 Gene-Specific PCR (GS-PCR) and Purification

Hands-on Time	30 – 45 min.
Total Time	3.25 – 4 hours

Amplify Transcript Targets

The following steps should be performed in a pre-PCR area.

For this portion of the protocol, prepare a pre-chilled benchtop cooler. The gene-specific PCR Master Mix (GS-PCR MMX) and the custom oligo pool should be kept in the cooler until needed.

- Prepare Gene-Specific PCR Mix 1:** Vortex and centrifuge the GS-PCR MMX and oligo pool before use. For each PCR reaction, the volume of each component is listed below.



Important: The gene-specific PCR master mix is viscous. Ensure the mix is fully homogenized before adding other reaction components. Vortexing is recommended and will not adversely affect enzyme activity.

GS-PCR Mix 1	
Reagent	Volume (μL)
Gene-Specific 2x PCR Master Mix	25.0
oncoReveal™ Multi-Cancer RNA Fusion v2 5x Oligo Pool	10.0
Subtotal	35.0

- Transfer:** Transfer 35 μL of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.
- Add input cDNA:** Add 15 μL of cDNA (diluted if necessary) to each sample well containing GS-PCR Mix 1. Add 15 μL of nuclease-free water to the no-template control well.

Up to 15 μL of undiluted cDNA reactions may be added to the GS-PCR reaction. It is recommended that the volume added to the reaction corresponds to at least 25 ng of RNA.

If using the entire cDNA reaction (5 μL or 10 μL reaction), the gene-specific PCR master mix and supplementing water may be added directly to the PCR plate. The reactions may proceed directly to GS-PCR. See previous section “cDNA Synthesis” for more information regarding input.

Final GS-PCR Mix	
Reagent	Volume (μL)
GS-PCR Mix 1	35.0
Diluted cDNA (or water)	15.0
Total	50.0

- Seal and mix:** Carefully seal the reactions and vortex for 10 – 15 seconds.
- Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.

6. **Perform PCR:** Perform the following program with the heated lid on:

GS-PCR Program		
Temperature	Time	Number of Cycles
95°C	15 min	1
95°C	1 min	5
58°C	1 min	
60°C	2 min	
64°C	30 sec	
72°C	1 min	
95°C	30 sec	24*
66°C	3 min	
8°C	Hold	1

* For samples prepared from cfRNA, additional cycles are needed. It is recommended that additional cycles be performed (up to 26 cycles) to achieve sufficient product yield.



Do not leave reactions on the thermocycler overnight at 8°C. Once cycling is complete the reactions can be stored at -20°C.



Optional Stopping Point: The gene-specific PCR reactions can be stored at -20°C after cycling is complete.

Gene-Specific Primer Digestion

Hands-on Time	10 – 15 min.
Total Time	40 – 50 min.

The following steps should be performed in a post-PCR area.

For this portion of the protocol prepare a pre-chilled benchtop cooler. The exonuclease should be kept in the cooler until it is needed. Keep the sample reactions at ambient temperature.

- Briefly centrifuge the reactions to remove droplets from the side walls. Carefully remove the seal or caps.
- Dilute Exonuclease:** Invert the Exonuclease I to mix and centrifuge briefly to remove any droplets from the lid. Prepare the Exonuclease Dilution as indicated below, pipette to mix, and place on in the cooler.

Exonuclease Dilution (per reaction)	
Reagent	Volume (µL)
Exonuclease I	3.0
Exonuclease I Buffer (or nuclease-free water)	2.0
Total	5.0



Important: The exonuclease solution is viscous and requires careful attention when pipetting. Ensure the dilution is mixed thoroughly.

3. **Add Exonuclease:** Add 5 µL of the diluted exonuclease to each well containing gene-specific PCR product. Pipette up and down to mix.
4. **Seal and mix:** Carefully seal the reactions. Pulse vortex the reactions on a medium setting for 5 – 10 seconds.
5. **Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.
6. **Perform the digestion:** Perform the following program with the heated lid on:

GS Primer Digestion Program		
Temperature	Time	Number of Cycles
37°C	20 min	1
80°C	10 min	1
8°C	Hold	1



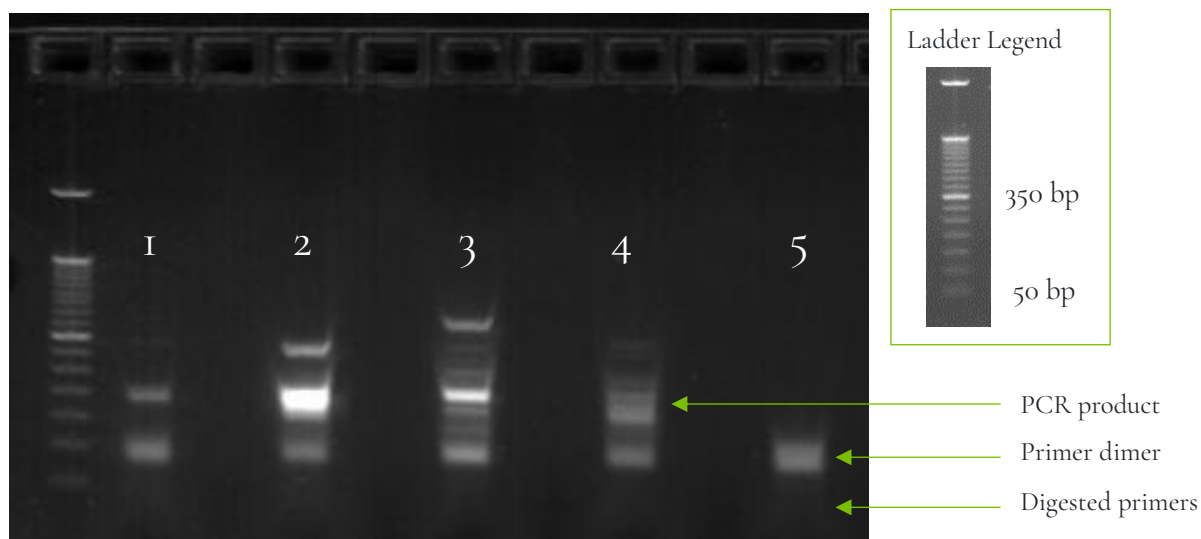
Do not leave reactions on the thermocycler overnight at 8°C. Once cycling is complete the reactions can be stored at -20°C.



