Leaders in Decision Medicine™

oncoReveal® Heme Fusion

USER MANUAL

UM-0084 version 1.0

For Research Use Only. Not for use in diagnostic procedures.

Revision History

Version 1.0	Initial release

Table of Contents

1. Product Introduction

The **oncoReveal® Heme Fusion Panel** is a robust NGS assay that interrogates fusion events in hematological malignancies. For more information on the panel specifications see the oncoReveal® Heme Fusion Panel product sheet *(Doc. No.: MK-0057)*.

Available to be run in parallel, the oncoReveal® Myeloid Panel (*Part No.: HDA-MY-1001-24*) and oncoReveal® Lymphoid Panel (*Part No.: HDA-LM-1001-24*) are designed for the detection of somatic mutations in DNA from myeloid and lymphoid cancers. For more information on the panel specifications for each assay, see the oncoReveal® Myeloid product sheet (*Doc. No.: MK-0007*) and the oncoReveal® Lymphoid product sheet (*Doc. No.: MK-0056*).

2. Product Description

The oncoReveal® Heme Fusion Panel utilizes our proprietary 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, the chimeric transcripts 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 primers undergo digestion, and the products are purified via size selection. This is followed by a second round of PCR that 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 an indexing kit, available in low or high throughput options; the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of 150 (2×150). The workflow for this panel can be completed within 11 hours. The protocol contains numerous stopping points for users who have time limitations.

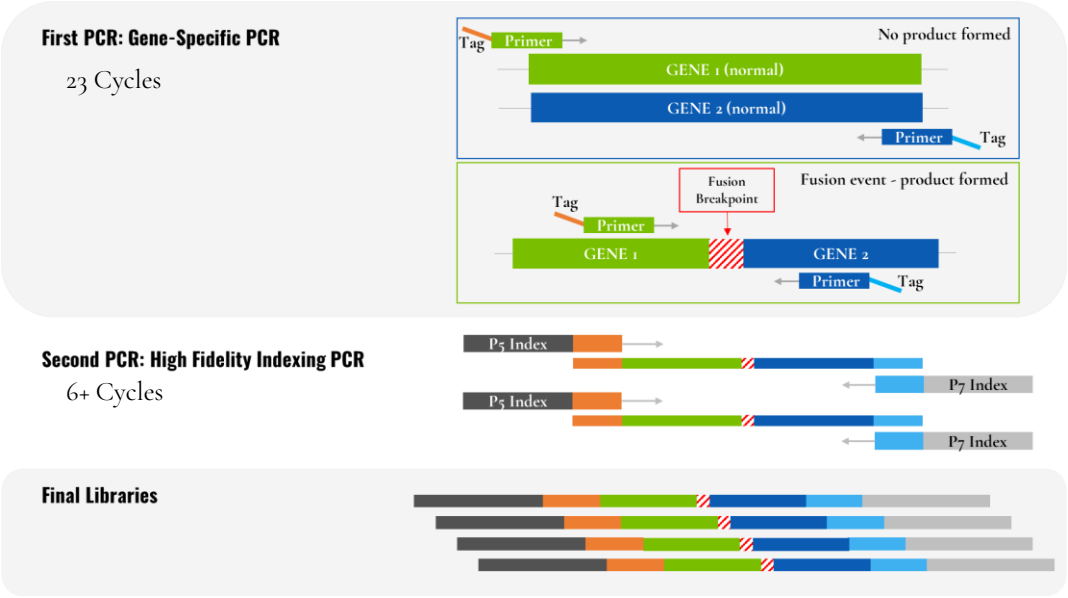

Figure 1. Overview of the oncoReveal® Heme Fusion Panel workflow library preparation using cDNA for fusion detection.

