

ONCO/Reveal™ Lung Cancer Fusion RNA Panel

Library Preparation User Guide

FOR RESEARCH USE ONLY

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Pillar Biosciences
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INTRODUCTION

The ONCO/Reveal Lung Cancer Fusion RNA Panel utilizes Pillar Biosciences' proprietary SLIMamp® (**S**tem-**L**oop **I**nhibition **M**ediated **a**mplification) technology, allowing researchers to detect common lung cancer fusion transcripts in a simple, multiplex reaction. The assay was designed for the detection of common fusion variants of ALK (NM_004304), NTRK1 (NM_002529), NRG1 (NM_013956), RET (NM_020975), ROS1 (NM_002944), FGFR3 (NM_000142), and MET (NM_000245). It can also be used to detect exon 14 skipping in MET, and it contains two housekeeping genes as internal controls. The ONCO/Reveal Lung Cancer Fusion RNA 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 on an Illumina sequencer using a paired-end read length of 150 (2x150).

The workflow of the ONCO/Reveal Lung Cancer Fusion RNA 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 Lung Cancer Fusion RNA 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 TruSeq Custom Amplicon index adaptors adds dual indices for sample tracking and sequencing. The final libraries are purified and sequenced on an Illumina sequencer (Figure 1).

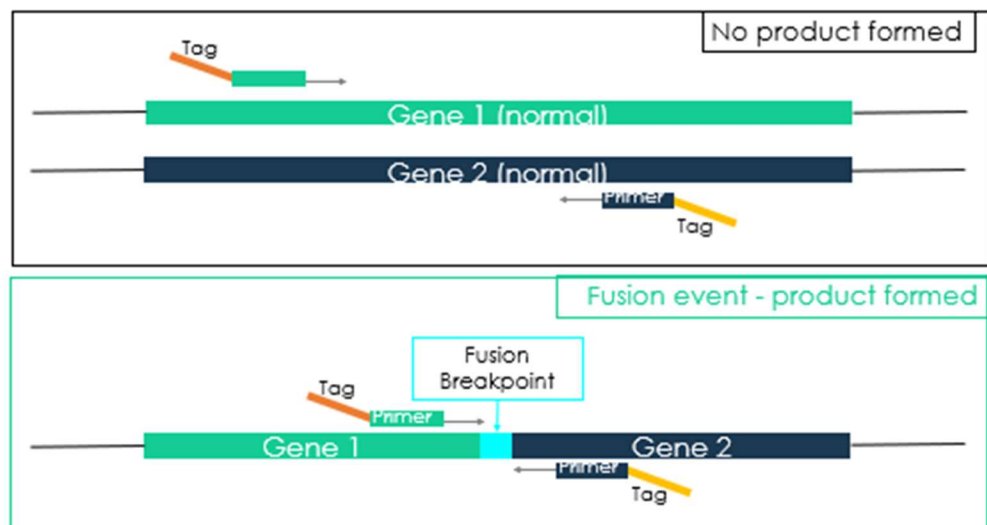
GS PCR**Indexing PCR****Final Product**

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

REVISION HISTORY

2017-10: User Guide created

2018-04: Corrections and added information about cDNA Master Mix

2018-06: Updated Figure 1 schematic

2019-04: Update product name and document format

2019-07: Index kit options and minor grammatical edits

2020-06: Updated figures and layout, added note about adaptor trimming in version 0.0.5

GETTING STARTED

This section describes the necessary equipment, reagents, and consumables needed before performing the protocol.

Components of the ONCO/Reveal Lung Cancer Fusion RNA Panel

Reagent	Use	Storage
<i>Gene-specific PCR Master Mix (2x)</i>	First PCR	-15° to -25°C
<i>Lung Fusion oligo pool</i>	First PCR	-15° to -25°C
<i>Exonuclease I</i>	Post PCR	-15° to -25°C
<i>Indexing PCR Master Mix</i>	Indexing PCR	-15° to -25°C

