Leaders in Decision Medicine™

oncoReveal™ Fusion LBx

USER MANUAL

UM-0078 version 1.0

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Revision History

Version 1.0: Initial Release

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1. Product Introduction

The **oncoReveal™ Fusion LBx Panel** is a robust NGS assay that interrogates multiple gene rearrangement regions across multiple solid tumor cancer types. The panel can detect fusion transcripts from cell-free total circulating nucleic acid (cfTNA) that has been extracted from plasma. Additionally, this panel can be used to detect exon 14 skipping in *MET* and contains two housekeeping genes as internal controls.

Available as a complementary assay, the *oncoReveal™ Core LBx Panel (Part No.: HLA-HS-1004-24)* is designed for the detection of somatic mutations from cell-free DNA (cfDNA) extracted from plasma. The oncoReveal™ Fusion LBx Panel in conjunction with the oncoReveal™ Core LBx Panel contain numerous gene regions of interest that allow for the detection of fusions (cfRNA) and the detection of somatic mutations (cfDNA) at very low frequencies. For more information on the panel specifications for each assay see the oncoReveal™ Fusion LBx product sheet *(Doc. No.: MK-0049)* and the oncoReveal™ Core LBx product sheet (*Doc. No.: MK-0044*).

2. Product Description

The oncoReveal™ Fusion LBx Panel utilizes our SLIMamp® (stem-loop inhibition mediated amplification) technology allowing researchers to amplify regions of interest in a simple, single-tube multiplex PCR reaction.

Primer and Amplicon Design
Gene-specific primers were designed for each major breakpoint between each driver and its partners.

Library Preparation
Using cDNA as input, transcripts of the driver genes are targeted in the first round of PCR. 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 products are purified via size selection, after which a second round of PCR adds unique dual barcode index adaptors for sample tracking and sequencing. The final libraries are further purified and can be sequenced on the Illumina sequencing platform.

The oncoReveal™ Fusion LBx Panel contains sufficient reagents to prepare 24 libraries. Use of this panel requires a unique dual barcode indexing kit (available separately; see section 4.2) and the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of at least 121 bp (2 × 121). The workflow of this panel can be processed in parallel with the oncoReveal™ Core LBx Panel workflow once cDNA synthesis is completed, allowing interrogation of both cfDNA and cfRNA derived from cfTNA extracted from a single sample. The libraries can be loaded onto the sequencing instrument within one day, and the protocols contain numerous stopping points for uses who have time limitations.

Note that as the average amplicon size is 80 bp, sequencing greater than 121 bp may lead to lower read quality scores. However, sequencing using read lengths longer than 121 bp does not affect downstream data analysis performed using PiVAT, Pillar Biosciences' proprietary secondary analysis pipeline.

3. Guidelines

3.1 cfTNA Sample Preparation and QC Guidelines

- **Blood collection:** This panel was developed using Streck Cell-Free DNA BCT® tubes *(Cat. No. 230469, 230470, 230471)*. Plasma should be isolated according to the manufacturer's guidelines. Plasma can be isolated up to 7 days after blood draw when the tubes are stored between 18°C to 25°C. Streck Cell-Free DNA BCT tubes have a capacity of 10 mL. Isolation of plasma from a full blood draw will typically produce between 4 and 5 mL; however, yields may vary due to differences between individual donors. Performance characteristics of other collection tube types, such as K_2EDTA, have not been evaluated with this assay.

- **cfTNA extraction:** The oncoReveal™ Fusion LBx panel was developed and extensively tested using the Qiagen QIAamp Circulating Nucleic Acid Kit *(Cat. No. 55114)* to extract cfTNA. Other extraction methods may be used, however their performance has not been assessed and results may vary.

- **Input volume:** The QIAamp Circulating Nucleic Acid Kit is compatible with sample volumes from 1 mL to 5 mL. To increase cfTNA yield, extraction from either 4 mL or 5 mL of plasma is recommended.

- **Elution volume:** The elution volume of the QIAamp Circulating Nucleic Acid Kit is 20 µL to 150 µL. An elution volume of 30 µL is recommended to increase cfDNA concentration.

