ONCO/REVEAL™ PillarHS Lung Panel (ONCO/Reveal Lung cfDNA Panel)

Library Preparation User Guide

FOR RESEARCH USE ONLY

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Pillar Biosciences
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INTRODUCTION

The ONCO/REVEAL™ PillarHS Lung 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 150 (2x150). The ONCO/Reveal PillarHS Lung 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.

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 workflow of the ONCO/Reveal PillarHS Lung 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.

SEQUENCING DEPTH

To appropriately utilize the information gained by using UIDs, appropriate sequencing depth is required for each sample. Inappropriate sequencing depth does not provide enough data to create a consensus. To take advantage of the UID and noise reduction, it is recommended that each sample obtain 5.05 million PE reads during sequencing.

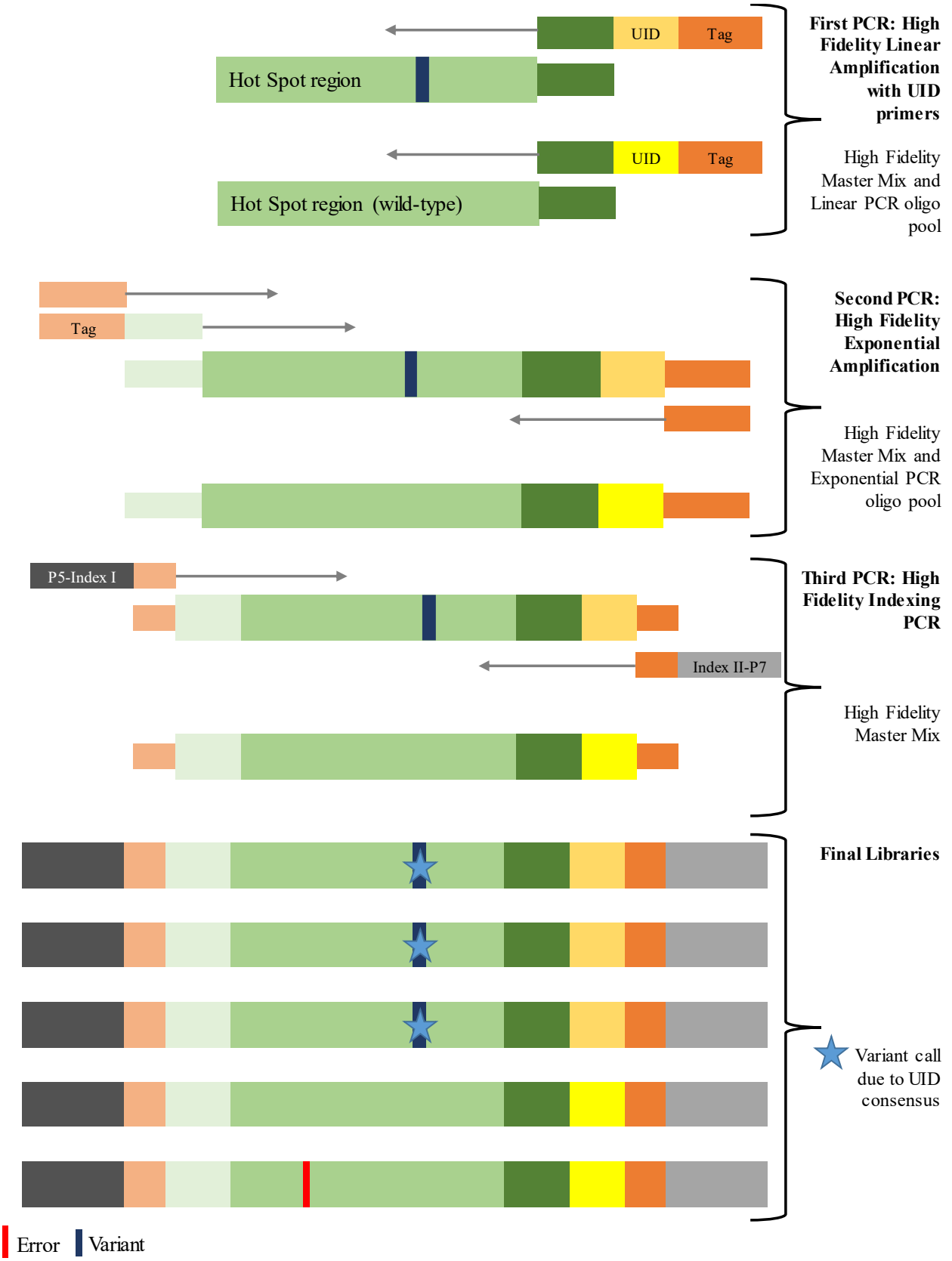


Figure 1. Pillar® High Sensitivity Panel workflow

REVISION HISTORY

2016-04: HS User Guide created
2016-09: Highlighted notes about performing setups on ice and updated reagent names
2016-09: Additional clarification for setup of exponential PCR
2016-11: Minor grammatical edits
2017-02: Updated linear PCR
2017-03: Update protocol for exponential PCR
2017-05: Added troubleshooting and sample sheet information
2017-10: Update protocol for indexing PCR and workflow image
2017-12: Workflow image update
2019-05: Product name and document format updates
2019-07: Index kit options and minor grammatical edits
2020-04: Renamed as ONCO/Reveal PillarHS Lung Panel

GETTING STARTED

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

Components of the ONCO/Reveal PillarHS Lung Panel

Reagent	Use	Storage
PCR High Fidelity 4x MasterMix	First, second, and indexing PCR	-15° to -25°C
L81 Linear PCR oligo pool v1	First PCR	-15° to -25°C
L81 Exponential PCR oligo pool v1	Second PCR	-15° to -25°C

ONCO/Reveal PillarHS Lung Panel Indexing Kits

Reagent and Part Number	Use	Area Use	Storage
Illumina TSCA Indexing Primers Kit A, indices AI501-8, AI701-4 (32 combinations - 96 reactions) PN: IDX-AI-1001-96	Indexing PCR	Pre-PCR	-15° to -25°C
Illumina TSCA Indexing Primers Kit B, indices AI501-8, AI705-8 (32 combinations - 96 reactions) PN: IDX-AI-1002-96	Indexing PCR	Pre-PCR	-15° to -25°C
Illumina TSCA Indexing Primers Kit C, indices AI501-8, AI709-12 (32 combinations - 96 reactions) PN: IDX-AI-1003-96	Indexing PCR	Pre-PCR	-15° to -25°C
Illumina TSCA Indexing Primers Kit D, indices AI501-8, AI701-12 (96 combinations - 192 reactions) PN: IDX-AI-1004-192	Indexing PCR	Pre-PCR	-15° to -25°C
Illumina TSCA Indexing Primers Kit E, indices AI501-8, AI701-12 (96 combinations - 384 reactions) 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. 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
10 N NaOH or 1 N NaOH	Post-PCR	General lab supplier
200 mM Tris-HCl, pH 7.0	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	Pre- and Post-PCR	Life Technologies, #Q32851/ #Q32854
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat#T7724
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 Illumina Sequencing Kits

MiSeq Reagent Kit v3 (600 cycles)	Post-PCR	Illumina, #MS-102-3003
NextSeq 500/550 Mid Output kit v2 (300 cycles)	Post-PCR	Illumina, #FC-404-2003
NextSeq 500/550 High Output kit v2 (300 cycles)	Post-PCR	Illumina, #FC-404-2004

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	Pre- and Post-PCR	Life Technologies, #Q32856