ONCO/Reveal Lung Cancer Fusion RNA Panel Indexing Kits

Reagent and Part Number	Use	Area Use	Storage
<i>Illumina TSCA Indexing Primers Kit A, indices AI501-8, AI701-4 (32 combinations - 96 reactions)</i> PN: IDX-AI-1001-96	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Illumina TSCA Indexing Primers Kit B, indices AI501-8, AI705-8 (32 combinations - 96 reactions)</i> PN: IDX-AI-1002-96	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Illumina TSCA Indexing Primers Kit C, indices AI501-8, AI709-12 (32 combinations - 96 reactions)</i> PN: IDX-AI-1003-96	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Illumina TSCA Indexing Primers Kit D, indices AI501-8, AI701-12 (96 combinations - 192 reactions)</i> PN: IDX-AI-1004-192	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Illumina TSCA Indexing Primers Kit E, indices AI501-8, AI701-12 (96 combinations - 384 reactions)</i> PN: IDX-AI-1005-384	Indexing PCR	Pre-PCR	-15° to -25°C

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

All reagents in the kit should be used in designated Pre-PCR areas to prevent amplicon contamination and kept free of RNases (see page 7 for handling RNA). Each area designated for pre- and post-PCR should have dedicated equipment, supplies, and reagents to prevent contamination.

User-supplied Reagents

Reagent	Area use	Supplier
cDNA Master Mix – (SuperScript VILO Master Mix)	cDNA synthesis	ThermoFisher #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	Life Technologies, #Q32851/ #Q32854
Qubit RNA Broad Range Assay kit	Pre-PCR	Life Technologies, #Q10211/ #Q10210
Qubit RNA High Sensitivity Assay kit	Pre-PCR	Life Technologies, #Q32852/ #Q32855
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

Compatible MiSeq Reagent Kits

MiSeq reagent Micro kit v2 (300 cycles)	Illumina, #MS-103-1002
MiSeq reagent Nano kit v2 (300 cycles)	Illumina, #MS-103-1001

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	Life Technologies, #Q32856

Equipment Requirements

Equipment	Area Use	Supplier
<i>Centrifuge adapted for PCR plates, tabletop</i>	Pre- and post-PCR	General lab supplier
<i>Gel electrophoresis apparatus (optional) or</i>	Post-PCR	General lab supplier
<i>2100 Bioanalyzer Instrument (optional)</i>	Post-PCR	Agilent. #G2939BA
<i>Magnetic stand for 96 wells</i>	Post-PCR	Life Technologies, #12331D/ #12027
<i>Microfuge</i>	Pre- and post-PCR	General lab supplier
<i>Thermal cycler, heated lid capability</i>	Post-PCR	General lab supplier
<i>Pipettes, 0.5-1000 μL capabilities</i>	Pre- and post-PCR	General lab supplier
<i>Qubit Fluorometer</i>	Post-PCR	Invitrogen, #Q33216/Q33218
<i>Vortexer</i>	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.

BEST PRACTICES FOR HANDLING RNA

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:** 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 reduce cross-contamination. Discard any tips that come into contact with surfaces (gloves, bench, and tube exteriors) outside of the sample area.

- **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 use racks that contain lids or covers.

ONCO/Reveal LUNG CANCER FUSION RNA WORKFLOW

The following chart (Figure 2) demonstrates the workflow for the ONCO/Reveal Lung Cancer Fusion RNA Panel library preparation protocol.

