

ONCO/Reveal™ PillarHS Multi-Cancer cfDNA Panel (ONCO/Reveal Multi-Cancer cfDNA Panel) Library Preparation User Guide

FOR RESEARCH USE ONLY

Table of Contents

INTRODUCTION	2
How Does the Pillar ONCO/Reveal Multi-Cancer cfDNA Panel Work?	2
REVISION HISTORY	4
GETTING STARTED	5
Components of the ONCO/Reveal Multi-Cancer cfDNA Panel	5
ONCO/Reveal Multi-Cancer cfDNA Panel Indexing Kits	5
User-supplied Reagents	6
Compatible Illumina Reagent Kits	6
Equipment Requirements	7
BEST PRACTICES	8
ONCO/Reveal Multi-Cancer cfDNA Panel Workflow	9
LIBRARY PREPARATION PROTOCOL	10
Linear PCR: High Fidelity Linear Amplification	10
Purify the Linear PCR Product.....	12
Exponential PCR: High Fidelity Exponential Amplification	16
Purify the Exponential PCR Product.....	19
Indexing PCR: Amplify the Libraries	22
Purify the Libraries	24
Qubit Quantitation of Purified Libraries	28
Prepare Diluted Libraries for Sequencing	29
Sequencing on MiSeq (MiSeq v3 Kit)	31
Sequencing on the NextSeq	33
Preparing a Sample Sheet for Sequencing	35
TROUBLESHOOTING	36

Pillar Biosciences
pillar-biosciences.com
version 0.2.5

INTRODUCTION

The ONCO/Reveal PillarHS Multi-Cancer Panel allows researchers to amplify regions of interest in a simple, multiplex PCR reaction for subsequent sequencing on an Illumina sequencer using a paired-end read length of 110 (2x110) or 150 (2x150). The ONCO/Reveal PillarHS Multi-Cancer Panel contains numerous gene regions of interest for researchers looking to detect somatic mutations at very low frequencies from cell-free DNA (cfDNA) samples in blood.

The workflow of the ONCO/Reveal PillarHS Multi-Cancer Panel can be completed from extracted DNA to libraries loaded onto the sequencing instrument in about ten hours. The protocol also contains multiple stopping points for users who have time limitations.

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

Because it is critical to reduce PCR errors in low-frequency variant detection, one of the gene-specific primers is tagged with a Unique Identifier (UID) containing 14 random bases, providing 4^{14} (268,435,456) unique combinations of UIDs. Using the UID-primers, a linear PCR is performed for multiple cycles to assign the UIDs from the original DNA. Subsequently, after removing the unused UID-primers, the UID-tagged molecules are then exponentially amplified with the other gene-specific primers and two universal primers. Therefore, each region contains multiple copies of uniquely tagged molecules that were created directly from the template during the linear PCR. To minimize the PCR error, both processes utilize a high-fidelity polymerase with an error rate >100-fold lower than that of Taq DNA Polymerase. Errors from PCR amplification and from the sequencing process are greatly reduced by calling the consensus bases across all reads within a UID family (See Figure 1).

The products from exponential PCR are subsequently purified via size selection. After purification, another round of PCR adds index adaptors and P5 and P7 sequences to each library for sample tracking and sequencing on Illumina's flow cells. Those products are further purified and sequenced (Figure 1).