3. 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 when handling 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® Heme Fusion Panel *Part No.: HRA-HM-1001-24*

Reagent	Use	Area Use	Storage
Gene-Specific PCR 2x Master Mix	Gene-Specific PCR	Pre-PCR	-25° to -15°C
oncoReveal® Heme Fusion 5x Oligo Pool	Gene-Specific PCR	Pre-PCR	-25° to -15°C
Exonuclease I	Gene-Specific PCR	Pre-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# 11755050
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 RNA High Sensitivity Assay kit	Post-PCR	Invitrogen, Cat# Q32852 or Q32855
Agarose gel, 2% (optional) [1]	Post-PCR	General lab supplier
DNA molecular weight markers (optional)[1]	Post-PCR	General lab supplier
TapeStation or equivalent	Post-PCR	Agilent Technologies, Cat # 5067-5584 and 5067-5585
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) [2]	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 TapeStation (or equivalent).

[2]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

‡ *Indicates the flowcells the oncoReveal® Heme Fusion 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, pre-chilled 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	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

4.5 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
TapeStation or equivalent*	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 either an agarose gel or TapeStation (or equivalent).*

5. Workflow

The following chart demonstrates the workflow for performing the oncoReveal® Heme Fusion Panel library preparation from blood RNA.

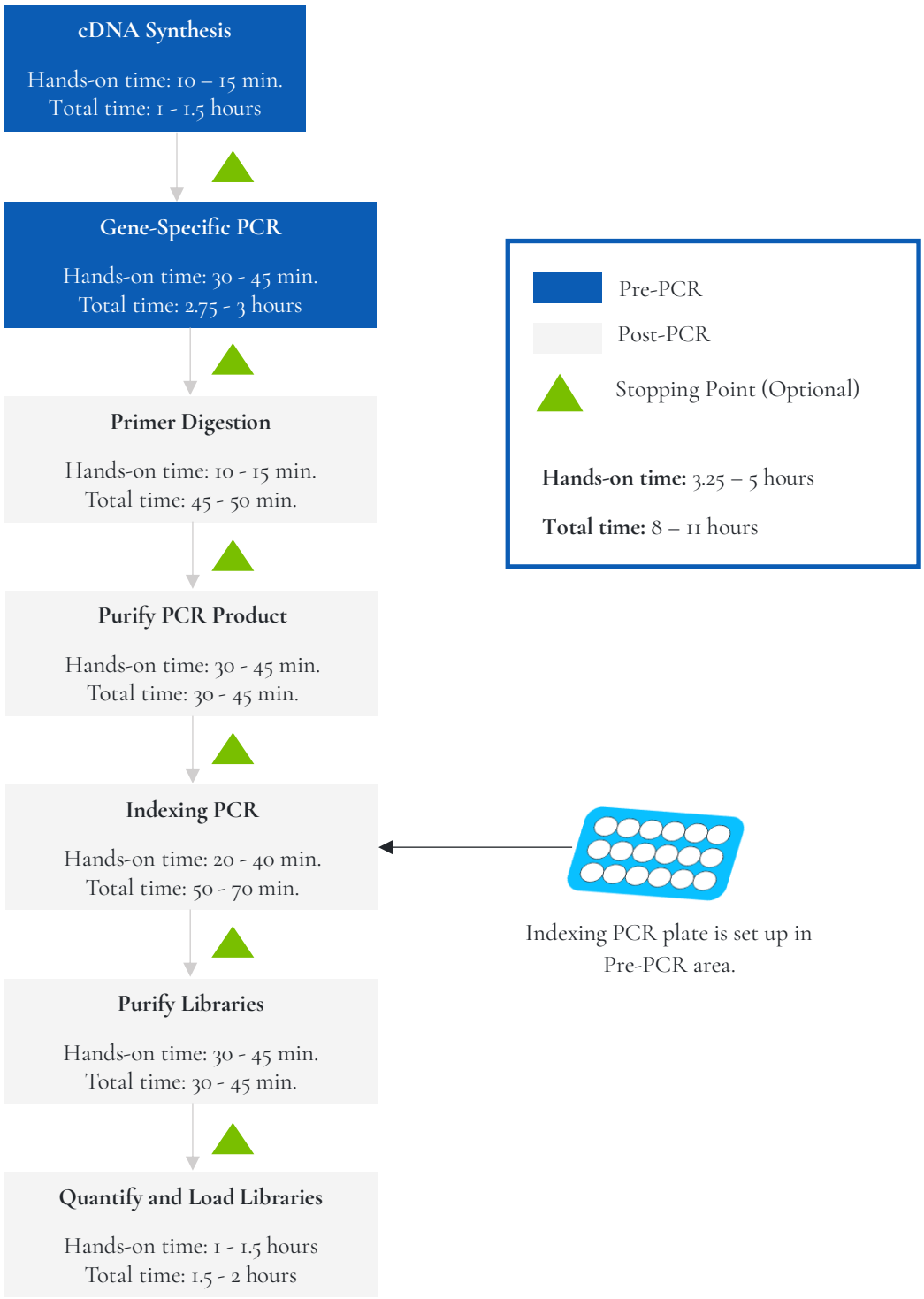

Figure 2. Library preparation workflow for oncoReveal® Heme Fusion Panel. This workflow contains multiple optional stopping points for users with time constraints.

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. 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 total RNA extracted from blood samples or cell lines. For the preparation from RNA, 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 **7.5 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.

Recommended input per sample:

- **Blood RNA: minimum 40ng**

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

cDNA Synthesis Example Reaction: *10 µL cDNA reaction mix*

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

1. **Add RNA:** Add **8 µL** (for a 10 µL reaction) of RNA* (diluted if necessary) 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 RNA and add cDNA Master Mix to the well containing the no-template control.

Example: cDNA Synthesis Mix	
Reagent	**Volume (µL) (10 µL reaction)**
cDNA Master Mix	2.0
RNA (or water)	8.0
Total	**10.0**

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

Example: 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.

6.2 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. Keep the gene-specific PCR Master Mix (GS-PCR MMX) and the oligo pool in the cooler until needed.