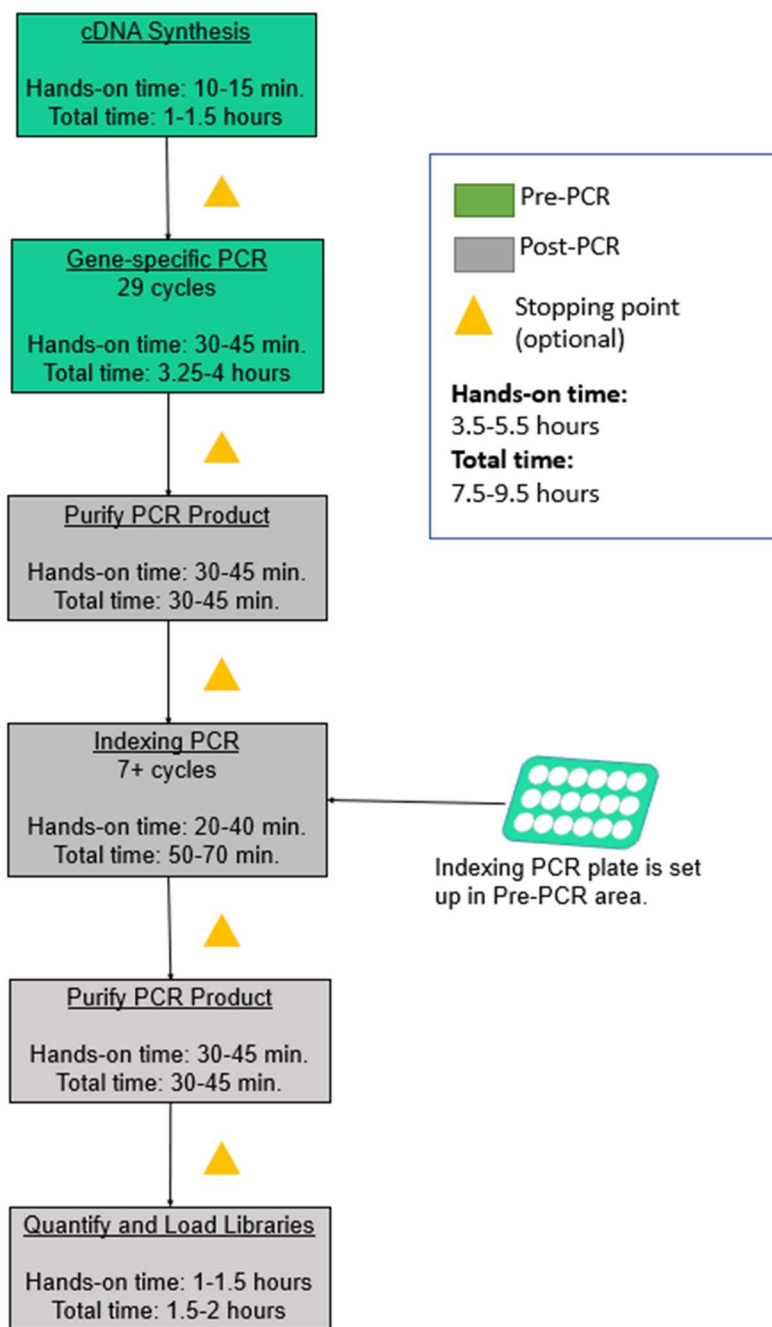


Figure 2. The ONCO/Reveal Lung Cancer Fusion RNA Panel workflow can be completed from extracted RNA in about ten hours but contains multiple stopping points for users with time constraints.

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. 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.

For the first PCR, the full cDNA reaction (10 or 20 μ L) can be added without inhibiting the PCR. Alternatively, with a higher RNA input, the cDNA reaction can be diluted with low TE or nuclease-free water. The recommend RNA input equivalent is 25 ng. For instance, using an initial RNA input of 50 ng into a 10 μ L cDNA reaction, 5 μ L would go into the initial PCR for an equivalent input of 25 ng of RNA.

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 μ L (for a 20 μ L reaction) or 8.0 μ L (for a 10 μ L 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). For degraded FFPE samples, the initial RNA should be increased to 75-100ng.*

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

Temperature	Time	No. of cycles
25°C	10 min	1
42°C	30 min	1
85°C	5 min	1
8°C	Hold	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: 3.5-5.5 hours

Total time: 7.5-9.5 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 below. Prepare enough PCR master mix for the number of samples being processed plus overage.

Reagent	Volume (μL)
Gene-specific PCR Master Mix	25.0
Fusion oligo pool (10x)	5.0
Sub-Total	30.0

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. **Dilute input:** Dilute cDNA* in nuclease-free water to a final volume 20.0 μL in a PCR plate. Add 20.0 μL of diluted cDNA to each sample well containing PCR master mix.

**The entire 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 FFPE RNA. If using the entire cDNA reaction (10 or 20 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.*

3. **Transfer:** Transfer 30.0 μL of master mix to each sample well in a PCR plate, strip tube, or PCR tube.

Reagent	Volume (μL)
PCR Master Mix	30.0
Diluted cDNA (or water)	20.0
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	No. of cycles
95°C	15 min	1
95°C	1 min	5
58°C	1 min	
60°C	2 min	
64°C	30 sec	
72°C	1 min	
95°C	30 sec	24
66°C	3 min	
8°C	Hold	Hold

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	No. of cycles
37°C	20 min	1
80°C	10 min	1
8°C	Hold	Hold

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 samples after the first round of PCR and primer digestion.