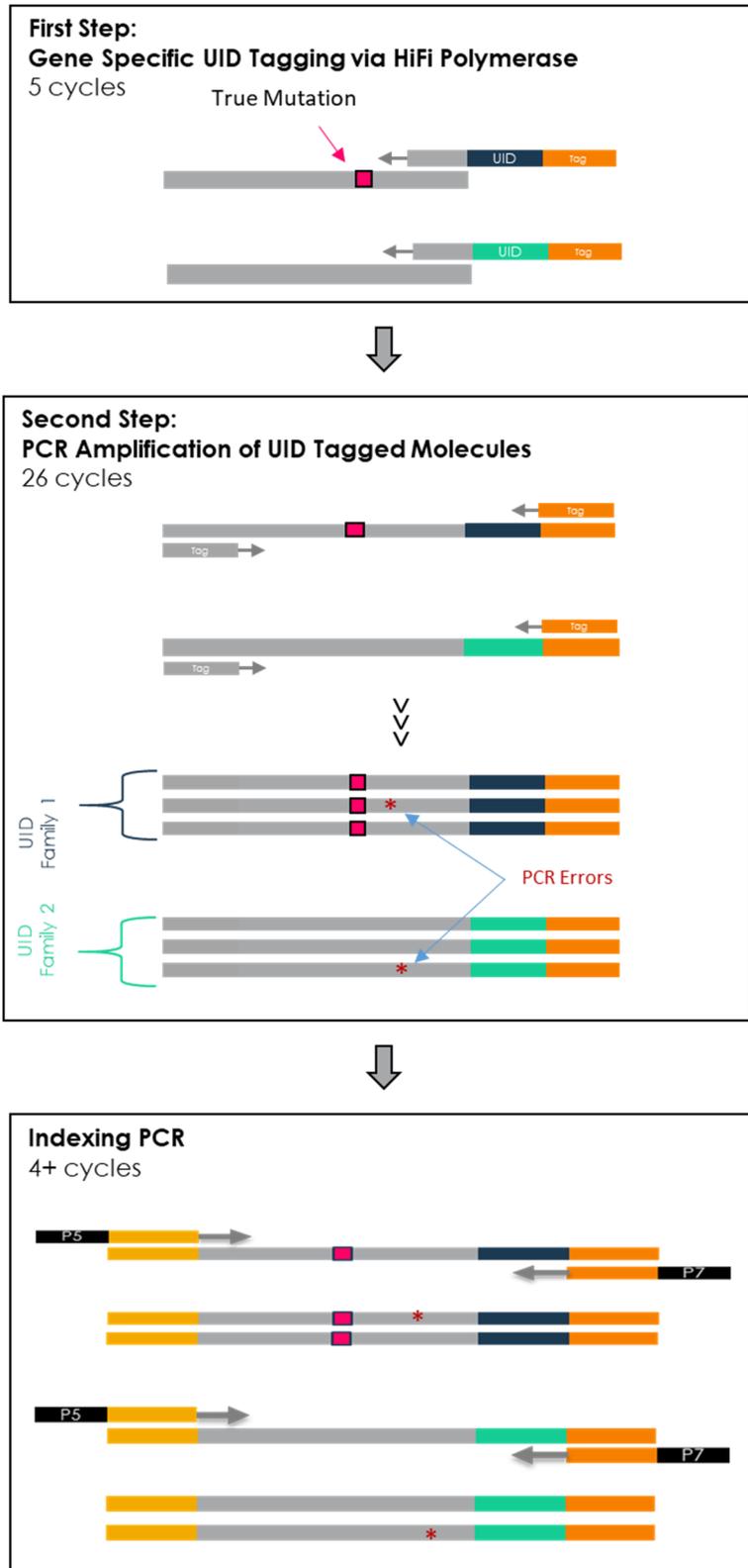


Figure 1. Overview of the ONCO/Reveal PillarHS Multi-Cancer library prep.

REVISION HISTORY

2019-05: Creation

2019-06: Tag primer information added

2019-07: Minor grammatical edits

2019-08: Updated ePCR Reagent list

2020-04: Note about sequencing read length added in sequencing section.
Note about indexing combinations added in indexing PCR section. Changes made in version 0.2.4.

2020-05: Clarified reagent names and updated Product Name

GETTING STARTED

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

Components of the ONCO/Reveal PillarHS Multi-Cancer Panel

Reagent	Use	Area Use	Storage
High Fidelity PCR Master Mix (4x) [Tube label: PCR Hifi 4x Master Mix]	All rounds of PCR	Pre-PCR	-15° to -25°C
MC cfDNA Linear PCR Oligo Pool	First PCR	Pre-PCR	-15° to -25°C
MC cfDNA Exponential PCR Oligo Pool [Tube label: MC cfDNA Exp oligo pool]	Second PCR	Pre-PCR	-15° to -25°C

ONCO/Reveal PillarHS Multi-Cancer Panel Indexing Kits

Reagent and Part Number	Use	Area Use	Storage
Pillar Custom Indexing Primers Kit A, indices PI501-8, PI701-4 (32 combinations - 96 reactions) PN: IDX-PI-1001-96	Indexing PCR	Pre-PCR	-15° to -25°C
Pillar Custom Indexing Primers Kit B, indices PI501-8, PI705-8 (32 combinations - 96 reactions) PN: IDX-PI-1002-96	Indexing PCR	Pre-PCR	-15° to -25°C
Pillar Custom Indexing Primers Kit C, indices PI501-8, PI709-12 (32 combinations - 96 reactions) PN: IDX-PI-1003-96	Indexing PCR	Pre-PCR	-15° to -25°C
Pillar Custom Indexing Primers Kit D, indices PI501-8, PI701-12 (96 combinations - 192 reactions) PN: IDX-PI-1004-192	Indexing PCR	Pre-PCR	-15° to -25°C
Pillar Custom Indexing Primers Kit E, indices PI501-8, PI701-12 (96 combinations - 384 reactions) PN: IDX-PI-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 or post-PCR areas to prevent amplicon contamination. Each area designated for pre-PCR and post-PCR should have dedicated equipment, supplies, and reagents to prevent contamination.