Equipment Requirements

Equipment	Area Use	Supplier
Centrifuge adapted for PCR plates, tabletop	Pre- and post-PCR	General lab supplier
Gel electrophoresis apparatus (optional) ***or***	Post-PCR	General lab supplier
2100 Bioanalyzer Instrument (optional)	Post-PCR	Agilent. #G2939BA
Magnetic stand for 96 wells	Post-PCR	Life Technologies, #12331D/ #12027
Microfuge	Pre- and post-PCR	General lab supplier
Thermal cycler, heated lid capability	Post-PCR	Bio-Rad T100, #186-1096 or equivalent
Pipettes, 0.5-1000 µL capabilities	Pre- and post-PCR	General lab supplier
PCR cooler	Pre- and post-PCR	General lab supplier
Qubit Fluorometer	Post-PCR	Invitrogen, #Q33216/Q33218
Vortexer	Pre- and post-PCR	General lab supplier

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

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

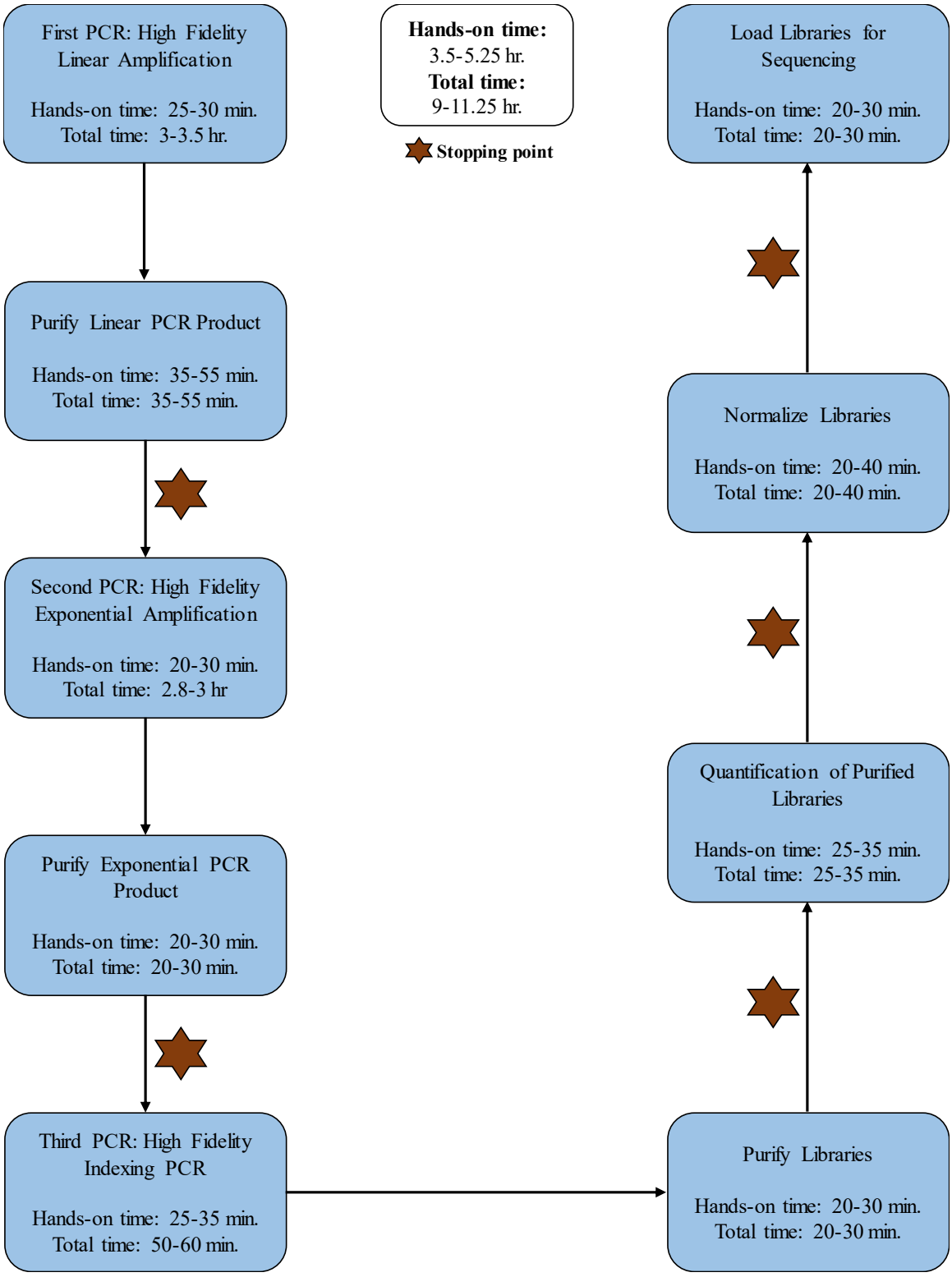
BEST PRACTICES

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 reduce 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 Lung Panel WORKFLOW

The following chart demonstrates the workflow for performing the ONCO/Reveal PillarHS Lung protocol.



LIBRARY PREPARATION PROTOCOL

Hands-on time: 3.5-5.25 hours
Total time: 9-11.25 hours

First PCR: High Fidelity Linear Amplification

Hands-on time: 25-30 minutes
Total time: 3-3.5 hours

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

IMPORTANT: Keep the High Fidelity 4x MasterMix and the Linear PCR oligo pool on ice during setup. Keep the PCR plate chilled on ice for the duration of the linear PCR setup.

1. **Prepare a PCR master mix:** For each PCR reaction, the volume of each component is listed in the table below. Briefly vortex (2-5 seconds) and spin down the HiFi MMX and Linear oligo pool before use.
    a. To prepare the PCR master mix, add all components except DNA to a 1.5 mL microtube and keep on ice.

       For example, to prepare enough mixture for 10 samples using a 50 µL reaction volume, mix 125 µL of HiFi PCR MMX, 50 µL of primer pool, and the appropriate amount of water. Add reagent overage appropriately.

    b. Mix the PCR master mix by pulse vortexing for 3-5 seconds, and spin down the solution in a microfuge. For a 50 µL reaction volume, transfer [50-n] µL of master mix to each well of a 96-well PCR plate *on ice*.

Reagent	**Volume (µL)**	**10 reactions (µL)**
High Fidelity 4x Master Mix	12.5	125
Linear PCR oligos v1 (10x)	5.0	50
cfDNA (15-50 ng)*	n	105
Nuclease-free water	32.5-n	
Total	**50.0**	

**The cfDNA concentration can be determined by the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. No. Q32851 or Q32854; Quantitation range 0.2-100 ng) or the Qubit dsDNA BR Assay Kit (Life Technologies, Cat. No. Q32850 or Q32853; Quantitation range 2-1,000 ng). The recommended input is at least 15 ng cfDNA, but the input can be as low as 5 ng for low-quantity samples.*

2. **Add DNA:** Keeping the reactions on ice, add (n) µL of cfDNA into each well.
3. **Seal plate and mix:** Carefully seal the plate with an Axygen sealing film (Axygen, Cat. No. PCR-TS) or an equivalent seal. Gently vortex the plate for 5-10 seconds to ensure sufficient mixing after sealing.
4. **Spin:** Briefly spin down the plate for 10-15 seconds in a microplate centrifuge to remove any bubbles within the reaction solutions.
5. **Perform PCR:** Place the PCR plate in a thermal cycler, and perform the following program:

Temperature	Time	Number of Cycles
95°C	10 seconds	1
95°C 58°C	10 seconds 10 minutes	15
8°C	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: 35-55 minutes
Total time: 35-55 minutes

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.