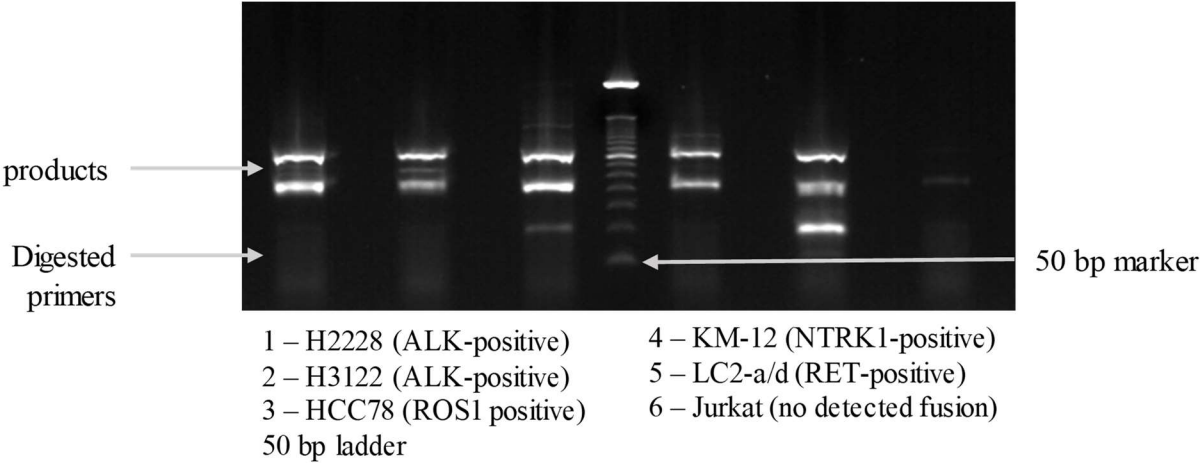


Figure 3. Gel analysis of GS PCR material on 2% agarose gel.

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 μ L 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 μ L 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 μ L 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 °C after elution.

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

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.

1. **Add indexing primers:** For each indexing reaction, add 4.0 μL of the appropriate forward and reverse indexing primer to each sample well being used.

Reagent	Volume (μL)
A700 TSCA Index	4.0
A500 TSCA 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 30.0 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)
Pi500 and Pi700 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	No. of cycles
95°C	2 min	1
95°C	30 sec	7*
66°C	30 sec	
72°C	60 sec	
72°C	5 min	1
8°C	Hold	Hold

*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.

TIP: Thaw the MiSeq reagent cartridge in a water bath or overnight at 4 °C per the MiSeq Reagent Kit Reagent Prep Guide, and prepare the sample sheet during indexing PCR (see page 32).

Gel Image after Indexing PCR

The following is a gel image of samples after the indexing PCR before purification.

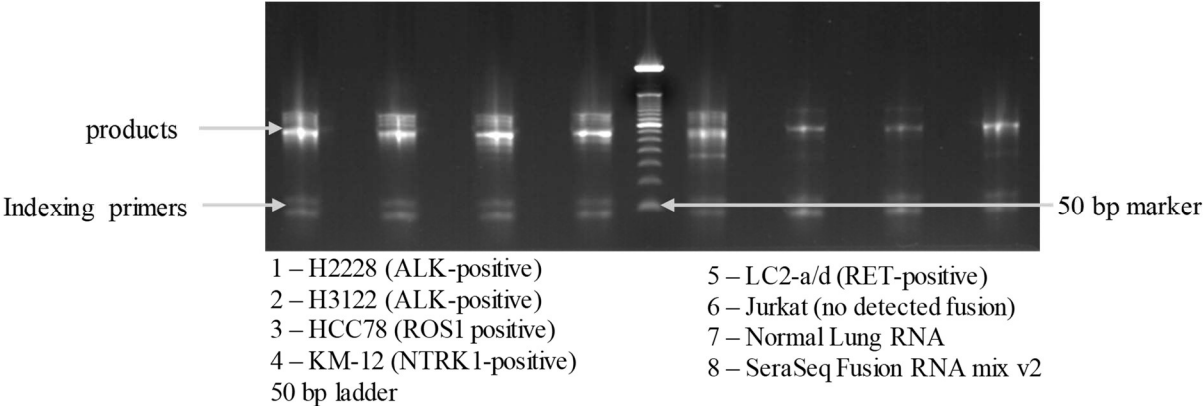


Figure 4. Gel analysis of Indexing PCR material on 2% agarose gel.