User-supplied Reagents

Reagent	Area use	Supplier
10 N NaOH or 1 N NaOH	Post-PCR	General lab supplier
Agencourt AMPure XP Beads	Post-PCR	Beckman Coulter, #A63881/ #A63880
DNA molecular weight markers	Post-PCR	General lab supplier
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
200 mM Tris-HCl, pH 7.0	Post-PCR	General lab supplier
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
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat#T7724

Compatible Illumina Reagent Kits

MiSeq reagent kit v3 (600 cycles)	Illumina, #MS-102-3003
NextSeq 500/550 Mid Output v2 kit (300 cycles)	Illumina, #FC-404-2003

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
<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

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

- **Work areas:** To reduce the risk of contamination from PCR amplicons, supplies should not be moved from one area to another. Separate storage areas (refrigerator, freezer) should also be designated for pre- and post-PCR products.
- **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 PillarHS Multi-Cancer Panel Workflow

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

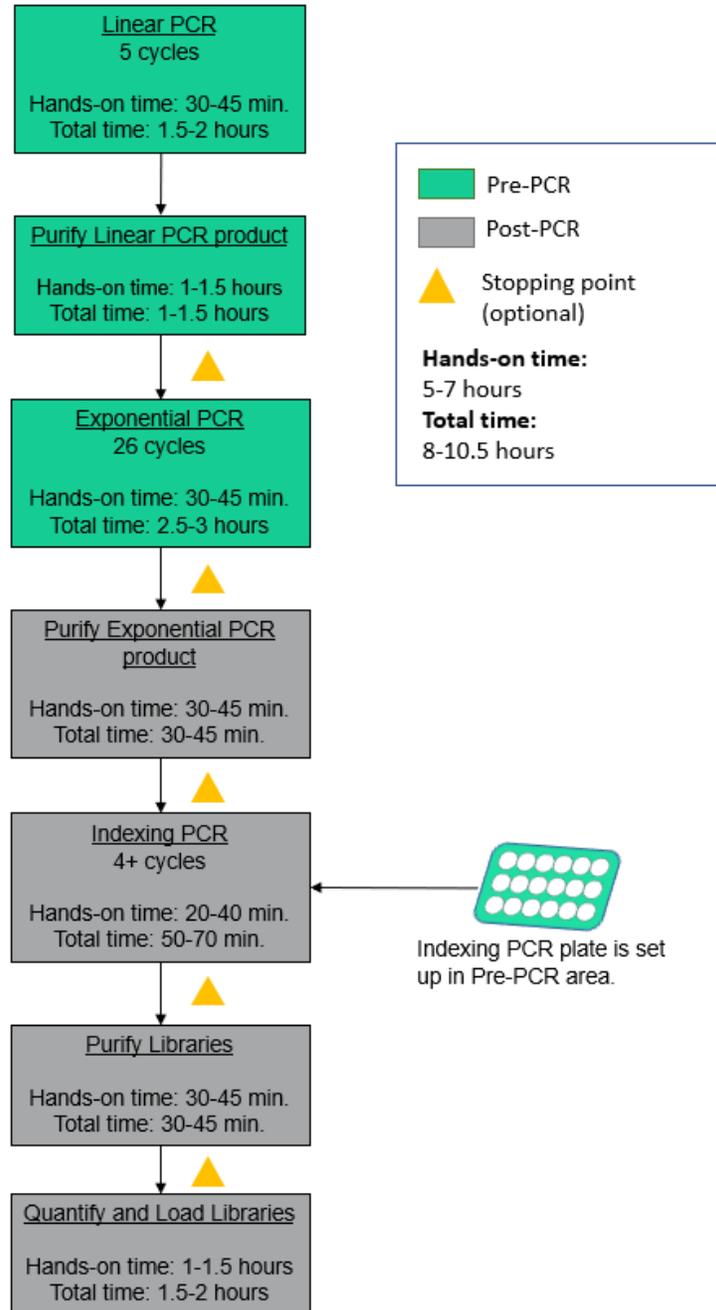


Figure 2. The ONCO/Reveal PillarHS Multi-Cancer Panel workflow can be completed in about ten hours but contains multiple stopping points for users with time constraints.