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.*
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 Part#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 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 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 6-8.*

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 beads. 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 Part#2

1. **Remove eluted product:** Remove the eluted product from the beads by pipetting 50 µL of the eluent 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 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 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 µL of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate 30 seconds, and then remove the supernatant from each well.

   IMPORTANT: *Do not allow the ethanol mixture to remain open to the air. The ethanol concentration will change over time, affecting the washing of the beads. Pour only enough solution for each wash.*

8. **Second wash:** Repeat step 7 for a second 70% ethanol wash. Remove supernatant from each well 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 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 24 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:** Place the plate with the first PCR product on the magnet to separate the beads from the eluent.

13. **Remove product from beads:** Remove the eluent 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. Alternately, after the elution, the products can be stored with the beads at -20°C for exponential PCR later.

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

Second PCR: High Fidelity Exponential Amplification

Hands-on time: 20-30 minutes
Total time: 2.8-3 hours

The following step should be performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. ***IMPORTANT:*** The HiFi 4x Master Mix, Exponential PCR oligo pool, and water should be kept on ice during setup. Prepare the exponential PCR components and plate on ice.

1. **Prepare the exponential PCR master mix:** For each PCR reaction, the volume of each component is listed in the table below. Briefly vortex and spin down the HiFi MMX and Exponential oligo pool before use.
    a. To prepare the PCR master mix, add all components except the linear PCR product to a 1.5 mL microtube and keep on ice.

       For example, to prepare enough mixture for 10 samples, mix 105 µL of chilled, nuclease-free water, 50 µL of the exponential primer pool, and 125 µL of the HiFi 4x MMX. Add reagent overage appropriately.

    b. Mix the PCR master mix by pulse vortexing the tube for 3-5 seconds, and spin down the solution in a microfuge.

Reagent	Volume (µL)	10 reactions (µL)
PCR High Fidelity 4x Master Mix	12.5	125
L81 Exponential PCR oligo pool v1 (10x)	5.0	50
Nuclease-free water	10.5	105
Linear PCR product, chilled and without beads	22.0	
Total	**50.0**	

IMPORTANT: The PCR plate with the linear product and the plate with the exponential PCR reagents should be kept on ice to keep all components of the reaction chilled prior to cycling. The linear PCR product should be chilled on ice for at least 5 minutes before mixing the linear product with the exponential PCR reagents.

2. **Add exponential PCR reagents and chilled linear product:**
    a. **Option 1:**
        i. **Aliquot exponential PCR reagents:** Aliquot 28 µL of the exponential mixture (step 1), which contains the HiFi 4x Master Mix, exponential PCR primers, and water to a PCR plate chilled on ice.

ii. **Add chilled linear PCR product:** Keeping the exponential PCR mixture on ice, add 22 µL of the chilled linear product to the corresponding wells of the PCR plate containing the exponential PCR reagents.

b. **Option 2:**

i. **Add exponential PCR reagents into chilled linear PCR product:** Chill 22 µL of the linear PCR product in a PCR plate. Keeping the exponential mixture (step 1) on ice, add 28 µL of the exponential mixture, which contains the HiFi 4x Master Mix, exponential primers, and water, to each well containing the linear PCR product.

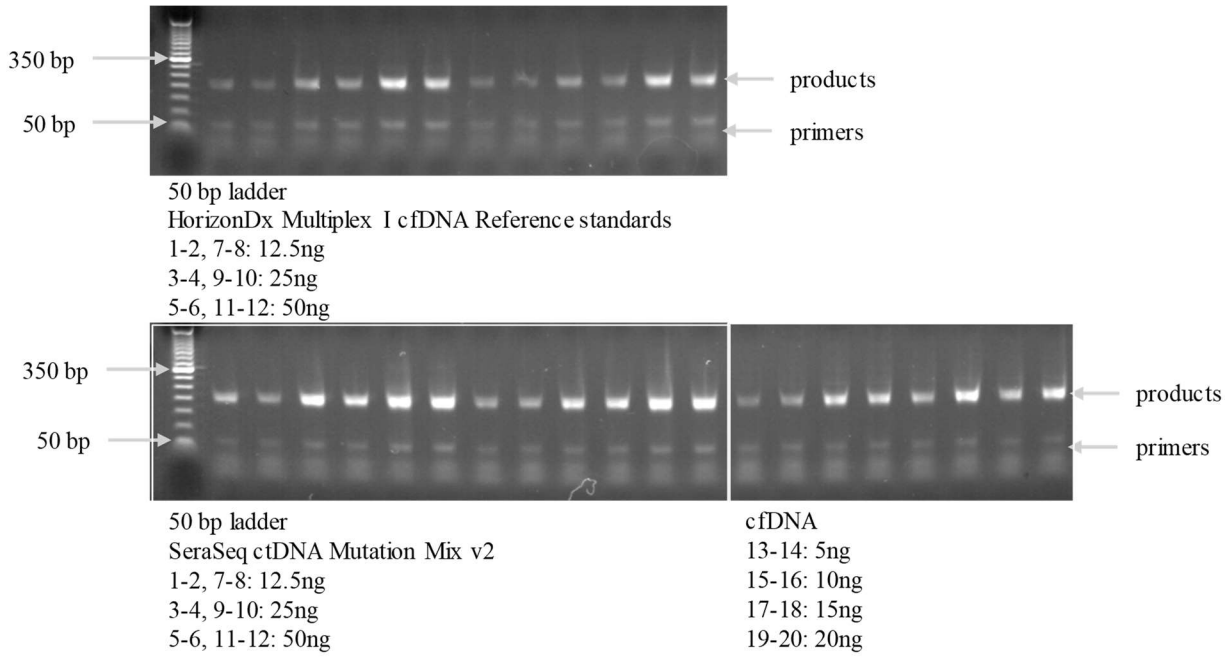
3. **Seal plate:** Carefully seal the plate with an Axygen sealing film (Axygen, Cat. No. PCR-TS) or an equivalent seal. Gently vortex the reactions for 3-5 seconds to ensure sufficient mixing after sealing.
4. **Spin:** Briefly spin down the plate for 10-15 seconds in a microplate centrifuge to remove any bubbles within the reaction solutions and place back on ice while transporting to the thermal cycler.
5. **Perform PCR:** Place the PCR plate in a thermal cycler, and perform the following program:

Temperature	Time	Number of Cycles
95°C 60°C	15 seconds 10 minutes	5
95°C 66°C	15 seconds 6 minutes	1
95°C 66°C	15 seconds 2 minutes	20
8°C	Hold	

IMPORTANT: *Do not leave the PCR plate at 8 °C. Purify the PCR product within 120 minutes.*
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 AMPure purification.

Quality Control

The following image is an example of samples after exponential PCR on a 2% agarose gel.



Purify the Exponential PCR Product

Hands-on time: 20-30 minutes
Total time: 20-30 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.

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.*
If the beads are not at ambient temperature once the cycling for exponential 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
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 60 µL beads (or 1.2x if the solution volume is less than 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 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 6-8.*

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

   TIP*: If needed, 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 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 µL of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate 30 seconds, and then remove the supernatant from each well.