- **Cross-contamination:** The oncoReveal™ Fusion LBx panel is extremely sensitive. As such, it is crucial that care must be taken during all steps of sample preparation to avoid inaccuracies in downstream results arising from pre-analytical errors. Consult section 3.2 for more details.

- **Quantification:** The cfRNA concentrations in plasma are low and its properties are not compatible with the dye in the Qubit RNA HS assay. Therefore, it is difficult to reliably quantify the cfRNA concentration in a cfTNA sample. For the purposes of this workflow, the cfTNA concentration, as measured using the Invitrogen Qubit dsDNA High Sensitivity Assay kit (*Thermo Fisher Scientific, Cat. No. Q32851, Q32854),* is used as the working value.

3.2 General Laboratory Guidelines for RNA Preparation

Due to the prevalence of ribonucleases (RNase enzymes) in the environment, RNA should be handled with care to avoid sample degradation.

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

- **Handling:** RNA is susceptible to degradation. When handing RNA, all components should be kept on ice and repeated freeze/thaw cycles should be avoided. Gloves should be worn to touch equipment used to process RNA samples.

- **Work areas:** 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).

- **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 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 the surrounding 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 & 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™ Fusion LBx Panel *Part No.: HLA-HS-1005-24*

Reagent	Use	Area Use	Storage
PCR HiFi 4x Master Mix	Gene-Specific PCR and Indexing PCR	Pre-PCR	-25°C to -15°C
oncoReveal™ Fusion LBx Oligo Pool	Gene-Specific PCR	Pre-PCR	-25°C to -15°C

4.2 Liquid Biopsy (LBx) Unique Dual Indexing kits

Reagent	Part Number	Use	Area Use	Storage
Pillar Biosciences LBx Indexing Kit A (24 combinations, 96 reactions)	IDX-PI-1013-96	Indexing PCR	Pre-PCR	-25°C to -15°C
Pillar Biosciences LBx Indexing Kit B (24 combinations, 96 reactions)	IDX-PI-1014-96	Indexing PCR	Pre-PCR	-25°C to -15°C

Only one index kit is needed per assay. Multiple options are available to meet various throughput needs. Kits A and B can be combined for a total of 48 unique dual index combinations.

4.3 User-Supplied Reagents

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
Negative control cfDNA	Pre-PCR	Refer to Section **Error! Reference s ource not found.** of this manual
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
Agarose gel, 2% (optional) [1]	Post-PCR	General lab supplier
DNA molecular weight markers (optional) [1]	Post-PCR	General lab supplier
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	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 an agarose gel or using the TapeStation DNA Analysis kit.*

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

‡ Indicates the flowcells the oncoReveal™ Fusion LBx Panel has been validated on. However, libraries generated using this protocol are compatible with all Illumina sequencers.

General Laboratory Consumables

In addition to the consumables listed below other general laboratory supplies needed to carry out the protocol include gloves, ice buckets, 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	Fisher Scientific, Cat# 14-222-334 or equivalent
Microplate sealing film	Pre- and Post-PCR	Fisher Scientific, Cat# 14-222-347 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

4.4 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
TapeStation or equivalent (optional)*	Post-PCR	Agilent Technologies
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 an agarose gel.*

5. Workflow

The following chart demonstrates the workflow for performing the oncoReveal™ Fusion LBx Panel library preparation.

