ONCO/Reveal™ TP53 Panel Reference Guide

Library Preparation User Guide

FOR RESEARCH USE ONLY

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INTRODUCTION

The ONCO/Reveal™ TP53 Panel targets the TP53 gene for use by researchers looking to explore the genetic sequences of both germline and formalin-fixed paraffin-embedded (FFPE) DNA samples from solid tumor samples. The panel utilizes Pillar Biosciences' proprietary SLIMamp® (stem-loop inhibition mediated amplification) technology, allowing researchers to target TP53 in a simple, multiplex reaction for subsequent sequencing on a sequencer using a paired-end read length of 150 (2x150).

The work flow of the ONCO/Reveal TP53 Panel can be performed and loaded onto the sequencing instrument by researchers within one day. The protocol also contains numerous stopping points for users who have time limitations.

How Does the ONCO/Reveal TP53 Panel Work?

A pair of DNA oligos was designed for each region of interest. Each region is amplified in the first round of gene-specific PCR (GS-PCR) and the products are subsequently purified via size selection. After purification, a second 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).

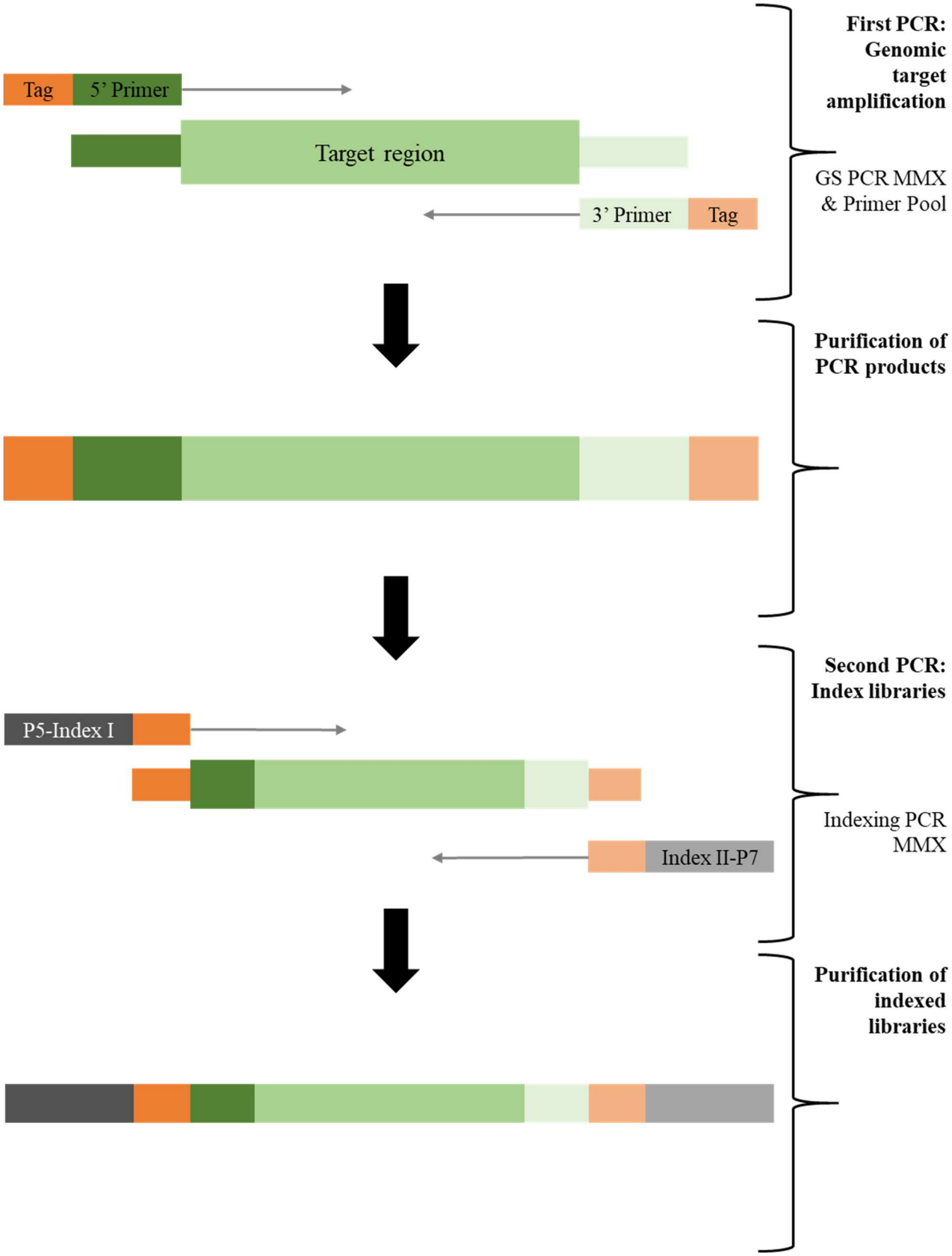


Figure 1. Overview of ONCO/Reveal TP53 Panel library preparation.

REVISION HISTORY

2018-03: Creation
2019-05: Update product name and document format
2019-07: Index kit options and minor grammatical edits

GETTING STARTED

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

Components of the ONCO/Reveal TP53 Panel

Reagent	Use	Area Use	Storage
Gene-Specific PCR Master Mix (2x)	Gene-specific PCR	Pre-PCR	-15° to -25°C
TP53 oligo pool (10x)	Gene-specific PCR	Pre-PCR	-15° to -25°C
Indexing PCR Master Mix (2x)	Indexing PCR	Pre-PCR	-15° to -25°C

ONCO/Reveal TP53 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 or post-PCR areas to prevent amplicon contamination. Each area designated for pre- and post-PCR should have dedicated equipment, supplies, and reagents to prevent contamination.