1. **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 PCR Master Mix	12.5
oncoReveal® Heme Fusion 5x Oligo Pool	5.0
Subtotal	**17.5**

2. **Transfer:** Transfer **17.5 µL** of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.

3. **Template addition:** Add 7.5 µL of cDNA (diluted if necessary) to each sample well containing GS-PCR Mix 1. Add 7.5 µL of nuclease-free water to the no-template control well.

Caution: Up to 7.5 µL of undiluted cDNA reaction volume may be added to the GS-PCR reaction. It is recommended that the volume added to the reaction should correspond to at least 30 ng of RNA input.

Final GS-PCR Mix	
Reagent	**Volume (µL)**
GS-PCR Mix 1	17.5
DNA (or water)	7.5
Total	**25.0**

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 PCR:** Perform one of the following programs with the heated lid on:

   If running the onocoReveal® Heme Fusion Pane on its own or in parallel with the oncoReveal® Myeloid Panel (*Part No. HDA-MY-1001-24*) by sharing the same thermocycler, perform the following program with the heated lid on:

GS-PCR Program		
Temperature	**Time**	**Number of Cycles**
95°C	15 min	1
98°C	1 min	3
72°C	1 min	
98°C	1 min	5
58°C	2 min	
60°C	4 min	
64°C	1 min	
72°C	1 min	
95°C	30 sec	18
66°C	3 min	
8°C	Hold	1

   If running in parallel with the oncoReveal® Lymphoid Panel (*Part No. HDA-LM-1001-24*) by sharing the same thermocycler, perform the following program with the heated lid on:

GS-PCR Program		
Temperature	**Time**	**Number of Cycles**
95°C	15 min	1
98°C	1 min	5
58°C	2 min	
60°C	4 min	
64°C	1 min	
72°C	1 min	
95°C	30 sec	18
66°C	3 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 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. Keep the exonuclease in the cooler. Keep the sample reactions at ambient temperature.

1. Briefly centrifuge the reactions to remove droplets from the side walls. Carefully remove the seal or caps.

2. **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 ice.

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 primer digestion is complete.

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 before 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 samples 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 before performing the purification. A non-uniform distribution can have unpredictable effects on the purification process.

3. **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.

4. **Add beads:** Add **60 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.

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

6. **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.

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

8. **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 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 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.

10. **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.

