ONCO/Reveal™ Multi-Cancer with CNV and RNA Fusion Panel for Ion Torrent Library Preparation

User Guide

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Pillar Biosciences
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INTRODUCTION

The ONCO/Reveal Multi-Cancer DNA and RNA Fusion Panel combines Pillar Biosciences' ONCO/Reveal Multi-Cancer v4 with CNV Panel and ONCO/Reveal Multi-Cancer RNA Fusion v2 Panel into one parallel workflow.

The ONCO/Reveal™ Multi-Cancer DNA Panel targets numerous, relevant gene regions of interest for researchers looking to explore the genetic sequences of both germline and formalin-fixed paraffin-embedded (FFPE) DNA samples from solid tumor samples. The panel utilizes Pillar Biosciences' proprietary SLIMamp® (stem-loop inhibition mediated amplification) technology, allowing researchers to amplify regions of interest in a simple, multiplex reaction for subsequent sequencing.

The workflow of the ONCO/Reveal Multi-Cancer Panel can be performed and loaded onto the sequencing instrument by researchers within one day. The protocol also contains numerous stopping points for users who have time limitations.

How Does the ONCO/Reveal Multi-Cancer DNA Panel Work?

A pair of DNA oligos was designed for each region of interest, or hot spot. Each region is amplified in the first round of gene-specific PCR (GS-PCR), the excess primers are digested, and the products are subsequently purified via size selection. After purification, indexing PCR using index adaptors adds indices for sample tracking and sequencing. The final libraries are purified and can be sequenced on the Ion Torrent (Figure 1) platform.

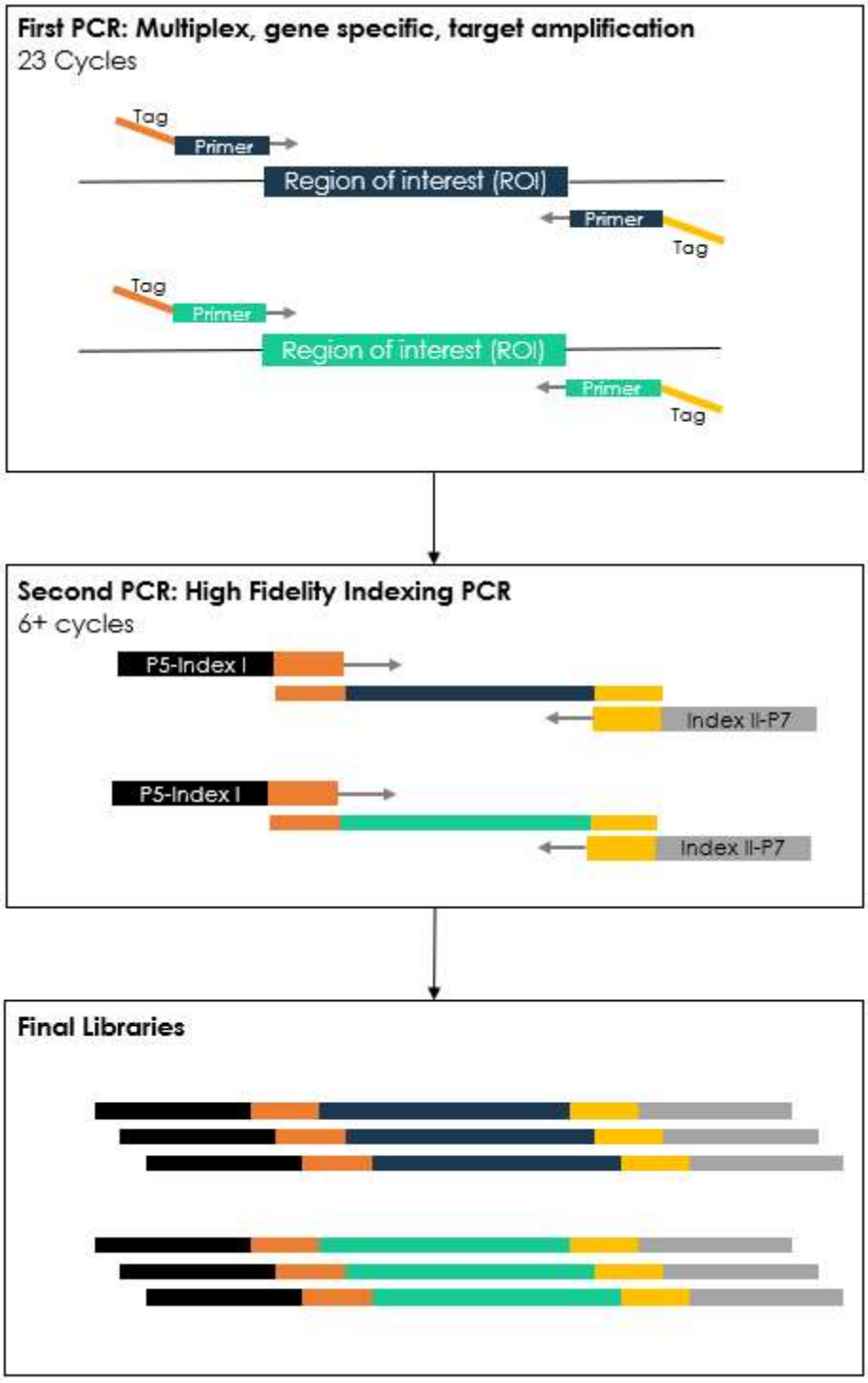


Figure 1. Overview of ONCO/Reveal Multi-Cancer Panel library preparation.

The ONCO/Reveal Multi-Cancer RNA Fusion Fusion v2 Panel utilizes Pillar Biosciences' proprietary SLIMamp® (**S**tem-**L**oop **I**nhibition **M**ediated **amp**lification) technology, allowing researchers to detect common Solid Tumor fusion transcripts in a simple, multiplex reaction. The assay was designed for the detection of common fusion variants including: ALK), NTRK1, NTRK2, NTRK3, NRG1, RET, ROS1, FGFR3 and MET among others. It can also be used to detect exon 14 skipping in MET, and it contains two housekeeping genes as internal controls. The ONCO/Reveal Multi-Cancer RNA Fusion Panel targets fusion variants of interest for researchers looking to explore fusion events using cDNA generated from FFPE (formalin-fixed paraffin-embedded) tissue, frozen tissue, or cell line samples. The prepared libraries are ready for sequencing.

The workflow of the ONCO/Reveal Multi-Cancer RNA Fusion Panel can be performed and loaded for sequencing within about ten hours. The workflow contains numerous stopping points for users who have time limitations.

How Does the ONCO/Reveal Multi-Cancer RNA Fusion Panel Work?

A gene-specific primer was designed for each major breakpoint in the fusion genes (e.g. ALK), and another primer was designed for each partner gene (e.g. EML4). Additionally, amplicons were designed for each side of the breakpoint for the major driver genes. Using cDNA as input, the chimeric transcripts and wild-type transcripts of the driver genes are targeted in the first round of PCR. In the absence of a fusion event, 5' and 3' balance amplicons amplify distal regions of the driver gene transcripts and no fusion PCR product is formed. When a fusion event occurs, fusion primer binding sites are present on the same RNA transcript and allow for chimeric fusion amplicons to amplify. After GS PCR, the primers undergo digestion and the products are purified via bead-based size selection. After purification, indexing PCR using index adaptors adds indices for sample tracking and sequencing. The final libraries are purified and can be sequenced on the Ion torrent (Figure 1) platform.