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 μ L 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 μ L 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 μ L 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 μ L 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 °C for up to 3 days or at -20 °C 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 °C for up to 3 days. Store the purified libraries at -20 °C for longer-term storage.

Final Library Gel Image

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

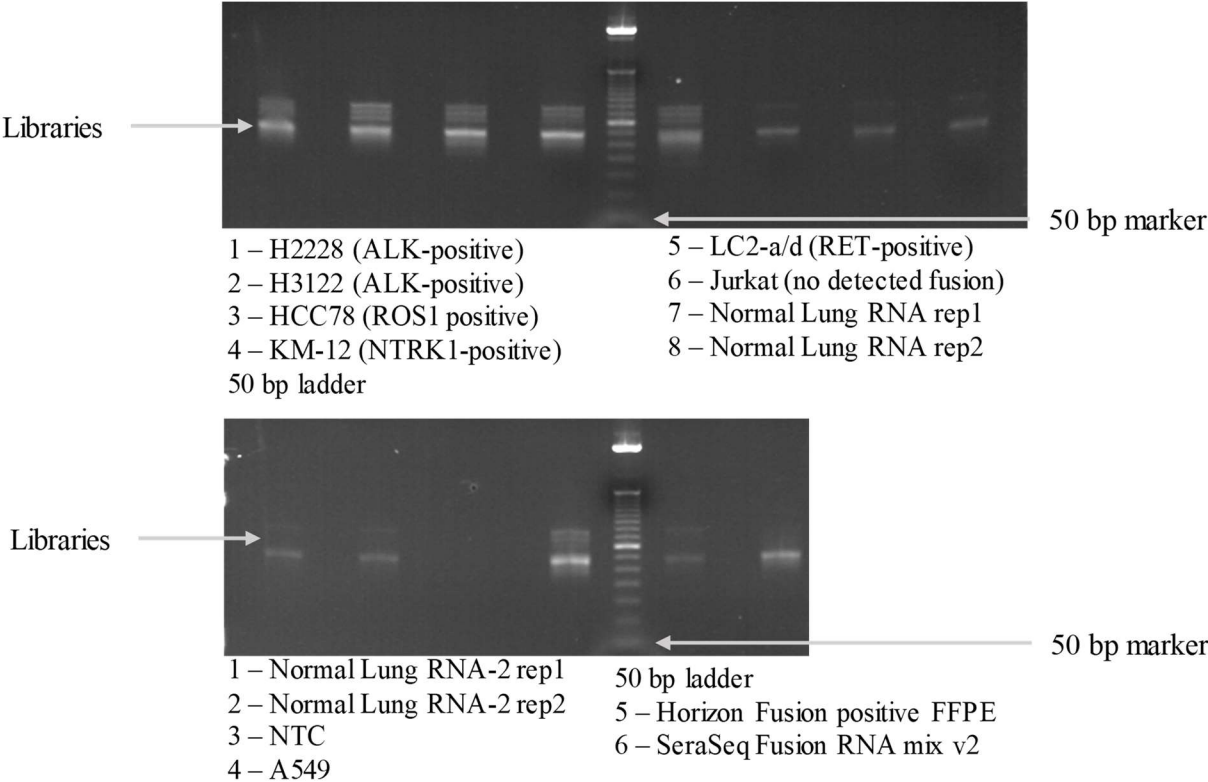


Figure 5. Gel analysis of Final Libraries on 2% agarose gel.

Qubit Quantitation 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/ μ L is equal to 5 nM. Example calculation is below. Adjust dilution factor accordingly.

2 μ L of library + 198 μ L Qubit solution:

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

STOPPING POINT: The undiluted libraries may be 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 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.

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

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

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 mix:** Label a new microtube for the library mix. Prepare a 5 nM mixture of libraries by combining each library at equal volume (i.e. mixing 5 µL of each 5 nM library). Quickly vortex the mix for 2-5 seconds and spin down.
4. **Quantify final library pool (recommended):** The libraries prepared using the ONCO/Reveal Lung Cancer Fusion RNA Panel 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 on the MiSeq

Hands-on time: 30-70 minutes

Total time: 30-70 minutes

The following steps should be performed in a post-PCR area. For this portion of the protocol, have an ice bucket prepared.