Optional Stopping Point: The reactions can be stored at -20°C after cycling is complete.

Gel Image After Gene-Specific PCR and Primer Digestion

The following is a gel image of FFPE RNA samples after GS-PCR and primer digestion.



- Lane 1:** Fusion negative sample, 25 ng
- Lane 2-4:** Fusion positive samples, 25 ng
- Lane 5:** No template control (NTC)

Figure 4. Analysis of GS-PCR material on 2% agarose gel.

Purify the Gene-Specific PCR Product

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

The following steps should be performed in a post-PCR area.

Before beginning the purification:

- Remove AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.



Caution: Ensure that the AMPure bead solution reaches room temperature before performing the purification. The temperature of the bead solution can have adverse effects on the purification process.

- If gene-specific PCR products were stored at -20°C, allow to thaw at room temperature and then proceed immediately to purification.
- Prepare 70% ethanol by mixing three parts of water with seven parts of absolute ethanol in a conical tube, invert tube a few times to mix, and dispense sufficient volume to a disposable trough for convenient dispensing using a multichannel pipette.



Caution: Fresh 70% ethanol is required for optimal results.

Gene-Specific Product Purification

- If condensation has formed or if reactions were stored at -20°C, briefly centrifuge the samples upon thawing. Carefully remove the seal.
- Mix Beads:** Vortex AMPure XP beads thoroughly until all beads are well-dispersed.



Caution: Ensure that the AMPure bead solution is homogenous before performing the purification. A non-uniform distribution can have unpredictable effects on the purification process.

- Add beads:** Add 66 μL AMPure beads (equivalent to a 1.2x bead ratio) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge the samples and mix again.
- Bind PCR product to beads:** Incubate for 5 minutes at room temperature.
- Separate beads containing PCR product:** Place the PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
- Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.
- Wash beads:** With the PCR plate still on the magnetic rack, add 150 μL of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate for 30 seconds and then remove the supernatant from each well.



Important: Do not allow the ethanol mixture to remain open to the air. The ethanol concentration will change over time, affecting the washing of the beads. Pour only enough solution for each wash.

- Second wash:** Repeat step 7 for a second 70% ethanol wash. Then remove the supernatant from each well. The unused ethanol solution can be used to purify the libraries after the indexing PCR is completed.

9. **Remove remaining ethanol wash:** Centrifuge the PCR plate for 10 – 15 seconds, place the plate back on the magnetic rack, and use a 10 or 20 μL tip to remove any trace amounts of ethanol from each well.
10. **Resuspend beads:** Remove the PCR plate from the magnetic rack and **immediately resuspend** the dried beads in each well using **64 μL** nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.

TIP: After resuspending the beads, cover the reactions and prepare for the indexing PCR step using the Indexing PCR Master Mix. The Indexing PCR Master Mix should be handled in the pre-PCR area. Alternatively, the purified gene-specific PCR product may be stored at -20°C after elution.

11. **Incubate and Elute:** Incubate at room temperature for 5 minutes.
12. **Separate GS-PCR product from beads:** Place the elution on a magnetic rack at room temperature until the solution appears clear. Transfer **62 μL** of clear supernatant to a new PCR plate.



Optional Stopping Point: The purified GS-PCR products can be stored at -20°C .

7.4 Index PCR and Purification

Indexing PCR: Amplify the Libraries

Hands-on Time	20 – 40 min.
Total Time	50 – 70 min.

The following steps should be performed in a pre-PCR area.

For this portion of the protocol, have a pre-chilled benchtop cooler prepared. The Indexing PCR Master Mix should be kept in the cooler until it is needed.

- Prepare Indexing Primer Mix:** In a new PCR plate, add **4 µL** each of the appropriate forward and reverse indexing primers to each sample well that will be used.

Indexing Primer Mix (per reaction)	
Reagent	Volume (µL)
Pi700 Pillar Index	4.0
Pi500 Pillar Index	4.0
Subtotal	8.0

- Prepare Indexing PCR Mix 1:** Briefly vortex and centrifuge the Indexing PCR Master Mix before use. To prepare the Indexing PCR Mix 1, combine the Indexing PCR Master Mix and water with sufficient overage.

Indexing PCR Mix 1 (per reaction)	
Reagent	Volume (µL)
Indexing PCR 2x Master Mix	25.0
Nuclease-free water	5.0
Subtotal	30.0

- Add Indexing PCR Mix 1 to Indexing Primer Mix:** Transfer **30 µL** of the Indexing PCR Mix 1 to each sample well of the PCR plate in step 1 that contains Indexing Primer Mix. To prevent cross-contamination of indices, be sure to change tips between each well.