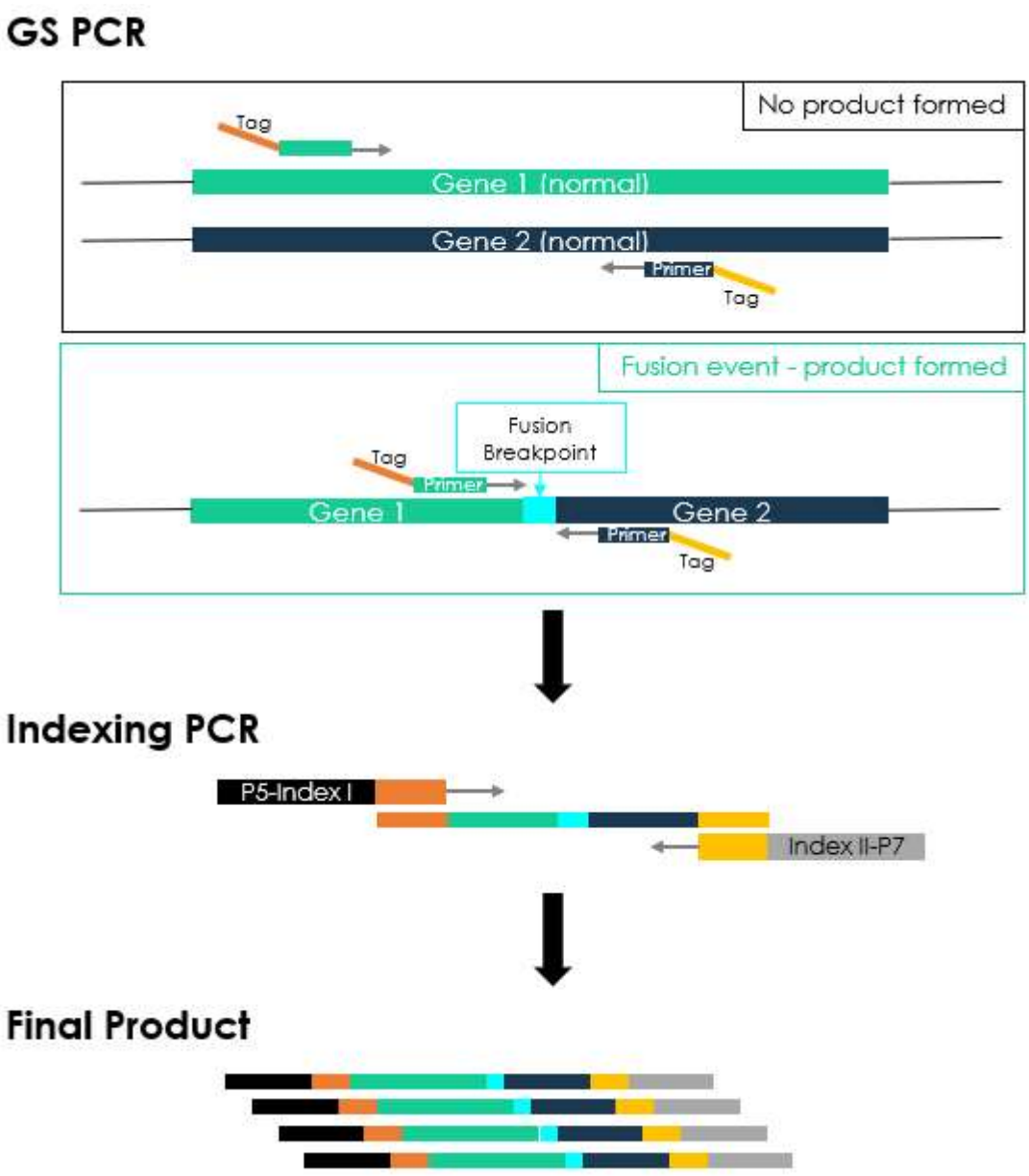


Figure 1. Overview of ONCO/Reveal Multi-Cancer RNA Fusion Panel library preparation.

BEST PRACTICES FOR DNA and RNA LIBRARY PREPARATION

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

Due to the prevalence of ribonucleases that will degrade RNA samples, RNA should be handled with care. RNases are not easily degraded and will quickly degrade precious RNA samples.

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

- **Handling:** RNA is susceptible to degradation. When handling RNA, keep all components on ice and avoid repeated freeze/thaw cycles. Always wear gloves to avoid touching equipment used for RNA work with bare hands.
- **Work areas:** To reduce the risk of contamination from PCR amplicons, supplies should not be moved from one area to another. Separate storage areas (refrigerator, freezer) should also be designated for pre- and post-PCR products. Work areas for RNA should be cleaned regularly and 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 working with RNA, use aseptic techniques. Change gloves regularly and spray them often with a laboratory cleaning solution of 70% alcohol. Avoid touching any parts or items on the body such as the face, hands, or glasses, as they can be sources of RNases.
- **Equipment and consumables:** All consumables used for RNA work should be ensured they are RNase-free. Equipment should be cleaned or treated to inactive RNases.
- **Lab cleanliness:** To further reduce the possibility of contamination, clean work areas 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. Gloves should also be changed after handling a contaminated item. If a sample tube or non-consumable item has fallen and remained capped, thoroughly clean the outside with a laboratory cleaning solution before use (70% alcohol or freshly-made 10% hypochlorite solution).
- **Aliquot reagents:** Aliquot frozen reagents into smaller volumes to prevent freeze/thaw cycles. For reagents stored at higher temperatures, aliquot from the stock and work from the aliquots to reduce the risk of stock contamination. In the case of contamination, aliquots can help to determine the source of contamination more quickly and easily.

- **Multichannel pipettes:** Use multichannel pipettes for consistency and efficiency among numerous samples.
- **Pipette tips:** Change tips between each sample to prevent cross-contamination. Discard any tips that may have become contaminated due to contact with gloves, lab bench, tube exteriors, etc.
- **Open containers and lids:** To prevent possible contamination from the air, keep tubes closed when not directly in use, avoid reaching over open containers, and cover plates with seals or lint-free laboratory wipes.

ONCO/Reveal Multi-Cancer DNA and RNA Fusion Panel Workflow

The following chart (Figure 2) demonstrates the workflow for performing the ONCO/Reveal Multi-Cancer DNA and RNA Fusion Panel library preparation.