Sequencing with Additional ONCO/Reveal Panels (Recommended)

Dilute Lung Cancer Fusion libraries to **5 nM**. Libraries should be loaded with libraries processed using Pillar's ONCO/Reveal Multi-Cancer or Lung & Colon Cancer Panel on a MiSeq Micro v2, MiSeq v2 kit, or MiSeq v3 kit. The final concentration of the libraries for sequencing is **15 pM** for v2 chemistry and **25 pM** for v3 chemistry.

1. **Prepare library mix:** Label a new 1.5 mL microtube for the library mix. Prepare a 5 nM mixture of libraries in the following manner:
 - a. **Pooling with Multi-Cancer and Lung & Colon Cancer libraries:** Pool equal volumes of each Multi-Cancer or Lung & Colon Cancer library (i.e. 5 μ L each library).
 - b. **Combine library pools:** Combine library pools for Multi-Cancer or Lung & Colon Cancer with the library pool for Fusion. An example of how library pools should be combined to account for differences in sequencing depth requirement is below.

ONCO/Reveal Panel	Volume of library pool (μ L)
Multi-Cancer or Lung & Colon	20.0
Lung Fusion	2.0
Total	22.0

2. Quickly vortex the mix for 2-5 seconds and spin down.

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 (\pm 10%) to prevent under- or over-clustering on the MiSeq.

The following steps can be found in greater detail in Illumina's "Preparing Libraries for Sequencing on the MiSeq" (part # 15039740).

3. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining 800 μ L nuclease-free water with 200 μ L of 1 N NaOH. Vortex the solution to mix.

Alternately, prepare a 1 N NaOH solution by combining 500 μ L 10 N NaOH into 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.

4. **Denature the library mix:** Label a new microtube for the denatured, 25 pM library mix.
 - a. Denature the library mix by combining 5 μ L of the library mix and 5 μ L of the freshly prepared 0.2 N NaOH.
 - b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add 990 μ L of Illumina's HT1 solution to the denatured library mix.
 - e. Invert the mixture several times, spin briefly, and place on ice.
5. **Dilute to 15 pM library mix (v2 chemistry only):** Label a new 1.5 mL microtube for the 15 pM library mix. Combine 360 μ L of the 25 pM library mix (step 4) with 240 μ L of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice.
6. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 594 μ L of the 15 pM (v2) or 25 pM (v3) library mix (step 5) with 6 μ L of a 12.5 pM PhiX library control. Briefly vortex, spin, and place on ice.
7. **Load MiSeq cartridge:** Using a clean 1000 μ L tip, puncture the foil cap above the sample loading tube on the MiSeq cartridge. Load the 600 μ L library mix and PhiX mixture (step 6) into the cartridge and ensure the solution has reached the bottom of the tube by lightly tapping the tube if liquid remains on the side wall or there is an air bubble at the bottom of the tube.
8. **Run the MiSeq:** Run the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150): "MiSeq System User Guide" (part #15027617). For instructions on preparing a sample sheet for the MiSeq, see page 32.
9. Store diluted libraries and mixtures at -20°C for long-term storage.

Sequencing ONCO/Reveal Lung Cancer Fusion RNA Libraries only

Dilute Lung Cancer Fusion libraries to **5 nM**. The final concentration of the libraries for sequencing using a MiSeq Nano v2, MiSeq Micro v2, or MiSeq v2 kit is **10 pM**.

The following steps can be found in greater detail in Illumina's "Preparing Libraries for Sequencing on the MiSeq" (part # 15039740).

1. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining 800 μL nuclease-free water with 200 μL of 1 N NaOH. Vortex the solution to mix.

Alternately, prepare a 1 N NaOH solution by combining 500 μL 10 N NaOH into 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.

2. **Denature the library mix:** Label a new microtube for the denatured, 25 pM library mix.
 - a. Denature the library mix by combining 5 μL of the library mix and 5 μL of the freshly prepared 0.2 N NaOH.
 - b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add 990 μL of Illumina's HT1 solution to the denatured library mix.
 - e. Invert the mixture several times, spin briefly, and place on ice.
3. **Dilute to 10 pM library mix:** Label a new 1.5 mL microtube for the 10 pM library mix. Combine 240 μL of the 25 pM library mix (step 2) with 360 μL of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice.
4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 540 μL of the 10 pM library mix (step 3) with 60 μL of a 12.5 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **Load MiSeq cartridge:** Using a clean 1000 μL tip, puncture the foil cap above the sample loading tube on the MiSeq cartridge. Load the 600 μL library mix and PhiX mixture (step 4) into the cartridge and ensure the solution has reached the bottom of the tube by lightly tapping the tube if liquid remains on the side wall or there is an air bubble at the bottom of the tube.