   IMPORTANT: *Do not allow the ethanol mixture to remain open to the air. The ethanol concentration will change over time, affecting the washing of the beads. Pour only enough solution for each wash.*

8. **Second wash:** Repeat step 7 for a second 70% ethanol wash. Remove supernatant from each well 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, spin the plate in a microplate centrifuge briefly 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 32 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.

    TIP*: After resuspending the beads, cover the plate and prepare the reactions for indexing the libraries in the Pre-PCR area.*

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

Indexing PCR: Amplify the Libraries

Hands-on time: 25-35 minutes
Total time: 50-60 minutes

The following step should be performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. The HiFi 4x MMX should be kept on ice. Briefly vortex (2-5 seconds) and spin down the HiFi MMX before use.

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.
2. **Prepare the master mix:** To prepare a master mix, add the HiFi 4x MMX and water to a 1.5 mL microtube and keep on ice. Mix the master mix by pulse vortexing for 3-5 seconds, and spin down the solution in a microfuge.

   For instance, to prepare enough mixture for 10 samples, mix 235 µL of chilled, nuclease-free water and 125 µL of the HiFi 4x MMX. Add reagent overage appropriately.

   Transfer 36 µL of the HiFi MMX and water mixture to each well containing the indexing primers. To prevent cross-contamination of indices, change tips between each well.

   Important: Before transferring from the pre-PCR area to the post-PCR area, cover or seal the plate.

Reagent	Volume (µL)	10 reactions (µL)
High Fidelity 4x Master Mix	12.5	125
A700 TruSeq Amplicon Index	4.0	
A500 TruSeq Amplicon Index	4.0	
Nuclease-free water	23.5	235
Exponential PCR product, without beads	6.0	
Total	**50.0**	

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

3. **Separate beads:** Place the plate with the exponential PCR product on the magnet to separate the beads from the eluent, which contains the exponential PCR product.
4. **Add exponential PCR product:** Add 6 µL of the exponential PCR product to the corresponding wells in the indexing PCR plate.

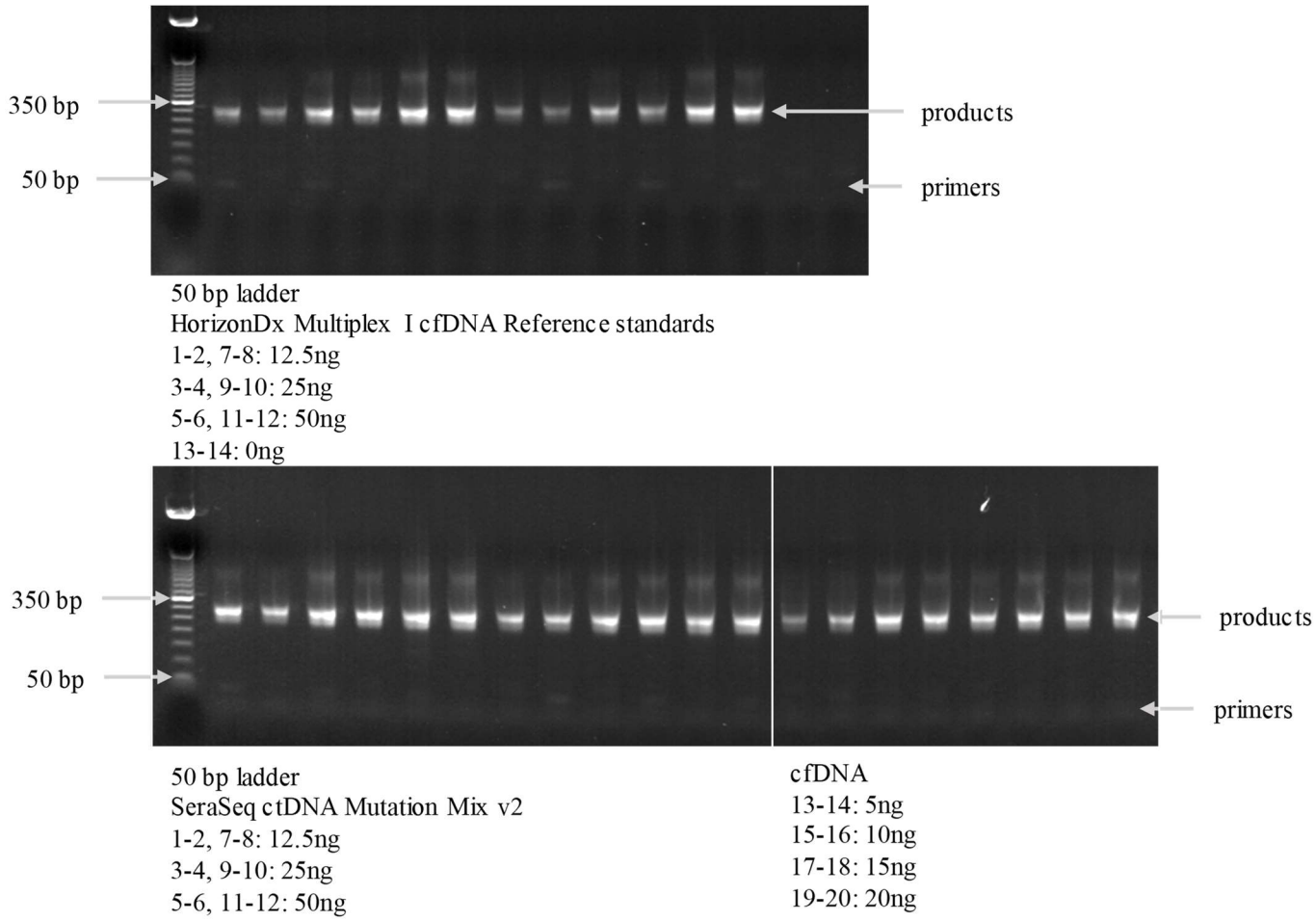
5. **Seal plate and mix:** Carefully seal the plate. After sealing, pulse vortex the reactions for 3-5 seconds.
6. **Spin:** Briefly spin down the reactions to remove any air bubbles from the bottom of the wells and spin down droplets from the seal or side walls.
7. **Perform PCR:** Place the PCR plate in a thermal cycler, and perform the following program:

Temperature	Time	Number of Cycles
95°C	2 minutes	1
95°C 66°C 72°C	30 seconds 30 seconds 60 seconds	5
72°C	5 minutes	1
8°C	Hold	

***TIP**: Thaw the sequencing reagent cartridge in a water bath per the corresponding Reagent Kit Reagent Prep Guide, and prepare the sample sheet during indexing PCR (see page 33 for the MiSeq or page 36 for the NextSeq).*

Quality Control

The following image is an example of samples after indexing PCR on a 2% agarose gel stained with ethidium bromide.



Purify the Libraries

Hands-on time: 20-30 minutes
Total time: 20-30 minutes

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.

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.*

Purification

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

1. If condensation has formed, 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 50 µL (or 1.0x if the reaction volume is less than 50 µL) beads 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 6-8.*

4. **Bind libraries to beads:** Incubate the PCR plate for 5 minutes at ambient temperature to bind the libraries to the beads.
5. **Separate libraries on beads:** 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 µL of the 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.

   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 µL tip to remove the remaining solution from wells.*

9. **Dry beads:** Keep the plate on the magnetic rack and let the beads air dry at ambient 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 plate from the magnetic rack and resuspend the dried beads in each well using 32 µL nuclease-free water. Gently pipette the beads suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin the plate and mix again.
11. **Elute libraries:** Incubate the PCR plate at ambient temperature for 5 minutes.
12. **Separate libraries from beads:** Place the PCR plate on the magnetic rack at ambient temperature until the solution appears clear. Transfer 30 µL of clear supernatant from each well of the PCR plate to the corresponding well of a new plate.