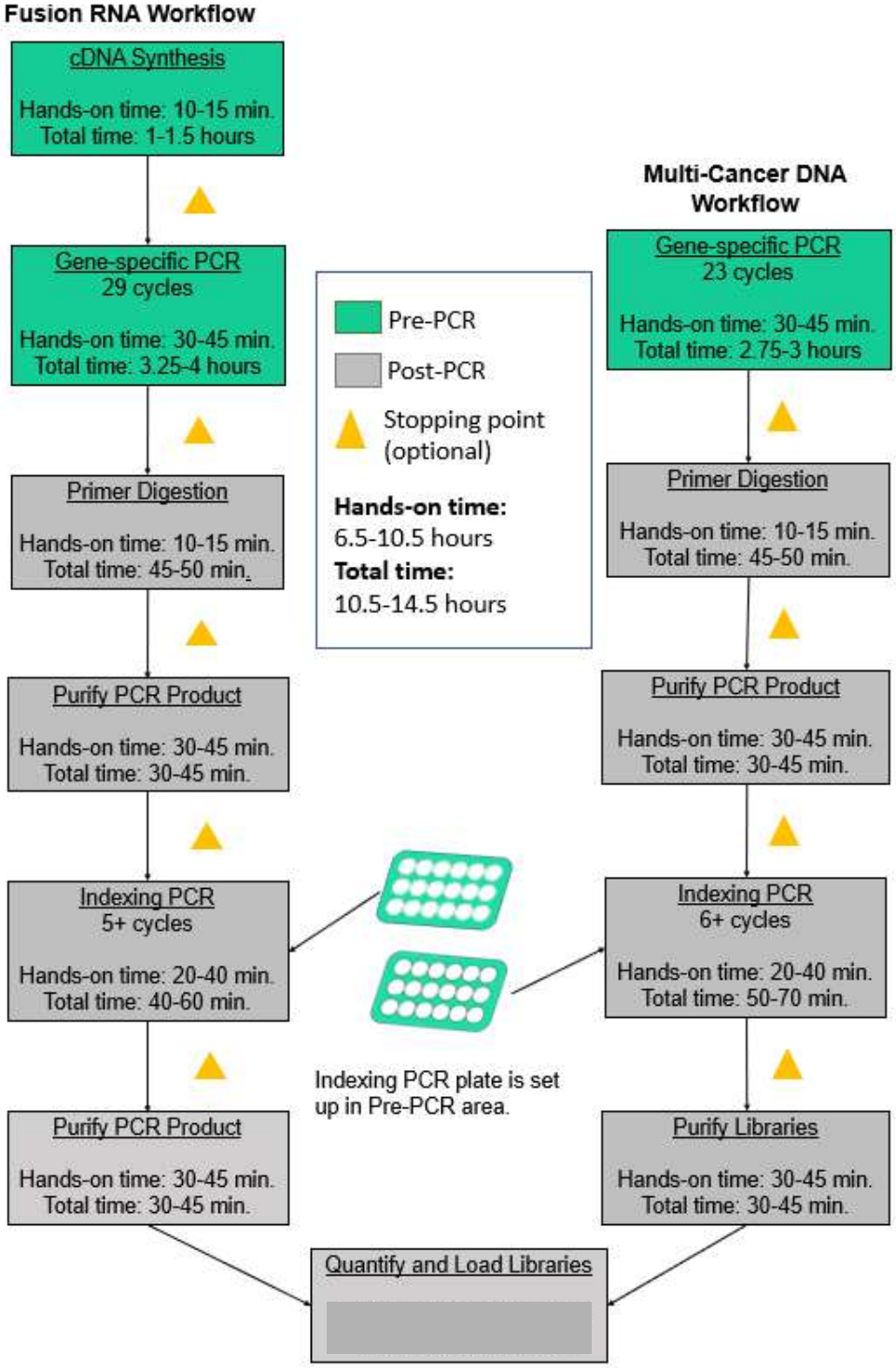


Figure 2. The ONCO/Reveal Multi-Cancer RNA Fusion Panel workflow can be completed from extracted RNA in about ten hours but contains multiple stopping points for users with time constraints.

REVISION HISTORY

2020-01: User Guide created

GETTING STARTED

This section describes the necessary equipment, reagents, and consumables needed before performing the protocol. All reagents in the kit 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, supplies, and reagents to prevent contamination.

Components of the ONCO/Reveal Multi-Cancer DNA/RNA Panel

Reagent	Use	Area Use	Storage
Multi-Cancer v4 (DNA) Oligo Pool	Gene-specific PCR	Pre-PCR	-15° to -25°C
SF v2 oligo pool (5x)	Gene-specific PCR	Pre-PCR	-15° to -25°C
Gene-specific PCR Master Mix (2x)	Gene-specific PCR	Pre-PCR	-15° to -25°C
Exonuclease I	Gene-specific PCR	Post-PCR	-15° to -25°C
Indexing PCR Master Mix (2x)	Indexing PCR	Pre-PCR	-15° to -25°C

ONCO/Reveal DNA and RNA Panel Indexing Kits

Reagent and Part Number	Use	Area Use	Storage
Pillar Biosciences IonXpress Indexing Kit A 24 combinations – 96 reactions *PN: IDX-TI-1001-96*	Indexing PCR	Pre-PCR	-15° to -25°C

User-supplied Reagents

Reagent	Area use	Supplier
****cDNA Master Mix – (SuperScript VILO Master Mix)*****	**cDNA synthesis**	**Thermo Fisher #11755500**
10 N NaOH or 1 N NaOH	Post-PCR	General lab supplier
Agencourt AMPure XP Beads	Post-PCR	Beckman Coulter, #A63881/ #A63880
Ethanol, 200 proof for molecular biology	Post-PCR	General lab supplier
Nuclease-free water	Pre- and Post-PCR	General lab supplier
Qubit dsDNA High Sensitivity assay kit	Post-PCR	Invitrogen, #Q32851/ #Q32854
Agarose gel, 2% (optional)	Post-PCR	General lab supplier
DNA molecular weight markers (optional)	Post-PCR	General lab supplier
***Or** Bioanalyzer High Sensitivity DNA Analysis (optional)*	Post-PCR	Agilent #5067-4627/ #5067-4626
Uracil-DNA glycosylase (UDG) (optional)	Pre-PCR	NEB, #M0280S or #M0280L
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat#T7724

Note: cDNA master mix (SuperScript VILO manufactured by Thermo Fisher) is not supplied in the kit as the manufacturer assigns a 6 month expiration date from the date of manufacture.

Consumables

Item	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	Axygen, #6551 or equivalent
Microplate sealing film	Pre- and post-PCR	Axygen, #PCR-TS or equivalent
Conical tubes, 15 mL	Pre- and post-PCR	General lab supplier
Conical tubes, 50 mL	Post-PCR	General lab supplier
Low retention, aerosol filter pipette tips	Pre- and post-PCR	General lab supplier
Solution basin (trough or reservoir)	Pre- and post-PCR	Fisher, #13-681-506 or equivalent
Qubit Assay tubes	Post-PCR	Invitrogen, #Q32856

Equipment Requirements

Equipment	Area Use	Supplier
Centrifuge adapted for PCR plates, tabletop	Pre- and post-PCR	General lab supplier
Gel electrophoresis apparatus (optional) ***or***	Post-PCR	General lab supplier
2100 Bioanalyzer Instrument (optional)	Post-PCR	Agilent. #G2939BA
Magnetic stand for 96 wells	Post-PCR	Life Technologies, #12331D/ #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, #Q33216/Q33218
Vortexer	Pre- and post-PCR	General lab supplier

Other general lab supplies needed to carry out the protocol include laboratory gloves, ice, ice buckets, tube racks, etc.

For reagents, consumables, and equipment required in both pre- and post-PCR processes, dedicated supplies (including gloves, lab coats, etc.) should be located in both areas.

Part 1:
Library Preparation from DNA

DNA INPUT INFORMATION

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

The recommended DNA input is 20-60 ng per PCR reaction for standard genomic DNA and 20-80 ng for FFPE DNA.

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

LIBRARY PREPARATION PROTOCOL

Hands-on time: 3-5 hours
Total time: 6.5-8.5 hours

Gene-specific PCR: Amplify Genomic DNA Targets

Hands-on time: 30-40 minutes
Total time: 2.75-3 hours

The following steps are performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the Gene-specific PCR Master Mix (GS PCR MMX) and Multi-Cancer oligo pool on ice.

1. **Prepare a PCR master mix:** Vortex and spin the GS PCR MMX and oligo pool before use. For each PCR reaction, the volume of each component is listed on the next page.

Reagent	Volume (µL) (without UDG)	Volume (µL) (with UDG)
Gene-specific PCR Master Mix	12.5	12.5
Multi-Cancer v4 (DNA) oligo pool	5.0	5.0
UDG (5 units/µL)	0.0	1.0
Sub-total	**17.5**	**18.5**

Note: *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.*

2. **Transfer:** Transfer 17.0 µL (or 18.0 µL if using UDG) to each sample well in a PCR plate, strip tube, or PCR tube.
3. **Dilute input DNA:** Dilute DNA in nuclease-free water to a final volume of 7.5 µL (or 6.5 µL if using UDG) of diluted DNA*. Add the diluted DNA to each sample well containing PCR master mix. Add 7.5 µL (or 6.5 µL if using UDG) of nuclease-free water to the no-template control well.

Reagent	Volume (uL) (without UDG)	Volume (uL) (with UDG)
PCR Master Mix	17.5	18.5
Diluted DNA (or water)	7.5	6.5
Sub-total	**25.0**	**25.0**

**The DNA concentration can be determined by the Qubit dsDNA BR Assay Kit (Life Technologies, Cat. No. Q32850 or Q32853; Quantitation range 2-1,000 ng) or the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. No. Q32851 or Q32854; Quantitation range 0.2-100 ng).*

The recommended DNA input is 20-60 ng per PCR reaction for standard genomic DNA and 20-80 ng for FFPE DNA. It is recommended that the quality of FFPE DNA be checked by qPCR (Taqman RNase P Detection Reagents Kit (Life Technologies, Cat. No. 4316831)) or a Bio-analyzer. If the FFPE DNA is not severely degraded, the DNA input can be as little as 5 ng. However, if the FFPE DNA is severely degraded, it is recommended to increase the DNA input quantitated by qPCR or Bio-analyzer when possible.