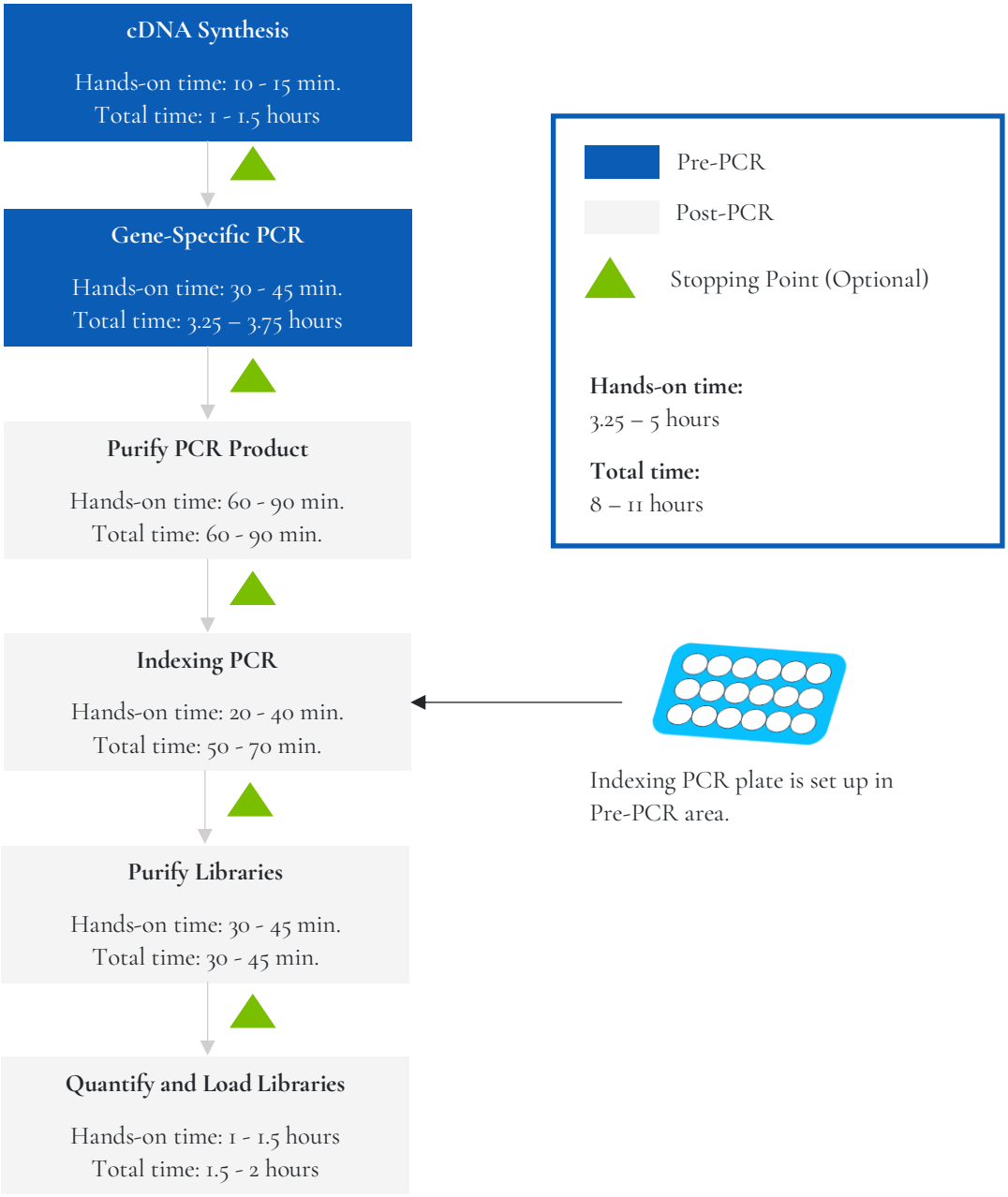

Figure 1. Library preparation workflow for oncoReveal™ Fusion LBx Panel. The workflow can be completed within a day but contains multiple optional stopping points for users with time constraints.

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. Library Preparation Protocol

Hands-on Time	3.25 – 5 hours
Total Time	8 – 11 hours

6.1 cDNA Synthesis

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

Before performing the library preparation, prepare cDNA from cfTNA extracted from plasma.

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

Up to **20 µL** of undiluted cDNA reaction can be added to the Gene-Specific PCR without inhibiting the reaction. Alternatively, with a higher cfTNA input, the cDNA reaction can be diluted with low TE or nuclease-free water.

The recommended input is 10 – 30 ng cfTNA (as measured by concentration of cfDNA from Qubit dsDNA high sensitivity assay kit).

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

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

cDNA Synthesis Example Reaction: *20 µL cDNA reaction mix*

Set up the reaction on ice and keep all components chilled.

1. **Add cfTNA:** Add **16 µL** of cfTNA (diluted if necessary) to each sample well in a PCR plate, strip tube, or PCR tube. Add nuclease-free water to the no-template control well.

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

cDNA Synthesis Mix	
Reagent	**Volume (µL)**
cDNA Master Mix	4.0
cfTNA (or water)	16.0
Total	**20.0**

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

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

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.