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
Ethanol, 200 proof for molecular biology	Post-PCR	General lab supplier
Nuclease-free water	Pre- and Post-PCR	General lab supplier
Qubit dsDNA High Sensitivity assay kit	Post-PCR	Invitrogen, #Q32851/ #Q32854
Agarose gel, 2% (optional)	Post-PCR	General lab supplier
DNA molecular weight markers (optional)	Post-PCR	General lab supplier
***Or** Bioanalyzer High Sensitivity DNA Analysis (optional)*	Post-PCR	Agilent #5067-4627/ #5067-4626
Uracil-DNA glycosylase (UDG) (optional)	Pre-PCR	NEB, #M0280S or #M0280L
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat#T7724

Compatible Illumina Reagent Kits

MiSeq reagent Nano kit v2 (300 cycles)	Illumina, #MS-103-1001
MiSeq reagent Micro kit v2 (300 cycles)	Illumina, #MS-103-1002
MiSeq reagent kit v2 (300 cycles)	Illumina, #MS-102-2002
MiSeq reagent kit v3 (600 cycles)	Illumina, #MS-102-3003

Consumables

Item	Area Use	Supplier
1.5 mL microcentrifuge tubes	Pre- and post-PCR	General lab supplier
96-well PCR plates, 0.2 mL	Pre- and post-PCR	Axygen, #6551 or equivalent
Microplate sealing film	Pre- and post-PCR	Axygen, #PCR-TS or equivalent
Conical tubes, 15 mL	Pre- and post-PCR	General lab supplier
Conical tubes, 50 mL	Post-PCR	General lab supplier
Low retention, aerosol filter pipette tips	Pre- and post-PCR	General lab supplier
Solution basin (trough or reservoir)	Pre- and post-PCR	Fisher, #13-681-506 or equivalent
Qubit Assay tubes	Post-PCR	Invitrogen, #Q32856

Equipment Requirements

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

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

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

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 TP53 Panel Workflow

The following chart (Figure 2) demonstrates the workflow for performing the ONCO/Reveal TP53 Panel library preparation.