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

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

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

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

***IMPORTANT:** Do not leave the reactions at 8°C overnight. Precipitation may occur when the reactions are incubated at 8°C overnight.*

***STOPPING POINT:** The gene-specific PCR reactions may be stored at -20°C after cycling.*

Gene-specific Primer Digestion

Hands-on time: 10-15 minutes
Total time: 50-55 minutes

The following steps are performed in a post-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the exonuclease on ice. Keep the sample reactions at ambient temperature.

1. Briefly spin the reactions to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Dilute Exonuclease:**
    a. Invert the Exonuclease I to mix and spin in a microfuge to remove any droplets from the lid.
    b. For 10 samples, dilute 30 µL of the exonuclease I in 20 µL nuclease-free water to prepare 50 µL of diluted exonuclease. Add reagent overage as appropriate. Any excess dilution can be stored at -20°C overnight for a second use.

    IMPORTANT: *The exonuclease solution is viscous and requires careful attention when pipetting. The diluted exonuclease I is good for a second use the next day if stored overnight at -20°C. Otherwise, freshly dilute the exonuclease before adding it to the samples.*

3. **Add Exonuclease:** Add 5 µL of the diluted exonuclease to each sample, pipetting 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 spin the reactions to remove any air bubbles from the bottom of the wells and spin down droplets from the seal or side walls.
6. **Perform digestion:** Perform the following program with the lid on:

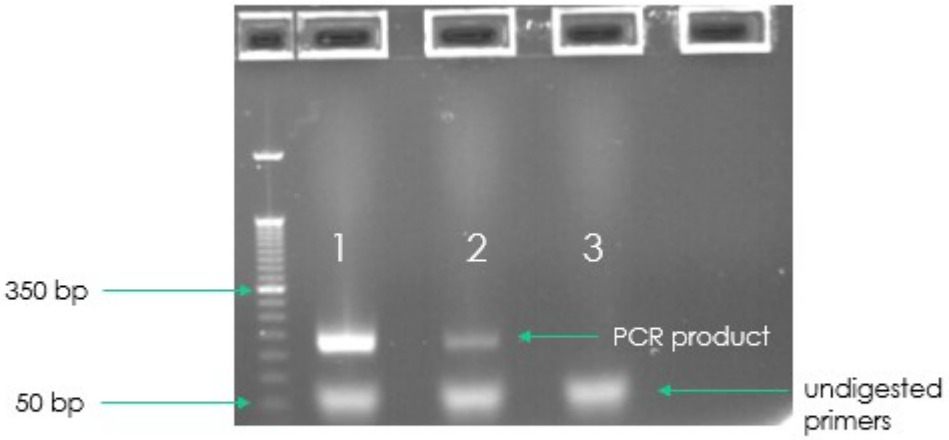
Temperature	Time	Number of Cycles
37°C	20 min	1
80°C	10 min	1
8°C	Hold	1

IMPORTANT: *Do not leave the reactions at 8°C overnight. Precipitation may occur when the reactions are incubated at 8°C overnight.*

STOPPING POINT: *The gene-specific PCR reactions may be stored at -20°C after primer digestion.*

Gel Image After Gene-specific PCR

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



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

Figure 3. Gel analysis of GS PCR material on 2% agarose gel.

Purify the Gene-specific PCR Product

Hands-on time: 30-45 minutes
Total time: 30-45 minutes

The following steps are performed in a post-PCR area.

Pre-purification
Warm AMPure beads: Take out Agencourt AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.

If samples were stored at -20°C, remove from the freezer to thaw to ambient temperature before purification.

IMPORTANT: *It is critical that the AMPure beads reach room temperature before performing the purification process. The temperature of the bead solution can alter the purification process.*

Gene-specific Product Purification

1. If the samples were stored at -20°C or condensation has formed, briefly spin the samples upon thawing to remove droplets from the side walls. Carefully remove the seal or caps.
2. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.

   IMPORTANT: *It is critical that the AMPure beads solution is homogeneous before performing the purification process. A non-uniform distribution can affect the purification process.*

3. **Add water to sample:** Add 20 µL of nuclease-free water to each well or add enough water to bring the volume to 50 µL.

   TIP*: Use a trough and multichannel pipette to quickly and easily add the water to each well. The same method can be applied to add the beads in step 4 and washes in steps 7-9.*

4. **Add beads:** Add 60 µL beads (1.2x beads if the volume is not currently 50 µL) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly the samples and mix again.
5. **Bind PCR product to beads:** Incubate the samples for 5 minutes at room temperature.

***TIP**: During the incubation time, prepare a 50 mL solution of 70% ethanol by combining 35 mL of ethanol and 15 mL of molecular biology grade water, which will be used to wash the beads in step 8.*

6. **Separate beads containing PCR product:** Place the samples 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 from the wall of each well.
8. **Wash beads:** Leave the samples 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 8 for a second 70% ethanol wash. Remove the supernatant from each well. The unused solution of ethanol can be used to purify the libraries after indexing PCR.
10. **Remove remaining ethanol wash:** Remove trace amounts of ethanol completely from each well. Spin the samples in a benchtop centrifuge for 10-15 seconds, place the samples 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.
11. **Resuspend beads:** Remove the samples from the magnetic rack, and immediately resuspend the dried beads in each well using 64 µL nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.

    ***Note:** Do not allow the beads to over-dry. During the purification, the beads tend to clump and "fan." Be sure to immediately resuspend the beads after removing the ethanol wash.*

12. Incubate the elution at room temperature for 5 minutes to fully elute the product.

    ***TIP**: After resuspending the beads, cover the samples and prepare the reactions for indexing the libraries using the Indexing PCR Master Mix in the Pre-PCR area. Alternately, the purified gene-specific PCR product (on beads) may be stored at -20 ℃ after elution.*

***STOPPING POINT:** The purified PCR product may be stored with the beads at -20 ℃.*

Indexing PCR: Amplify the Libraries

Hands-on time: 20-40 minutes
Total time: 50-70 minutes

The following steps should be performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. The Indexing PCR Master Mix should be kept on ice.

***IMPORTANT**: The following protocol is for preparing libraries to be sequenced on the Ion Torrent platform.*

1. **Add indexing primers:** For each indexing reaction, add 4 µL of the appropriate forward and reverse indexing primer to each sample well being used.

Reagent	Volume (µL)
IonXpress Barcode Index	4.0
Common Index	4.0
Total	**8.0**

2. **Prepare a master mix:** Vortex and spin the Indexing PCR Master Mix before use. To prepare the PCR master mix, combine the Indexing PCR Master Mix and water sufficient for the samples being processed with overage.

Reagent	Volume (µL)
Indexing PCR Master Mix (2x)	25.0
Nuclease-free water	11.0
Total	**36.0**

3. **Add master mix to wells:** Add prepared master mix to wells that contain indices from step 1. Transfer 36 µL of master mix to each sample well in a PCR plate, strip tube, or PCR microtube. To prevent cross-contamination of indices, be sure to change tips between each well.

Reagent	Volume (µL)
IonXpress and Common Indices	8.0
Master Mix	36.0
Total	**44.0**

4. **Add gene-specific PCR product:** The following steps should be performed in a post-PCR area. Important: Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area. Aliquot 6 µL of the separated supernatant (Gene-Specific PCR product) into the appropriate wells containing indices and PCR Master Mix, being sure that no beads are transferred.

Reagent	Volume (µL)
Indices and PCR Master Mix	44.0
Gene-specific PCR product	6.0
Total	**50.0**

5. **Mix and spin:** Pulse vortex the sealed reactions on a medium setting for 5-10 seconds to mix. Briefly spin down the reactions to remove any bubbles within the reaction solutions.