6.2 Gene-Specific Target Amplification (GS-PCR) & Target Amplicon Purification

Hands-on Time	30 – 45 min.
Total Time	3.25 – 3.75 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 cooler and keep the PCR HiFi 4x Master Mix and the custom oligo pool in the cooler until needed.

1. **Prepare Gene-Specific PCR Mix 1 (GS-PCR Mix 1):** Vortex and centrifuge the PCR HiFi 4x Master Mix and the oligo pool before use. For each PCR reaction, the volume of each component is listed below.

Important: The gene-specific PCR master mix reagent 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)**
PCR HiFi 4x Master Mix	12.5
Fusion LBx Oligo Pool	5.0
Nuclease-free water	12.5
Subtotal	**30.0**

2. **Transfer:** Transfer **30 µL** of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.

3. **Dilute input DNA**: Add **20 µL** of DNA (diluted if necessary) to each sample well containing GS-PCR Mix. Add **20 µL** of nuclease-free water to the no-template control well.

   If using the entire cDNA reaction (**20 µL** reaction), the PCR master mix and supplementing water may be added directly to the PCR plate. The reactions may proceed directly to GS-PCR.

Final GS-PCR Mix	
Reagent	**Volume (µL)**
GS-PCR Mix 1	30.0
DNA (or water)	20.0
Total	**50.0**

4. **Seal and mix:** Carefully seal the reactions and vortex for 10–15 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.

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

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

GS-PCR Program		
Temperature	**Time**	**Number of Cycles**
95°C	15 sec	1
95°C	1 min	10
58°C	2 min	
60°C	4 min	
64°C	1 min	
72°C	1 min	
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 gene-specific PCR reactions may be stored at -20°C after cycling is complete.

Purify the Gene-Specific PCR Product

Hands-on Time	60 – 90 min.
Total Time	60 – 90 min.

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

Before beginning the purification:

- Remove the 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

1. If condensation has formed or if reactions were stored at -20°C briefly centrifuge the reactions upon thawing. Carefully remove the seal.

2. **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.

3. **Add beads:** Add **75 µL** of AMPure beads (equivalent to a 1.5x 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.

4. **Bind GS-PCR product to beads:** Incubate for 5 minutes at room temperature.

5. **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.

6. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.

7. **Resuspend beads:** Remove the PCR plate from the magnetic rack and resuspend the beads in each well using **53 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.

8. **Incubate:** Incubate for 5 minutes at room temperature.

9. **Separate GS-PCR product from beads:** Place the PCR plate on a magnetic rack at room temperature until the solution appears clear. Transfer **50 µL** of clear supernatant from each well of the PCR plate to a new PCR plate.

10. **Add beads:** With the eluted product from step 9, repeat steps 3-6 for a second round of purification.

11. **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.

12. **Second wash:** Repeat step 11 for a second 70% ethanol wash. Remove the supernatant from each well.

13. **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 the remaining ethanol solution at the bottom of the wells.

14. **Dry beads:** Keep the PCR plate on the magnetic rack and 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 among the entire bead ring or they are flaky, they are over-dried. Beads that are too dry may be difficult to resuspend.

15. **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 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 PCR HiFi 4x Master Mix. The PCR HiFi 4x 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.*

16. **Incubate and Elute:** Incubate for 5 minutes at room temperature.

17. **Separate GS-PCR product from beads:** Place the PCR plate on a magnetic rack at room temperature until the solution appears clear. Transfer **30 µL** of clear supernatant from each well of the PCR plate to a new PCR plate.

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

6.3 Index PCR & Purification

Indexing PCR

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 cooler. The PCR HiFi 4x Master Mix should be kept in the cooler until it is needed.

1. **Prepare Indexing PCR Mix 1:** Briefly vortex and centrifuge the PCR HiFi 4x Master Mix before use. To prepare Indexing PCR Mix 1 combine the HiFi Master Mix and water with sufficient overage.