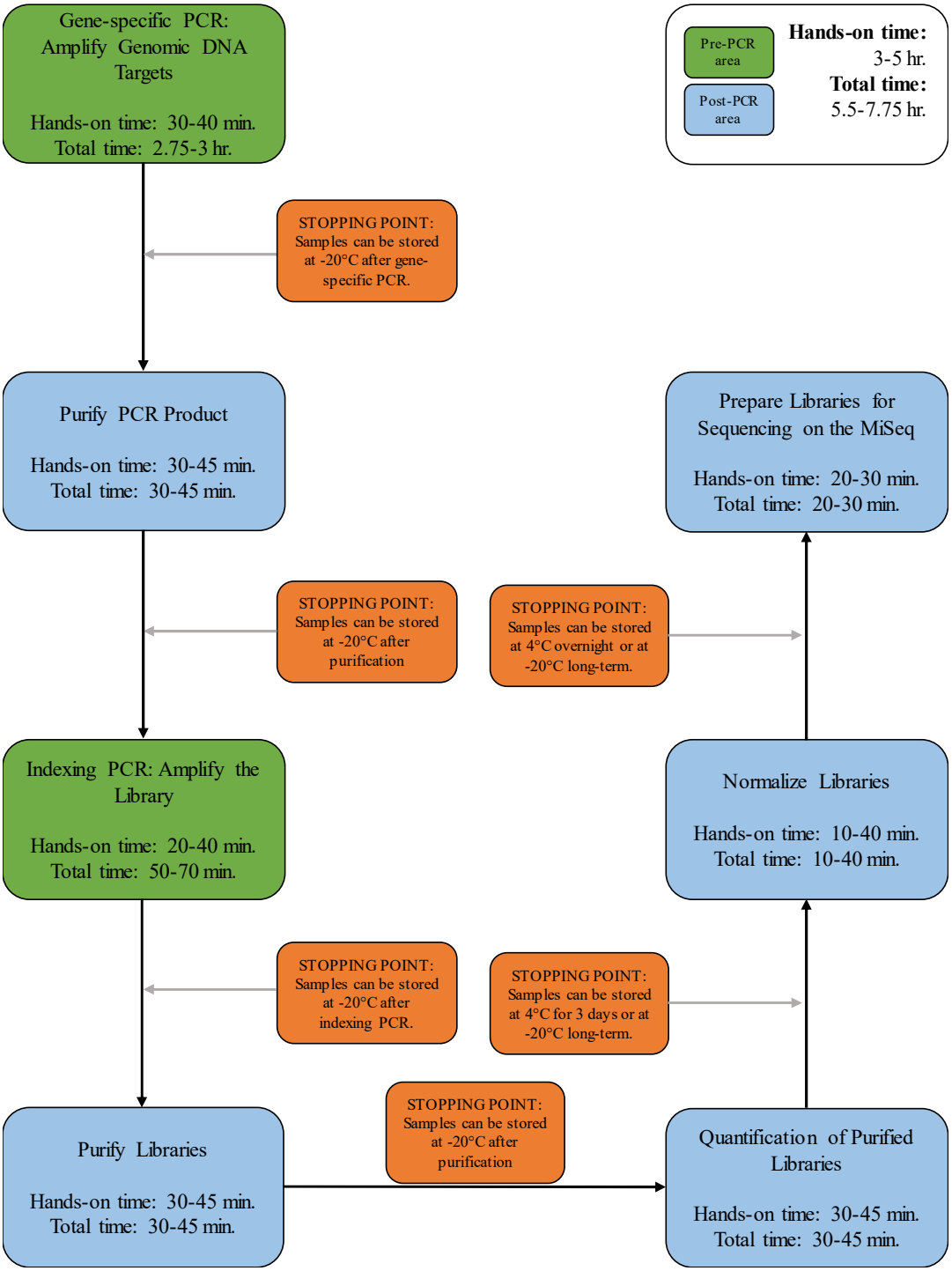


Figure 2. The ONCO/Reveal TP53 Panel workflow can be completed within a day but contains multiple stopping points for users with time constraints.

DNA INPUT INFORMATION

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

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

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

LIBRARY PREPARATION PROTOCOL

Hands-on time: 3-5 hours
Total time: 5.5-7.5 hours

Gene-specific PCR: Amplify Genomic DNA Targets

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

The following steps are performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the gene-specific PCR Master Mix (GS PCR MMX) and TP53 oligo pool on ice.

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

   Note: *The gene-specific PCR Master Mix is viscous. Ensure the mix is fully homogenized before adding other reaction components. Vortexing is recommended and will not adversely affect enzyme activity.*

   a. To prepare the PCR master mix, add components except genomic DNA to a 1.5 mL microtube and keep on ice.