11. **Resuspend beads:** Remove the PCR plate from the magnetic rack, and **immediately resuspend** the dried beads in each well using **32 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.*

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

13. **Separate GS-PCR product from beads:** Place the elution on a magnetic rack at room temperature until the solution appears clear. Transfer **30 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, have a pre-chilled benchtop cooler prepared. The Indexing PCR Master Mix should be kept in the cooler until it is needed.

1. **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**

2. **Prepare Indexing PCR Mix 1:** Briefly vortex and centrifuge the Indexing PCR Master Mix before use. Prepare Indexing PCR Mix 1 by combining Indexing PCR Master Mix and water with sufficient overage.

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

3. **Add Indexing PCR Mix 1 to Indexing Primer Mix:** Transfer **36 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.

Indexing PCR Mix 2 (per reaction)	
Reagent	**Volume (µL)**
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 purification, ensure that they have been thawed at room temperature 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 (volumes are per reaction)	
Reagent	**Volume (µL)**
Index PCR Mix 2	44.0
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 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	6*
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.

- 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.

- 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.

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.

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 TapeStation, 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.

   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 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:** 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 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 **4.3 nM**. Example calculation is below. Adjust dilution factor accordingly.

   2 µL of library + 198 µL Qubit working solution:

$$\frac{Qubit\ reading\ \left(\frac{ng}{mL}\right)}{1{,}000}\ x\ dilution\ factor\ (100)\ x\ conversion\ factor\ (4.3) = nM$$

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:

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

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

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 an equimolar 5 nM mixture of libraries by combining each library at equal volume (e.g., mixing 5 µL of each 5 nM library). 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.

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.

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 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.

It is recommended that Heme Fusion libraries are sequenced on the MiSeq with libraries generated using Pillar's oncoReveal® Myeloid Panel *(Part No.HDA-MY-1001-24)*. Please refer to the oncoReveal® Myeloid Panel User Manual *(Doc. No.: UM-0033)*, section 7 for sequencing information.

When sequencing Heme Fusion libraries only, see sequencing instructions below.

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

Table 1. Sequencing recommendations.

Sequencing Configuration	Index	Recommended Reads Per Sample
2×150	**Dual** (8 bp each)	100,000 PE reads

Table 2. Multiplexing recommendations for Illumina sequencers if sequencing oncoReveal® Heme Fusion libraries only.

Sequencing Flow Cell	Estimated Instrument Output (million PE reads)	Estimated Maximum Sequencing Batch Size
MiSeq Nano	2	12
MiSeq Micro	8	48

Calculations assume 60% flow cell usage and ideal cluster density.

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'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 it in the cooler.

5. **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 it in the cooler.

6. **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 150 (2 × 150) 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 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 i5 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 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 (_). 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".

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. 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.

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

8. Troubleshooting

Low Yield of Gene-Specific Product

Potential Cause	Solution
Nucleic acid quantity or quality	The recommended minimum input for the assay is 40ng of blood RNA for cDNA synthesis or 30ng cDNA for gene-specific PCR input. 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.
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 is necessary 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
Nucleic acid quantity or quality	The recommended minimum input for the assay is 20–60 ng of blood RNA and genomic DNA. 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 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	Improper library quantitation may result in artificially high or low yields, which affects downstream normalization. Re-quantify 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 for the 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 that fresh 70% ethanol was used for the wash.

9. 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 & 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

10. Appendix B: Fusion Caller Using PiVAT®

The *oncoReveal® Heme Fusion* 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-0057)* 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 and understanding the output in PiVAT®.

10.1 KMT2A Partial Tandem Duplication (PTD) Calling

Signals of low level partial tandem duplications (PTDs) in KMT2A may be observed in normal, healthy controls[1]. Customers are encouraged to set their own threshold for calling PTDs in their samples based on their own testing experience and data.

An example of how the customer can calculate a threshold is below:

$$PTD\ calling\ threshold\ for\ sample = \frac{\#\ reads\ called\ in\ sample\ PTD}{\#\ reads\ called\ in\ normal\ PTD}$$

[1]Huang, Jiajie, et al. "Detection of KMT2A Partial Tandem Duplications (KMT2A-PTDs) in Healthy Donors Using Next Generation Sequencing." Blood 142.Supplement 1 (2023): 5986-5986.