Index PCR Mix 2	
Reagent	Volume (µL)
Indexing Primer Mix	8.0
Indexing PCR Mix 1	30.0
Subtotal	38.0

The following steps should be performed in a post-PCR area. Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area.

If the purified GS-PCR products were stored at -20°C after the GS-PCR purification, ensure that they have been thawed at room temperature before proceeding.

4. **Add purified GS-PCR product:** Aliquot 12 µL of purified GS-PCR product into the appropriate wells containing Indexing PCR Mix 2.

Indexing PCR Final Mix (volumes are per reaction)	
Reagent	Volume (µL)
Index PCR Mix 2	38.0
Gene-Specific PCR product	12.0
Total	50.0

5. **Seal and mix:** Carefully seal the reactions and vortex for 10-15 seconds.
6. **Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.
7. **Perform PCR:** Perform the following program with the heated lid on:

Indexing PCR Program		
Temperature	Time	Number of Cycles
95°C	2 min	1
95°C	30 sec	5*
66°C	30 sec	
72°C	1 min	
72°C	5 min	1
8°C	Hold	1

* Additional PCR cycles can be performed if the final library yield is low or initial input is below recommended minimum.



Optional Stopping Point: The indexed libraries can be stored at -20°C after cycling is complete.

Indexing PCR: Purify the Libraries

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

Before beginning the purification:

- Keep AMPure XP beads at room temperature while the Indexing PCR is being performed unless reactions will be stored at -20°C after the program is complete.



Caution: Ensure that the AMPure bead solution reaches room temperature before performing the purification. The temperature of the bead solution can have adverse effects on the purification process.

- If the indexed libraries were stored at -20°C, remove them from the freezer to thaw to ambient temperature before purification. After samples have thawed, briefly centrifuge to remove any droplets from the side walls.

Library Purification

The following steps should be performed in a post-PCR area.

1. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.



Caution: Ensure that the AMPure bead solution is homogenous before performing the purification. A non-uniform distribution can have unpredictable effects on the purification process.

2. **Add beads:** Add 50 µL beads (equivalent to a 1.0x bead ratio) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.
3. **Bind libraries to beads:** Incubate for 5 minutes at room temperature.
4. **Separate libraries on beads:** Place the PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
5. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.
6. **Wash beads:** With the PCR plate still on the magnetic rack, add 150 µL of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove the supernatant from each well.



Caution: Do not allow the ethanol mixture to remain open to the air. The ethanol concentration will change over time, affecting the washing of the beads. Pour only enough solution for each wash.

7. **Second wash:** Repeat step 6 for a second 70% ethanol wash. Then remove the supernatant from each well.
8. **Remove remaining ethanol wash:** Centrifuge the PCR plate for 10 – 15 seconds, place the plate back on the magnetic rack, and use a 10 or 20 µL tip to remove any trace amounts of ethanol from each well.
9. **Dry beads:** Let the beads air dry at room temperature for 2 – 5 minutes.



Important: To mitigate product loss, do not allow the beads to over-dry. The beads have sufficiently dried when the bead mass has small cracks in the middle. If large cracks have appeared throughout the entire bead ring or they are flaky, they are over-dried. Beads that are too dry may be difficult to resuspend.

10. **Resuspend beads:** Remove the PCR plate from the magnetic rack and resuspend the dried beads in each well using 32 µL nuclease-free water. Gently pipette the beads suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.
11. **Elute libraries:** Incubate at room temperature for 5 minutes.

12. **Separate libraries from beads:** Place the bead suspensions on the magnetic rack at room temperature until the solution appears clear. Transfer 30 µL of clear supernatant from each well to a new plate.

TIP: During the incubation and magnetic separation of the beads, cover the samples with microplate sealing film and prepare the solutions needed for quantitation in the next section.

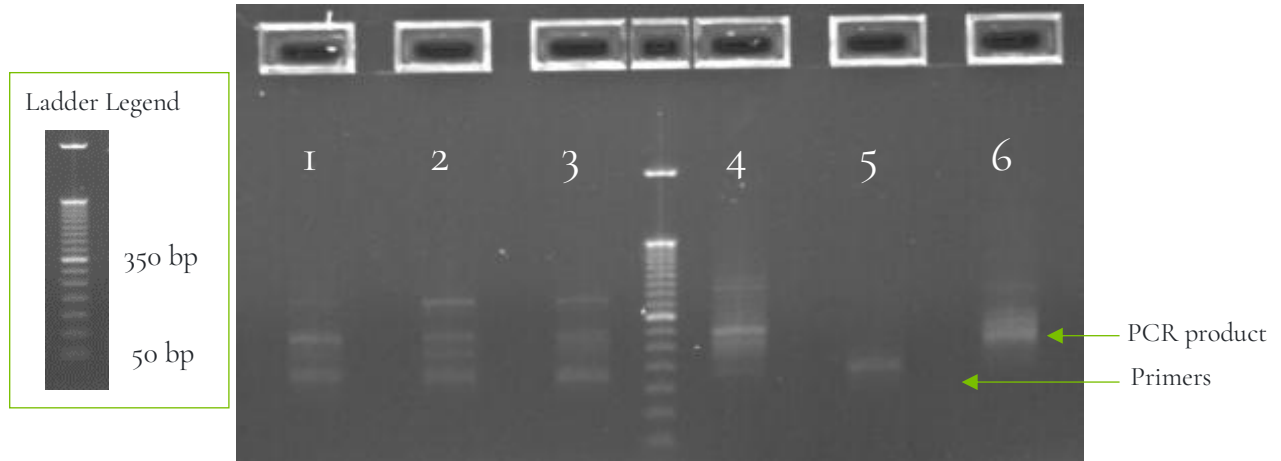
13. **Quantification:** Analyze an aliquot of each library per the instructions in the next section.