Indexing PCR Mix 1 (per reaction)	
Reagent	**Volume (µL)**
PCR HiFi 4x Master Mix	12.5
Nuclease-free water	23.5
Subtotal	**36.0**

2. Transfer **36 µL** of the Indexing PCR Mix 1 to each sample well of a PCR plate.
3. Transfer **8uL** of unique, pre-mixed Index Mix P5xx & P7xx to each sample well of the PCR plate. Ensure no well-to-well cross-contamination during indexing primer transfer.

Indexing PCR Mix 2 (per reaction)	
Reagent	**Volume (µL)**
Indexing PCR Mix 1	36.0
Index Mix P5xx & P7xx	8.0
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 GS-PCR products were stored at -20°C after the GS-PCR purification, ensure that the samples have been thawed at room temperature thoroughly before proceeding.

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

Indexing 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 66°C 72°C	30 sec 30 sec 1 min	18*
72°C	5 min	1
8°C	Hold	1

* *Additional Indexing PCR cycles can be performed if final library yield is low or if initial DNA 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 samples will be stored at -20°C after the program is complete.

- 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 to remove any droplets from the side walls. 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.

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 **60 µL** 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 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. 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 among 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** of nuclease-free water. Gently pipette the bead 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 of the PCR plate 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.

6.4 Quantitation and Normalization of Purified Libraries

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 Bioanalyzer, may be used according to the manufacturer's protocol.

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 µL is sufficient buffer for 10 readings (8 samples + 2 standards). Combine 1990 µL of Qubit dsDNA HS buffer and 10 µL HS reagent. Add reagent overage appropriately.

   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 µL** of Qubit working solution into two tubes for standard 1 and standard 2, and then add **10 µL** 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 µL** of Qubit working solution to each 0.5 mL tube, and then add **2 µL** 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 libraries.

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

8. **Calculate concentration**: 1 ng/µL of library is equal to **6 nM**. Example calculation is below. Adjust dilution factor accordingly.

   2 µL of library + 198 µL Qubit working solution:

$$\frac{Qubit\ reading\ \left(\frac{ng}{mL}\right)}{1{,}000}\ x\ dilution\ factor\ (100) x\ conversion\ factor\ (6) = nM$$

Optional Stopping Point: Once libraries have been quantified either proceed with normalization and pooling or else 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.

Important: It is likely that the final library concentration of the normal samples with lower input mass will be less than 5 nM. In this case, skip the normalization step and proceed to pooling (step 3 below) with undiluted libraries.

Libraries from samples with higher input mass or from samples with positive fusion variants are expected to have a yield above 5 nM. For these libraries, proceed from step 1 below to normalize each to 5 nM.

1. **Normalize libraries to 5 nM (only applicable to libraries with a concentration greater than 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. For NTCs, use the same dilution scheme as the library with the lowest measured concentration. An example calculation is as follows:

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

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

2. **Mix and spin:** Mix the 5 nM libraries thoroughly by vortexing and then centrifuge briefly.

3. **Prepare library pool:** Label a new 1.5 mL microtube for the library pool. Prepare a mixture of libraries by combining each library at equal volume (e.g., mixing 5 µL of each 5 nM library or 5uL of undiluted libraries with concentrations less than 5 nM). Gently pipette the entire solution up and down 10 times to mix thoroughly. Quickly vortex the pool and then briefly centrifuge.

4. **Quantify library pool (recommended):** 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 (± 10%) to prevent poor sequencing performance. If the final dilution is not 5 nM (±10%), adjust the dilution for loading the sequencer accordingly to obtain the desired concentration.

If the Fusion LBx library pools are sequenced alone (i.e., not sequenced alongside libraries generated from the oncoReveal™ Core LBx workflow) with concentrations well below 5 nM, the final concentration of the libraries loaded on the sequencing flow cell might not be reached, in which case a lower cluster density would be expected.

Optional Stopping Point: The normalized libraries can be stored at 4°C overnight for sequencing the next day. For longer storage, the normalized libraries can be stored at -20°C.

7. Sequencing

Prepare Diluted Libraries for Sequencing

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

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 samples that can be multiplexed together is dependent on several factors, among which are the estimated throughput of the flowcell and sequencing platform, the desired sequencing depth, as well as the number of unique index combinations available.

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

The recommended reads per sample and the estimated instrument output reported in Table 1 and Table 2 respectively are equivalent to the number of sequencing clusters multiplied by 2.