6. **Run the MiSeq:** Run the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150): "MiSeq System User Guide" (part #15027617). For instructions on preparing a sample sheet for the MiSeq, see page 32.
7. Store diluted libraries and mixtures at -20°C for long-term storage.

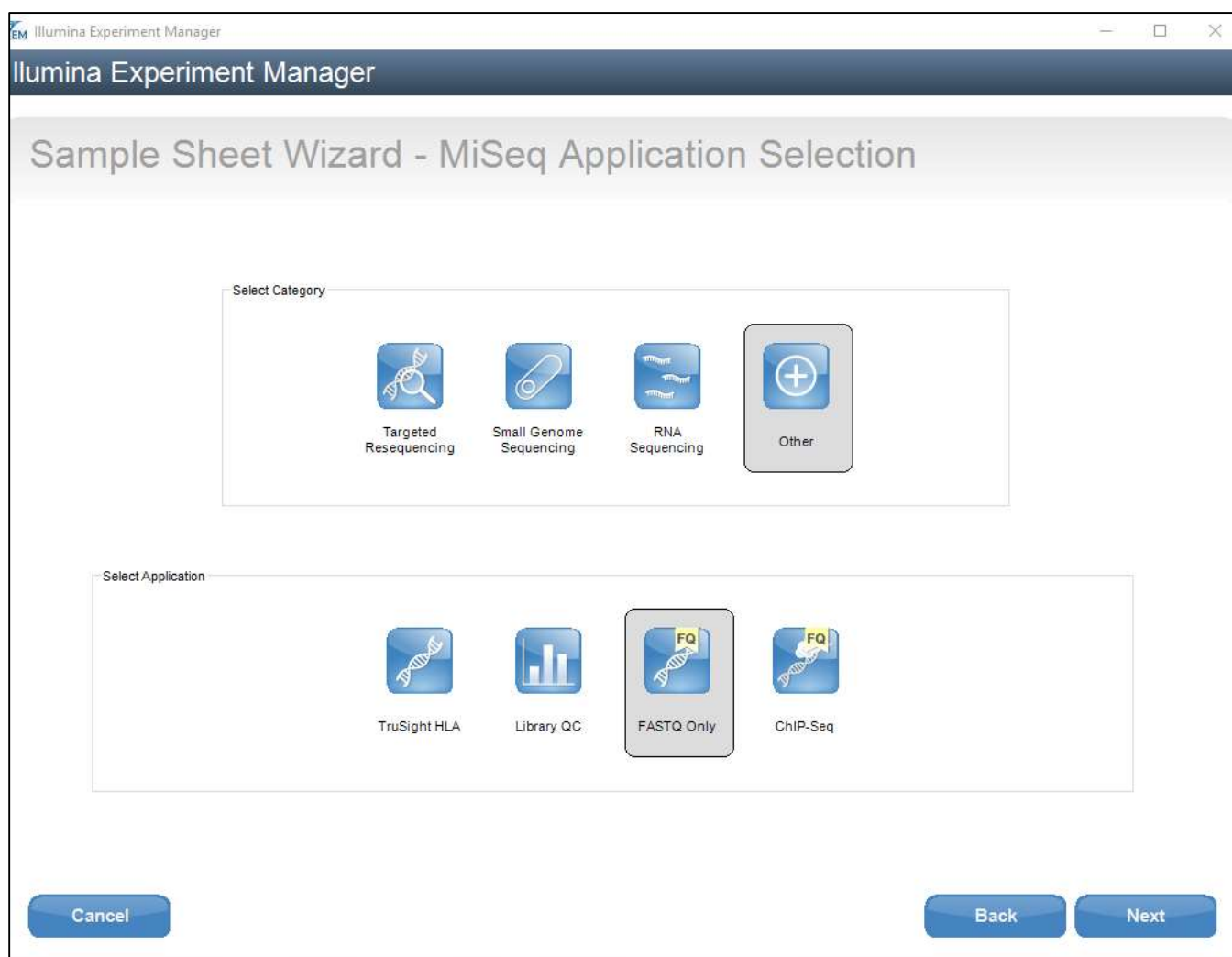
Preparing a Sample Sheet Using Illumina Experiment Manager

NOTE: When creating the sample sheet and running BLC2FQ, do not perform adapter trimming as this will interfere with analysis in PIVAT version 2020.1

In the Illumina Experiment Manager, prepare a sample sheet that contains the information for the samples that are being loaded.