Optional Stopping Point: The purified libraries can be stored at 4°C for up to 3 days, or at -20°C for longer-term storage.

Final Library Gel Image

The following image is an example of final libraries after all rounds of PCR and purification on a 2% agarose gel.



Lane 1-2: Fusion negative sample, 25 ng
Lane 3-4,6: Fusion positive samples, 25 ng
Lane 5: No template control (NTC)

Figure 5. Gel analysis of final library material.

8. Quantitation and Normalization of Purified Libraries

For the remainder of the workflow, final DNA and RNA libraries can be processed simultaneously.

Prior to sequencing, libraries must be quantified, normalized, and then pooled together. The following section describes how to quantify libraries using the Qubit system. Other library quantification methods, such as qPCR quantification kits or TapeStation, may be used according to the manufacturer's protocol.

Libraries from the DNA and RNA workflow may be pooled and loaded onto the sequencer together.

After quantification, libraries are normalized and then pooled together. The volume of each normalized library to pool depends on the desired coverage depth for the libraries prepared with each panel.

Qubit Quantitation

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

The following steps should be performed in a post-PCR area.

1. **Prepare Qubit working solution:** Dilute the Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer. Vortex briefly to mix the Qubit working solution.

For example, 2000 μ L is sufficient buffer for 10 readings (8 samples + 2 standards). Combine 1990 μ L of Qubit dsDNA HS buffer and 10 μ L HS reagent.



Important: Fluorescent dyes are sensitive to light. Protect the Qubit working solution from light.

2. **Label tubes:** Set up 0.5 mL Qubit tubes for standards and samples. Label the tube lids.
3. **Prepare standards:** Transfer **190 μ L** of Qubit working solution into two tubes for standard 1 and standard 2, and then add **10 μ L** of each standard to the corresponding tube.



Caution: New standard dilutions should be prepared with the libraries to be quantified. Do not re-use standard dilutions from previous experiments.

4. **Prepare samples:** Transfer **198 μ L** of Qubit working solution to each tube, and then add **2 μ L** of each library to its corresponding Qubit tube (1:100 dilution).
5. **Mix and spin:** Vortex to mix and then centrifuge the tubes briefly.
6. Incubate the tubes at room temperature for 2 minutes.
7. **Measure concentration:** Measure the concentration of each library on the Qubit Fluorometer per the manufacturer's instructions. Use the dsDNA High Sensitivity assay to read standards 1 and 2 followed by the samples.

If any concentration is above the linear range of the instrument, prepare a new dilution by combining 199 μ L Qubit working solution and 1 μ L library (1:200 dilution). Repeat steps 5–7.

8. **Calculate concentration:** 1 ng/μL of library is equal to 5 nM. Example calculation is below. Adjust dilution factor accordingly.

2 μL of library + 198 μL Qubit working solution:

$$\frac{\text{Qubit reading } \left(\frac{\text{ng}}{\text{mL}}\right)}{1,000} \times \text{dilution factor (100)} \times \text{conversion factor (5)} = \text{nM}$$



Optional Stopping Point: Once libraries have been quantified, either proceed with normalization and pooling or keep them stored at 4°C for up to 3 days. Store libraries at -20°C for long-term storage.

Normalization and Pooling

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

The following steps should be performed in a post-PCR area.



If sequencing on the MiniSeq, libraries should be normalized to 1 nM prior to pooling. For all other Illumina sequencing platforms, libraries should be normalized to 5 nM prior to pooling.

1. **Normalize libraries to 5 nM:** Dilute an aliquot (e.g., 4 μL) of each sample library to 5 nM using nuclease-free water or 10 mM Tris-HCl with 0.1% Tween-20, pH 8.5. An example calculation is as follows:


$$\frac{\text{Library concentration (nM)} \times 4 \mu\text{L library}}{5 \text{ nM}} = \text{final volume of library}$$

$$\text{Final volume of library} - 4 \mu\text{L library} = \text{volume of diluent}$$

2. **Mix and spin:** Mix the 5 nM libraries thoroughly by vortexing and centrifuging briefly.
3. **Prepare library pool:** Label two new 1.5 mL microtubes to prepare two library pools, one for each panel.
 - a. *DNA Pool:* Combine equal volumes (e.g., mixing 5 μL of each 5 nM library) of each normalized library for the **Multi-Cancer v4 with CNV** panel
 - b. *RNA Pool:* Combine equal volumes (e.g., mixing 5 μL of each 5 nM library) of each normalized library for the **Multi-Cancer RNA Fusion v2** panel
4. Briefly vortex each library pool for 2 – 5 seconds and then centrifuge.
5. **Combine library pools:** Combine library pools prepared for each panel (step 3) into one final library pool for sequencing. Library pools should be combined according to the volumes listed in the table below.

DNA-RNA Library Mix	
oncoReveal™ Reveal Panel	Volume of library pool (μL)
Multi-Cancer v4 with CNV (DNA)	24.0
Multi-Cancer RNA Fusion v2 (RNA)	2.0
Total	26.0

NOTE: Libraries from the Multi-Cancer RNA Fusion v2 Panel are pooled at a lower volume than the Multi-Cancer v4 with CNV Panel to account for the difference in sequencing depth needed.