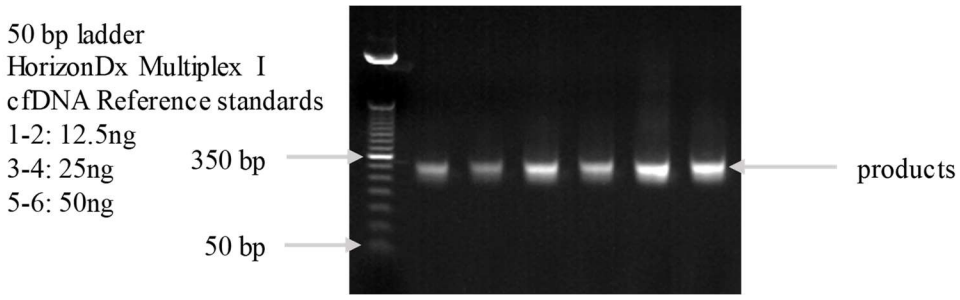
    TIP*: During the incubation and magnetic separation of the beads, cover the plate and prepare the solutions needed for quantitation in the next section. Alternately, store the purified libraries at 4℃ for up to 3 days or at -20℃ for longer-term storage.*

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

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

Quality Control

The following image is an example of final libraries after all rounds of PCR and purification.



Qubit Quantitation of Purified Libraries

Hands-on time: 25-35 minutes
Total time: 25-35 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, warmed to ambient temperature, 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 or inversion and then spin the tubes briefly.
6. Incubate the tubes at ambient temperature for 2 minutes.

7. **Measure concentration:** Measure the concentration of each sample on the Qubit 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:** Calculate the concentration (ng/mL) of each undiluted sample. Convert the concentration of each sample to nM (1 ng/µL of library is equal to 5 nM of library for the Lung cfDNA panel).

    2 in 200: [X ng·mL^{-1}] x [1 mL·1000 µL^{-1}] x dilution factor [100] = [Y ng·µL^{-1}]

    [Y] ng·µL^{-1} x [5 nM·ng^{-1}·µL] = [Z nM]

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

Prepare Libraries for Sequencing

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

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

Depending on the number of samples, samples can be multiplexed and sequenced using either MiSeq v3 chemistry or a NextSeq. Please choose the appropriate workflow based on the number of samples and the desired sequencing depth. To take advantage of the UID families, the recommended mean depth is 28,000x. The total recommended paired-end reads per sample is at least 5.05 million.

The following table provides a general guideline for the performance of the ONCO/Reveal PillarHS Lung Panel.

Kit	Cycles	Estimated PE reads	Estimated Mean amplicon coverage (x)[a]	Est. Minimum amplicon coverage (x)[b]	Estimated PE reads/sample	Est. Maximum # libraries
MiSeq v3	2x150	50 Million	30,000	4600	5.56 million	9
NextSeq Mid	2x150	260 Million	28,000	4200	5.10 million	51
NextSeq High	2x150	800 Million	46,000	6900	8.33 million	96

[a] In paired end sequencing, each amplicon is sequenced from both the forward and reverse directions, generating two reads. Therefore, an amplicon coverage of 1x requires a read coverage of 2x.
[b] The minimum coverage estimate is based on obtaining a minimum coverage of 15% of the mean amplicon coverage.

Sequencing on the MiSeq (MiSeq v3 Kit)

For sequencing on the MiSeq, dilute libraries to **5 nM.** The final concentration of the libraries for sequencing is **25 pM**.

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.

   The calculation uses the following equation:
   $Concentration_{initial} * Volume_{initial} = Concentration_{final} * Volume_{final}$

   (Z nM) * (4 µL library) / (5 nM) = Final volume of sample library
   Final volume of sample library – 4 µL library = volume of diluent

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

2. **Mix and spin:** Mix the 5 nM libraries thoroughly by vortexing followed by spinning 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 4 µL 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.

   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 to prevent over- or under-clustering on the MiSeq. If the final dilution is not 5 nM (±10%), adjust the dilution in step 6 accordingly with HT1 to obtain the desired concentration.

The following steps can be found in greater detail in Illumina's "MiSeq System: Denature and Dilute Libraries Guide" (part # 15039740).

4. **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.

5. **Denature the library mix:** Label a new 1.5 mL 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.
6. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 594 µL of the 25 pM library mix (step 5) with 6 µL of a 20 pM PhiX library control. Briefly vortex, spin, and place on ice.
7. **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 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 33.
9. 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 **2.0 pM**.

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.

   The calculation uses the following equation:
   $Concentration_{initial} * Volume_{initial} = Concentration_{final} * Volume_{final}$

   (Z nM) * (4 µL library) / (5 nM) = Final volume of sample library
   Final volume of sample library – 4 µL library = volume of diluent

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

2. **Mix and spin:** Mix the 5 nM libraries thoroughly by vortexing followed by spinning 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 4 µL 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.

   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 to prevent over- or under-clustering on the NextSeq. If the final dilution is not 5 nM (±10%), adjust the dilution in step 6 accordingly to obtain the desired concentration.

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

4. **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.

5. **Denature the library mix:** Label a new 1.5 mL 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.