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

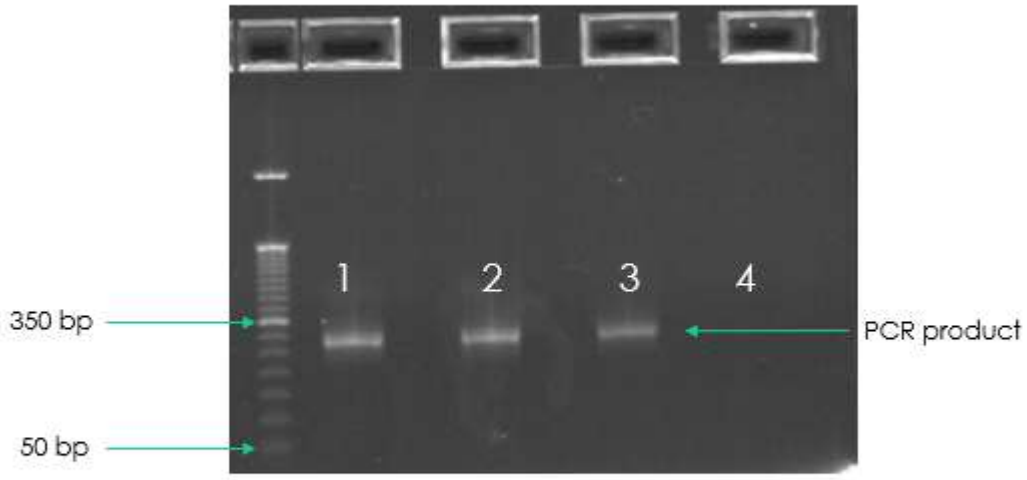
Temperature	Time	Number of Cycles
95°C	2 min	1
95°C 66°C 72°C	30 sec 30 sec 60 sec	6*
72°C	5 min	1
8°C	Hold	1

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

STOPPING POINT: *The indexed libraries may be stored at -20 ℃.*

Gel Image After Indexing PCR

The following image is an example of libraries for sequencing after indexing PCR on a 2% agarose gel.



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

Figure 4. Gel analysis of Index PCR material on 2% agarose gel.

Purify the Libraries

Hands-on time: 30-45 minutes
Total time: 30-45 minutes

Pre-purification
Keep Agencourt AMPure XP beads at room temperature while the indexing PCR is being performed unless samples are going to be stored at -20°C.

If samples were stored at -20°C remove the samples from the freezer to thaw to ambient temperature before purification. Remove Agencourt AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.

IMPORTANT: *It is critical that the AMPure beads reach room temperature before performing the purification process. The temperature of the bead solution can alter the purification process.*

Library Purification

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

1. If the samples were stored at -20°C or condensation has formed, briefly spin the samples once thawed to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.

   IMPORTANT: *It is critical that the AMPure beads solution is homogeneous before performing the purification process. A non-uniform distribution can affect the purification process.*

3. **Add beads:** Add 50 µL beads (1.0x beads if reaction is not at 50 µL) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.

   TIP*: Use a trough and multichannel pipette to quickly and easily add the beads to each well. The same method can be applied to the washes in steps 6-8.*

4. **Bind libraries to beads:** Incubate the samples for 5 minutes at room temperature to bind the libraries to the beads.
5. **Separate libraries on beads:** Place the samples 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 from the wall of each well.
7. **Wash beads:** Leave the samples 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.*

8. **Second wash:** Repeat step 7 for a second 70% ethanol wash. Remove the supernatant from each well.

   IMPORTANT: *Remove trace amounts of ethanol completely from each well. If ethanol drops are attached to the sidewall of some wells, spin the samples in a benchtop centrifuge for 10-15 seconds and use a 10 or 20 µL tip to remove the remaining solution from wells.*

9. **Dry beads:** Let the beads air dry at room temperature for 2-5 minutes.

   IMPORTANT: *Do not over-dry the beads. 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 samples 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 spin and mix again.
11. **Elute libraries:** Incubate the resuspended beads at room temperature for 5 minutes to elute the final libraries.
12. **Separate libraries from beads:** Place the elutions 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 or tubes to the corresponding well of a new plate or tube.

    TIP*: During the incubation and magnetic separation of the beads, cover the samples and prepare the solutions needed for quantitation in the next section. The purified libraries may also be stored at 4 ℃ for up to 3 days or at -20 ℃ for longer-term storage.*

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

STOPPING POINT: *The purified libraries may be stored at 4 ℃ for up to 3 days. Store the purified libraries at -20 ℃ for longer-term storage.*

Final Library Image

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

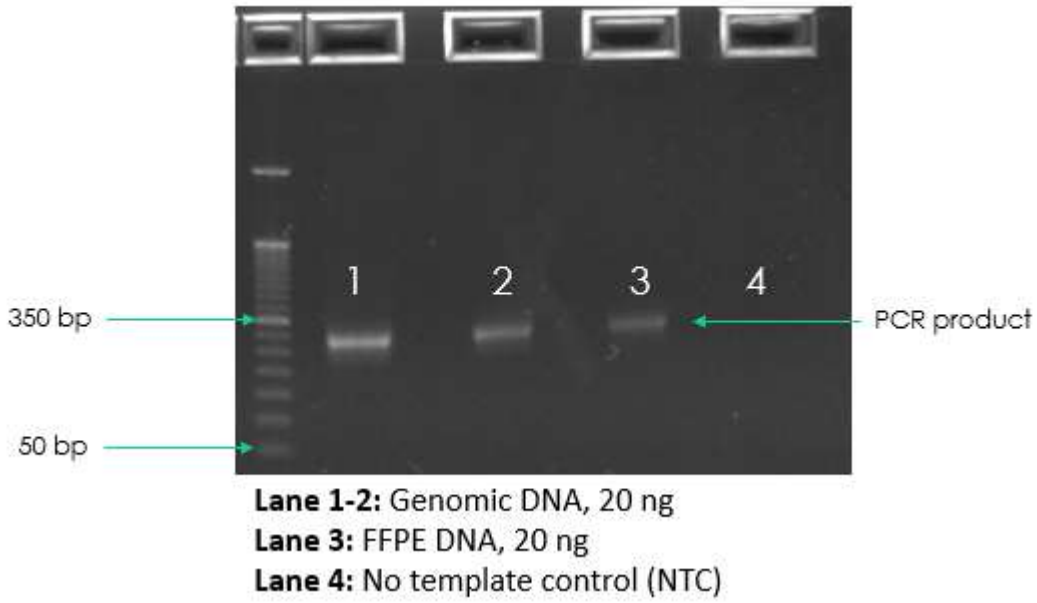


Figure 5. Gel analysis of Final Libraries on 2% agarose gel.

Part 2:
Library Preparation from RNA

cDNA SYNTHESIS

Hands-on time: 10-15 minutes
Total time: 1.5-1.75 hours

Before performing the library preparation, prepare cDNA from total RNA extracted from FFPE samples, tissue, or cell lines. Additionally, cDNA may be prepared from total circulating nucleic acid extracted from blood. For the preparation from RNA, the cDNA should be prepared using random primers, not exclusively oligo d(T). Using only oligo d(T) may result in low or no coverage of the 5' end of transcripts. The cDNA Master Mix provided in the kit uses random hexamers for the synthesis of cDNA.

Up to 15 µL of undiluted cDNA reaction can be added to the Gene-Specific PCR without inhibiting the reaction. Alternatively, with a higher RNA input, the cDNA reaction can be diluted with low TE or nuclease-free water. The recommend minimum input equivalent of FFPE RNA is 25 ng. For instance, using an initial RNA input of 50 ng into a 10 µL cDNA reaction, 5 µL would go into the initial PCR for an equivalent input of 25 ng of RNA. The recommended minimum input equivalent of cfRNA is 20 ng.

An example of the cDNA synthesis follows (see manufacturer's protocol for complete details). Set up the reaction on ice and keep all components chilled.

1. **Dilute RNA:** Dilute RNA* in nuclease-free water to a final volume of 16.0 µL (for a 20 µL reaction) or 8.0 µL (for a 10 µL reaction) to each sample well in PCR plate, strip tube, or PCR tube. Add nuclease-free water to the no-template control well.
2. **Add cDNA Master Mix:** Add cDNA Master Mix to each sample well containing diluted RNA and no-template control well.