6. Briefly vortex each library pool for 2 – 5 seconds and then centrifuge.
 7. **Quantify library pool (recommended):** The libraries prepared using the Multi-Cancer v4 with CNV Panel and Multi-Cancer RNA Fusion v2 cluster very efficiently on the MiSeq. It is recommended that the library pool be quantified using Qubit or another library quantitation method (qPCR) to ensure the pool is at 5 nM ($\pm 10\%$) to prevent poor sequencing performance. If the final dilution is not 5 nM ($\pm 10\%$), adjust the dilution for loading the sequencer accordingly to obtain the desired concentration.
-  **Optional Stopping Point:** The normalized libraries can be stored at 4°C overnight for sequencing the next day. For longer storage, the normalized samples can be stored at -20°C.

9. Sequencing

Prepare Diluted Libraries for Sequencing

Hands-on Time	30 – 40 mins.
Total Time	30 – 40 mins.

The libraries generated using this protocol can be multiplexed and sequenced on Illumina® sequencers. Table 1 outlines the sequencing parameters and the recommended per-sample sequencing coverage.

The number of libraries that can be multiplexed together is dependent on several factors, among them are the estimated throughput of the flow cell and sequencing platform, the desired sequencing depth, as well as the number of unique index combinations available.

The estimated **maximum** number of libraries that can be multiplexed on a single flow cell using each kit is displayed in Table 2. Select the appropriate sequencing kit based on the number of libraries to be sequenced.

Table 1. Sequencing recommendations.

Sequencing Configuration	Index	Recommended Coverage	Recommended Reads Per Library	
2 x 150	Dual (8 bp each)	Mean: 2000X Min: 400X	DNA >1.364 million PE reads	RNA 50,000 PE reads

Table 2. Multiplexing recommendations for Illumina sequencers.

Sequencing Flow Cell	Estimated Instrument Output (million PE reads)	Estimated Maximum Sequencing Batch Size
MiSeq Micro	8	5
MiSeq v2	30	19
MiSeq v3	50	31
NextSeq 500/550 Mid Output	260	165

Calculations assume ≥ 90% effective on-target rate after read mapping and minimum segment coverage > 20% of the mean coverage.

The estimated maximum number of samples in the table above corresponds to the number of samples from each workflow that can be pooled together.

For example: Using the MiSeq Micro (v2), the maximum number of samples that can be loaded onto that flow cell are 3 samples processed through the *Multi-Cancer v4 with CNV* panel workflow **and** 3 samples processed through the *Multi-Cancer RNA Fusion v2* panel workflow.

The following steps should be performed in a post-PCR area.

For this portion of the protocol, prepare a pre-chilled benchtop cooler.

Sequencing Using Illumina MiSeq™ v2 Reagents

MiSeq v2 recommended final library pool loading concentration: 15 pM

MiSeq v2 recommended PhiX spike-in concentration: 12.5 pM

The following steps can be found in greater detail in Illumina's "MiSeq System: Denature & Dilute Libraries Guide" (Doc# 15039740).

1. **Normalize:** Dilute libraries to 5 nM, as demonstrated in the section "Quantification and Normalization of Purified Libraries."
2. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining **800 µL** of nuclease-free water with **200 µL** of 1 N NaOH. Vortex the solution to mix.

NOTE: Alternatively, prepare a 1 N NaOH solution by combining 500 µL of 10 N NaOH with 4.5 mL of nuclease-free water. Vortex the solution to mix. If 1 N NaOH has not been prepared within the last week from a 10 N solution, prepare a new 1 N NaOH solution.
3. **Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.
 - a. Denature the 5 nM library pool by combining **5 µL** of the library pool and **5 µL** of the freshly prepared 0.2 N NaOH.
 - b. Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add **990 µL** of Illumina's HT1 hybridization buffer to the denatured library pool.
 - e. Invert the mixture several times, centrifuge briefly, and place on in the cooler.
4. **Dilute library pool to 15 pM:** Label a new 1.5 mL microtube for the 15 pM library mix. Combine **360 µL** of the 25 pM library pool with **240 µL** of Illumina's HT1 hybridization buffer. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, centrifuge briefly, and place in the cooler.
5. **Combine library pool and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded onto the sequencer. Combine **594 µL** of the 15 pM library pool with **6 µL** of 12.5 pM PhiX library control. Briefly vortex, centrifuge, and place in the cooler.
6. **Load MiSeq cartridge:** Using a clean 1000 µL tip puncture the foil cap above the sample loading well on the MiSeq cartridge. Load the **600 µL** library pool and PhiX mixture into the reagent cartridge, ensuring that the solution has reached the bottom of the well.
7. **Run the MiSeq:** Sequence the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each. See "MiSeq System User Guide" (part #15027617).
8. **Store** diluted libraries and mixtures at -20°C for long-term storage.

Sequencing Using Illumina MiSeq™ v3 Reagents

MiSeq v3 recommended final library pool loading concentration: 25 pM

MiSeq v3 recommended PhiX spike-in concentration: 20 pM

The following steps can be found in greater detail in Illumina's "MiSeq System: Denature & Dilute Libraries Guide" (Doc# 15039740).