LIBRARY PREPARATION PROTOCOL

Hands-on time: 5-7 hours

Total time: 8-10.5 hours

Linear PCR: High Fidelity Linear Amplification

Hands-on time: 30-45 minutes

Total time: 1.5-2 hours

The following steps are performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the High Fidelity PCR Master Mix (HiFi PCR MMX) and Linear PCR oligo pool on ice.

1. **Dilute input DNA:** Dilute cell-free DNA (cfDNA) in nuclease-free water in a final volume of 35.5 μ L of diluted cfDNA. The recommended input range is 10-50 ng of cfDNA*. If using good quality cfDNA, the recommended minimum input is 10 ng. If using poor quality cfDNA, then the recommended minimum input is 25 ng.

**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). Recommended input range is 10-50 ng of cfDNA.*

2. **Prepare a PCR master mix:** Vortex and spin the HiFi PCR MMX and oligo pool before use. For each PCR reaction, the volume of each component is listed below. Prepare a master mix that is sufficient for the number of samples being processed plus overage.

Reagent	Volume (μ L)
HiFi PCR Master Mix (4x)	12.5
Linear PCR oligo pool	2.0
Sub-Total	14.5

Note: The High Fidelity 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.

- Transfer:** Keep the diluted DNA on ice while transferring the master mix. Transfer 14.5 μ L of master mix to each sample well in a PCR plate, strip tube, or PCR tube.

Reagent	Volume (μ L)
PCR master mix	14.5
Diluted DNA (or water)	35.5
Total	50.0

- Seal and mix:** Carefully seal the reactions and vortex for 10-15 seconds.
- 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.
- Perform PCR:** Perform the following program with the heated lid on:

Temperature	Time	No. of cycles
95°C	10 sec	x1
95°C	10 sec	x5
58°C	10 min	
8°C	Hold	Hold

IMPORTANT: Do not leave the PCR plate at 8°C. Purify the PCR product within 120 minutes after cycling has finished.

Once the reaction has been removed from the cycler, keep it chilled on an ice block until just before AMPure purification. Bring the reactions to ambient temperature on the bench for 3-5 minutes once the user is ready to perform the AMPure purification. Leaving reactions at ambient temperature for an extended period may result in product loss.

Purify the Linear PCR Product

Hands-on time: 1-1.5 hours

Total time: 1-1.5 hours

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. Allow Agencourt AMPure XP beads to come to room temperature for at least 30 minutes before use.

If the beads are not at ambient temperature once the cycling for linear PCR is finished, DO NOT store the plate at ambient temperature. Keep the plate chilled until the AMPure beads are ready. At that point, bring the plate to ambient temperature on the bench for 3-5 minutes.

Purification Round #1:

1. Briefly spin the plate to remove any droplets from the side walls before removing the seal. Carefully remove the seal from the PCR plate.
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 75 μ L beads (1.5x) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly spin the plate 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 6-0.

4. **Bind PCR product to beads:** Incubate the PCR plate for 5 minutes at ambient 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 PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
6. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads from the wall of each well.
7. **Wash beads:** Leave the PCR plate 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 ethanol wash 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. Pour only enough solution for each wash.

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

IMPORTANT: Remove trace amounts of ethanol completely from each well of the plate. If ethanol drops are attached to the sidewall of some wells, briefly spin the plate in a microplate centrifuge, and use a 10 or 20 μL tip to remove the remaining ethanol solution.

9. **Dry beads:** Keep the plate on the magnetic rack and let the beads air dry at ambient temperature for 2-5 minutes or until residual ethanol has dried.

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 plate from the magnetic rack and resuspend the dried beads in each well using 52 μL nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin the plate and mix again.
11. **Elute product:** Incubate the PCR plate at ambient temperature for 5 minutes to elute the product.
12. **Separate beads:** Separate the beads from the eluted product by placing the PCR plate on a magnetic rack until the solution appears clear.

Purification Round #2

1. **Remove eluted product:** Remove the eluted product from the beads by pipetting 50 μ L of the elution to the corresponding well in a new PCR plate.
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 75 μ L beads (1.5x beads if the volume is not currently 50 μ L) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly spin the plate and mix again.
4. **Bind PCR product to beads:** Incubate the PCR plate for 5 minutes at ambient temperature.
5. **Separate beads containing PCR product:** Place the PCR plate on a magnetic rack until the solution appears clear, which may 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 PCR plate 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 of the plate. The unused solution of ethanol can be used to purify the libraries after the second and third PCR.