Reagent	Volume (µL) (20 µL reaction)	Volume (µL) (10 µL reaction)
cDNA Master Mix	4.0	2.0
Diluted RNA (or water)	16.0	8.0
Total	**20.0**	**10.0**

**The RNA concentration can be determined by the Qubit RNA BR Assay kit (Life Technologies, Cat. Q10211 and Q10210; Quantitation range 20-1,000 ng) or Qubit RNA HS Assay kit (Life Technologies, Cat. Q32852 and Q32855;*

Quantitation range 5-20 ng). The recommended minimum RNA input of FFPE RNA is 25 ng. For degraded FFPE samples, the initial RNA should be increased to 75-100ng.

For cfRNA, the RNA concentration can be assumed to be equal to the DNA concentration of the total circulating nucleic acid sample. The DNA concentration can be determined by the Qubit dsDNA HS Assay kit ((Life Technologies, Cat. No. Q32851 or Q32854; quantitation range 0.2-100 ng). The recommended minimum input of cfRNA is 20 ng. For poor quality samples, a higher input amount may be necessary.

3. Perform the reverse transcription in a thermal cycler with the lid on according to the conditions below.

Temperature	Time
25°C	10 min
42°C	30 min
85°C	5 min
8°C	Hold

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

1. **Dilute RNA:** Dilute RNA in nuclease-free water to a final volume of 4.0 µL in each sample well of a PCR plate, strip tube, or PCR tube. Add nuclease-free water to the no-template control well.

Reagent	Volume (µL)
cDNA Master Mix	1.0
Diluted RNA (or water)	4.0
Total	**5.0**

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

3. Perform the reverse transcription in a thermal cycler with lid on according to the conditions below.

Temperature	Time
25°C	10 min
50°C	10 min
85°C	5 min
8°C	Hold

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

LIBRARY PREPARATION PROTOCOL

Hands-on time: 3-5 hours
Total time: 6.25-8.75 hours

Gene-specific PCR: Amplify Transcript Targets

The following steps are performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. Vortex and spin the Gene-specific PCR Master Mix (GS PCR MMX) and Fusion oligo pool before use.

1. **Prepare a PCR master mix:** Vortex and spin the GS PCR MMX and oligo pool before use. For each PCR reaction, the volume of each component is listed in the table below.

Reagent	Volume (µL)
Gene-specific PCR Master Mix	25.0
SF v2 oligo pool (5x)	10.0
Sub-Total	**35.0**

Note: *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.*

2. **Transfer:** Transfer 35.0 µL of master mix to each sample well in a PCR plate, strip tube, or PCR tube.
3. **Dilute input:** Dilute cDNA in nuclease-free water to a final volume 15.0 µL of dilute cDNA*. Add 15.0 µL of diluted cDNA to each sample well containing PCR master mix. Add 15.0 µL of nuclease-free water to the no-template control well.

   **Up to 15 µL of undiluted cDNA reaction may be added to the GS-PCR reaction. It is recommended that the volume added to the reaction corresponds to at least 25 ng of RNA. If using the entire cDNA reaction (5 or 10 µL reaction), the PCR master mix and supplementing water may be added directly to the cDNA reaction plate. The plate may proceed directly to GS-PCR. See previous section "*cDNA SYNTHESIS*" for more information regarding input.*

Reagent	Volume (µL)
PCR Master Mix	35.0
Diluted cDNA (or water)	15.0
Sub-Total	**50.0**

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

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

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

IMPORTANT: *Do not leave the reactions at 8°C overnight. Precipitation may occur when the reactions are incubated at 8°C overnight.*

STOPPING POINT: *The gene-specific PCR reactions may be stored at -20°C after cycling.*

Gene-specific Primer Digestion

Hands-on time: 10-15 minutes
Total time: 50-55 minutes

The following steps are performed in a post-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the exonuclease on ice. Keep the sample reactions at ambient temperature.

1. Briefly spin the samples to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Dilute Exonuclease:**
    a. Invert the exonuclease I to mix and spin in a microfuge to remove any droplets from the lid.
    b. For 10 samples, dilute 30 µL of the exonuclease I in 20 µL nuclease-free water to prepare 50 µL of diluted exonuclease. Add reagent overage as appropriate. Any excess dilution can be stored at -20°C overnight for a second use.

    IMPORTANT: *The exonuclease solution is viscous and requires careful attention when pipetting. The diluted exonuclease I is good for a second use the next day if stored overnight at -20°C. Otherwise, freshly dilute the exonuclease before adding it to the samples.*

3. **Add Exonuclease:** Add 5 µL of the diluted exonuclease to each sample, pipetting 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 spin the reactions to remove any air bubbles from the bottom of the wells and spin down droplets from the seal or side walls.
6. **Perform digestion:** Perform the following program with the lid on:

Temperature	Time	Number of Cycles
37°C	20 min	1
80°C	10 min	1
8°C	Hold	1

IMPORTANT: *Do not leave the reactions at 8°C overnight. Precipitation may occur when the reactions are incubated at 8°C overnight.*

STOPPING POINT: *The gene-specific PCR reactions may be stored at -20°C after primer digestion.*

Gel Image after Gene-Specific PCR

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

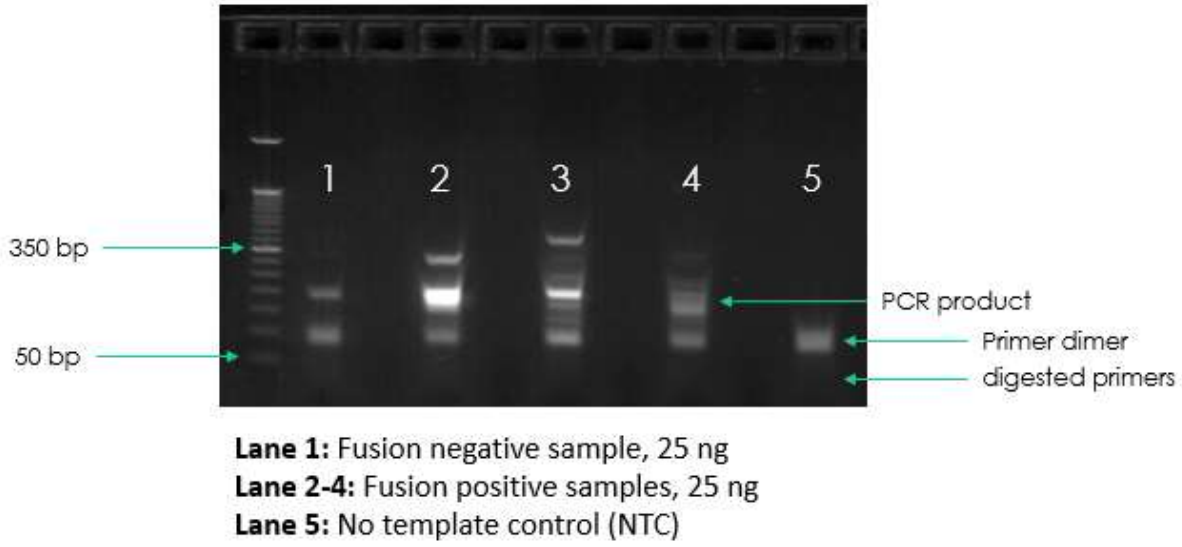


Figure 3. Gel analysis of GS PCR material on 2% agarose gel.

Purify the Gene-specific PCR Product

Hands-on time: 30-45 minutes
Total time: 30-45 minutes

The following steps are performed in a post-PCR area.

Pre-purification
Warm AMPure beads: Take out Agencourt AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.

If samples were stored at -20°C, remove from the freezer to thaw to ambient temperature before purification.

IMPORTANT: *It is critical that the AMPure beads reach room temperature before performing the purification process. The temperature of the bead solution can alter the purification process.*

Gene-specific Product Purification

1. If the samples were stored at -20°C or condensation has formed, briefly spin the samples upon thawing to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.