1. **Normalize:** Dilute libraries to 5 nM, as demonstrated in the previous section "Quantification and Normalization of Purified Libraries."
2. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining **800 µL** nuclease-free water with **200 µL** of 1 N NaOH. Vortex the solution to mix.
NOTE: Alternatively, prepare a 1 N NaOH solution by combining 500 µL of 10 N NaOH with 4.5 mL of nuclease-free water. Vortex the solution to mix. If 1 N NaOH has not been prepared within the last week from a 10 N solution, prepare a new 1 N NaOH solution.
3. **Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.
 - a. Denature the 5 nM library pool by combining **5 µL** of the library pool and **5 µL** of the freshly prepared 0.2 N NaOH.
 - b. Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add **990 µL** of Illumina's HT1 hybridization buffer to the denatured library pool.
 - e. Invert the mixture several times, centrifuge briefly, and place on in the cooler.
4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded onto the sequencing cartridge. Combine **594 µL** of the 25 pM library pool with **6 µL** of 20 pM PhiX library control. Briefly vortex, centrifuge, and place in the cooler.
5. **Load MiSeq cartridge:** Using a clean 1000 µL tip puncture the foil cap above the sample loading well on the MiSeq cartridge. Load the **600 µL** library pool and PhiX mixture into the cartridge, ensuring that the solution has reached the bottom of the well.
6. **Run the MiSeq:** Sequence the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each. See "MiSeq System User Guide" (part #15027617).
7. **Store** diluted libraries and mixtures at -20°C for long-term storage.

Sequencing Using Illumina's NextSeq™ 500/550 Reagents

Mid-Output recommended library pool concentration: 1.8 pM

Mid-Output recommended PhiX spike-in concentration: 1.8 pM

1. **Normalize:** Dilute libraries to 5 nM, as demonstrated in the previous section "Quantification and Normalization of Purified Libraries."
2. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining **800 µL** of nuclease-free water with **200 µL** of 1 N NaOH. Vortex the solution to mix.

NOTE: Alternatively, prepare a 1 N NaOH solution by combining 500 µL of 10 N NaOH with 4.5 mL of nuclease-free water. Vortex the solution to mix. If 1 N NaOH has not been prepared within the last week from a 10 N solution, prepare a new 1 N NaOH solution.

3. **Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.
 - a. Denature the 5 nM library pool by combining 5 µL of the library pool and 5 µL of the freshly prepared 0.2 N NaOH.
 - b. Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add 5 µL of 200 mM Tris-HCl, pH 7.0.
 - e. Vortex briefly and centrifuge for 1 minute.
 - f. Add 985 µL of Illumina's HT1 hybridization buffer to the denatured library mix.
 - g. Vortex briefly and centrifuge for 1 minute.
4. **Dilute 25 pM library pool to 1.8 pM:** Dilute the denatured library to 1.8 pM in 1400 µL by combining 101 µL of the 25 pM denatured library mix with 1299 µL of Illumina's HT1 hybridization buffer. Invert to mix and centrifuge briefly.
5. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 1287 µL of the 1.8 pM library mix with 13 µL of a 1.8 pM PhiX library control. Briefly vortex, centrifuge, and place in the cooler.
6. **Load NextSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading well on the NextSeq cartridge. Load 1300 µL library mix and PhiX mixture into the cartridge and ensure the solution has reached the bottom of the cartridge well.
7. **Run the NextSeq:** Sequence the libraries on the NextSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each. See "NextSeq System User Guide" (part #15046563 or 15069765).
8. **Store** diluted libraries and mixtures at -20°C for long-term storage.

Preparing a Sample Sheet for Sequencing

TIP: Prepare the sample sheet prior to loading the MiSeq, NextSeq, or MiniSeq cartridge. If an error has been made during the indexing PCR where samples have the same indices, it can be remedied before loading the samples on the sequencer.

The available Pillar indexing primers and their barcode sequences are listed in Appendix A. For the 15 indexing primers, indexing on the NextSeq or the MiniSeq requires the reverse complement of the barcode sequence. The correct barcode sequences for sequencing on the MiSeq and the NextSeq or MiniSeq are provided in Appendix A. Additionally, the Pillar sample sheet generator will automatically populate the correct barcode sequence when the indexing primer is selected.

In Appendix A, note that indexing primers highlighted in yellow have the same barcode sequences as Illumina TruSeq Custom Amplicon (TSCA) indices.

In the Pillar sample sheet generator, prepare a sample sheet that contains the information for the samples that are being loaded. Ensure that the appropriate sample sheet is being made for the MiSeq, NextSeq, or MiniSeq.

Open the Pillar sample sheet generator and enter user input in the shaded cells. Cells that are shaded blue are required and cells that are shaded grey are optional.

1. Enter the "Sample_ID" for each sample. Each Sample_ID must be unique and contain only alphanumeric characters, dashes (-), and underscores (_). All other characters are not allowed. To check that the Sample_ID meets all requirements click "Reset Sample_ID color" and then click "Check Sample_ID".

2. If text is green, the Sample_ID is acceptable. If text is red, Sample_ID is not acceptable. Change Sample_ID accordingly and repeat step 2 until all text is green.
3. Next, enter indices into appropriate fields. Index sequences will be populated once the index_ID is entered.
4. Check that the index combination for each sample is unique. If “Check_index_uniqueness” column is green, then all index combinations are unique. If the column is red for a sample, then the index combination is not unique. Do not load samples together in the same run that have the same index combination.
5. Once all requirements for the sample sheet are met, export the sample sheet as a comma-separated values (.csv) file by clicking “Export”.