      For example, to prepare enough mixture for 10 samples, mix 125 µL of GS-PCR MMX, 50 µL of primer pool, 10 µL UDG (optional; see DNA input note), and the appropriate amount of water. Add reagent overage appropriately.

b. Mix the PCR master mix by vortexing on medium speed for 10 seconds and spin down the solution in a microfuge. Transfer (25-n) µL of master mix to each sample well in a PCR plate, strip tube, or PCR tube.

Reagent	Volume (µL) (without UDG)	Volume (µL) (with UDG)
Gene-specific PCR MasterMix	12.5	12.5
TP53 Oligo Pool (10x)	2.5	2.5
DNA (20-60 ng*)	n	n
UDG (5 units/µL)	0.0	1.0
Nuclease-free water	10.0-n	9.0-n
Total	**25.0**	**25.0**

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

The recommended DNA input is 20-60 ng per PCR reaction for standard genomic DNA and 20-80 ng for FFPE DNA. It is recommended that the quality of FFPE DNA be checked by qPCR (Taqman RNase P Detection Reagents Kit (Life Technologies, Cat. No. 4316831)) or a Bio-analyzer. If the FFPE DNA is not degraded, the input can be as low as 5 ng. If using lower than 20 ng of DNA, up to 8 cycles of ***indexing PCR*** *can be used to improve the final library yield. Using 8 cycles of indexing PCR may increase primer-dimer formation, however the Pillar PiVAT software is able to differentiate on-target reads from primer-dimer artifacts and thus the data analysis will be unaffected. If the FFPE DNA is severely degraded, it is recommended to increase the DNA input and to quantitate the sample by qPCR or Bio-analyzer when possible.*

Note about FFPE samples: *For FFPE samples, it is recommended that UDG be added to the reaction to reduce artifacts from fixation and storage. The deamination of cytosine is a common cause of the presence of artificial C>T (or G>A) variants.*

2. **Add DNA:** Add (n) µL of genomic DNA (or water for a no-template control).
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	Number of Cycles (without UDG)*	Number of Cycles (with UDG)
37°C	10 minutes	0	1
95°C	15 minutes	1	1
95°C 58°C 60°C 64°C 72°C	1 minute 2 minutes 4 minutes 1 minute 1 minutes	5	5
95°C 66°C	30 seconds 3 minutes	22	22
8°C	Hold	1	1

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

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

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

Purify the Gene-specific PCR Product

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

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

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

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

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

Gene-specific Product Purification

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

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

3. **Add water to sample:** Add 25 µL of nuclease-free water to each well or add enough water to bring the volume to 50 µL.

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

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

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

6. **Separate beads containing PCR product:** Place the samples on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
7. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads from the wall of each well.
8. **Wash beads:** Leave the samples on the magnetic rack. Add 150 µL of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate 30 seconds, and then remove the supernatant from each well.

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

9. **Second wash:** Repeat step 8 for a second 70% ethanol wash. Remove the supernatant from each well. The unused solution of ethanol can be used to purify the libraries after indexing PCR.
10. **Remove remaining ethanol wash:** Remove trace amounts of ethanol completely from each well. Spin the samples in a benchtop centrifuge for 10-15 seconds, place the samples back on the magnetic rack, and use a 10 or 20 µL tip to remove the remaining ethanol solution at the bottom of the wells.
11. **Dry beads:** Keep the samples on the magnetic rack and let the beads air dry at room temperature for 2-5 minutes or until residual ethanol has dried.

    ***Note:** To prevent product loss, do not allow the beads to over-dry. The beads have sufficiently dried when the bead mass has small cracks in the middle. If large cracks have appeared 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 64 µL nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.
13. Incubate the elution at room temperature for 5 minutes to fully elute the product.

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

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

Indexing PCR: Amplify the Libraries

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

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

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 a master mix:** Vortex and spin the Indexing PCR Master Mix before use. To prepare the PCR master mix, combine the Indexing PCR Master Mix and water sufficient for the samples being processed with overage.
    a. For example, to prepare enough mixture for 10 samples, mix 250 µL of Indexing PCR MMX and 110 µL of water. Add reagent overage appropriately.
    b. Mix the PCR master mix by vortexing on medium speed for 10 seconds and spin down the solution in a microfuge. Transfer 36 µL of master mix to each sample well in a PCR plate, strip tube, or PCR microtube. To prevent cross-contamination of indices, be sure to change tips between each well.