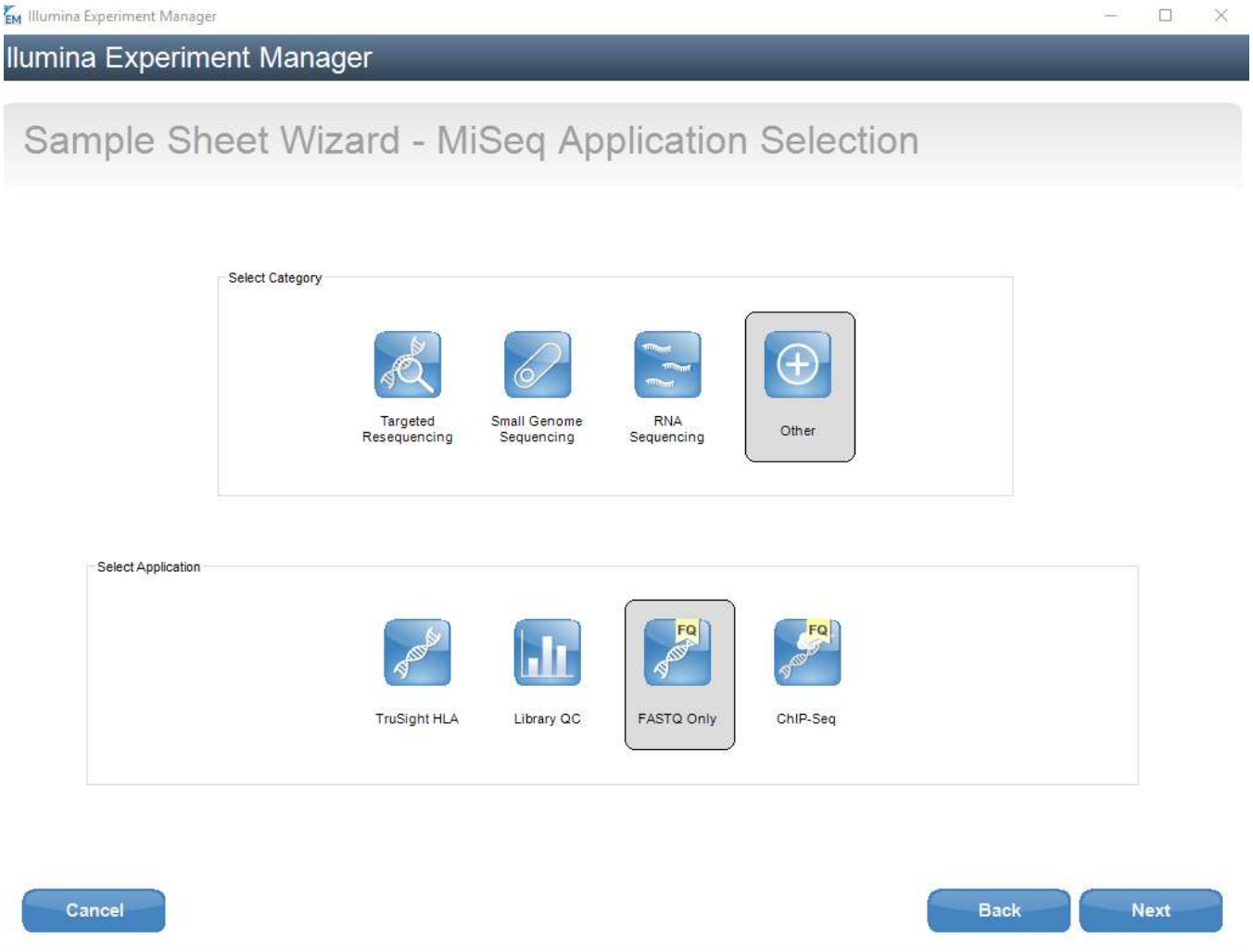
6. **Dilute 25 pM library mix to 2.0 pM:** Dilute the denatured library to 1300 µL of a 2.0 pM solution by combining 104 µL of the 25 pM denatured library mix with 1196 µL of Illumina's HT1 solution. Invert to mix and spin briefly.
7. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 1287 µL of the 2.0 pM library mix (step 6) with 13 µL of a 1.8 pM PhiX library control. Briefly vortex, spin, and place on ice.
8. **Load NextSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading well on the NextSeq cartridge. Load the 1300 µL library mix and PhiX mixture (step 7) into the cartridge and ensure the solution has reached the bottom of the cartridge well.
9. **Run the NextSeq:** Run the libraries on the NextSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) 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 36.
10. Store diluted libraries and mixtures at -20°C for long-term storage.

Preparing a Sample Sheet for the MiSeq Using Illumina Experiment Manager

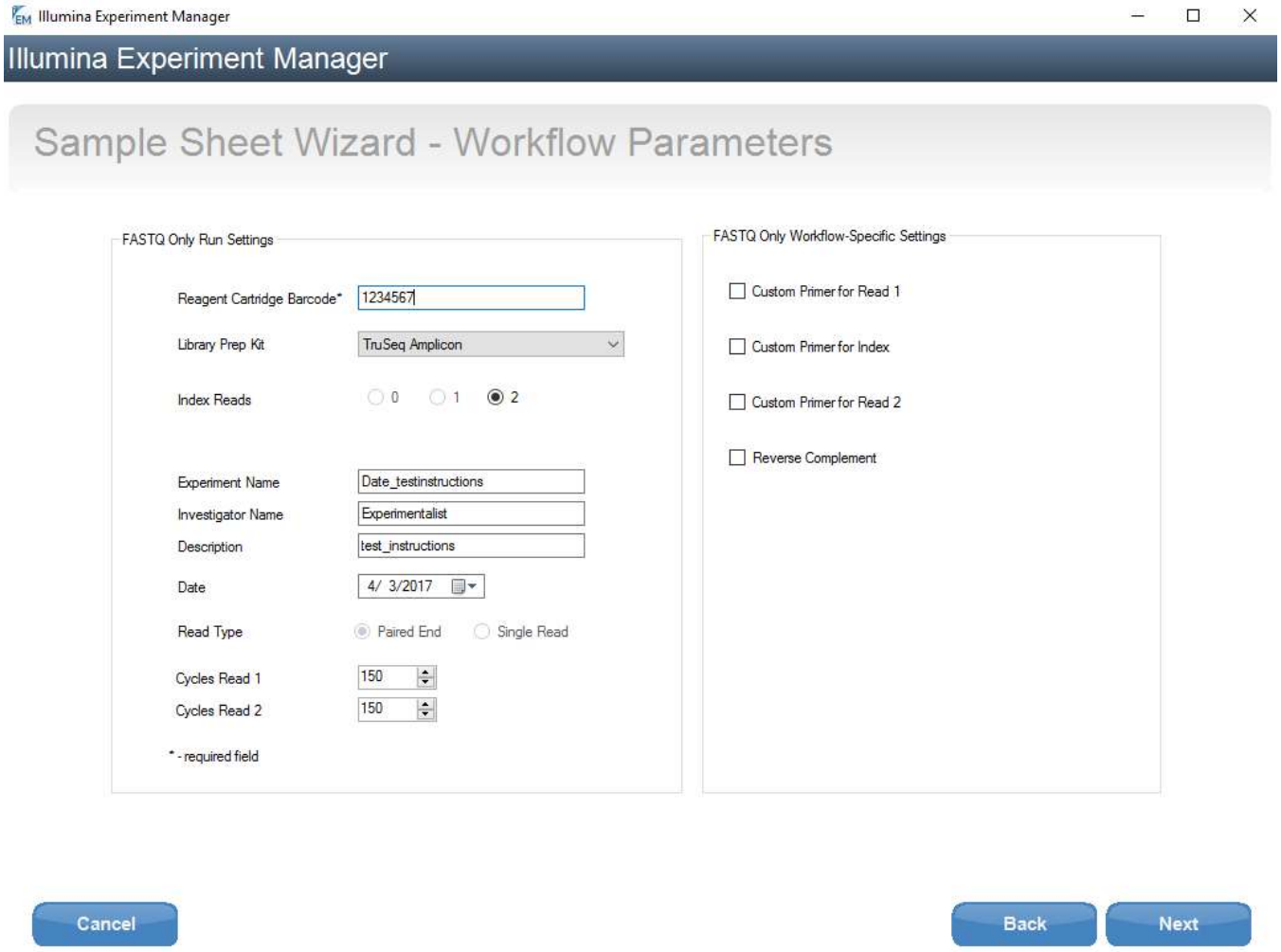
In the Illumina Experiment Manager, prepare a sample sheet that contains the information for the samples that are being loaded.

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.