10. Troubleshooting

Low Yield of Gene-Specific Product

Potential Cause	Solution
DNA quantity or quality	The recommended input for the assay is 20–60 ng of genomic DNA and 20 – 80 ng for FFPE DNA. Higher quantities may be necessary for low- or poor-quality FFPE samples.
RNA quantity or quality	The recommended minimum input for the assay is 25 ng of FFPE RNA or 20 ng of cfRNA. Higher quantities may be necessary for low- or poor-quality FFPE or cfRNA samples.
Improper cycling	Check that the cycling protocol performed is the appropriate protocol for gene-specific amplification.

Low Indexing Efficiency

Potential Cause	Solution
Improper AMPure purification	Incomplete AMPure purification or loss of gene-specific product will affect the indexing PCR reaction. The purified gene-specific product can be checked on an agarose gel to ensure the product was not lost, and that the clean-up was sufficient to remove excess primers.
	The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and fresh 70% ethanol was used for the wash.
	Leftover ethanol from the wash steps can hinder the PCR reaction. Remove as much of the ethanol as possible during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated.
Partial primer digestion	Poor digestion of the gene-specific primers can hinder the indexing efficiency of the indexing PCR reaction. Check the primer digestion using an agarose gel.
Incomplete deactivation of exonuclease	The inactivation of the nuclease and AMPure purification are necessary steps before performing indexing PCR. Leftover active exonuclease can digest the indexing PCR primers, reducing the yield of the indexing PCR reaction.

Low Yield of Final Library

Potential Cause	Solution
DNA quantity or quality	The recommended input for the assay is 20–60 ng of genomic DNA and 20 – 80 ng for FFPE DNA. Higher quantities may be necessary for low- or poor-quality FFPE samples.
	Run the product from the gene-specific PCR on an agarose gel to check the yield.
RNA quantity or quality	The recommended minimum input for the assay is 25 ng of FFPE RNA or 20 ng of cfRNA. Higher quantities may be necessary for low- or poor-quality FFPE or cfRNA samples.
	Run the product from the gene-specific PCR on an agarose gel to check the yield.
Improper AMPure purification	Incomplete AMPure purification or loss of product will affect the final yield. The purified product can be checked on an agarose gel to ensure that the product was not lost during PCR clean-up.
	The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and fresh 70% ethanol is used for the wash.

Amplicon Contamination in No-Template Control

Potential Cause	Solution
Cross-contamination	Make sure to change tips between samples and avoid waving tips over tubes or plates.
	Poor sealing or residual liquid in tips can cause contamination of nearby samples. If possible, leave adjacent wells empty between samples.
	Workspaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination.
	Periodically clean the workspace, floor, equipment, and instrumentation with a laboratory cleaning solution to break down amplicons on surfaces. Recommended cleaning solutions are 10% bleach, 70% isopropanol, or 70% ethanol.

Sequencing Performance

Potential Cause	Solution
Improper normalization and pooling of libraries	Confirm that the appropriate loading concentration was used for the applicable sequencing instrument.
	Check the 5 nM (or 1 nM if sequencing on the MiniSeq) library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration.
Improper library quantitation	<p>Improper library quantitation may result in artificially high or low yields, which affects downstream normalization.</p> <p>Re-quantify the final libraries and/or the normalized libraries to check for the expected values.</p>
Improper AMPure purification	<p>Changing the ratio of AMPure beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering.</p> <p>The final libraries can be checked on an agarose gel for the proper product size and for the presence of primer dimers.</p>
	The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and that fresh 70% ethanol was used for the wash.

II. Appendix A: Index Sequences

Indexing primers highlighted in yellow have the same barcode sequences as Illumina TruSeq Custom Amplicon (TSCA) indices.

i7 Index Sequences	
Index ID	Index Barcode Sequence
Pi701	ATTACTCG
Pi702	TCCGGAGA
Pi703	CGCTCATT
Pi704	GAGATTCC
Pi705	ATTCAGAA
Pi706	GAATTCGT
Pi707	CTGAAGCT
Pi708	TAATGCGC
Pi709	ATCACGAC
Pi710	ACAGTGGT
Pi711	CAGATCCA
Pi712	ACAAACGG
Pi713	GAAACCCA
Pi714	TGTGACCA
Pi715	AGGGTCAA
Pi716	AGGAGTGG

i5 Index Sequences		
Index ID	Index Barcode Sequence for MiSeq	Index Barcode Sequence for NextSeq and MiniSeq
Pi501	TATAGCCT	AGGCTATA
Pi502	ATAGAGGC	GCCTCTAT
Pi503	CCTATCCT	AGGATAGG
Pi504	GGCTCTGA	TCAGAGCC
Pi505	AGGCGAAG	CTTCGCCT
Pi506	TAATCTTA	TAAGATTA
Pi507	CAGGACGT	ACGTCCTG
Pi508	GTACTGAC	GTCAGTAC
Pi509	TGAACCTT	AAGGTTCA
Pi510	TGCTAAGT	ACTTAGCA
Pi511	TGTTCTCT	AGAGAACA
Pi512	TAAGACAC	GTGTCTTA
Pi513	CTAATCGA	TCGATTAG
Pi514	CTAGAACA	TGTTCTAG
Pi515	TAAGTTCC	GGAACTTA
Pi516	TAGACCTA	TAGGTCTA

12. Appendix B: CNV Analysis using PiVAT®

See the *PiVAT® User Manual (Doc. No. UM-0073)* for detailed instructions on initializing the analysis and understanding the output in PiVAT®.