Reagent	Volume (µL)
Indexing PCR Master Mix (2x)	25.0
A700 TruSeq Amplicon Index	4.0
A500 TruSeq Amplicon Index	4.0
Nuclease-free water	11.0
Gene-specific PCR product, without beads	6.0
Total	**50.0**

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.

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 gene-specific PCR product:** Add 6 µL of the gene-specific PCR product with the beads to the corresponding wells and carefully seal the plate or cap the tubes.
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.

Perform PCR: Perform the following program with the heated lid on:

Temperature	Time	Number of Cycles
95°C	2 minutes	1
95°C 66°C 72°C	30 seconds 30 seconds 60 seconds	6
72°C	5 minutes	1
8°C	Hold	1

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

TIP*: Thaw the sequencing reagent cartridge in a water bath per the MiSeq Reagent Kit Reagent Preparation Guide, and prepare the sample sheet during indexing PCR (see page 25).*

Purify the Libraries

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

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

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

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

Library Purification

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

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

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

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

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

4. **Bind libraries to beads:** Incubate the samples for 5 minutes at room temperature to bind the libraries to the beads.
5. **Separate libraries on beads:** Place the samples on a magnetic rack until the solution appears clear, which can take up to 5 minutes.

6. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads from the wall of each well.
7. **Wash beads:** Leave the samples on the magnetic rack. Add 150 µL of freshly-prepared 70% ethanol to each well without disturbing the beads. Incubate 30 seconds, and then remove the supernatant from each well.

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

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

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

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

   IMPORTANT: *Do not over-dry the beads. The beads have sufficiently dried when the bead mass has small cracks in the middle. If large cracks have appeared among the entire bead ring or they are flaky, they are over-dried. Beads that are too dry may be difficult to resuspend.*

10. **Resuspend beads:** Remove the samples from the magnetic rack and resuspend the dried beads in each well using 32 µL nuclease-free water. Gently pipette the beads suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.
11. **Elute libraries:** Incubate the resuspended beads at room temperature for 5 minutes to elute the final libraries.
12. **Separate libraries from beads:** Place the elutions on the magnetic rack at room temperature until the solution appears clear. Transfer 30 µL of clear supernatant from each well of the PCR plate or tubes to the corresponding well of a new plate or tube.

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

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

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

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.

   ***IMPORTANT**: New standard dilutions should be prepared with the samples. Do not re-use standard dilutions from previous experiments.*

4. **Prepare samples:** Transfer 198 µL of Qubit working solution to each tube, and then add 2 µL of each sample to the tube (1:100 dilution).
5. **Mix and spin:** Mix the tubes by vortexing and then spinning the tubes briefly.
6. Incubate the tubes at room temperature for 2 minutes.
7. **Measure concentration:** Measure the concentration of each sample on the Qubit 2.0 Fluorometer per the Qubit User Guide. Use the dsDNA High Sensitivity assay to read standards 1 and 2 followed by the samples.
   a. If any sample concentrations are above the linear range of the instrument, prepare a new dilution using 199 µL Qubit buffer with dye and 1 µL sample (1:200 dilution). Repeat steps 5-7.
8. **Calculate concentration:** Calculate the concentration (in ng/µL) 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 TP53 Panel).

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

   [Y] ng $\cdot$µL^{-1} x [5 nM $\cdot$ng^{-1} $\cdot$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 Diluted Libraries for Sequencing

Hands-on time: 30-70 minutes
Total time: 30-70 minutes

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

Depending on the number of samples, samples can be multiplexed and sequenced using MiSeq v2 chemistry or MiSeq v3 chemistry. Please choose the appropriate workflow based on the number of samples and the desired sequencing depth.

The following table provides a general guideline for the performance of the ONCO/Reveal TP53 Panel. The total number of sequenced reads obtained is a function of the cluster density and the read quality passing filter. Generally, as the cluster density increases, the passing filter decreases.