IMPORTANT: *Remove trace amounts of ethanol completely from each well of the plate. If ethanol drops are attached to the sidewall of some wells, spin the plate in a microplate centrifuge briefly and use a 10 or 20 μ L tip to remove the remaining ethanol solution.*

9. **Dry beads:** Keep the plate on the magnetic rack and let the beads air dry at ambient temperature for 2-5 minutes or until residual ethanol has dried.

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 plate from the magnetic rack and resuspend the dried beads in each well using 25 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 the plate and mix again.
11. **Elute product:** Incubate the PCR plate at ambient temperature for 5 minutes to elute the product.
12. **Separate beads:** Separate the beads from the eluted product by placing the PCR plate on a magnetic rack until the solution appears clear.
13. **Remove product from beads:**
 - a. Remove 22 μ L of the elution from the beads to a new plate. Seal the plate and place it on ice for at least 5 minutes during the setup of exponential PCR.
 - b. Alternatively, after the elution, the products can be stored with the beads at -20°C for exponential PCR later. When exponential PCR is set up, place plate containing purified Linear PCR product on the magnet to separate elution for beads. Then transfer 22 μ L of elution to exponential PCR reaction plate.

STOPPING POINT: Purified Linear PCR products can be stored at -20°C .

Exponential PCR: High Fidelity Exponential Amplification

Hands-on time: 1-1.5 hours

Total time: 1-1.5 hours

The following steps are performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the High Fidelity PCR Master Mix (HiFi PCR MMX) and Exponential PCR oligo pool on ice.

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

Reagent	Volume (μL)
HiFi PCR Master Mix (4x)	12.5
Exponential PCR oligo pool	2.0
Water	13.5
Sub-Total	28.0

Note: The High Fidelity 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 reagents to PCR plate:** Keep the master mix on ice until it is ready to be added to the PCR plate.
 - a. With the PCR plate on ice, transfer 28 μL of master mix to each sample well in a PCR plate, strip tube, or PCR tube. Then, add 22 μL of purified Linear PCR product (without beads) to the corresponding wells.
 - b. Alternatively, 28 μL of the master mix may be added directly to the chilled plate containing 22 μL of purified Linear PCR product in each well.

Reagent	Volume (μL)
PCR master mix	28.0
Purified Linear PCR product	22.0
Total	50.0

3. **Seal and mix:** Carefully seal the reactions and vortex for 10-15 seconds.
4. **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.
5. **Perform PCR:** Perform the following program with the heated lid on:

Temperature	Time	No. of cycles
95°C	15 sec	x5
60°C	10 min	
95°C	15 sec	x1
66°C	6 min	
95°C	15 sec	x20
66°C	2 min	
8°C	hold	hold

IMPORTANT: Do not leave the PCR plate at 8 °C overnight.

STOPPING POINT: Exponential PCR plate can be stored at -20 °C.

Gel Image After Exponential PCR

The following image is an example of samples after Linear and Exponential PCR using various inputs and quality of cfDNA.

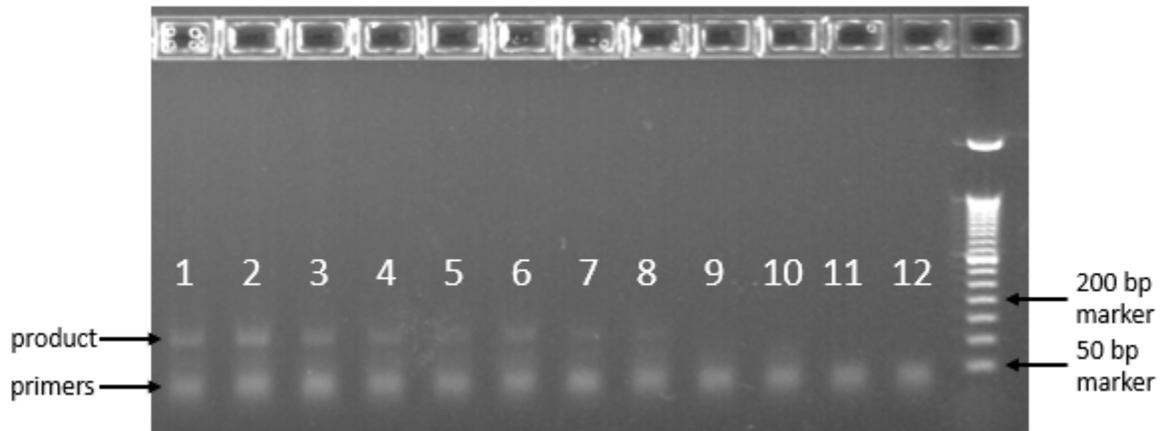


Figure 3. Gel analysis of product after Linear and Exponential PCR. Lanes 1-8 are cfDNA of various input and quality. Lanes 9 and 10 are no template controls (NTC) that went through Linear and Exponential PCR. Lanes 11 and 12 are no template controls (NTC) that only went through Exponential PCR.