   IMPORTANT: *It is critical that the AMPure beads solution is homogeneous before performing the purification process. A non-uniform distribution can affect the purification process.*

3. **Add beads:** Add 66 µL beads (1.2x beads if the volume is not currently 55 µL) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly the samples and mix again.

   TIP*: Use a trough and multichannel pipette to quickly and easily add the beads to each well. The same method can be applied to the washes in steps 6-9.*

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

   TIP*: During the incubation time, prepare a 50 mL solution of 70% ethanol by combining 35 mL of ethanol and 15 mL of molecular biology grade water, which will be used to wash the beads in step 7.*

5. **Separate beads containing PCR product:** Place the samples 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 from the wall of each well.
7. **Wash beads:** Leave the samples 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.*

8. **Second wash:** Repeat step 7 for a second 70% ethanol wash. Remove supernatant from each well. The unused solution of ethanol can be used to purify the libraries after indexing PCR.
9. **Remove remaining ethanol wash:** Remove trace amounts of ethanol completely from each well. Spin the samples in a benchtop centrifuge for 10-15 seconds, place the samples 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.
10. **Resuspend beads:** Remove the samples from the magnetic rack, and immediately resuspend the beads in each well using 64 µL nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.
11. Incubate the elution at room temperature for 5 minutes to elute the product.

    TIP*: After resuspending the beads, cover the samples and prepare the reactions for indexing the libraries using the Indexing PCR Master Mix in the Pre-PCR area. Alternately, the purified gene-specific PCR product (on beads) may be stored at -20 ℃ after elution.*

STOPPING POINT: *The purified PCR product may be stored with the beads at -20 ℃.*

Indexing PCR: Amplify the Libraries

Hands-on time: 20-40 minutes
Total time: 50-70 minutes

The following step should be performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. The Indexing PCR Master Mix should be kept on ice.

***IMPORTANT**: The following protocol is for preparing libraries to be sequenced on the Ion Torrent platform.*

1. **Add indexing primers:** For each indexing reaction, add 4 µL of the appropriate forward and reverse indexing primer to each sample well being used.

Reagent	Volume (µL)
IonXpress Barcode Index	4.0
Common Index	4.0
Total	**8.0**

2. **Prepare a master mix:** Vortex and spin the Indexing PCR Master Mix before use. To prepare the PCR master mix, combine the Indexing PCR Master Mix and water sufficient for the samples being processed with overage. Transfer 36 µL of master mix to each sample well in a PCR plate, strip tube, or PCR microtube. To prevent cross-contamination of indices, be sure to change tips between each well.

Reagent	Volume (µL)
Indexing PCR Master Mix (2x)	25.0
Nuclease-free water	5.0
Total	**30.0**

3. **Add master mix to wells:** Add prepared master mix to wells that contain indices from step 1.

Reagent	Volume (µL)
IonXpress and Common Indices	8.0
Master Mix	30.0
Total	**38.0**

4. **Add gene-specific PCR product:** The following steps should be performed in a post-PCR area. ***Important***: Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area. Aliquot 12.0 µL of the separated supernatant (Gene-specific PCR product) into the appropriate wells containing indices and PCR Master Mix, being sure that no beads are transferred.

Reagent	Volume (µL)
Indices and PCR Master Mix	38.0
Gene-specific PCR product	12.0
Total	**50.0**

5. **Mix and spin:** Pulse vortex the sealed reactions on a medium setting for 5-10 seconds to mix. Briefly spin down the reactions to remove any bubbles within the reaction solutions.
6. **Perform PCR:** Perform the following program with the heated lid on:

Temperature	Time	Number of Cycles
95°C	2 min	1
95°C 66°C 72°C	30 sec 30 sec 60 sec	5*
72°C	5 min	1
8°C	Hold	1

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

STOPPING POINT: *The indexed libraries may be stored at -20°C.*

Purify the Libraries

Hands-on time: 30-45 minutes
Total time: 30-45 minutes

Pre-purification
Keep Agencourt AMPure XP beads at room temperature while the indexing PCR is being performed unless samples are going to be stored at -20°C.

If samples were stored at -20°C remove the samples from the freezer to thaw to ambient temperature before purification. Remove Agencourt AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.

IMPORTANT: *It is critical that the AMPure beads reach room temperature before performing the purification process. The temperature of the bead solution can alter the purification process.*

Library Purification

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

1. If the samples were stored at -20°C or condensation has formed, briefly spin the samples once thawed to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.

   IMPORTANT: *It is critical that the AMPure beads solution is homogeneous before performing the purification process. A non-uniform distribution can affect the purification process.*

3. **Add beads:** Add 50 µL beads (1.0x beads if reaction is not at 50 µL) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.

   TIP*: Use a trough and multichannel pipette to quickly and easily add the beads to each well. The same method can be applied to the washes in steps 6-8.*

4. **Bind libraries to beads:** Incubate the samples for 5 minutes at room temperature to bind the libraries to the beads.
5. **Separate libraries on beads:** Place the samples 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 from the wall of each well.
7. **Wash beads:** Leave the samples 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.*

8. **Second wash:** Repeat step 7 for a second 70% ethanol wash. Remove supernatant from each well.

   IMPORTANT: *Remove trace amounts of ethanol completely from each well. If ethanol drops are attached to the sidewall of some wells, spin the samples in a benchtop centrifuge for 10-15 seconds and use a 10 or 20 µL tip to remove the remaining solution from wells.*

9. **Dry beads:** Let the beads air dry at room temperature for 2-5 minutes.

   IMPORTANT: *Do not over-dry the beads. 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:** Removing the samples from the magnetic rack, 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 spin and mix again.
11. **Elute libraries:** Incubate the resuspended beads at room temperature for 5 minutes to elute the final libraries.
12. **Separate libraries from beads:** Place the elutions 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 or tubes to the corresponding well of a new plate or tube.

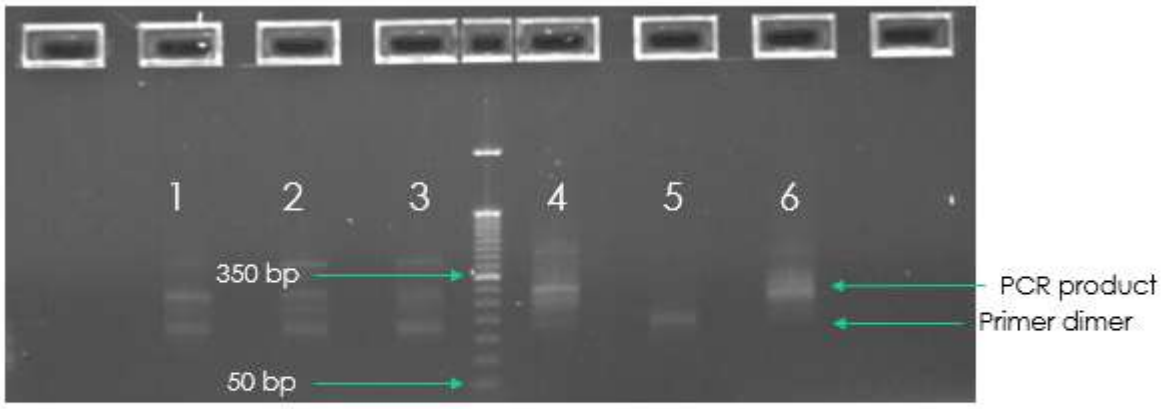
    TIP*: During the incubation and magnetic separation of the beads, cover the samples and prepare the solutions needed for quantitation in the next section. The purified libraries may also be stored at 4 ℃ for up to 3 days or at -20 ℃ for longer-term storage.*

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

STOPPING POINT: *The purified libraries may be stored at 4 ℃ for up to 3 days. Store the purified libraries at -20 ℃ for longer-term storage.*

Final Library Gel Image

The following is a gel image of final libraries for sequencing after all rounds of PCR and purification.



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

Figure 4. Gel analysis of Final Libraries on 2% agarose gel.