Kit	Cycles	Estimated PE reads	Est. Mean amplicon coverage (x)[a]	Est. Min. amplicon coverage (x)[b]	Estimated PE reads/sample	Est. Maximum # libraries[c]
Nano v2	2x151	2 Million	2500	500	100,00	18
Micro v2	2x151	8 Million	2500	500	100,000	75
v2	2x151	30 Million	7400	1400	312,000	96
v3	2x151	50 Million	12000	2400	520,000	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 20% of the mean amplicon coverage.
[c] The number of maximum libraries is limited by the number of TruSeq Amplicon indices.

Sequencing Using v2 Chemistry (MiSeq Micro v2 or MiSeq v2 Kit)

For running a MiSeq kit using v2 chemistry (MiSeq Micro v2 or MiSeq v2 kits), dilute libraries to **5 nM.** The final concentration of the libraries for sequencing is **10 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.
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 µL of each 5 nM library). Quickly vortex the mix for 2-5 seconds and spin down.

   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-clustering on the MiSeq. 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 "Preparing Libraries for Sequencing on the MiSeq" (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 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. **Dilute to 10 pM library mix:** Label a new 1.5 mL microtube for the 10 pM library mix. Combine 240 µL of the 25 pM library mix (step 5) with 320 µL of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice.
7. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 570 µL of the 10 pM library mix (step 6) with 30 µL of a 12.5 pM PhiX library control. Briefly vortex, spin, and place on ice.
8. **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 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 there is an air bubble at the bottom of the tube.
9. **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 25.
10. Store diluted libraries and mixtures at -20°C for long-term storage.

Sequencing Using v3 Chemistry (MiSeq v3 Kit)

For v3 chemistry (MiSeq v3 kit), dilute libraries to **5 nM.** The final concentration of the libraries for sequencing is **15 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.
3. **Prepare library mix:** Label a new microtube for the library mix. Prepare a 5 nM mixture of libraries by combining each library at equal volume (i.e. mixing 5 µL of each 5 nM library). Quickly vortex the mix for 2-5 seconds and spin down.

   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-clustering on the MiSeq. If the final dilution is not 5 nM (±10%), adjust the dilution in step ***Error! Reference source not found.*** *accordingly to obtain the desired concentration.*

The following steps can be found in greater detail in Illumina's "Preparing Libraries for Sequencing on the MiSeq" (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. **Dilute to 15 pM library mix:** Label a new 1.5 mL microtube for the 15 pM library mix. Combine 360 µL of the 25 pM library mix (step 5) with 240 µL of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice.
7. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 570 µL of the 15 pM library mix (step 6) with 30 µL of a 20 pM PhiX library control. Briefly vortex, spin, and place on ice.
8. **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 **Error! Reference source not found.**) 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.
9. **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 25.
10. Store diluted libraries and mixtures at -20°C for long-term storage.