13. Appendix C: Fusion Caller on PiVAT®

The *oncoReveal® Multi-Cancer RNA Fusion v2* panel detects common fusion transcripts in a simple, multiplex reaction. The output provides likely fusion transcripts by gene and exon pairs. Only fusions described in the product sheet (*Doc. No. MK-0024*) will be reported. Fusions detected which do not match any described in the product sheet, will be filtered from results and recorded in the debug log.

Fusion Sample Setup

Control samples are not required for this panel.

Fusion Analysis Parameters Setup

Navigate to the ① **Secondary Analysis Parameters** page to access the following list of adjustable parameters and default values (in parentheses) by tab.

Tab	QC Parameters [default]
Fusion BWA Alignment	<p>Minimum Distance to Breakpoint (12)</p> <p>-T parameter input into BWA. Sets a different T value when running BWA, which can help get a shorter alignment as a secondary alignment. Decreasing this parameter may increase noise but may help rescue fusion calls for shorter read lengths.</p>
	<p>Fusion Cutoff Threshold (200)</p> <p>Minimum supporting reads for a positive fusion call. Usually far below what would be needed for a positive call due to the normalized fusion count cutoff. May need to be adjusted if the normalized cutoff is significantly decreased.</p>
Fusion Sample Summary	<p>Normalized Fusion Cutoff Threshold (250)</p> <p>Minimum normalized reads for a positive fusion call.</p> <p>NormalizedCount = (Fusion count * Constant) / mapped read count</p> <p>Constant = 10,000</p> <p>Decreasing this value increases sensitivity but may result in more false positive calls. Increasing this value will decrease sensitivity, resulting in possible false negative calls.</p>
	<p>Total Read Count Threshold (100,000)</p> <p>Samples with less total reads than this parameter will fail PiVAT's QC. Decrease this value if sample input is low. May result in poor quality samples with fusion calls.</p>
	<p>Internal Control Threshold (1,500)</p> <p>The sum of the counts for TBP HBMS in the IMBALANCE RATIO sheet. Samples with less than 1500 reads for both targets combined will fail PiVAT's QC.</p>

PIVAT Output: CUSTOMER_RESULTS File

Definitions and/or descriptions of results columns reported in REPORT_FUSION file sheets are provided below.

Sheet Tab	Column/Row Name	Definition/Description
Fusions	SAMPLE_NAME	Name of sample
	FusionName	Fusion ID = LeftGene(LeftExon):RightGene(RightExon)
	Count	Number of reads traversing fusion breakpoint
	NormalizedCount	Count / MappedReads * Constant
	HGVS Notation	Fusion ID in HGVS Nomenclature
	Breakpoint	Fusion breakpoint LeftPosition, RightPosition
	LeftStrand	Orientation of left gene. 1 positive strand, -1 negative strand
	RightStrand	Orientation of right gene. 1 positive strand, -1 negative strand
IMBALANCE RATIO	SAMPLE_NAME	Name of sample
	Gene	Target gene for balance reads
	Type	Internal control or 3'/5' comparison
	Count	Number of reads on left Number of reads on right
	Ratio	Imbalance score = (3' balance reads - 5' balance reads) / reads in control gene
		“Positive” or “Negative” or “No Call” for evidence of fusion
	Detection	<i>NOTE: This is not factored into the Fusions sheet. If positive calls exist, this provides support for a fusion event with this gene. The partner should be in the fusion sheet if it was included in the panel's targets.</i>
RUN STATS	Target_Name	Name of left target Name of right target
	Read Count	The total raw reads in the sample's fastq files
	Assembled	Total count of forward and reverse reads that were paired and merged during paired end assembly. Each pair counts as a value of 2.
	% Assembled	Percentage of Assembled reads out of Read Count : <ul style="list-style-type: none"> (Assembled / Read Count) * 100
	Mapped Reads	Total number of reads that were successfully aligned by BWA.
	Mapped Reads (%)	Percentage of Mapped Reads out of Total Reads Count : <ul style="list-style-type: none"> (Mapped Reads / Read Count) * 100
		Reads that have a secondary alignment are considered fusion reads because they are candidates for a junction point between the primary and secondary alignment.
	Fusion Reads	These reads are counted as the following: <ul style="list-style-type: none"> Assembled Reads: If there is a secondary alignment, this counts as a value of 2. Paired End Reads: Each read with a secondary alignment will count as a value of 1.
	FILTERED: Single Side Fusions With Unassembled paired end reads where the forward read detected a different junction point than the reverse read.	

Sheet Tab	Column/Row Name	Definition/Description
	Mate Aligned On Different Gene	
	FILTERED: Dimers	Reads filtered because fusion was detected to be a dimer.
	FILTERED: Misprimings	Reads filtered because the aligned read was detected to be a mispriming.
	Total Valid Fusion Sequences	Total number of reads that cover a junction after filtering.
	Balance Reads	The total number of balance reads.
	FILTERED: Unique Reads Mapped Off Target	All reads that are aligned to untargeted coordinates.
	Total Valid Balance Sequences	Total number of balance reads that are not filtered. Reads are filtered if they are not on targeted regions.
	Total Reads After Filtering	Total reads that were not filtered
	% Total Unfiltered Reads	Percentage of Total Unfiltered Reads out of Total Reads Count . (Total Unfiltered Reads / Total Reads Count) * 100
QC FILTERED SAMPLES	Reason	Reason sample did not pass QC thresholds.