Part 3:
Quantify Libraries for Pooling and Sequencing

Prior to loading the sequencer, libraries must be quantified, normalized, and then pooled together. The following protocol 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. Final libraries from both the DNA and RNA workflow may be quantified at the same time.

After quantification, libraries are normalized to ensure that libraries achieve a similar number of desired reads. Normalized libraries are then pooled together. Libraries from the DNA and RNA workflow may be pooled and loaded onto the sequencer together. The volume of each normalized library to pool depends on the desired coverage depth for the libraries prepared with each panel.

Qubit Quantification of Purified Libraries

Hands-on time: 30-45 minutes
Total time: 30-45 minutes

The following steps should be performed in a post-PCR area. Ensure to prepare enough buffer for the number of samples being processed with overage.

1. **Prepare buffer with dye:** Dilute the Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer. Vortex briefly to mix 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 buffer mixture with dye 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.

   ***IMPORTANT**: New standard dilutions should be prepared with the samples. 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 sample to the tube (1:100 dilution).
5. **Mix and spin:** Mix the tubes by vortexing and then spinning the tubes briefly.
6. Incubate the tubes at room temperature for 2 minutes.
7. **Measure concentration:** Measure the concentration of each sample on the Qubit 2.0 Fluorometer per the Qubit User Guide. Use the dsDNA High Sensitivity assay to read standards 1 and 2 followed by the samples.
   a. If any sample concentrations are above the linear range of the instrument, prepare a new dilution using 199 µL Qubit buffer with dye and 1 µL sample (1:200 dilution). Repeat steps 5-7.
8. **Calculate concentration:** 1 ng/ µL is equal to 5 nM. Example calculation is below. Adjust dilution factor accordingly.

   2 µL of library + 198 µL Qubit solution:

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

STOPPING POINT: *The undiluted libraries may be stored at 4 ℃ for up to 3 days. Store libraries at -20 ℃ for long-term storage.*

Normalization and Pooling

Hands-on time: 30-45 minutes
Total time: 30-45 minutes

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

1. **Normalize libraries to 5 nM:** Dilute an aliquot (i.e. 4 µL) of each sample library to 5 nM using nuclease-free water or 10 mM Tris-Cl with 0.1% Tween-20, pH 8.5.

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

$$Final\ volume\ of\ library - 4\ uL\ library = volume\ of\ diluent$$

STOPPING POINT: *The normalized library products can be stored at 4°C overnight for loading the next day. For longer storage, the normalized samples can be stored at -20°C.*

2. **Mix and spin:** Mix the 5 nM libraries thoroughly by vortexing followed by spinning.
3. **Prepare library pools for each panel:** Retrieve and label two 1.5 mL microtubes to prepare two library pools, one for each panel. Combine equal volumes (i.e. 5 µL of each 5 nM library) of each normalized library for the Multi-Cancer Panel into one pool. Combine equal volumes of each normalized library for the Multi-Cancer RNA Fusion panel into another pool. Quickly vortex each library pool for 2-5 seconds and spin down.
4. **Combine library pools:** Combine library pools prepared for each panel from step 3 into one final library pool for sequencing. Library pools should be combined according to the volumes listed in the table below.

ONCO/Reveal Panel	Volume of library pool (µL)
Multi-Cancer	24.0
Solid Tumor Fusion	2.0
Sub-total	**26.0**

Note: *Libraries from the Multi-Cancer RNA Fusion Panel are pooled at a different volume than the Multi-Cancer Panel to account for the difference in coverage depth needed.*

5. Quickly vortex the final library pool for 2-5 seconds and spin down.
6. **Quantify final library pool (recommended):** The libraries prepared using the Multi-Cancer Panel and Multi-Cancer RNA Fusion cluster very efficiently on the MiSeq. It is recommended that the library mix be quantified using Qubit or another library quantitation method (qPCR) to ensure the mix is at 5 nM to prevent over-clustering or under-clustering on the MiSeq. If the final dilution is not 5 nM (±10%), adjust the dilution accordingly when loading the sequencer to obtain the desired concentration.

Prepare Diluted Libraries for Sequencing

Continue with template generation and sequencing following Ion torrent protocol

Preparing a Sample Sheet for Sequencing

IMPORTANT: *See the attached Appendix A for information regarding Pillar's IonXpress Indexing primers.*

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of gene-specific product	DNA quantity or quality	The recommended input for the assay is 20-80ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples.
	Improper cycling	Check that the cycling protocol performed is the appropriate protocol for gene-specific amplification.
Low indexing efficiency	Improper Ampure purification	Incomplete Ampure purification or loss of gene-specific product will affect the indexing PCR reaction. The purified product can be checked on an agarose gel to ensure the gene-specific product was not lost or that clean-up was sufficient to remove excess primers.
		The Ampure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct Ampure concentration was used for cleanup and fresh, 70% ethanol is 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 library yield	DNA quantity or quality	The recommended input for the assay is 20-60ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples. Run the product from the gene-specific PCR on agarose gel to check the yield. The product can also be checked on an agarose gel after indexing PCR before and after Ampure purification.
	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 the product was not lost during PCR cleanup. The Ampure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct Ampure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
The libraries over-cluster or under-cluster on the MiSeq	Normalization and mix of libraries is not 15 pM (v2) 25 pM (v3), or 1.8 pM (NextSeq)	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-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 Ampure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct Ampure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
		The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.
No-template control contains amplicons	Cross-contamination	Make sure to change tips between samples and avoid waving over tubes or plates. When liquid handling, be careful to avoid waving used tips over samples. Poor sealing or residual liquid in tips can cause contamination of nearby samples. If possible, leave adjacent wells empty between samples. Work spaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination. Periodically clean the work space, floor, equipment, and instrumentation with a laboratory cleaning solution (10% bleach, 70% isopropanol, or 70% ethanol) to break down amplicons on surfaces.

Appendix A

Index Name	Index Barcode Sequence
IonXpress_1	CTAAGGTAAC
IonXpress_2	TAAGGAGAAC
IonXpress_3	AAGAGGATTC
IonXpress_4	TACCAAGATC
IonXpress_5	CAGAAGGAAC
IonXpress_6	CTGCAAGTTC
IonXpress_7	TTCGTGATTC
IonXpress_8	TTCCGATAAC
IonXpress_9	TGAGCGGAAC
IonXpress_10	CTGACCGAAC
IonXpress_11	TCCTCGAATC
IonXpress_12	TAGGTGGTTC
IonXpress_13	TCTAACGGAC
IonXpress_14	TTGGAGTGTC
IonXpress_15	TCTAGAGGTC
IonXpress_16	TCTGGATGAC
IonXpress_17	TCTATTCGTC
IonXpress_18	AGGCAATTGC
IonXpress_19	TTAGTCGGAC
IonXpress_20	CAGATCCATC
IonXpress_21	TCGCAATTAC
IonXpress_22	TTCGAGACGC
IonXpress_23	TGCCACGAAC
IonXpress_24	AACCTCATTC

Index Name	Index Barcode Sequence
IonXpress_25	CCTGAGATAC
IonXpress_26	TTACAACCTC
IonXpress_27	AACCATCCGC
IonXpress_28	ATCCGGAATC
IonXpress_29	TCGACCACTC
IonXpress_30	CGAGGTTATC
IonXpress_31	TCCAAGCTGC
IonXpress_32	TCTTACACAC
IonXpress_33	TTCTCATTGAAC
IonXpress_34	TCGCATCGTTC
IonXpress_35	TAAGCCATTGTC
IonXpress_36	AAGGAATCGTC
IonXpress_37	CTTGAGAATGTC
IonXpress_38	TGGAGGACGGAC
IonXpress_39	TAACAATCGGC
IonXpress_40	CTGACATAATC
IonXpress_41	TTCCACTTCGC
IonXpress_42	AGCACGAATC
IonXpress_43	CTTGACACCGC
IonXpress_44	TTGGAGGCCAGC
IonXpress_45	TGGAGCTTCCTC
IonXpress_46	TCAGTCCGAAC
IonXpress_47	TAAGGCAACCAC
IonXpress_48	TTCTAAGAGAC