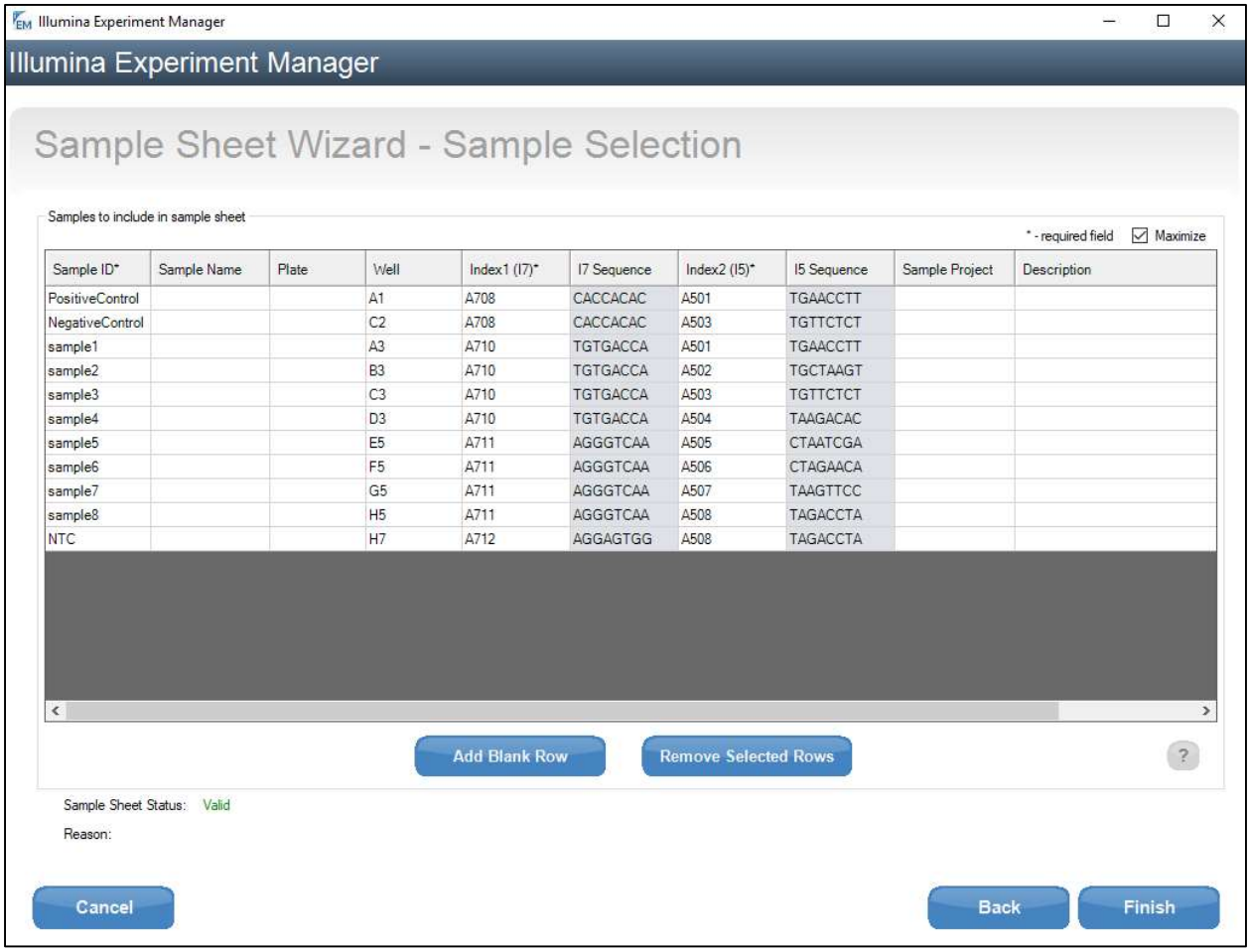
1. Open Illumina Experiment Manager, select "Create Sample Sheet."
2. **Instrument selection:** Select "MiSeq" and "Next."
3. **Application selection:** Under "Category," select "Other." Under "Select Application," select "FASTQ only" and "Next."



4. **Reagent barcode:** Enter the reagent barcode number found on the cartridge being loaded onto the instrument. For MSXXXXXXX-600v3, 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."



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.
    a. Name the samples in the column labeled "Sample ID." Illegal characters include spaces, periods, and other special characters.
    b. (Optional) The plates and well numbers can be added in "Plate" and "Well." ***Note:*** *Information in the "Sample Name" column will be used to name the corresponding FASTQ files in BCL2FASTQ.*
    c. For each sample, indicate the TruSeq Custom Amplicon indices used during indexing PCR.