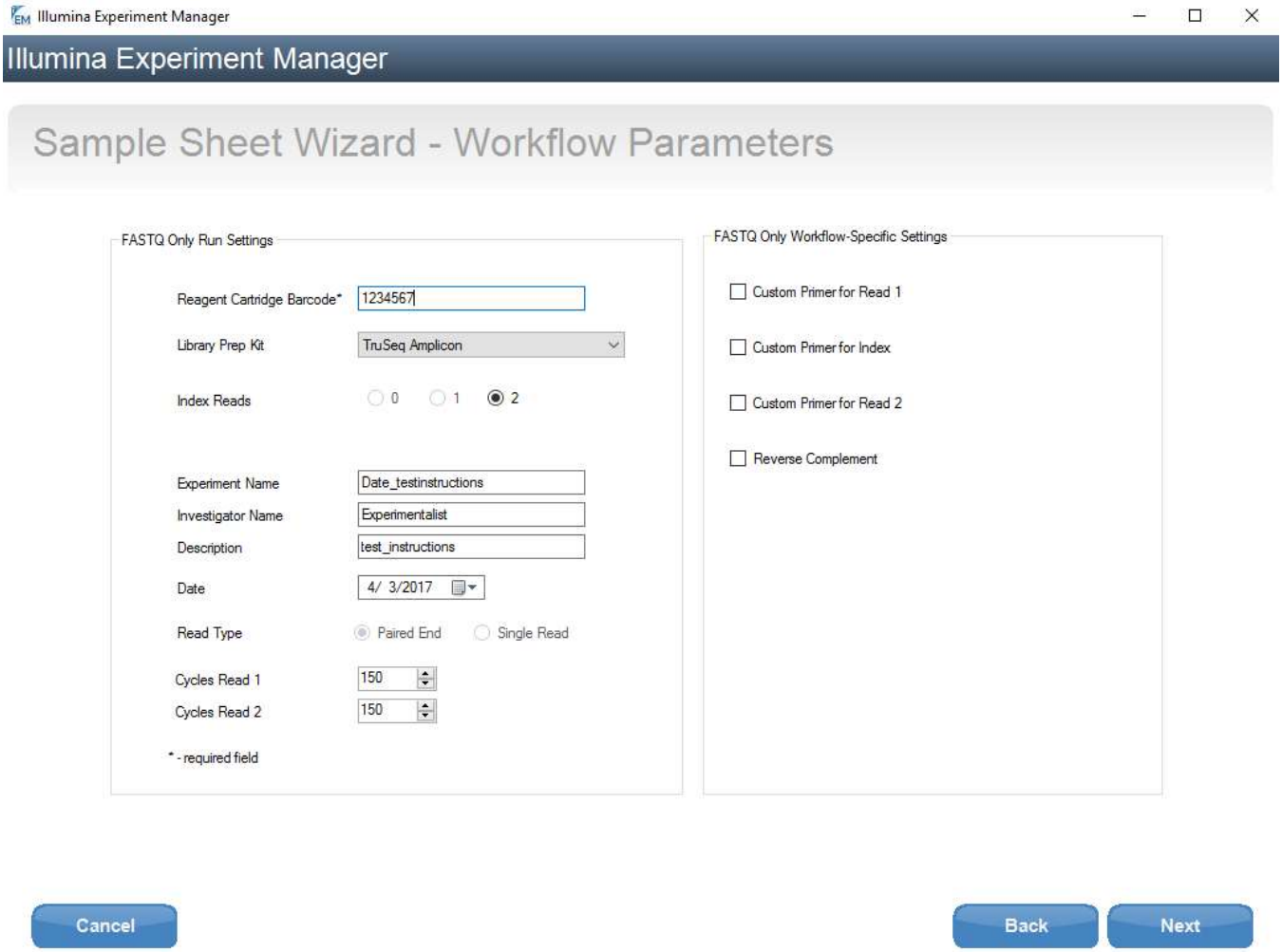
Preparing a Sample Sheet for the MiSeq Using Illumina Experiment Manager