For best practice, prepare the sample sheet prior to loading the MiSeq cartridge. If an error has been made during indexing PCR where samples have the same indices, it can be remedied before loading the samples on the MiSeq.

1. Open Illumina Experiment Manager, select "Create Sample Sheet."
2. **Instrument selection:** Select "MiSeq" and "Next."
3. **Application selection:** Under "Category," select "Other." Under "Select Application," select "FASTQ only" and "Next."



4. **Reagent barcode:** Enter the reagent barcode number found on the cartridge being loaded onto the instrument. Example: For MSXXXXXXX-300v2, enter the 7-digit number following MS.
5. **Library Prep Kit:** Use the menu to select "TruSeq Amplicon."
6. **Index Reads:** Select to perform two index reads if not already selected.
7. **Experiment Name, Investigator Name, Description:** These fields can be filled out by the user per the laboratory's standard operator procedure.
8. **Read type:** Select Paired End reads if not already selected.
9. **Cycle numbers:** Cycle numbers for both Read 1 and Read 2 should be 150. Select "Next."

The screenshot shows the 'Sample Sheet Wizard - Workflow Parameters' window in the Illumina Experiment Manager. The window is divided into two main sections: 'FASTQ Only Run Settings' and 'FASTQ Only Workflow-Specific Settings'.

FASTQ Only Run Settings:

- Reagent Cartridge Barcode*: 1234567
- Library Prep Kit: TruSeq Amplicon (dropdown menu)
- Index Reads: 0 (radio), 1 (radio), 2 (selected radio)
- Experiment Name: Date_testinstructions
- Investigator Name: Experimentalist
- Description: test_instructions
- Date: 4/ 3/2017 (calendar icon)
- Read Type: Paired End (selected radio), Single Read (radio)
- Cycles Read 1: 150 (spinner)
- Cycles Read 2: 150 (spinner)
- * - required field

FASTQ Only Workflow-Specific Settings:

- ☐ Custom Primer for Read 1
- ☐ Custom Primer for Index
- ☐ Custom Primer for Read 2
- ☐ Reverse Complement

At the bottom of the window, there are three buttons: 'Cancel', 'Back', and 'Next'.

10. **Sample Information:** Prepare the sample sheet with the sample identifiers, indices, and other pertinent information and save the sample sheet to a MiSeq-accessible folder.
- Name the samples in the column labeled "Sample ID." Illegal characters include spaces, periods, and other special characters.
 - (Optional) The plates and well numbers can be added in "Plate" and "Well."
 - For each sample, indicate the TruSeq Custom Amplicon indices used during indexing PCR.

EM Illumina Experiment Manager

Illumina Experiment Manager

Sample Sheet Wizard - Sample Selection

Samples to include in sample sheet

* - required field ☒ Maximize

Sample ID*	Sample Name	Plate	Well	Index1 (I7)*	I7 Sequence	Index2 (I5)*	I5 Sequence	Sample Project	Description
PositiveControl			A1	A708	CACCACAC	A501	TGAACCTT		
NegativeControl			C2	A708	CACCACAC	A503	TGTTCTCT		
sample1			A3	A710	TGTGACCA	A501	TGAACCTT		
sample2			B3	A710	TGTGACCA	A502	TGCTAAGT		
sample3			C3	A710	TGTGACCA	A503	TGTTCTCT		
sample4			D3	A710	TGTGACCA	A504	TAAGACAC		
sample5			E5	A711	AGGGTCAA	A505	CTAATCGA		
sample6			F5	A711	AGGGTCAA	A506	CTAGAACA		
sample7			G5	A711	AGGGTCAA	A507	TAAGTTCC		
sample8			H5	A711	AGGGTCAA	A508	TAGACCTA		
NTC			H7	A712	AGGAGTGG	A508	TAGACCTA		

< >

Add Blank Row Remove Selected Rows ?

Sample Sheet Status: Valid

Reason:

Cancel Back Finish