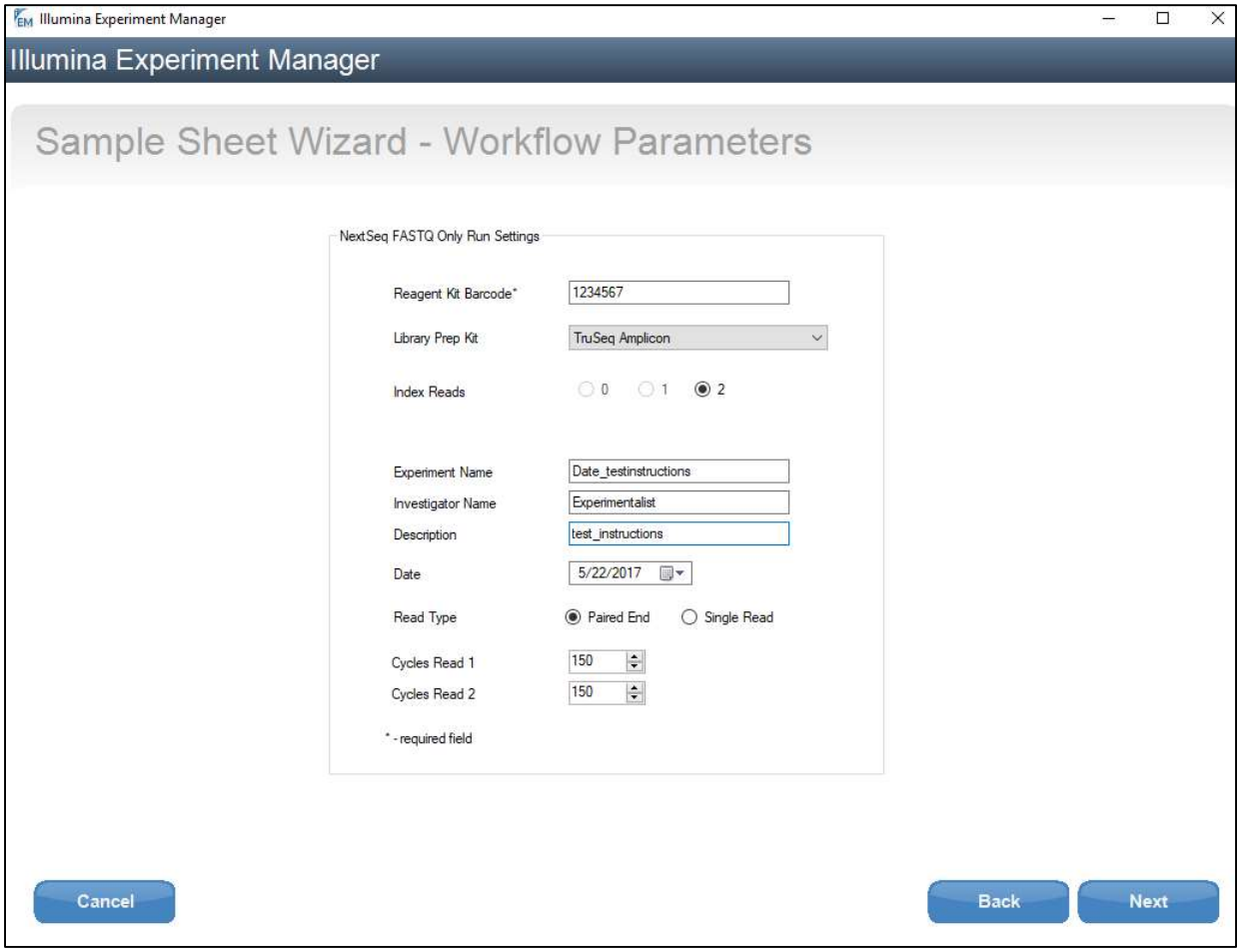


Preparing a Sample Sheet for the NextSeq Using Illumina Experiment Manager

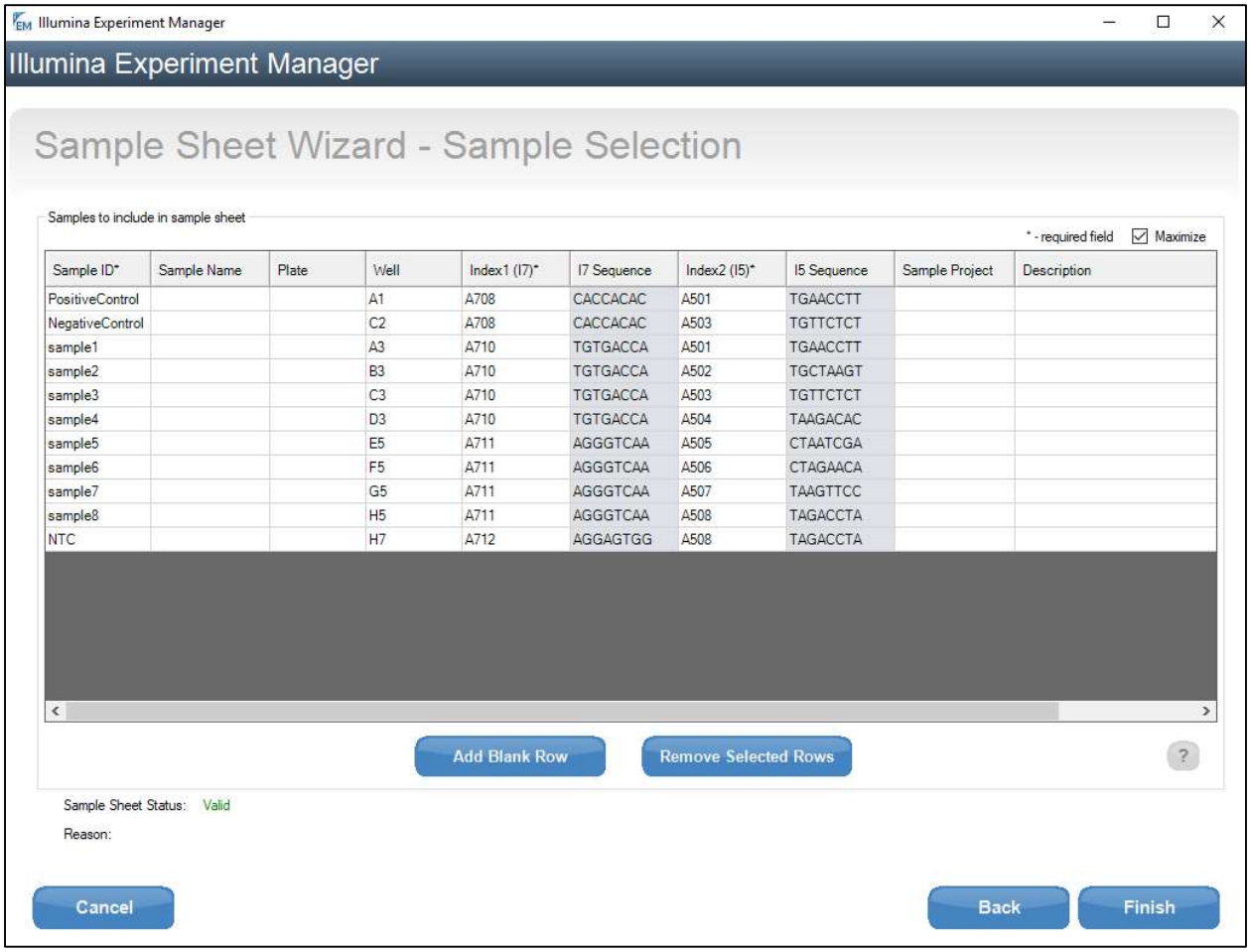
In the Illumina Experiment Manager, prepare a sample sheet that contains the information for the samples that are being loaded.

For best practice, prepare the sample sheet prior to loading the NextSeq 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 NextSeq.

1. Open Illumina Experiment Manager, select "Create Sample Sheet."
2. **Instrument selection:** Select "NextSeq" and "Next."
3. **Application selection:** Select "NextSeq FASTQ only" and "Next."
4. **Reagent barcode:** Enter the reagent barcode number found on the cartridge being loaded onto the instrument. For NSXXXXXXX, enter the 7-digit number following NS.
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."



10. **Sample Information:** Prepare the sample sheet with the sample identifiers, indices, and other pertinent information.
    a. Name the samples in the column labeled "Sample ID." Illegal characters include spaces, periods, and other special characters.
    b. (Optional) The plates and well numbers can be added in "Plate" and "Well." ***Note:*** *Information in the "Sample Name" column will be used to name the corresponding FASTQ files in BCL2FASTQ.*
    c. For each sample, indicate the TruSeq Custom Amplicon indices used during indexing PCR. ***Note:*** *The A500 indices are the reverse complement in the NextSeq workflow.*


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

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of product after exponential PCR	cfDNA quantity	The recommended input for the assay is 5-50ng of cfDNA. Check the input of the cfDNA with the Qubit dsDNA High Sensitivity or Qubit dsDNA Broad Range assay.
	Improper cycling	Check that the cycling protocol performed is the appropriate protocol for exponential amplification.
	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 (usually 75 µL), and AMPure purification is performed at ambient temperature. The temperature of the beads and reactions will also affect the purification process. AMPure purification is performed at ambient temperature. The incorrect ethanol concentration can affect the washing steps. Use fresh, 70% ethanol for washing the beads.
	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.
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	Changing the ratio of AMPure beads affects the purification of the products. For linear PCR, ensure the ratio of AMPure XP is 1.5x of the reaction volume (usually 75 µL), and AMPure is performed twice to remove excess primers from linear PCR. For

		exponential PCR, the ratio for AMPure XP beads is 1.2x. The temperature of the beads and reactions will also affect the purification process. AMPure purification is performed at ambient temperature. The incorrect ethanol concentration can affect the washing steps. Use fresh, 70% ethanol for washing the beads.
	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 library yield	Improper AMPure purification	Incomplete AMPure purification or loss of product will affect the final yield. The purified product can be checked on an agarose gel to ensure the exponential PCR product was not lost during PCR cleanup.
		The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash. AMPure purification is performed at ambient temperature.
The libraries over-cluster or under-cluster	Normalization and mix of libraries is not 25 pM (MiSeq) or 2.0 pM (NextSeq)	Check the 5 nM library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration.
	Improper library quantitation	Improper library quantitation may result in artificially high or low yields, which affects downstream normalization. Re-quantate the final libraries and/or the normalized libraries to check for the expected values.

	Improper AMPure purification	Changing the ratio of AMPure beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering. The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash. AMPure purification is performed at ambient temperature. The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.
No-template control contains amplicons	Cross-contamination	Make sure to change tips between samples, and avoid waving over tubes or plates. When liquid handling, be careful to avoid waving used tips over samples. Poor sealing or residual liquid in tips can cause contamination of nearby samples. If possible, leave adjacent wells empty between samples. Work spaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination. Periodically clean the work space, floor, equipment, and instrumentation with a laboratory cleaning solution (10% bleach, 70% isopropanol, or 70% ethanol) to break down amplicons on surfaces.