Purify the Exponential 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.*

Exponential PCR 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:** If reaction volume is not already 50 µL, add nuclease-free water to each well 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 75 µL beads (1.5x 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 spin 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 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.

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 μ L tip to remove the remaining ethanol solution at the bottom of the wells.
11. **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.

12. **Resuspend beads:** Remove the samples from the magnetic rack, and immediately resuspend the dried beads in each well using 32 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.
13. **Elute:** Incubate the elution at room temperature for 5 minutes to fully elute the product.

- 14. Separate supernatant containing PCR product:** Place the samples on a magnetic rack until the solution appears clear, which can take up to 5 minutes.

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 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

NOTE: To prevent crosstalk between indexing primers at the coverage depth needed to sequence ONCO/Reveal PillarHS Multi-Cancer Panel samples, it is recommended that a P5 and P7 sequence be used only once per sequencing run. For example, if one sample is indexed using Pi501 and Pi701, then Pi501 should not be used again with any other P7 sequence. Additionally, Pi701 should not be used again with any other P5 sequence.

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.

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)
Pi700 Pillar Index	4.0
Pi500 Pillar Index	4.0
Total	8.0

2. **Prepare a Master Mix:** Vortex and spin the High Fidelity PCR Master Mix before use. To prepare the PCR master mix, combine the High Fidelity 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)
HiFi PCR Master Mix (4x)	12.5
Nuclease-free water	23.5
Total	36.0

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 Master Mix	8.0 36.0
Total	44.0

4. **Add purified GS 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 (Exponential 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 Gene-specific PCR product	44.0 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	30 sec	
66°C	30 sec	4*
72°C	60 sec	
72°C	5 min	1
8°C	Hold	1

* Additional Indexing PCR cycles can be performed if final library yield is low.

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 60 µL beads (1.2x 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: The purified libraries may also be stored at 4 $^{\circ}\text{C}$ for up to 3 days or at -20 $^{\circ}\text{C}$ for longer-term storage.

13. **Quantitation:** 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 Image

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

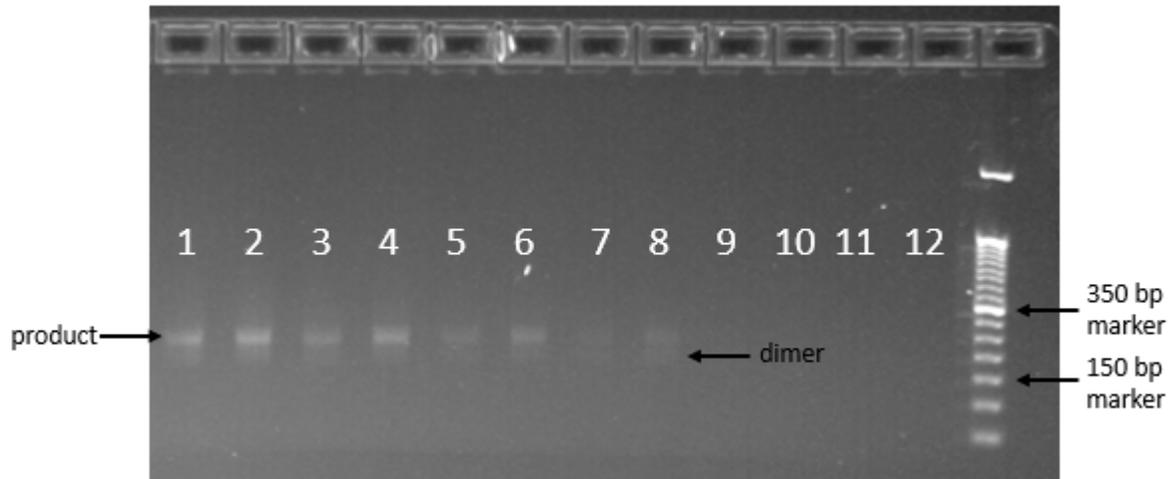


Figure 4. Gel analysis of final libraries. Lanes 1-8 are cfDNA of various input and quality. Lanes 9 and 10 are no template controls (NTC) that went through Linear and Exponential PCR. Lanes 11 and 12 are no template controls (NTC) that only went through Exponential PCR.