Caution: Fusion LBx libraries sequenced alone must be combined with at least 40% PhiX.

Sequencing Configuration	Index	Recommended Reads Per Sample
2 × 121	**Unique Dual Indices** (8 bp each)	200,000 PE reads

Table 1. Sequencing recommendations.

Sequencing Flowcell	Estimated Instrument Output (million PE reads)	Estimated Maximum Sequencing Batch Size
MiSeq Nano	2	6
MiSeq Micro	8	24

Table 2. Multiplexing recommendations for Illumina sequencers. Calculations assume 60% flowcell usage and ideal cluster density if sequencing Fusion LBx libraries alone.

It is recommended that Fusion LBx libraries are sequenced with libraries generated using Pillar's oncoReveal™ Core LBx Panel *(Part No. HLA-HS-1004-24)*, **and the fusion libraries should make up 1% of the sequencing loading percentage:**

- Given an equal number of Core LBx libraries and Fusion LBx libraries, all of which are normalized to 5 nM, combine the libraries at a volume ratio of 99:1 (Core LBx libraries:Fusion LBx libraries).

For combined sequencing with oncoReveal™ Core LBx libraries, see Table 3 below and refer to the oncoReveal™ Core LBx Panel User Manual *(Doc. No.: UM-0075)*, section 7 for multiplexing and sequencing information.

Sequencing Flowcell	Estimated Instrument Output (million PE Reads)	Estimated Maximum Sequencing Sample Size	
		oncoReveal™ Core LBx	oncoReveal™ Fusion LBx
NextSeq 500/550 Mid Output	260	7	7
NextSeq 500/550 High Output	800	24	24

Table 3. Multiplexing recommendations for combined loading of oncoReveal™ Fusion LBx libraries with oncoReveal™ Core LBx libraries. Calculations presume Core LBx libraries were prepared with out-of-batch normal.

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

For this portion of the protocol have a pre-chilled benchtop cooler prepared.

Sequencing Using Illumina's MiSeq™ Reagents

MiSeq recommended final library pool loading concentration: 12.5 pM
MiSeq 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 "*Quantitation 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 in the cooler.

4. **Dilute library pool to 12.5 pM:** Label a new 1.5 mL microtube for the 12.5 pM library mix. Combine **300 µL** of the 25 pM library pool with **300 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 **360 µL** of the 12.5 pM library pool with **240 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 121 (2 × 121) 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.

Preparing a Sample Sheet for Sequencing

TIP: *Prepare the sample sheet prior to loading the sequencing reagent 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. The Pillar sample sheet generator will automatically populate the correct barcode sequence when the indexing primer is selected.

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 sequencing instrument used.

1. 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.

2. Enter the "Sample_ID" for each sample. Each Sample_ID must be unique and contain only alphanumeric characters, dashes (-), and underscores (_). No other characters are allowed. To check that the Sample_ID meets all requirements click "Reset Sample_ID color" and then click "Check Sample_ID".

3. 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.

4. Next, enter indices into appropriate fields. Index sequences will be populated once the index_ID is entered.

5. Library preparation using the oncoReveal™ Fusion LBx panel requires the use of unique dual barcodes. Using Pillar Bioscience LBx Indexing Kit A and LBx Indexing Kit B a maximum of 48 index combinations are available. If sequencing more than 48 libraries, do not load samples together in the same run that have the same index combination.

6. Once all requirements for the sample sheet are met, export the sample sheet as a comma-separated values (.csv) file by clicking "Export".

7. The recommended read length for oncoReveal™ Fusion LBx libraries is at least 2×121 bp. Open the .csv file and input the read length that will be used during the sequencing run.

8. Troubleshooting Library Preparation

Low Yield of Gene-Specific Product

Potential Cause	Solution
cfTNA quantity or quality	The recommended input for the assay is 10–30 ng of cfTNA. Higher quantities may be necessary for low- or poor-quality 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 during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated.

Low Yield of Final Library

Potential Cause	Solution
cfTNA quantity or quality	The recommended input for the assay is 10–30 ng of cfTNA. Higher quantities may be necessary for low- or poor-quality 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 was 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 library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration.
Improper library quantitation	Improper library quantitation may result in artificially high or low yields, which affects downstream normalization. Re-quantitate the final libraries and/or the normalized libraries to check for the expected values.