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

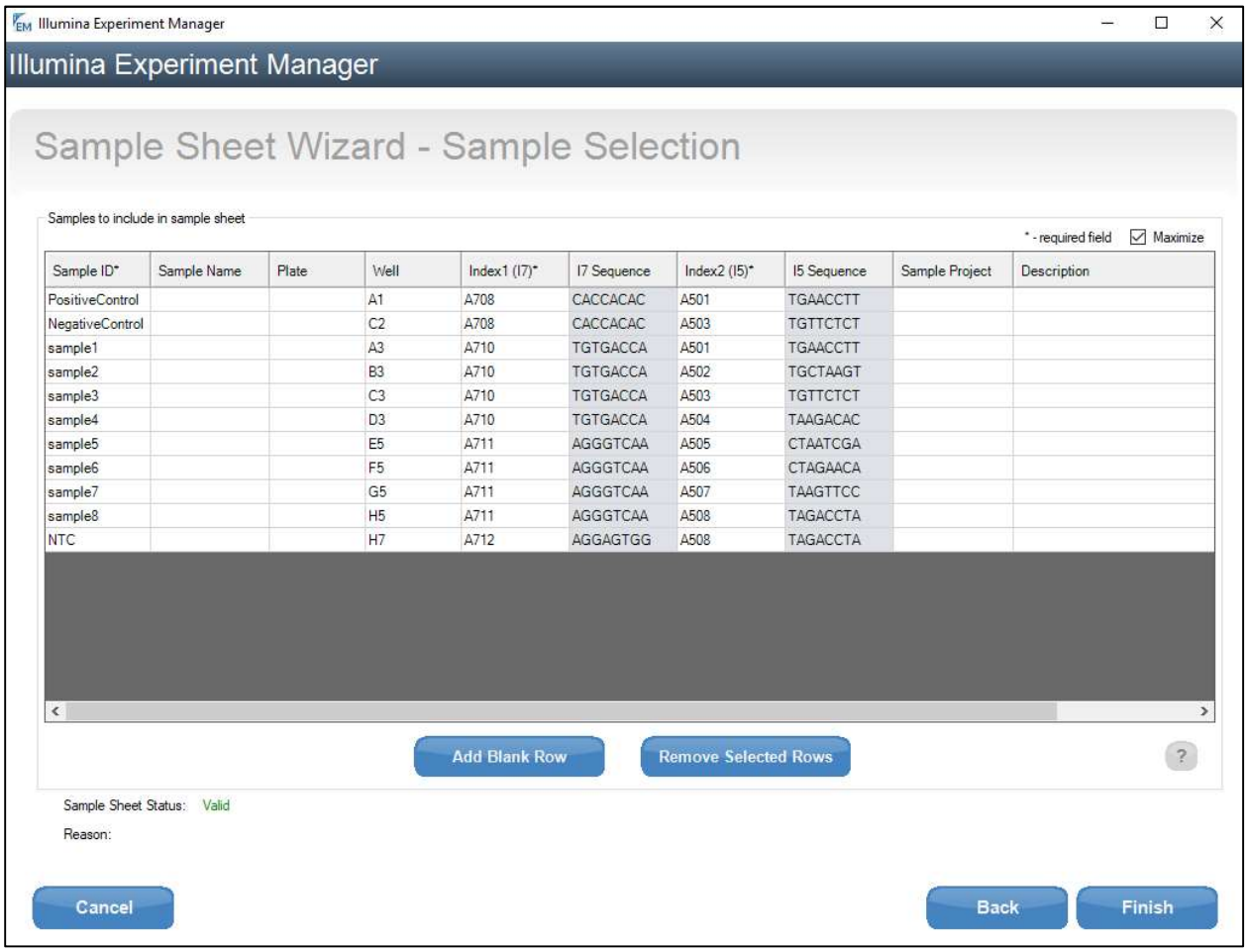
For best practice, prepare the sample sheet prior to loading the MiSeq cartridge. If an error has been made during indexing PCR where samples have the same indices, it can be remedied before loading the samples on the MiSeq.

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. Example: For MSXXXXXXX-300v2 or 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."
    c. For each sample, indicate the TruSeq Custom Amplicon indices used during indexing PCR.



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		

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of gene-specific product	DNA quantity or quality	The recommended input for the assay is 20-80ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples.
	Improper cycling	Check that the cycling protocol performed is the appropriate protocol for gene-specific amplification.
Low indexing efficiency	Improper AMPure purification	Incomplete AMPure purification or loss of gene-specific product will affect the indexing PCR reaction. The purified product can be checked on an agarose gel to ensure the gene-specific product was not lost or that clean-up was sufficient to remove excess primers.
		The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
		Leftover ethanol from the wash steps can hinder the PCR reaction. Remove as much of the ethanol during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated.
Low library yield	DNA quantity or quality	The recommended input for the assay is 20-60ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples. Run the product from the gene-specific PCR on agarose gel to check the yield.

		The product can also be checked on an agarose gel after indexing PCR before and after AMPure purification.
	Improper AMPure purification	Incomplete AMPure purification or loss of product will affect the final yield. The purified product can be checked on an agarose gel to ensure the product was not lost during PCR cleanup. The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
The libraries over-cluster or under-cluster on the MiSeq	Normalization and mix of libraries is not 10 pM (v2) or 15 pM (v3)	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-quantitate the final libraries and/or the normalized libraries to check for the expected values.
	Improper AMPure purification	Changing the ratio of AMPure beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering. The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.

		The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.
No-template control contains amplicons	Cross-contamination	Make sure to change tips between samples and avoid waving over tubes or plates. When liquid handling, be careful to avoid waving used tips over samples. Poor sealing or residual liquid in tips can cause contamination of nearby samples. If possible, leave adjacent wells empty between samples. Workspaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination. Periodically clean the workspace, floor, equipment, and instrumentation with a laboratory cleaning solution (10% bleach, 70% isopropanol, or 70% ethanol) to break down amplicons on surfaces.