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.

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.
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 of library is equal to **7 nM**. Example calculation is below. Adjust dilution factor accordingly.

2 μL of library + 198 μL qubit solution:

$$\frac{\text{Qubit reading} \left(\frac{\text{ng}}{\text{mL}} \right)}{1,000} \times \text{dilution factor (100)} \times \text{conversion factor (7)} = \text{nM}$$

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 μ L) of each sample library to 5 nM using nuclease-free water or 10 mM Tris-Cl with 0.1% Tween-20, pH 8.5. An example calculation is as follows:

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

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

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 briefly.
3. **Prepare library mix:** Label a new 1.5 mL microtube for the library mix. Prepare a 5 nM mixture of libraries by combining each library at equal volume (i.e. mixing 5 μ L of each 5 nM library). Gently pipette the entire solution up and down 10 times to mix thoroughly. The mixture can also be quickly vortexed and spun.
4. **Quantify library pool (recommended):** It is recommended that the library mix be quantitated using Qubit or another library quantitation method (qPCR) to ensure the mix is at 5 nM (\pm 10%) to prevent over- or under-clustering on the MiSeq. If the final dilution is not 5 nM (\pm 10%), adjust the dilution for loading the sequencer accordingly to obtain the desired concentration.

Prepare Diluted Libraries for Sequencing

Hands-on time: 30-70 minutes

Total time: 30-70 minutes

Samples can be multiplexed and sequenced on the MiSeq using the v3 chemistry or the NextSeq. The number of samples that can be loaded is dependent on the number of paired-end reads per sample and sequencing

depth. To take advantage of the UID families, the recommended paired-end reads per sample is at least 5.0 million.

The recommended maximum number of samples that can be loaded on each kit is displayed in the table below. Please choose the appropriate sequencing workflow and kit based on the number of samples to be sequenced.

Kit	Cycles*	Estimated PE reads (million)	Mean Amplicon Coverage	Minimum Amplicon Coverage	Estimated PE reads/sample (million)	Estimated maximum # libraries
MiSeq v3	2x110	50	28,000	5,600	8.3	6
NextSeq Mid	2x110	260	28,000	5,600	8.3	31
NextSeq High	2x110	800	28,000	5,600	8.1	98

***NOTE:** *If samples processed with the ONCO/Reveal PillarHS Multi-Cancer Panel are to be loaded alone, then sequencing can be performed using a paired-end read length of 110 (2x110). If samples are to be loaded together with samples processed using other ONCO/Reveal panels, then sequencing can be performed using a paired-end read length of 150 (2x150) to accommodate the appropriate read length of the other ONCO/Reveal panels.*

Sequencing on MiSeq (MiSeq v3 Kit)

For v3 chemistry (MiSeq v3 kit), dilute libraries to **5 nM**. The final concentration of the libraries for sequencing is **20 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 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.

2. **Denature the library mix:** Label a new 1.5 mL microtube for the denatured, 20 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.
3. **Dilute to 20 pM library mix:** Label a new 1.5 mL microtube for the 20 pM library mix. Combine 480 μ L of the 25 pM library mix (step 5) with 120 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.
4. **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 20 pM library mix (step 6) with 6 μ L of a 20 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **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 7) 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 if 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 110 (2x110) and two indexing reads of 8 cycles each: "MiSeq System User Guide" (part #15027617). For instructions on preparing a sample sheet for the MiSeq, see page 35.
7. Store diluted libraries and mixtures at -20°C for long-term storage.

Sequencing on the NextSeq

For sequencing on the NextSeq, dilute libraries to **5 nM**. The final concentration of the libraries for sequencing is **1.8 pM**.

The following steps can be found in greater detail in Illumina's "NextSeq System: Denature and Dilute Libraries Guide" (part #15048776).