Improper AMPure purification	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. The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.
	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.

9. Appendix A: Index Sequences

i7 Index Sequences	
Index ID	Index Barcode Sequence for NextSeq Sample Sheet
Pi713	GAAACCCA
Pi714	TGTGACCA
Pi715	AGGGTCAA
Pi716	AGGAGTGG
Pi717	ACGTTACC
Pi718	CTGTGTTG
Pi719	TGAGGTGT
Pi720	GATCCATG
Pi721	GCCTATCA
Pi722	AACAACCG
Pi723	ACTCGTTG
Pi724	CCTATGGT
Pi725	TGTACACC
Pi726	GTATGCTG
Pi727	TGATGTCC
Pi728	GTCCTTCT
Pi729	ATAAGGCG
Pi730	CTTACCTG
Pi731	CGTTGCAA
Pi732	GATTCAGC
Pi733	TCACGTTC
Pi734	TGTGCGTT
Pi735	TAGTTGCG
Pi736	ACAGCTCA
Pi737	GTTAAGGC
Pi738	AAGCCACA
Pi739	ACACGGTT
Pi740	CAGCGATT
Pi741	TAGTGACC
Pi742	CGAGACTA
Pi743	GACATGGT
Pi744	GCATGTCT
Pi745	ACTCCATC
Pi746	CGAAGAAC
Pi747	GGTGTCTT
Pi748	AAGAAGGC
Pi749	CATGTTCC
Pi750	GTGCCATA

i7 Index Sequences	
Index ID	**Index Barcode Sequence for NextSeq Sample Sheet**
Pi751	CCTTGTAG
Pi752	GCTGGATT
Pi753	TAACGAGG
Pi754	ATGGTTGC
Pi755	CCTATACC
Pi756	TTAGGTCG
Pi757	GCAAGATC
Pi758	AGAGCCTT
Pi759	GCAATGGA
Pi760	CTGGAGTA

i5 Index Sequences	
Index ID	**Index Barcode Sequence for NextSeq Sample Sheet**
Pi513	TCGATTAG
Pi514	TGTTCTAG
Pi515	GGAACTTA
Pi516	TAGGTCTA
Pi517	TTGTCGGT
Pi518	TTGCCACT
Pi519	AGTCTGTG
Pi520	AAGTGTCG
Pi521	CACAAGTC
Pi522	AGTCTCAC
Pi523	CATGGAAC
Pi524	CTCAGCTA
Pi525	TTGCGAAG
Pi526	CATACCAC
Pi527	CTACAGTG
Pi528	TAGCGTCT
Pi529	TGGAGTTG
Pi530	AGCGTGTT
Pi531	ACCATCCA
Pi532	GCTTCGAA
Pi533	GTGGTGTT
Pi534	ACAGCTCA
Pi535	TTCCTGTG
Pi536	GGTTGTCA
Pi537	TCTCTAGG
Pi538	CGTTATGC
Pi539	AAGCACTG
Pi540	GAGATACG
Pi541	TCTTGACG
Pi542	GTTCATGG
Pi543	GAAGTACC
Pi544	ATAGCGGT
Pi545	ACCTGGAA
Pi546	AGGTTCGA
Pi547	TGGCACTA
Pi548	TTCGTACC
Pi549	CGATGCTT
Pi550	GTATTGGC
Pi551	GCATACAG
Pi552	GTCCTAAG
Pi553	TGGCATGT
Pi554	ACTCCATC
Pi555	ATCGATCG
Pi556	CTGGAGTA
Pi557	TGGTAGCT

i5 Index Sequences	
Index ID	**Index Barcode Sequence for NextSeq Sample Sheet**
Pi558	CTTGTCGA
Pi559	CGGTCATA
Pi560	AGTTGGCT

10. Appendix B: Fusion Caller Using PiVAT®

The *oncoReveal™ Fusion LBx* 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-0022)* for a given panel will be reported by that panel. See the *PiVAT User Manual (Doc. No. UM-0073)* for detailed instructions on initializing the analysis & understanding the output in PiVAT®.