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 5 μL of 200 mM Tris-HCl, pH 7.0.
 - e. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
 - f. Add 985 μL of Illumina's HT1 solution to the denatured library mix.
 - g. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
3. **Dilute 25 pM library mix to 1.8 pM:** Dilute the denatured library to 1400 μL of a 1.8 pM solution by combining 101 μL of the 25 pM denatured library mix with 1299 μL of Illumina's HT1 solution. Invert to mix and spin briefly.
4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 1287 μL of the 1.8 pM library mix (step 3) with 13 μL of a 1.8 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **Load NextSeq cartridge:** Using a clean 1000 μL tip, puncture the foil cap above the sample loading well on the NextSeq cartridge. Load 1300 μL library mix and PhiX mixture (step 4) into the cartridge and ensure the solution has reached the bottom of the cartridge well.
6. **Run the NextSeq:** Run the libraries on the NextSeq per the manufacturer's instructions using a paired-end read length of 110 (2x110) and two indexing

reads of 8 cycles each: "NextSeq System User Guide" (part #15046563 or 15069765). For instructions on preparing a sample sheet for the NextSeq, see page **Error! Bookmark not defined..**

7. **Store Libraries:** Store diluted libraries and mixtures at -20°C for long-term storage.

Preparing a Sample Sheet for Sequencing

IMPORTANT: See the attached Appendix A for information regarding Pillar Indexing primers.

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

The available Pillar indexing primers and their barcode sequences are listed in the attached Appendix A. For the i5 indexing primers, indexing on the NextSeq requires the reverse complement of the barcode sequence. The correct barcode sequence for sequencing on the MiSeq and the NextSeq is provided in Appendix A. Additionally, the Pillar sample sheet generator will automatically populate the correct barcode sequence when the indexing primer is selected.

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

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

1. Open the Pillar sample sheet generator and enter user input in the shaded cells. Cells that are shaded blue are required and cells that are shaded grey are optional.
2. Enter the "Sample_ID" for each sample. Each Sample_ID must be unique and contain only alphanumeric characters, dashes (-), and underscores (_). All other characters are not allowed. To check that the Sample_ID meets all requirements click "Reset Sample_ID color" and then click "Check Sample_ID".
3. If text is green, the Sample_ID is acceptable. If text is red, Sample_ID is not acceptable. Change Sample_ID accordingly and repeat step 2 until all text is green.
4. Next, enter indices into appropriate fields. Index sequences will be populated once the index_ID is entered.
5. Check that all index combinations for each sample is unique. If "Check_index_uniqueness" column is green, then all index combinations are unique. If the column is red for a sample, then index combination is not unique. Do not load samples together in the same run that have the same index combination.
6. Once all requirements for the sample sheet are met, export the sample sheet as a comma-separated values (.csv) file by clicking "Export".

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of exponential PCR product	DNA quantity or quality	The recommended input for the assay is 10-50 ng of cell-free DNA. Higher quantities may be necessary for low- or poor-quality samples.
	Improper cycling	Check that the cycling protocols performed were the appropriate protocols for linear and exponential amplification.
Large ratio of primer dimers compared to product	Improper cycling	Check that the cycling protocols performed were the appropriate protocols for linear and exponential amplification.
	Improper AMPure purification	Check that the bead ratio is correct (1.5x for linear PCR and exponential PCR). Ensure the concentration of ethanol for the wash is 70%. AMPure purification should be performed at ambient temperature.
	Reactions not prepared on ice	To hinder enzyme activity before PCR, reactions and components should be kept on ice. Before exponential PCR, the linear PCR product and exponential PCR reagents should be kept on ice to prevent changes in temperature before performing PCR.
Low indexing efficiency	Improper AMPure purification	The loss of linear PCR product during the AMPure purification will affect the yield after exponential PCR. Ensure the ratio of AMPure XP is 1.5x of the reaction volume (75 μ L beads for 50 μ L reaction volume), and AMPure purification is performed at ambient temperature.

		<p>The ethanol concentration can affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.</p> <p>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.</p>
Low library yield	DNA quantity or quality	<p>The recommended input for the assay is 10-50 ng of cell-free DNA. Higher quantities may be necessary for low- or poor-quality samples.</p> <p>Run the product from the exponential PCR on agarose gel to check the yield.</p> <p>The product can also be checked on an agarose gel after indexing PCR before and after AMPure purification.</p>
	Improper AMPure purification	<p>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.</p> <p>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.</p>
The libraries over-cluster or under-cluster on the MiSeq	Normalization and mix of libraries is not 25 pM (MiSeq), 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.

	<p>Improper library quantitation</p>	<p>Improper library quantitation may result in artificially high or low yields, which affects downstream normalization.</p> <p>Re-quantitate the final libraries and/or the normalized libraries to check for the expected values.</p>
	<p>Improper AMPure purification</p>	<p>Changing the ratio of AMPure beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering.</p> <p>The 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.</p> <p>The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.</p>
<p>No-template control contains amplicons</p>	<p>Cross-contamination</p>	<p>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.</p> <p>Work spaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination.</p> <p>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.</p>